ON THE ROLE OF SycE IN TARGETING YopE INTO HeLa CELLS

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Yersinia enterocolitica inject toxic proteins (effector Yops) into the cytosol of eukaryotic cells by a mechanism requiring the type III machinery. Previous work mapped a signal sufficient for the targeting of fused reporter proteins to amino acids 1–100 of YopE. Targeting requires the binding of SycE to YopE residues 15–100 in the bacterial cytoplasm. We asked whether SycE functions only to stabilize YopE in the bacterial cytoplasm, or whether the secretion chaperone itself contributes to substrate recognition by the type III machinery. Fusions of glutathione S-transferase to either the N or C terminus of SycE resulted in hybrid proteins that bound YopE but prevented targeting of the export substrate into HeLa cells. As compared with wild-type SycE, glutathione S-transferase-SycE bound and stabilized YopE in the bacterial cytoplasm but failed to release the polypeptide for export by the type III machinery. Thus, it appears that SycE functions to deliver YopE to the type III secretion machinery. A model is presented that accounts for substrate recognition of effector Yops, a group of proteins that do not share amino acid sequence or physical similarities.

Yersinia infect human and animal hosts to cause a variety of intestinal and septicemic diseases. Three pathogenic species, Y. enterocolitica, Y. pseudotuberculosis, and Y. pestis, share a tropism for lymphoid tissues but differ in their mode of host entry and the sites of the resulting pathological lesions (1, 2). To evade phagocytic killing by immune cells, Yersinia bind to the surface of macrophages and inject several proteins (effector Yops) into the eukaryotic cytosol via a mechanism requiring the type III secretion machinery (3–5). Genes specifying the type III machinery and Yop proteins are located on a 70-kilobase pair virulence plasmid (6). In the absence of calcium, Yersinia carrying this plasmid are triggered to secrete massive amounts of all Yops into the extracellular medium (7). Some 14 secreted Yops have been identified, and their specific role during animal infection is currently being investigated. Several different assays have been established that permit localization of Yops during infection of either HeLa cells or cultured macrophages (5, 8, 9). YopE, YopH, YopM, YopN, YopO (YpkA), YopP (YopJ), and YopT are injected into the eukaryotic cytosol (5, 10–14), whereas YopB, YopD, and YopR are found in the extracellular milieu (9, 15). Other type III secretion substrates such as YopQ (YopK) and LcrV remain associated with the bacteria during infection, and these proteins have been implicated in regulating the injection of effector Yops (16, 17). Yop proteins display neither amino acid sequence homology nor physical similarity (2).

Yersinia mutants lacking any one of the structural components of the type III machinery are defective for the export of all Yops both during tissue culture infection and when induced by low calcium (18–20). In contrast, Yersinia mutants that lack a small cytoplasmic protein, SycE, can not inject YopE into eukaryotic cells but retain the ability to secrete YopE into the medium of low calcium-induced cultures (9, 21). Mapping of the signals for YopE secretion revealed that this polypeptide can be exported in two ways (21). One pathway recognizes a signal located in YopE mRNA specifying the first 15 amino acids of the polypeptide (22–24). The second signal is provided by amino acid residues 15–100 and is absolutely dependent on the presence of SycE (9). The mRNA encoded signal of YopE does not appear to result in secretion under these conditions because polypeptides generated by fusions of this signal to a reporter are located in the bacterial cytoplasm (9).

The role of SycE in targeting YopE into the eukaryotic cytosol has not yet been established. Because YopE is not exported by the type III machinery in the absence of SycE, we wondered whether this chaperone contributes to substrate recognition. SycE itself remains in the bacterial cytoplasm and, after delivery of YopE to the type III machinery, must be released from the bound substrate (9, 25, 26). Thus, identification of SycE mutants that abolish targeting of YopE but do not interfere with polypeptide binding would provide evidence that the chaperone contributes to substrate recognition. Here we demonstrate the existence of such mutants and suggest a molecular explanation for their defect in substrate recognition.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—Y. enterocolitica strains W22703 (wild-type), LC2 (sycE1), and KUM1 (lerD1) have been described elsewhere (21). Wild-type SycE, GST-SycE, and SycE-GST were cloned on a low copy number vector and transformed by electroporation into

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The abbreviations used are: GST, glutathione S-transferase; GnHCl, guanidine hydrochloride; Ni-NTA, nickel nitrilotriacetic acid; NPT, neomycin phosphotransferase; PAGE, polyacrylamide gel electrophoresis; TSB, tryptic soy broth; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
Yersinia. Recombinant genes were assembled from polymerase chain reaction-amplified DNA fragments via abutted restriction sites and cloned between the PstI and BamHI sites of pHSG576 (27). All constructs used the SacE promoter and upstream untranslated sequences that were derived from primers Pst-SacE (5'-AACTATTTATTCCCTTGGCTAT-3') and SacE of pHSG576 (5'-AACATATGTTATCTAATTAGTTGATTATAATAT-3'). Coding sequences were immediately following the AUG start codon were amplified with primer pairs and cut with Ndel-KpnI. The primers used for these reactions were as follows: for GST, Nde-Nst-GST (5'-AACTATTCTCTCTATAGAATG- TATTGG-3') and Nst-Gst-Pro (5'-AACTATCTACACACCAACTG- CGACC-3'); for SacE, Nde-SacE-N (5'-AACTATGTACGCACAACCACCAATGGTGATGGTGATGGTGAC-3') and SacE-Nst (5'-AACTATCTTCTTCCATTCTGTT-3'); for YopE, Nde-YopE-N (5'-AACTATTTTCTTCCATTCTGTT-3') and SacE-Nst (5'-AACTATCTTCTTCCATTCTGTT-3'). Coding sequences at the 3' end were amplified with primers and cut with KpnI-BamHI. The primers used for these reactions were as follows: for SacE, Kpn-SacE-C (5'-AACTATTTTCTTCCATTCTGTTAACACACACCAATGGTGATGGTGATGGTGAC-3') and SacE-Bam (5'-AACTATTTTCTTCCATTCTGTTAACACACACCAATGGTGATGGTGATGGTGAC-3'); for GST, Kpn-Gst-C (5'-AACTATTTTCTTCCATTCTGTTAACACACACCAATGGTGATGGTGATGGTGAC-3') and GST-Tac (5'-AACTATTTTCTTCCATTCTGTTAACACACACCAATGGTGATGGTGATGGTGAC-3'). The polymerase chain reaction-amplified fragments described above were cloned between the Ndel and BamHI sites of pETDuet to generate plasmids pLC331 (tac SacE-GST), pLC332 (tac YopE2-GST), and pLC333 (tac YopE1-GST). Purified GST-SacE and GST-YopE proteins driven by the tac promoter were cloned on GST-SacE and GST-YopE expression vectors, respectively.

**RESULTS**

**Role of SacE in Type III Secretion of YopE**

SacE was overexpressed in E. coli by polymerase chain reaction amplifying wild-type yopE with abutted Ndel and BamHI sites using SacE-B (5'-AACTATTTTCTTCCATTCTGTTAATGGTGATGGTGATGGTGAC-3') and SacE-N (5'-AACTATTTTCTTCCATTCTGTTAATGGTGATGGTGATGGTGAC-3'). The DNA fragment was cut with Ndel-BamHI and cloned into pET9a to yield pHT1. One liter of E. coli BL21 (DE3), pHT1 was grown to mid-log phase and induced for 4 ml of 50% solution Ni-NTA resin and incubated at room temperature for 15 min, suspended in 1 ml of buffer (50 mM Tris-HCl, 20% sucrose, and 1 mM dithiothreitol, pH 7.5), and lysed by one passage through a French press at 14,000 p.s.i. Extracts were centrifuged twice at 35,000 g for 15 min, and proteins in 20 ml of supernatant were precipitated with 45% ammonium sulfate by incubation at 4 °C for 2 h and centrifugation at 33,000 x g for 15 min. The pellets were suspended in 50 mM Tris-HCl, pH 7.5, and subjected to chromatography on a Sephacryl S-200HR (Amersham Pharmacia Biotech) column. The purification of GST-SacE protein has been described elsewhere (21).

**Binding of YopE to GST-SacE Hybrid Proteins**—Overnight cultures of SacE LC2 (sacE1) carrying pGST-SacE, pGST-SacE2, or pGST-SacE3 grown in TSB supplemented with 20 μg/ml chloramphenicol were diluted into fresh media (10 ml of culture:500 ml of TSB). Bacteria were grown for 2 h at 26 °C and induced for 3 h at 37 °C. Cells were harvested and suspended in 10 ml of F buffer and broken by a single passage through a French pressure cylinder at 14,000 p.s.i. Unbroken cells, debris, and membranes were removed by centrifugation at 6,000 x g for 15 min. Supernatants were subjected to affinity chromatography on glutathione-Sepharose (Amersham Pharmacia Biotech) pre-equilibrated with F buffer. The column was washed with 30 column volumes of wash buffer (50 mM Tris-HCl, 150 mM NaCl, and 15% glycerol, pH 7.5), and proteins were eluted with 4 ml of the same buffer containing 10 mM glutathione. Proteins in the eluate were precipitated with 10% trichloroacetic acid using 50 μl of 24% deoxycholate/ml as a carrier. Precipitates were washed with acetone and air-dried, and proteins were suspended in 100 μl of sample buffer containing 3 μl urea.

**Cell Fractionations**—Overnight cultures of Yersinia were diluted 1:50 to 250 ml of fresh TSB media, grown for 2 h at 26 °C, and induced for 37 °C for 3 h. Cells were harvested and suspended in 10 ml of F buffer and broken by a single passage through a French pressure cylinder at 14,000 p.s.i. Unbroken cells, debris, and membranes were removed by centrifugation at 6,000 x g for 15 min. Supernatants were subjected to affinity chromatography on glutathione-Sepharose (Amersham Pharmacia Biotech) pre-equilibrated with F buffer. The column was washed with 30 column volumes of wash buffer (50 mM Tris-HCl, 150 mM NaCl, and 15% glycerol, pH 7.5), and proteins were eluted with 4 ml of the same buffer containing 10 mM glutathione. Proteins in the eluate were precipitated with 10% trichloroacetic acid using 50 μl of 24% deoxycholate/ml as a carrier. Precipitates were washed with acetone and air-dried, and proteins were suspended in 100 μl of sample buffer containing 3 μl urea.

**Pulse-Chase Analysis**—Overnight cultures of Yersinia were diluted into M9 minimal media lacking methionine and cysteine and grown for 2 h at 26 °C (21). Cultures were induced for 3 h at 37 °C, and 3 ml of culture was pulse-labeled with 300 μCi of Pro-Mix™ (1′′-Smethionine and cysteine) for 2 min. Labeling was quenched by the addition of 150 μl of 15% glycerol, pH 7.5), and bacteria were broken in a French pressure cell at 14,000 p.s.i., and intact cells were removed by centrifugation at 6,000 x g for 10 min. Two 3-ml aliquots of crude bacterial extract were centrifuged at 180,000 x g for 30 min. The supernatant (ua1) was separated from the membrane sediment, which was suspended in 3 ml of buffer (UE1). The sediment of the other aliquot was washed with 3 ml of 1 M KOAc and suspended in 180,000 x g for 30 min, and the supernatant (ua2) was separated from the sediment (uca2). At each step of the fractionation, 500-μl aliquots were withdrawn and precipitated with 50 μl of 10% trichloroacetic acid and washed with acetone. The precipitate was solubilized by adding 50 μl of buffer B and 50 μl of sample buffer before boiling. Samples were separated on 15% SDS-PAGE and immunoblotted with anti-YopE and anti-SacE, and the developed signals were quantified by densitometry scanning.

**K<sub>d</sub> Determination**—500 ng of purified, denatured YopE<sub>SacE</sub> in 10 μl of eluate (6 μg GluHCl, 0.1 μM NaH<sub>2</sub>PO<sub>4</sub>, and 0.1 μM Tris-HCl, pH 6.0) were diluted into 1 ml of buffer (50 mM Tris, pH 7.0, 100 mM KCl, 5 mM MgOAc, 0.25% Tween 20, and 50 μg/ml bovine serum albumin) containing 50% slurry Ni-NTA resin and incubated at room temperature for 20 min. The beads were washed three times and suspended at a concentration of 1 pmol of YopE<sub>SacE</sub>/μl of beads. Increasing amounts of YopE<sub>SacE</sub> (0–150 pmol) were added to a 200-μl suspension of YopE<sub>SacE</sub>-Ni-NTA-Sepharose (100 pmol of YopE<sub>SacE</sub>). Purified GST-SacE was first bound to glutathione-Sepharose (Amersham Pharmacia Biotech) at a concentration of 1 pmol of protein/μl of beads. Increasing concentrations of YopE<sub>SacE</sub> (0–120 pmol) were then added to a 200-μl suspension of GST-SacE-glutathione-Sepharose (100 pmol of GST-SacE). Samples were incubated for 20 min and centrifuged at 15,000 x g for 5 min. An aliquot of the supernatant was removed, mixed with sample buffer, separated on 15% SDS-PAGE, and immunoblotted with anti-SacE and anti-YopE (1:2000 dilution). Immune complexes were detected with 12 μCi of 125I-labeled protein A/Abot, and signals were quantified by PhosphorImager. Each SDS-PAGE blot was calibrated with a dilution series of known concentrations of YopE<sub>SacE</sub> or SacE.

**RESULTS**

**GST-SacE and SacE-GST Fail to Complement the YopE Targeting Defect of sacE<sup>−</sup> Yersinia.** To identify SacE sequence
Role of SycE in Type III Secretion of YopE

Elements required for YopE binding and/or targeting, we constructed both N- and C-terminal fusions of GST with SycE and asked whether the hybrid proteins were functional in delivering polypeptide to the type III machinery. This was tested by infecting HeLa cells with either wild-type Y. enterocolitica W22703 or strain LC2 (sycE1) carrying either no plasmid, pSycE, pGST-SycE, or pSycE-GST. After incubation for 3 h at 37 °C, the tissue culture medium (M) was decanted and centrifuged to separate secreted proteins from those present within nonadherent bacteria. HeLa cells as well as adherent Yersinia were extracted with digitonin (D), a detergent that solubilizes the eukaryotic plasma membrane but not the bacterial envelope. Extracts were centrifuged to separate proteins solubilized from the HeLa cytoplasm from those that sediment with the bacteria. Proteins in each fraction were precipitated with chloroform/methanol and analyzed by SDS-PAGE and immunoblotting with antibodies directed against YopE, SycE, or YopH. Targeting was measured as the percentage amount of protein solubilized by digitonin extraction divided by the total amount of protein. As a control for solubilization of all membranes, HeLa cells and adherent bacteria were extracted with SDS (S). P, pellet.

When infected with wild-type Yersinia strain W22703, YopE and YopH were located in the supernatant of digitonin extracts, indicating that 35% (YopE) and 48% (YopH) of these proteins had been injected into the cytosol of HeLa cells (Fig. 1). SycE remained inside of bacterial cells and was found only in the sediment of digitonin extracts. The sycE- strain LC2 infected 54% of YopH into the eukaryotic cytosol. However, YopE was found only in the bacterial sediment of digitonin extracts, indicating that this polypeptide had not been targeted into HeLa cells. Complementation of the sycE- mutation with plasmid-encoded sycE restored YopE targeting (29%). Neither plasmid-encoded gdst-sycE nor sycE-gst complemented the YopE targeting defect of strain LC2. The targeting defect of strains carrying the gdst-sycE and sycE-gst alleles was limited to YopE because the mutant Yersinia injected YopH in a manner similar to wild-type bacteria.

GST-SycE and SycE-GST Bind YopE and Do Not Affect Low Calcium-induced Secretion of YopE—One explanation for the targeting defect of GST-SycE and SycE-GST could be that the hybrids do not bind YopE and thus cannot deliver substrate to the type III machinery. To examine this possibility, we asked whether YopE could co-purify with GST-SycE. Bacterial extract supernatants representing soluble cytoplasmic contents of Yersinia were subjected to affinity chromatography on glutathione-Sepharose. Eluted samples were analyzed by SDS-PAGE and immunoblotting for the presence or absence of GST-SycE (lane 1), SycE-GST (lane 2), GST-SycE, (lane 3), and GST-SycEGST (lane 4) as well as bound YopE. B. Y. enterocolitica LC2 (sycE1) carrying either no plasmid, pSycE, pGST-SycE, or pSycE-GST was grown in the presence or absence of calcium at 37 °C for 3 h. Cultures were centrifuged to separate proteins secreted into the culture medium (S) from those sedimenting with the bacteria (P). Samples were precipitated with trichloroacetic acid and analyzed by SDS-PAGE and immunoblotting for expression and secretion of YopE, YopM, and SycE. Secretion is indicated as the percentage amount of polypeptide present in the culture medium divided by the total amount of polypeptide.

![Fig. 1. Type III targeting of YopE polypeptide into the cytoplasm of HeLa cells.](Image 77x535 to 269x729)

![Fig. 2. Binding of GST-SycE and SycE-GST to YopE.](Image 341x575 to 521x729)

![Fig. 2A. Binding of GST-SycE and SycE-GST to YopE.](Image 341x575 to 521x729)

![Fig. 2B. Binding of GST-SycE and SycE-GST to YopE.](Image 341x575 to 521x729)
subjected to gel filtration chromatography on Sephacryl S-200HR. Dstained with Coomassie Brilliant Blue. The majority of SycE eluted in collected fractions were separated on SDS-PAGE as indicated and indicated in thousands.

The binding of wild-type SycE was determined as the amount that sedimented with YopE6His: Ni-NTA. The collected data are shown as a Scatchard plot. SycE bound to YopE6His with a $K_d$ of $3.3 \times 10^{-10}$ M. Each YopE6His molecule appears to bind two SycE polypeptides.

Fig. 3. Purification of YopE6His and SycE. A, YopE6His carrying a C-terminal six histidine tag was expressed under the control of T7 polymerase in E. coli BL21 (DE3), pLysS. Cells were lysed in 6 M GnHCl and subjected to affinity purification on Ni-NTA-Sepharose. The figure shows the Coomassie Brilliant Blue-stained SDS-PAGE of YopE6His, eluted with 6 M GnHCl at pH 4.0 (lanes 1–5). B, SycE was expressed under the control of T7 polymerase in E. coli BL21 (DE3). Cells were lysed in a French pressure cell (φ), and SycE was precipitated with 45% ammonium sulfate and sedimented by centrifugation (P). No SycE was found in the supernatant of the centrifugation step at 32,000 × g for 15 min (S). C, SycE was suspended in 50 mM Tris-HCl, pH 7.5 (P), and subjected to gel filtration chromatography on Sephacryl S-200HR. D, collected fractions were separated on SDS-PAGE as indicated and stained with Coomassie Brilliant Blue. The majority of SycE eluted in fraction 37–41. Left, the molecular weight markers (lane MW) are indicated in thousands.

The YopE mRNA signal.

Binding of SycE and GST-SycE to YopE—We used purified protein components to measure the dissociation constants of wild-type SycE and GST-SycE for YopE. YopE carrying a C-terminal six histidine tag was expressed in E. coli under the control of T7 polymerase in E. coli BL21 (DE3), pLysS. Cells were lysed in 6 M GnHCl and subjected to affinity purification on Ni-NTA-Sepharose. The figure shows the Coomassie Brilliant Blue-stained SDS-PAGE of YopE6His, eluted with 6 M GnHCl at pH 4.0 (lanes 1–5). B, SycE was expressed under the control of T7 polymerase in E. coli BL21 (DE3). Cells were lysed in a French pressure cell (φ), and SycE was precipitated with 45% ammonium sulfate and sedimented by centrifugation (P). No SycE was found in the supernatant of the centrifugation step at 32,000 × g for 15 min (S). C, SycE was suspended in 50 mM Tris-HCl, pH 7.5 (P), and subjected to gel filtration chromatography on Sephacryl S-200HR. D, collected fractions were separated on SDS-PAGE as indicated and stained with Coomassie Brilliant Blue. The majority of SycE eluted in fraction 37–41. Left, the molecular weight markers (lane MW) are indicated in thousands.

We wished to test whether SycE-GST could also stabilize YopE. Previous work showed that YopE is rapidly degraded in the absence of SycE and that GST-SycE can complement this defect of sycE mutant Y. pseudotuberculosis (32). We wished to test whether SycE-GST could also stabilize YopE polypeptide. Yersiniae were pulse-labeled with $[^{35}S]$methionine, and culture aliquots were analyzed at timed intervals by

![Figure 3](image-url)

**Fig. 3.** Purification of YopE6His and SycE. A, YopE6His carrying a C-terminal six histidine tag was expressed under the control of T7 polymerase in E. coli BL21 (DE3), pLysS. Cells were lysed in 6 M GnHCl and subjected to affinity purification on Ni-NTA-Sepharose. The figure shows the Coomassie Brilliant Blue-stained SDS-PAGE of YopE6His, eluted with 6 M GnHCl at pH 4.0 (lanes 1–5). B, SycE was expressed under the control of T7 polymerase in E. coli BL21 (DE3). Cells were lysed in a French pressure cell (φ), and SycE was precipitated with 45% ammonium sulfate and sedimented by centrifugation (P). No SycE was found in the supernatant of the centrifugation step at 32,000 × g for 15 min (S). C, SycE was suspended in 50 mM Tris-HCl, pH 7.5 (P), and subjected to gel filtration chromatography on Sephacryl S-200HR. D, collected fractions were separated on SDS-PAGE as indicated and stained with Coomassie Brilliant Blue. The majority of SycE eluted in fraction 37–41. Left, the molecular weight markers (lane MW) are indicated in thousands.

![Figure 4](image-url)

**Fig. 4.** Affinity of SycE and GST-SycE for YopE secretion substrate. A, purified SycE was incubated with purified YopE6His bound to Ni-NTA. YopE6His:SycE and Ni-NTA complexes were sedimented by centrifugation, and the binding of wild-type SycE was determined as the amount that sedimented with YopE6His:Ni-NTA. The collected data are shown as a Scatchard plot. SycE bound to YopE6His with a $K_d$ of $3.3 \times 10^{-10}$ M. Each YopE6His molecule appears to bind two SycE polypeptides. B, for the binding of GST-SycE to YopE6His, purified polypeptide was added to GST-SycE bound to glutathione-Sepharose, and the bound complexes were sedimented by centrifugation. GST-SycE bound to YopE6His with a $K_d$ of $3.6 \times 10^{-10}$ M. In contrast to wild-type SycE, about four molecules of GST-SycE appeared to be bound to each YopE6His polypeptide.
Role of SycE in Type III Secretion of YopE

To measure the secretion of YopE bound to SycE or GST-SycE, we incubated pulse-labeled Yersinia for 10 min to allow type III secretion of polypeptide via the mRNA pathway. The bacteria were collected by centrifugation, suspended in fresh medium, and incubated over a period of 20 min. Post-translational export of YopE was measured on a PhosphorImager after SDS-PAGE separation of pulse-labeled YopE that had been immunoprecipitated from culture supernatants. Wild-type Yersinia secreted 18% of all pulse-labeled YopE, whereas sycE mutants did not secrete YopE. This secretion defect could not be rescued by expressing GST-SycE in sycE mutants, indicating that although the mutant chaperone binds and stabilizes the polypeptide, GST-SycE cannot release YopE for secretion by the type III secretory pathway.

YopE Requires SycE or GST-SycE for Solubility within the Cytoplasm of Yersinia—We wished to determine the fate of SycE-YopE complexes in bacterial cells and fractionated Yersinia grown under low calcium conditions (Fig. 6). Bacteria were collected by centrifugation and lysed in a French pressure cell. Extracts were ultracentrifuged to sediment membranes, whereas soluble cytoplasmic components remained in the supernatant. In wild-type Yersinia, 95% of all YopE was soluble in the bacterial cytoplasm. The subcellular distribution of YopE changed dramatically when analyzed for sycE - cells: all YopE (100%) sedimented with the membranes, suggesting that these polypeptides were insoluble in the bacterial cytoplasm. Expression of GST-SycE and SycE-GST in sycE - cells restored YopE solubility to 75% and 47%, respectively. Thus, the binding of hybrid GST-SycE and SycE-GST to YopE in the Yersinia cytoplasm solubilized the polypeptide.

Membrane fractions were extracted with 1 M KOAc to release proteins peripherally associated with the lipid bilayer (Fig. 6). In wild-type Yersinia, most YopE species that sedimented with the membranes could not be extracted with 1 M KOAc (80%). Membrane-bound YopE species might represent polypeptides in the process of translocation by the type III machinery in a manner that cannot be extracted with salt. If so, mutants of the type III pathway that cannot export Yop proteins should not contain YopE species that are resistant to salt...
Role of SycE in Type III Secretion of YopE

FIG. 7. Expression of GST-SycE interferes with the SycE-dependent type III secretion of YopE. Plasmid pDA141 encodes YopE, a fusion protein between full-length YopE and NPT. The first 15 codons (45 nucleotides) of YopE carry a +1 frameshift mutation (insertion of A immediately after the AUG start codon) of the mRNA signal that is suppressed at codon 15 via the removal of a nucleotide. The defective mRNA signal is tethered to the nucleotide sequence (codons 16–220) encoding the SycE binding site of YopE. The sycE allele is located on a p15A replicon plasmid and expressed from the IPTG-inducible tac promoter. Bacteria were grown at 37 °C in TSB supplemented with 0.01 mM EDTA and 1 mM IPTG. Cells were harvested by centrifugation (P) and separated from the culture supernatant (S). Proteins in both fractions were precipitated with trichloroacetic acid (TCA) by centrifugation (P) and separated from the culture supernatant (S). Proteins in both fractions were precipitated with TCA by centrifugation (P) and separated from the culture supernatant (S).

FIG. 8. Expression of GST-SycE interferes with the SycE-dependent type III targeting of YopE into HeLa cells. Wild-type Y. enterocolitica W22703 expressing GST-SycE from the IPTG-inducible tac promoter was used to infect HeLa cells in the presence or absence of IPTG. Type III targeting was measured as described in the legend to Fig. 1 and detected by the immunoblotting of collected samples. Overexpression of GST-SycE interfered with the SycE-dependent type III targeting of YopE. In contrast, GST-SycE expression did not reduce the injection of YopH into the cytosol of eukaryotic cells. Results similar to those for YopH were obtained when samples were immunoblotted for YopM (data not shown).

DISCUSSION

To investigate the targeting pathway of YopE, we examined the role of SycE in binding to YopE polypeptide. Initial experiments aimed at the purification of YopE species that had been secreted into the extracellular medium of low calcium-induced Yersinia cultures (data not shown). We were surprised to find that most, if not all, extracellular YopE was insoluble after extraction. This was tested, and Yersinia carrying a null allele of lcrD (strain KUM1) (21), a cytoplasmic membrane protein absolutely required for all type III export (33), were fractionated as described above. Almost all YopE (94%) was soluble after French press lysis. Of the 6% of polypeptide that sedimented with the membranes, 83% could be extracted with 1 M KOAc. Thus, in the lcrD1 strain KUM1, 99% of YopE is soluble, suggesting that the salt-resistant, membrane-associated YopE species represent an export intermediate of the type III pathway. Export intermediates were observed in LC2 cells (sycE) (38% of all YopE) because the mRNA signal initiates YopE into the export pathway, even in the absence of SycE. Similarly, GST-SycE- and SycE-GST-expressing Yersinia also harbored significant amounts of membrane-associated, salt-resistant YopE species. GST-SycE Competes with SycE for YopE Secretion Substrate—If GST-SycE cannot release polypeptide for membrane translocation, GST-SycE should compete with SycE binding to YopE, thereby preventing type III secretion in wild-type Yersinia (Fig. 7). To test this prediction, we used plasmid pDA141 encoding a yopE-npt translational fusion with a frameshift mutation of codons 2–15 that abolishes the function of the secretion signal located in yopE mRNA (YopE1-NPT). When expressed in wild-type cells, YopE1-NPT protein was secreted into the extracellular milieu (Fig. 7A). In contrast, yscE1 strain LC2 failed to export this polypeptide, indicating that secretion of the fusion protein is absolutely dependent on binding to SycE polypeptide. Expression of GST-SycE did not complement the secretion defect of the sycE1 strain for YopE1-NPT. When overexpressed in wild-type Yersinia, GST-SycE interfered with the secretion of YopE1-NPT, suggesting that GST-SycE competes with SycE for secretion substrate (Fig. 7A). If so, increased expression of GST-SycE via the IPTG-inducible tac promoter should lead to an increased ratio of GST-SycE/SycE concentration and to a decreased secretion of YopE1-NPT by wild-type Yersinia. This was tested, and Yersinia grown in the absence of IPTG secreted 47% YopE1-NPT, whereas the addition of 0.1, 0.5, and 1 mM IPTG to the growth medium caused an increase in the ratio of GST-SycE/SycE and a stepwise reduction in the percentage amount of secreted fusion protein (Fig. 7B). As a control for the function of the yop mRNA signaling pathway, both wild-type and yscE1 mutant Yersinia exported wild-type YopE. Thus, when bound to GST-SycE, YopE is irreversibly retained from the type III secretory pathway. If GST-SycE expression interferes with the SycE-dependent export of YopE1-NPT in low calcium-induced cultures, it should also interfere with the targeting of YopE into the eukaryotic cytosol during Yersinia infection of HeLa cells. To examine this possibility, HeLa cells were infected with wild-type Yersinia expressing GST-SycE from the IPTG-inducible tac promoter in either the absence or presence of increasing concentrations of IPTG (Fig. 8). Increased expression of GST-SycE led to decreased targeting of YopE in the presence of wild-type SycE, indicating that GST-SycE competed with SycE for binding to YopE. Overexpression of GST-SycE did not affect the targeting of YopH into HeLa cells. Because the targeting of YopH is thought to be dependent on its binding to the SycH chaperone, this observation suggests that GST-SycE2:YopE complexes do not compete with YopH:SycH for recognition by the type III machinery.
centrifugation at 100,000 × g. This observation is consistent with the notion that some secreted Yops aggregate in the extracellular milieu to form macroscopically visible precipitates (7). Thus, it appears that YopE cannot fold properly unless the polypeptide is in the appropriate environment, i.e. the eukaryotic cytoplasm. To examine this prediction, we report here the purification of denatured YopE which, when diluted into aqueous buffer, did not acquire solubility even after prolonged incubation. Binding of purified SycE to denatured YopE resulted in solubility, even when the complex was diluted in the absence of detergent, suggesting that SycE binding is required for intrabacterial solubility. This hypothesis was tested in vivo; indeed, soluble YopE could not be detected in the cytoplasm of Yersiniae lacking the SycE chaperone. SycE remains in the bacterial cytoplasm, and once YopE is injected into eukaryotic cells, this polypeptide likely depends on host cell factors for folding and function.

SycE binding to YopE may not only provide solubility in the bacterial cytoplasm but may also allow recognition of the SycE2:YopE complex by the type III machinie. We sought to separate polypeptide binding and type III delivery functions of the SycE chaperone by mutational analysis. Hybrid GST-SycE and SycE-GST fusion proteins were designed with the rationale to facilitate binding measurements with YopE. We were surprised to find that fusion of the GST domain to either the N or C terminus of SycE abolished type III targeting of YopE without affecting binding to the polypeptide. These results suggest that SycE does interact with the type III machinery during delivery of YopE and that fusion of the bulky GST domain may prevent this interaction. An alternative explanation is that type III machines recognize Yops while they are bound to Syc chaperones. However, this appears somewhat unlikely, because Yop proteins do not display sequence or physical similarity (2).

If type III machines recognize export substrate as a property of the SycE2:YopE complex, is there an element that is shared to SecA than for SecB alone. ATP binding of SecA is thought to promote both translocation of the released precursor substrate and displacement of SecB, presumably via conformational changes of SecA that alter the SecB binding site (44). Released SecB is then once again available to bind another polypeptide precursor. Perhaps delivery of YopE to the type III machinery occurs by a similar cycle in which the SycE-YopE complex is dissociated by a component of this machine, thereby initiating polypeptide substrate into the targeting pathway and releasing SycE chaperone.

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