Hierarchical Delivery of an Essential Host Colonization Factor in Enteropathogenic Escherichia coli*

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Many significant bacterial pathogens use a type III secretion system to inject effector proteins into host cells to disrupt specific cellular functions, enabling disease progression. The injection of these effectors into host cells is often dependent on dedicated chaperones within the bacterial cell. In this report, we demonstrate that the enteropathogenic Escherichia coli (EPEC) chaperone CesT interacts with a variety of known and putative type III effector proteins. Using pull-down and secretion assays, a degenerate CesT binding domain was identified within multiple type III effectors. Domain exchange experiments between selected type III effector proteins revealed a modular nature for these CesT binding domains, as demonstrated by secretion, chaperone binding, and infection assays. The CesT-interacting type III effector Tir, which is crucial for in vivo intestinal colonization, had to be expressed and secreted for efficient secretion of other type III effectors. In contrast, the absence of other CesT-interacting type III effectors did not abrogate effector secretion, indicating an unexpected hierarchy with respect to Tir for type III effector delivery. Coordinating the expression of type III effectors with CesT in the absence of tir partially restored total type III effector secretion, thereby implicating CesT in secretion events. Collectively, the results suggest a coordinated mechanism involving both Tir and CesT for type III effector injection into host cells.

Bacterial pathogens often have multiple systems to disrupt and subvert host cellular processes that are involved in disease production. One such system is the type III secretion complex (T3SS) that is widespread among Gram-negative pathogens of animals and plants. T3SSs are composed of multipartite complexes in the bacterial membrane that mediate the rapid injection of type III effector proteins directly into host cells (1).

Enteropathogenic Escherichia coli (EPEC) is a human diarrheal attaching and effacing (A/E) pathogen that attaches to intestinal microvilli and then injects type III effector proteins directly into host cells (2, 3). After bacterial attachment, dramatic cytoskeletal rearrangements result in the effacement of microvilli (4). The A/E phenotype has been linked to the locus of enterocyte effacement (LEE) (5), a pathogenicity island that is involved in EPEC virulence (6). The LEE encodes components of the T3SS, transcriptional regulators, chaperones and type III effector proteins, the latter of which are translocated directly into host cells (7).

EPEC and other related strains have multiple type III effectors that are encoded within the LEE, in addition to non-LEE-encoded effectors that are located in distinct pathogenicity islands throughout the chromosome (3, 8, 9). One of the best studied EPEC type III effectors is Tir (translocated intimin receptor), a protein that is injected into host cells, modified by host kinases, and localized to the host membrane (10, 11). Furthermore, the amino- and carboxyl-terminal regions of Tir interact with a number of host proteins, causing dramatic host cytoskeletal rearrangements, which result in actin-rich lesions termed pedestals (12–16). Remarkably, host membrane-localized Tir also serves as a receptor for EPEC via intimin (10), a bacterial outer membrane adhesin also encoded by the LEE of A/E pathogens. The direct importance of Tir in the virulence of A/E pathogens has been convincingly demonstrated in three in vivo animal models of infection, where tir mutants do not colonize the host intestine or cause clinical symptoms of disease (7, 17, 18). Other well studied LEE encoded type III effectors include Map, EspF, and EspG (19–22), which have been shown to have multifunctional disruptive properties within host cells.

The translocation of type III effectors into host cells is often dependent on a dedicated family of proteins termed type III chaperones (NCBI conserved domain data base, pfam05932). These proteins are cytosolic or membrane-associated, generally small (~20 kDa), soluble, and negatively charged (pl 4–5) (23). Type III secretion chaperones typically form homodimers that bind to the amino-terminal region of effectors and remain in the bacterial cell following translocation of effectors into the host cell. The crystal structures of many type III secretion chaperones have been solved, some in a complex with an effector (24–29). Collectively, these studies have revealed remarkable chaperone structural similarity, even in the absence of primary sequence sim-

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¶ The abbreviations used are: T3SS, type III secretion system; EPEC, enteropathogenic E. coli; A/E, attaching and effacing; LEE, locus of enterocyte effacement; CBD, CesT binding domain; aa, amino acids; GST, glutathione S-transferase.

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TABLE 1

Strains and plasmids used in this study

| Strains                  | Description                                                                 | Reference or source |
|-------------------------|-----------------------------------------------------------------------------|---------------------|
| Wild type EPEC          | EPEC strain E2348/69, streptomycin-resistant                                 | Ref. 36             |
| Δtir                    | tir deletion mutant                                                         | Ref. 10             |
| ΔsepD                   | Nonpolar deletion of sepD                                                   | Ref. 38             |
| ΔsepDΔtir               | Double mutant, derived from ΔsepD                                            | This study          |
| ΔsepDΔnleA              | Double mutant, derived from ΔsepD                                            | This study          |
| ΔsepDΔepsF              | Double mutant, derived from ΔsepD                                            | This study          |
| ΔsepDΔescN              | Double mutant, derived from ΔescN                                            | This study          |
| ΔsepDΔcesT              | Double mutant, derived from ΔsepD                                            | Ref. 33             |
| BL21(ADE3)              | E. coli host for overexpression of recombinant proteins                      | Novagen             |
| DH5α                    | General E. coli strain for cloning purposes                                  | Life Technologies   |

| Plasmids                | Description                                                                 | Reference or source |
|-------------------------|-----------------------------------------------------------------------------|---------------------|
| pHisCesT                | pET28 expressing His-CesT                                                   | Ref. 33             |
| pGST-CesT               | Encodes GST-CesT fusion in pGEX                                              | Ref. 30             |
| pTir(ΔCBD)              | Encodes Tir missing its CBD                                                  | Ref. 39             |
| pSepD                   | pACYC184 encoding EPEC SepD                                                 | Ref. 38             |
| pTir                    | pACYC184 encoding EPEC Tir                                                  | Ref. 30             |
| pTir(Δ2–38)             | pACYC184 encoding EPEC Tir (Δ2–38)                                          | This study          |
| pNT242                  | pACYC184 encoding CesT-FLAG from a cloned tir promoter                      | Ref. 33             |
| pNT244                  | nleH and upstream promoter region cloned as a blunt KpnI fragment, encodes NleH-FLAG fusion | This study          |
|                         | from its native promoter in pACYC184                                        |                     |
| pNT245                  | Encodes NleH (aa 1–30) fused to FLAG, transcribed from the nleH promoter in pACYC184 | This study          |
| pNT246                  | Encodes NleH (aa 1–100) fused to FLAG, transcribed from the nleH promoter in pACYC184 | This study          |
| pNT247                  | Encodes NleH (aa 1–150) fused to FLAG, transcribed from the nleH promoter in pACYC184 | This study          |
| pNT248                  | Encodes NleH (aa 1–200) fused to FLAG, transcribed from the nleH promoter in pACYC184 | This study          |
| pNT249                  | Encodes NleH (aa 1–250) fused to FLAG, transcribed from the nleH promoter in pACYC184 | This study          |
| pNT250                  | Encodes NleH(Δ2–50) aa) fused to FLAG, transcribed from the nleH promoter in pACYC184 | This study          |
| pNT251                  | Encodes Tir variant such that the Tir(CBD) is replaced with the CBD from NleH in pACYC184 | This study          |
| pNT252                  | Encodes NleH variant such that the NleH CBD is replaced with the CBD from Tir in pACYC184 | This study          |
| pNT253                  | Encodes NleH-FLAG from the tir promoter in pACYC184                        | This study          |
| pNT254                  | Encodes NleH-FLAG from the tac promoter in pACYC184                        | This study          |
| pNT255                  | Encodes NleA-FLAG from the tac promoter in pFLAG-CTC                       | This study          |
| pNT256                  | ΔnleA deletion construct in suicide vector pRE112                           | This study          |

**Experimental Procedures**

**Bacterial Strains and Growth Conditions**—EPEC 2348/69 (36) and relevant mutant strains were used for all experiments unless otherwise indicated (Table 1). Cultures were routinely grown in Luria broth (10 g of Bacto-peptone, 5 g of yeast extract, 10 g of NaCl) and then subcultured into Dulbecco’s modified Eagle’s medium (HyClone). *E. coli* DH5α served as a cloning strain and *E. coli* BL21(ADE3) was used as an overexpression strain for selected proteins.

**Recombinant DNA Techniques and Mutant Strain Construction**—Full-length nleH was amplified from genomic DNA in a PCR using primers 162 (GGAAATCCATATGT-TATCGGCCTCTTCTATAATTTGG) and 169 (CGGG-TACCATCTTACTTATATACAC). The PCR fragment was restriction-digested with Ndel and KpnI and cloned into the respective restriction sites of pFLAG-CTC (Sigma) to create an in-frame fusion with a sequence encoding the FLAG peptide. The resulting construct (pNT243) encodes NleH-FLAG from the recombinant tac promoter of pFLAG-CTC.

To create a plasmid for nleH expression from its native promoter, primers 163 (TGGAAATCCATTGATTTGGCTGG) and 169 were used in a PCR with genomic DNA. The resulting PCR fragment encompassed 419 bp upstream of the nleH start codon and the complete nleH open reading frame. The fragment was restriction-digested with KpnI followed by directional cloning into NruI/KpnI-treated pNT242 to create pNT244, which expresses NleH-FLAG from the nleH promoter. Truncated forms of NleH-FLAG were created by using pNT244 as template in a PCR with primer 163 individually...

Ilarity. Certain type III secretion chaperones are thought to bind a single effector, whereas others have been demonstrated to have multivalent properties and bind more than one effector.

In the case of EPEC, the LEE encoded type III secretion chaperone CesT was initially shown to bind and stabilize Tir within the bacterial cell (30, 31); however, additional studies have demonstrated interactions with the effectors Map, EspF, and the bacterial cell (30, 31); however, additional studies have demonstrated interactions with the effectors Map, EspF, and the bacterial cell. CesT is also required for the efficient in vitro type III secretion of other LEE and non-LEE type III effectors (33), suggesting that other chaperone-effector interactions may occur within the bacterium. Furthermore, we have previously demonstrated that CesT interacts with the membrane-associated ATPase EscN of the T3SS, in a role probably serving to recruit and target type III effectors for translocation into host cells (33, 35). Interestingly, cesT mutants do not cause overt disease in a mouse model of attaching and effacing pathogenesis (7), indicating that this type III secretion chaperone has a central role in disease.

In this study, we demonstrate that CesT interacts with at least eight EPEC type III effectors. A degenerate CesT binding domain within multiple type III effectors was identified and experimentally confirmed by pull-down and protein domain-exchanging experiments. A variety of complementary genetic and biochemical assays were used to demonstrate a modular nature for the CesT binding domain. Last, using multiple EPEC strains in secretion and infection assays, it is demonstrated that a coordinated Tir–CesT interaction is required for the efficient injection of other type III effectors into host cells. The results highlight events leading to the hierarchical injection of the critical host colonization factor Tir, which probably precedes the delivery of subsequent effectors.
Hierarchical Type III Effector Delivery into Host Cells

A plasmid construct expressing NleH-FLAG from the tir promoter was created by amplifying a DNA fragment with primers 175 (CGGTCGAGGTCTGTAGAAATAATGAGG) and 169 in a PCR with pNT244 serving as template, followed by cloning of the product into XhoI/KpnI-digested pNT242, to create pNT253. The same primers were used in a PCR with pNT250 as template to create pNT254 expressing NleH[ΔCBD]-FLAG from the tir promoter. A plasmid construct expressing NleA-FLAG from a tac promoter was generated by amplifying a DNA fragment from EPEC genomic DNA with primers 157 (GGATTCCCATTAGACATTACACCGATGTAAC) and 158 (CGGGTACCGACTCTTGTT-TCTTGAGATTATAC) followed by cloning into Ndel/KpnI-digested pFLAG-CTC to create pNT255.

To create a plasmid expressing Tir (Δ2–38), an inverse PCR strategy was used. Briefly, primer 186 (CACATTATATCTTTTATTTTAGAAAATTGTACAGC) and primer 191 (AGCCTTGACCCGGATCTCGCG) were used in a PCR with pTir as template DNA. The resulting product was treated with T4 polynucleotide kinase, followed by ligation and transformation into DH5α, creating pTir(Δ2–38). The plasmid was then transformed into Δtir for phenotypic analyses.

A nonpolar in frame nleA deletion mutant was generated by allelic exchange with an internal in-frame deletion. Briefly, primers 159 (CGGAGTGTACATTTCATCGCGG) and 160 (CCGCTAGCGGAGGAGG) were used in a PCR with pNT250 as template to create pNT254 expressing Tir[ΔCBD]-FLAG from the tir promoter. A plasmid construct expressing NleH-FLAG from the tir promoter was created by amplifying a DNA fragment with primers 175 (CGGTCGAGGTCTGTAGAAATAATGAGG) and 169 in a PCR with pNT244 serving as template, followed by cloning of the product into XhoI/KpnI-digested pNT242, to create pNT253. The same primers were used in a PCR with pNT250 as template to create pNT254 expressing NleH[ΔCBD]-FLAG from the tir promoter. A plasmid construct expressing NleA-FLAG from a tac promoter was generated by amplifying a DNA fragment from EPEC genomic DNA with primers 157 (GGATTCCCATTAGACATTACACCGATGTAAC) and 158 (CGGGTACCGACTCTTGTT-TCTTGAGATTATAC) followed by cloning into Ndel/KpnI-digested pFLAG-CTC to create pNT255.

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ice-cold trichloroacetic acid (final concentration 10% (v/v)) and incubated on ice for 30 min. The precipitated proteins were pelleted by centrifugation at 16,000 × g and then washed with ice-cold acetone. Precipitated proteins were resuspended in 2× ESB (0.0625 M Tris- HCl (pH 6.8), 1% (w/v) SDS, 10% glycerol, 2% 2-mercaptoethanol, 0.001% bromphenol blue) and subjected to SDS-PAGE and Western blotting analyses.

CesT Affinity Column Binding and FLAG Immunoprecipitation Pull-down Assays—A CesT affinity column was generated by purifying overexpressed His-CesT from an E. coli BL21 (ADE3) lysate using Ni²⁺-nitrilotriacetic acid affinity chromatography (33). Purified CesT (200 μg) was equilibrated with binding buffer (20 mM Tris (pH 7.9), 0.5 M NaCl), followed by the addition of various filtered EPEC culture supernatants. The column was extensively washed with binding buffer, followed by a higher stringency wash buffer (20 mM Tris (pH 7.9), 0.5 M NaCl, 60 mM imidazole). Retained proteins were co-eluted with His-CesT using elution buffer (20 mM Tris (pH 7.9), 0.5 M NaCl, 1 M imidazole). For mass spectrometry identification of CesT-interacting proteins, the procedure was modified to specifically exclude CesT from the elution fraction. Briefly, after CesT-interacting EPEC proteins were column-bound, the column was treated with denaturing binding buffer (20 mM Tris (pH 7.9), 0.5 M NaCl, 6 M urea). This served to denature and elute proteins from the column. The eluate was then passed over a separate Ni²⁺-nitrilotriacetic acid column, pre-equilibrated with denaturing binding buffer, to specifically retain denatured His-CesT. The eluate from this column was then trichloroacetic acid-precipitated, followed by mass spectrometry analyses (see below).

To evaluate a direct interaction between NleH and CesT, FLAG-tagged NleH expressed in DH5α was immunoprecipitated from a cell lysate (5-ml culture, A₆₀₀ = 0.8) onto FLAG beads as previously described (33). Purified GST-CesT or GST only was added to the NleH-containing beads, followed by incubation at 4 °C. The beads were then washed three times with phosphate-buffered saline containing protease inhibitors (Roche Applied Science complete mini-EDTA, one tablet per 10 ml of phosphate-buffered saline). NleH-FLAG was competitively eluted from the beads using FLAG peptide (final concentration of 90 ng/μl). The eluted protein fraction was then subjected to SDS-PAGE and immunoblotting analyses. An additional control for GST-CesT demonstrated that it did not bind to FLAG-beads exposed to a DH5α lysate, ruling out CesT interacting with the beads or any nonspecific bead-interacting proteins from DH5α.

In Vitro Cell Culture EPEC Infection Assays—for immunofluorescence microscopy experiments, HeLa cells were infected with overnight LB-cultured EPEC strains at a multiplicity of infection of 100. All infections were carried out at 37 °C, 5% CO₂ for 3 h in Dulbecco’s modified Eagle’s medium containing fetal bovine serum unless otherwise indicated. Infected HeLa monolayers were washed with phosphate-buffered saline, followed by fixation with paraformaldehyde (2.5%, v/v). The samples were processed for immunofluorescence microscopy as previously described (37). Monoclonal antibodies were used to stain fixed samples: anti-FLAG (1:2000) (Sigma), anti-Tir (1:500), and anti-PY (1:500) clone 4G10 (Upstate Biotechnology, Inc.), followed by extensive washing and staining with secondary antibodies (Alexa 488- or 568-conjugated anti-mouse (Molecular Probes), 1:400). For visualization of polymerized actin, Alexa 488-conjugated phalloidin (Molecular Probes) was included with the secondary antibodies at a 1:100 dilution. Images were detected using a Zeiss Axioskop microscope, captured with an Empix DVC1300 digital camera, and analyzed using Northern Eclipse imaging software.

Mass Spectrometry Analyses—20 ml of a filtered EPEC ΔsepD secreted protein preparation was applied to a CesT affinity column. Retained proteins were extensively washed and eluted as described above. The eluted proteins were trichloroacetic acid-precipitated and washed in acetone before SDS-PAGE, followed by Sypro Ruby Red staining. Well defined dominant protein bands were excised, whereas less obvious protein-containing regions within the same gel lane were excised according to apparent molecular weight. Excised gel slices were subjected to trypsin digestion and peptide recovery using a Montage in-gel digest kit (Millipore) as directed by the manufacturer. The peptides were analyzed on an API Q STAR PULSARi Hybrid liquid chromatograph/tandem mass spectrometer at the University of British Columbia Michael Smith Laboratories/Laboratory of Molecular Biophysics Proteomics Core Facility. The data were analyzed using Mascot software (39), and peptide sequences were...
searched within the nonredundant Proteobacteria data base at the NCBI. In addition, matches to peptides were also searched within the assembled EPEC 2348/69 genome data base at the Welcome Trust Sanger Institute.

RESULTS

CesT Interacts with Diverse Type III Effector Proteins Encoded on Different Pathogenicity Islands in EPEC—CesT is known to interact with four effector proteins, Tir, Map, EspF, and NleA (30–34). Recently, we demonstrated that the efficient secretion of many other EPEC type III effector proteins also required CesT (32, 33), suggesting a possible interaction of these proteins with CesT. To identify new CesT interacting type III effector proteins, we took advantage of a \( \text{H9004}\ sepD \) strain of EPEC that is known to hypersecrete type III effector proteins but not translocator proteins (38). \( \text{H9004}\ sepD \) was cultured under conditions that promote type III effector secretion, and the culture supernatant was collected and passed over a CesT affinity column. After extensive washing, bound protein species were eluted and subjected to mass spectrometry analyses.

Tir was completely depleted from the \( \text{H9004}\ sepD \) supernatant flow-through fraction, indicating a strong interaction with CesT (Fig. 1A). NleA was reduced in the flow-through, as well as many other protein species. A \( \Delta sepD\Delta escN \) strain defective for type III secretion due to the absence of the T3SS ATPase did not secrete known type III effectors and served as a control on a separate CesT affinity column. A column without CesT did not bind supernatant proteins to any significant extent (data not shown). Notably, EspC, a large type V secreted protein (110 kDa) (40), was not depleted to any significant extent from the \( \text{H9004}\ sepD \) flow-through, further indicating the specificity of the column for CesT interactions (Fig. 1A). As expected, mass spectrometry analyses of gel-extracted proteins from the \( \text{H9004}\ sepD \) supernatant identified three known CesT binding partners, Tir, NleA, and EspF. Five other type III effectors (EspG, EspZ, NleG, NleH, and NleH2) (Table 2; see “Experimental Procedures”) were also identified as CesT-interacting proteins. These proteins are known to be substrates of the type III secretion system, and some require CesT for their efficient secretion in EPEC (33). Thus, their observed interaction with the CesT affinity column is consistent with their dependence on CesT for efficient secretion. The novel effectors are encoded in different pathogenicity islands within EPEC (Table 2), implicating CesT as a multivalent chaperone for diverse type III effector proteins.

Identification of the CesT Binding Domain of NleH—The retention of multiple type III effectors on the CesT affinity column could be mediated by direct CesT-type III effector interactions.
actions or bridging interactions between proteins. To examine the possibility that CesT affinity column interactions were due to bridging proteins, NleH-FLAG was expressed from a plasmid in
coli DH5α that does not encode EPEC type III effectors. Notably, NleH-FLAG could be expressed as a soluble protein in the absence of CesT, which was not the case for all type III effectors tested (see “Discussion”). NleH-FLAG was immunoprecipitated onto FLAG beads from a cell lysate and then mixed with purified recombinant GST-CesT (30), followed by extensive washing and elution with FLAG peptide. GST-CesT co-eluted with NleH-FLAG, whereas GST alone did not bind to the NleH-FLAG-containing beads (Fig. 1B). Next, we set out to further characterize the CesT interaction with NleH. Plasmid-encoded NleH was expressed in ΔsepD or ΔsepDΔcesT and examined for protein secretion. NleH was highly secreted in ΔsepD but only to a minimal extent in ΔsepDΔcesT, as demonstrated by Western blotting total secreted protein preparations, confirming the requirement of CesT for efficient NleH secretion (Fig. 2A). Carboxyl-terminal truncated versions of NleH and a deletion mutant (Δ2–50 NleH) were then expressed in

**FIGURE 2. Functional determination of the NleH chaperone binding domain and secretion requirements.** A, plasmid-encoded NleH expressed in ΔsepD requires CesT for efficient secretion as demonstrated by Western blotting of total secreted proteins. M, protein standards are those indicated in B. B, Coomassie stain of total secreted proteins from ΔsepD culture supernatants. D, Western blot of the respective ΔsepD whole cell lysates expressing NleH variants. Note the absence of secretion for NleH(ΔCBD) (C) and its accumulation in the whole cell lysate. E, CesT affinity column demonstrating that ΔsepD secreted NleH(aa 1–100) interacts with CesT.
Hierarchical Type III Effector Delivery into Host Cells

A Putative Degenerate CesT-Interacting Domain Is Located at the Amino-Terminal Region of Multiple Type III Effectors of EPEC. A, ClustalW alignment of Tir (aa 40–122) (which overlaps the known CesT binding domain of aa 1–100) with the amino-terminal region of NleH and NleH2. The parameters were as follows: BLOSUM matrix, open gap penalty = 5, end gap penalty = 5, extension gap penalty = 0.05, separating gap penalty = 0.05. Multiple sequence alignment of the amino-terminal region of EPEC type III effectors from three different pathogenicity islands in EPEC (NleH (O-island 122), NleI (O-island 71), and EspF (LEE)). Identical residues in at least two sequences are denoted by an asterisk, and conserved substitutions are denoted by a colon.

ΔsepD to characterize the type III secretion requirements of NleH. All of the ΔsepD strains harboring plasmids had similar secretion profiles (Fig. 2B). NleH (aa 1–50) was not detectable in lysate or secreted protein samples, whereas the longer versions were all secreted at high levels from ΔsepD strains harboring plasmids (Fig. 2C). The amino-terminal deletion mutant (Δ2–50 NleH) was not secreted, and the protein remained in the bacterial cell, as demonstrated by Western blotting of ΔsepD whole cell lysates (Fig. 2D). Next, the culture supernatant from EPEC ΔsepD expressing NleH (aa 1–100) was collected and passed over the CesT affinity column. NleH (aa 1–100) was depleted from the flow-through fraction and remained bound after extensive washing. The protein was co-eluted with CesT (Fig. 2E), indicating that the CesT binding domain of this effector resides within the first 100 amino acids.

Collectively, these experiments demonstrate an interaction between CesT and the amino-terminal region of NleH. Furthermore, the first 100 amino acids of NleH are necessary and sufficient to mediate CesT-dependent secretion of this non-LEE-encoded type III effector in EPEC.

A Putative Degenerate CesT Binding Domain Is Located at the Amino Terminator of Diverse Type III Effectors—The ability of column-bound CesT to retain multiple type III effectors suggested that each protein has a functional CBD. In order to determine if this binding domain was conserved between the type III effectors, the known CBD of Tir was used as a “scaffold” to identify similar sequences in other type III effectors. Amino acid sequences of other known and putative EPEC type III effectors were evaluated by alignment or multialignment analyses (e.g. BLAST or Clustal, default settings); however, no significant sequence similarities were observed (data not shown). Altering the ClustalW parameters revealed an alignment with the amino-terminal region of NleH and NleH2 of EPEC (Fig. 3A). The CesT binding domain of this effector resides within the first 100 amino acids.

The protein was co-eluted with CesT (Fig. 2E), indicating that the CesT binding domain of this effector resides within the first 100 amino acids. However, the first 100 amino acids of NleH are necessary and sufficient to mediate CesT-dependent secretion of this non-LEE-encoded type III effector in EPEC.

Collectively, these experiments demonstrate an interaction between CesT and the amino-terminal region of NleH. Furthermore, the first 100 amino acids of NleH are necessary and sufficient to mediate CesT-dependent secretion of this non-LEE-encoded type III effector in EPEC.
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The presence of Tir is important for efficient type III effector secretion. A, total secreted protein profiles of various EPEC strains. Note that the deletion of tir from the ΔsepD background dramatically reduces type III effector secretion. The ΔsepDΔtir double mutant was transcomplemented with a plasmid expressing SepD to return the total secreted protein profile back to that of Δtir. Transcomplementation of ΔsepDΔtir with plasmid-expressed Tir partially restored type III effector secretion to ΔsepD levels. The arrowheads point to protein species in the last lane, which are lacking in the ΔsepDΔtir secretion profile. M, protein standards. B, deletion of other effector genes encoded within the LEE (espZ) or outside the LEE (nleA) does not alter total type III effector secretion. The arrowheads indicate protein species that are not present in the ΔsepDΔtir secretion profile. C, transcriptional fusion analyses using a plasmid nleA reporter construct harbored within different EPEC strains.

FIGURE 6. The presence of Tir is important for efficient type III effector secretion. A, total secreted protein profiles of various EPEC strains. Note that the deletion of tir from the ΔsepD background dramatically reduces type III effector secretion. The ΔsepDΔtir double mutant was transcomplemented with a plasmid expressing SepD to return the total secreted protein profile back to that of Δtir. Transcomplementation of ΔsepDΔtir with plasmid-expressed Tir partially restored type III effector secretion to ΔsepD levels. The arrowheads point to protein species in the last lane, which are lacking in the ΔsepDΔtir secretion profile. M, protein standards. B, deletion of other effector genes encoded within the LEE (espZ) or outside the LEE (nleA) does not alter total type III effector secretion. The arrowheads indicate protein species that are not present in the ΔsepDΔtir secretion profile. C, transcriptional fusion analyses using a plasmid nleA reporter construct harbored within different EPEC strains.

The Degenerate CesT Binding Domain Is Modular in Nature and Can Be Functionally Exchanged between Effectors—Based on the CesT-dependent protein secretion of multiple type III effectors and CesT interaction data presented here, it was hypothesized that the CBD is modular in nature and serves to target effectors for type III secretion via interaction with CesT. To test this hypothesis, the CBD of NleH and Tir were swapped (see “Experimental Procedures” for chimeric protein construction). The respective plasmid constructs were transformed into EPEC for characterization studies.

Deletion of the CBD from NleH produced a stable protein species that was not secreted to any extent (Fig. 2B). Fusing the CBD of Tir (amino acids 39–83) to NleH lacking its own CBD resulted in the recombinant NleH protein being secreted at levels comparable with wild type NleH (Fig. 4A). This indicated that the Tir CBD, when fused to NleH (missing its native CBD), can support the type III secretion of the recombinant protein in EPEC.

Since the CBD of Tir is located ~38 amino acids into the protein, the CBD of NleH (aa 1–40) was used to replace amino acids 38–83 of Tir. Deletion of the CBD of Tir destabilizes the protein, consistent with Tir stability being strictly dependent on the presence of CesT in EPEC (30). The recombinant Tir protein harboring the NleH CBD was rendered stable and was efficiently secreted by Δtir EPEC (Fig. 4A), indicating that the CBD of NleH functionally served to stabilize cytoplasmic Tir and supported its secretion. A ΔescN mutant defective for type III secretion did not secrete the NleH- and Tir-interchanged CBD fusion proteins (data not shown). This latter result indicated that the secretion of the fusion proteins remained dependent on a functional type III secretion system.

The ability of each fusion protein to interact with CesT was examined using the aforementioned CesT affinity binding assay. The Tir-NleH(CBD)-Tir fusion protein secreted from Δtir bound to the CesT column at levels similar to native Tir secreted by wild type EPEC (Fig. 4B). The Tir(CBD)-NleH fusion was also observed to interact with CesT.

Bacteria expressing the recombinant proteins were then tested in an infection assay for their ability to deliver the domain-exchanged type III effectors into HeLa cells. Tir injection into HeLa cells results in the formation of actin-rich pedestals that can be observed as punctate actin staining by immunofluorescence. Since the Tir-NleH(CBD)-Tir hybrid protein was stable and efficiently secreted in secretion assays, we evaluated the efficiency of pedestal formation for a tir mutant expressing the recombinant Tir-NleH(CBD)-Tir hybrid protein. The Δtir mutant expressing a Tir derivative lacking its CBD did not produce pedestals or focus actin to any significant extent, whereas the tir mutant harboring pNT251 encoding Tir-NleH(CBD)-Tir produced pedestals and focused actin similar to wild type EPEC (Fig. 5). Wild type EPEC harboring a plasmid encoding epitope-tagged NleH or the Tir(CBD)-NleH fusion translocated the respective NleH proteins into HeLa...
cells (Fig. 5). NleH and Tir(CBD)-NleH both localized immediately underneath adherent bacteria, close to regions of considerable actin focusing. These experiments indicate that the degenerate CesT binding domain is modular in nature and can be functionally exchanged between type III effectors.

**Tir Deletion Abrogates Type III Effector Secretion but Not Gene Expression**—The tir and cesT genes are co-transcribed as part of a multicistronic transcript from the LEE5 promoter (41). The co-transcription and adjacent arrangement of many effector-chaperone pairings are common features among pathogens, and it is believed that the coordinated expression perhaps allows for the productive interaction of the respective proteins. It is now known that not all effector genes are co-transcribed with their partner type III secretion chaperone, since some are located within different pathogenicity islands within the chromosome. We asked the question whether CesT could carry out its function for other effectors in the absence of Tir protein expression. The ΔsepD strain hypersecretes effectors that are readily detectable by Coomassie staining and thus represents an ideal genetic background to address the role of Tir with respect to other type III effectors. Therefore, a ΔsepDΔtir strain was generated and evaluated for effector secretion. Surprisingly, total effector secretion was dramatically reduced for the ΔsepDΔtir strain compared with the ΔsepD parent (Fig. 6A). Notably, abundant levels of NleA were missing from the culture supernatant as well as other type III effector proteins. The ΔsepDΔtir strain was trans-complemented with a plasmid expressing Tir, which restored total effector secretion. This experiment also served to rule out polar transcriptional effects on chromosomal cesT gene expression. Deletion of either a type III effector gene encoded outside of the LEE (nleA) or within the LEE (espZ) from the ΔsepD background did not alter secreted type III effector levels (Fig. 6B), indicating that Tir protein expression is important to sustain total type III effector secretion. A transcriptional fusion to nleA was used to evaluate the expression level of this effector gene in different EPEC strain backgrounds. nleA gene expression was not significantly different in ΔsepD or ΔsepDΔtir strains (Fig. 6C), indicating that the absence of NleA secretion in ΔsepDΔtir was not due to a lack of nleA transcription.

A Coordinated CesT-Type III Effector Interaction Rescues the ΔsepDΔtir Secretion Defect—Based on these results, it was hypothesized that by unlinking tir from cesT gene expression, the CesT protein would be temporally unbound and that free CesT would act as a feedback signal to negatively modulate the secretion of other type III effectors. It was therefore hypothesized that any type III effector that interacts with CesT should return total type III effector secretion to the ΔsepDΔtir strain, provided that its expression was linked with CesT. To address this hypothesis, NleA-FLAG was expressed within ΔsepDΔtir from a recombinant tir promoter, which is active during typical Tir expression conditions. NleA-FLAG (encoded by pNT255) was found to be secreted and partially restored the secretion of other type III effectors (Fig. 7). Another plasmid encoding NleH from the tir promoter (pNT253) was transformed into the ΔsepDΔtir strain and evaluated for effector secretion. NleH expressed from the tir promoter also partially restored type III effector secretion minus Tir (Fig. 7). Notably, NleA secretion was restored. Last, a plasmid encoding NleH without its CBD (pNT254) did not restore effector secretion, suggesting that a coordinated CesT-type III effector protein interaction is important for efficient type III effector secretion.

**Tir Secretion Is Required to Efficiently Activate Type III Effector Secretion in EPEC**—The ability of any effector to rescue total effector secretion in the ΔsepDΔtir double mutant could be due to an interaction with CesT. Alternatively, other type III effectors may require the action of Tir secretion from the cytoplasm to support their subsequent secretion. To address these questions, we constructed a plasmid that expresses a CesT-interacting Tir variant without its putative signal sequence (Tir Δ2–38 aa). This region of Tir encodes the putative Tir secretion signal (aa 1–26) (42) up to the beginning of the Tir CBD. Immunoblotting of cell lysates and the secreted protein preparations revealed that the Tir variant was stable within the EPEC cytoplasm, suggestive of an interaction with CesT. In addition, this interaction is important for efficient type III effector secretion.
completely restored type III effector secretion as visualized by staining total secreted proteins.

The Putative Tir Secretion Signal Cannot Impose Type III Effector Secretion Hierarchy—Interestingly, amino acids 38–75 of Tir aligns with amino acids 2–40 of NleH (Fig. 3A). This region of both proteins corresponds to the CBD, as determined by the domain exchange experiments presented here. The absence of a similar secretion signal within NleH (and other type III effectors) combined with the observed hierarchy of Tir secretion over other type III effectors led us to hypothesize that amino acids 1–38 of Tir (which overlap with the putative signal sequence) (42) mediate Tir secretion hierarchy. Therefore, a plasmid construct that expresses Tir (aa 1–38) fused to NleH transcribed from the nleH promoter was created and tested in secretion assays. ΔsepD was found to secrete comparable levels of NleH and Tir (aa 1–38)-NleH as determined by immunoblotting of total secreted protein preparations (Fig. 8B). In addition, Tir (aa 1–38)-NleH did not restore total type III effector secretion to ΔsepDΔtir (Fig. 8C), indicating that amino acids 1–38 of Tir alone cannot impose secretion hierarchy to other type III effectors.

DISCUSSION

Type III secretion chaperones bind effector proteins, promoting their rapid and efficient translocation into host cells. Here, it is demonstrated that a degenerate protein domain found in multiple EPEC type III effectors is involved in CesT binding. Interestingly, the data demonstrate that the coordinated interaction of the type III effector Tir with its chaperone CesT, followed by Tir secretion, appears to be a sequence of events that is absolutely critical for the efficient secretion of other type III effectors. This finding is in agreement with Tir being a critical in vivo colonization factor and for the first time elucidates a hierarchy for Tir delivery over other EPEC type III effectors.

A pressing question in bacterial pathogenesis is how pathogens regulate their multiple virulence determinants to create a productive infection leading to disease. For extracellular intestinal pathogens, it is absolutely critical to attach and colonize gut tissues before being prematurely shed into the environment. We show here with genetic and biochemical evidence that the receptor for intimate attachment, Tir, is the hierarchical type III effector in EPEC. The data suggest that once Tir-mediated intimate attachment has been achieved, then other type III effectors involved in subverting the host's intracellular processes are injected. The results are in direct accordance with animal infection experiments where Tir-mediated adherence of A/E pathogens is important for host colonization and disease progression. This point has been directly and robustly demonstrated in three animal models of infection: (i) EHEC tir mutants do not efficiently colonize the infant rabbit intestine and do not show clinical signs of disease (18); (ii) Citrobacter rodentium tir mutants do not colonize the mouse intestine and do not cause disease (43); and (iii) rabbit

FIGURE 8. Tir secretion is required to efficiently activate type III effector secretion in EPEC. A, total secreted protein preparations from various EPEC strains were subjected to SDS-PAGE, followed by Coomassie Blue staining. The lower panel shows an anti-Tir immunoblot using one-tenth of the sample amount loaded for Coomassie Blue staining. Note that Tir (Δ2–38) is minimally secreted by the ΔsepDΔtir strain and does not return the secretion profile back to ΔsepD levels. M, protein standards. B, fusion of the putative Tir secretion signal (aa 1–38) to the amino terminus of NleH does not increase secretion levels of the recombinant NleH protein, as demonstrated by SDS-PAGE of total secreted proteins (top) and immunoblotting (bottom). C, NleH or a Tir (aa 1–38)-NleH fusion expressed from the nleH promoter did not rescue total type III effector secretion within ΔsepDΔtir, as demonstrated by SDS-PAGE of total secreted proteins followed by Coomassie Blue staining.
EPEC tir mutants do not cause diarrhea in weaned rabbits and do not intimately adhere to host intestinal cells (17).

Previously, a comprehensive screen of LEE-interacting proteins using the yeast two-hybrid assay identified a strong CesT-Map and a weak CesT-EspF interaction (34) but failed to identify EspG and EspZ as interacting proteins. Interestingly, EspF is reported to have its own chaperone CesF (44) (rorf10 within the LEE). We cannot rule out the possibility that EspF interacts with both CesF and CesT. Our results independently confirm a CesT-EspF interaction using a different approach (Table 2). We also identify a putative CesT binding domain on EspF through comparative analyses (Fig. 3).

There could be additional CesT-interacting type III effectors that were not secreted at high enough levels within our EPEC secretion assays or were in an improper conformation to interact with immobilized His-CesT (Fig. 1). Indeed, it has been reported that the CesT-interacting type III effector Map is expressed at very low levels within EPEC (32), which may explain why it was not detected in our analyses. Nonetheless, from our observations and those from other reports (32–34), CesT physically interacts or is involved in the secretion of every type III effector described to date, further strengthening its central role in EPEC pathogenesis. Pairwise protein interaction studies between CesT and each of its partner effectors will help to identify specific binding affinities for this multivalent chaperone of EPEC.

The multivalent binding property of CesT with different type III effectors suggests that there is a common feature among the effectors that mediates CesT binding. A study by Lilic et al. (26) suggests that a number of type III effectors have a conserved structural β-fold that type III secretion chaperones interact with. This does not appear to be the case for all of the EPEC type III effectors that interact with CesT, since the presumed structural β-folds are not in a proper spatial context for dimeric CesT binding. From our CBD swapping experiments with normal CesT levels (data not shown). This suggests that CesT alone does not mediate regulated type III effector secretion. We hypothesize that the action of Tir secretion results in CesT being associated with the secretory apparatus. This is in agreement with the observed inner membrane association of CesT within EPEC, a localization that is enhanced in the presence of Tir (33). After Tir secretion, other effectors can bind to membrane-associated or cytoplasmic CesT, which then supports their secretion through the secretory apparatus (Fig. 9). This model is similar to a proposed model for Yersinia pestis type III effector secretion, where it is thought that LcrQ and its chaperone SycH function together to mediate a hierarchy of secretion (45).

Prior to this study, genetic evidence for type III effector hierarchy was lacking, although convincing evidence for the hierarchical assembly of the type III translocase has been reported (46). From the studies presented here, it is evident that certain type III effectors are hierarchical over others. The data presented here also implicate type III secretion chaperones to be involved with hierarchy, although additional studies are required to elucidate the mechanisms and dynamics underlying this process. The role of multivalent secretion chaperones, such as CesT, are particularly interesting in this regard, given their multiple interactions with diverse type III effectors.

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