Pore-forming Activity of the *Escherichia coli* Type III Secretion System Protein EspD*

Received for publication, February 25, 2015, and in revised form, August 13, 2015 Published, JBC Papers in Press, August 31, 2015, DOI 10.1074/jbc.M115.648204

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**Background:** The membrane protein EspD is critical for pathogenic *E. coli* to inject virulence factors into host mammalian cells.

**Results:** EspD inserts into membranes and forms an ∼2.5-nm pore.

**Conclusion:** Pore assembly is dependent on anionic phospholipids and acidic pH.

**Significance:** Elucidating the structural mechanisms of pore formation advances understanding of the T3SS function in EPEC and EHEC infections.

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*Escherichia coli* is a Gram-negative bacterium present in the intestinal microbiota of healthy humans (1, 2). Certain strains of *E. coli*, however, have developed a sophisticated mechanism for adhesion and injection of bacterial virulence factors into gut epithelial cells. Enteropathogenic (EPEC)3 and enterohemorrhagic (EHEC) *E. coli*, such as the strain O157:H7, are pathogens responsible for gastrointestinal infections (3, 4). When EPEC or EHEC cells come in close proximity to the host epithelial cells lining the intestinal tract, they express a set of proteins involved in the assembly of a structural organelle known as the type III secretory system (T3SS) (5). The T3SS is capable of translocating proteins directly from the bacteria into the host cell cytosolic compartment, an event that is critical for pathogenesis (6). Bacterial effector proteins injected into gut enterocytes manipulate a variety of cellular functions; however, the most striking phenotype associated with EHEC and EPEC is a remodeling of cytoskeleton and rearrangement of the actin filaments underneath the bacterial adhesion site resulting in a deformation of microvilli into a dysfunctional pedestal-like structure designated the site of attachment and effacement (7, 8).

The proteins responsible for the attachment and effacement lesion are encoded on a pathogenicity island of ∼35 kb referred to as the locus of enterocyte effacement (9, 10). In EPEC, the locus of enterocyte effacement has been estimated to contain 41 open reading frames that encode effector molecules such as EspF, EspG, EspH, Map, regulatory proteins LcrV, and GrlA/GrlR (11–13), an outer membrane adhesin known as intimin and its receptor Tir (14), as well as the translocation apparatus that consists of the proteins EspA, EspB, and EspD (15–20). Insertion of EspD into the cell membrane has been postulated to be an essential step in the formation of a translocation pore through which bacterial effector proteins are injected into the host cell (19).

In EPEC and EHEC, espB and espD have been genetically demonstrated to be important virulence factors as deletion of either of these genes impaired bacterial adhesion and infectivity (20–26). Additionally, it has been suggested that EspB and EspD participate in pore formation, as loss of these proteins

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8 This work was supported by Natural Science and Engineering Research Council of Canada Grant RGPIN 238294-11, Canada Foundation for Innovation New Opportunities Program, and a Regroupements Strategiques de la Societe Que´becoise de Recherche en Biologie. The authors declare that they have no conflicts of interest with the contents of this article.

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3 The abbreviations used are: EPEC, enteropathogenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; T3SS, type III secretion system; LUV, large unilamellar vesicles; SUV, small unilamellar vesicle; PL, phospholipid; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPG, dioleoylphosphatidylglycerol; 10-DN, 10-doxyl nonadecane; DOPE, dioleoylphosphatidylethanolamine; SM, sphingomyelin; Chl, cholesterol; SRB, sulforhodamine B, 10-DN, 10-doxyl nonadecane; POPS, palmitoylphosphatidylserine; POPC, palmitoylphosphatidylcholine; CBD, chitin binding domain.
abolished pore assembly and injection of effector protein (19, 21, 23, 27, 28).

The EHEC bacterial strain EDL933 (O157:H7) espD encodes a 374-amino acid protein (39 kDa) predicted to contain two coiled-coil motifs located near the N (COIL I) and C (COIL II) termini, regions postulated to mediate EspD oligomerization (Fig. 1) (29, 30), and two transmembrane helices that form a hairpin structure that inserts into the host membrane. Although EspD is recognized to be a critical EPEC and EHEC virulence factor, little is known about the mechanism associated with membrane binding, oligomerization, and pore formation by this protein. Previous studies have demonstrated that the N-terminal region of EspD contains two amphipathic helices that mediate the initial anchoring of this protein to the lipid bilayer (29). Whether EspD alone in vivo is sufficient for translocon formation is not clear as previous studies detected EspB and EspD in plasma membranes (19). Here, we show that recombinant EspD, like native EspD secreted by EHEC, inserts into anionic lipid bilayers containing phosphatidylserine and forms a pore with an inner diameter of ~2.5 nm.

**Experimental Procedures**

**Chemicals and Reagents**—Restriction endonuclease DNA-modifying enzymes and cell culture reagents were obtained from Invitrogen. The pTYB2 vector and chitin affinity beads were purchased from New England Biolabs (Ipswich, MA). Electrophoresis and chromatography products were obtained from Bio-Rad, and clostripain was purchased from Promega. All other reagents were of the highest quality commercially available.

**Purification of Recombinant EspD**—The EHEC espD open reading frame was amplified from *E. coli* O157:H7 EDL933 genomic DNA and cloned into NdeI and EcoRI restriction endonuclease sites in the pTYB2 plasmid (New England Biolabs) to generate the pTYB2-espD expression vector used to express the EspD-chitin binding domain (CBD) fusion protein. For protein expression, the *E. coli* strain C41 (DE3) transformed with the pTYB2-espD construct was grown in Terrific Broth (1 liter) containing 100 µg/ml ampicillin and 1% glucose at 37 °C with vigorous shaking to an *A*₆₀₀ of 0.8, and then benzyl alcohol was added (10 mM final concentration) and the culture incubated at 20 °C for 30 min prior to the addition of isopropyl thiogalactoside (0.5 mM). Cultures were incubated for 75 min at 37 °C to induce EspD-CBD protein expression. The cell pellet was resuspended in 80 ml of Buffer A (10 mM Na₃HPO₄, 1.8 mM KH₂PO₄, 500 mM NaCl, pH 7.2, 10% glycerol, containing a protease inhibitor mixture tablet (Roche Applied Science)), and cells were lysed by sonication. Clarified supernatants were applied to a chitin column (2.0 ml) equilibrated in Buffer B (10 mM Na₃HPO₄, 1.8 mM KH₂PO₄, 500 mM NaCl, pH 7.2, 10% glycerol, 0.2% Triton X-100) and incubated with end-on-end mixing for 16 h at 4 °C. The chitin column was washed with 400 ml of Buffer A, and the EspD fusion was cleaved with 50 mM dithiothreitol in Buffer B for 48–72 h at 4 °C. EspD was eluted, and the solution was made up to 1% Triton X-114. EspD was isolated in the detergent phase following phase separation performed at 37 °C (31). The detergent phase containing EspD was collected by centrifugation at 18,000 × g at 20 °C for 10 min, and the Triton X-114 was removed by acetone precipitation. The purified protein was stored at −20 °C as pellet and dissolved in 4.0 M urea in phosphate-buffered saline immediately prior to use. Protein concentrations were determined spectrophotometrically as described previously (32).

**HeLa Cell Culture and Infection**—HeLa cells (8 × 10⁵ cells/well) grown to 80% confluence in 6-well tissue culture plates (Corning, Lowell MA) in DMEM with 10% heat-inactive bovine serum albumin were co-cultured with a 100-µl aliquot of an overnight EPEC E2348/69 culture for 4 h at 37 °C (29). The adherent cells were washed three times with phosphate-buffered saline (PBS) to remove bacteria, and the HeLa cells were gently scraped and resuspended in 1.0 ml of PBS containing protease inhibitor mixture and vigorously mixed using a vortex mixer to remove adherent bacteria. HeLa cells were lysed using a Dounce homogenizer (Kimble-Kontes, Vineland, NY), and crude membrane and cytosolic fractions were prepared by centrifugation at 14,000 rpm for 20 min at 4 °C. The crude membrane preparation was extracted with 200 µl of 1% sodium dodecyl sulfate for 30 min at 0 °C. The insoluble material was removed, and the native EspD complex in the extract was analyzed by rate zonal ultracentrifugation.

**Unilamellar Vesicle Preparation**—The phospholipids dioleoylphosphatidylserine (DOPS), dioleoylphosphatidylycholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylglycerol (DOPG), palmitoyleoleoylphosphatidylserine (POPS), palmitoyleoleoylphosphatidylcholine (POPC), sphingomyelin (SM), cholesterol (Chl), or mixtures of these lipids dissolved in chloroform were evaporated using nitrogen gas to generate thin films. Residual solvent was removed by incubating films under reduced pressure for 16 h. The lipid films were dispersed in 40 mM sodium phosphate, 150 mM NaCl buffer, pH 7.2 (PBS), by vigorous mixing to form multilamellar vesicles that were incubated at 25 °C for 1 h prior to extruding the mixture 30 times through a 200 nm polycarbonate filter using an extruder (Avanti Polar Lipids) to generate large unilamellar vesicles (LUVs). To prepare small unilamellar vesicles (SUVs), the multilamellar vesicles were sonicated with a probe sonicator on ice for 2–5 min until the lipid solution became clear. The resulting SUVs were annealed for 1 h at 37 °C. SUVs were used in intrinsic fluorescence studies to minimize the degree of light scattering.

**Membrane Binding Assay**—LUVs (500 µg) were incubated with recombinant EspD or espD mutant proteins (5 µg) in 300 µl of 40 mM sodium acetate, pH 4.5, 150 mM NaCl (SAS), or PBS, pH 7.2, for 15 min at 25 °C and then diluted with 1.2 ml of 66% sucrose, 350 mM NaCl in PBS buffer. These preparations were overlaid with 3.0 ml of PBS, supplemented 350 mM NaCl and 35% sucrose and then 0.7 ml of PBS, pH 7.2. Samples were subjected to centrifugation at 28,000 rpm for 16 h at 4 °C in a Beckman SW55 rotor. Fractions (0.75 ml) were collected from the top of the gradient, and proteins were precipitated with 12% trichloroacetic acid (TCA). Protein pellets were resuspended in SDS-PAGE sample buffer, and the distribution of EspD was examined by Western blot analysis.
To determine the apparent EspD membrane binding affinities, 667 μM phospholipid in the form of SUV with composition SM/DOPC/DOPS (54:26:20), SM/DOPC/DOPS/Chl (44:24:12:20), or SM/DOPC/DOPe/Chl (44:24:12:20) was mixed with increasing concentrations of EspD (46–1330 nM) in 75 μl of SAS or PBS buffer and incubated at 25 °C for 10 min. The reaction mixture was made to 40% sucrose and transferred to a thick-walled 1.5-ml tube and then overlaid with 1.0 ml of 25% sucrose and 150 μl of PBS. Samples were centrifuged at 49,000 rpm in a TLA100.3 rotor for 2.5 h at 4 °C on a Beckman Coulter tabletop ultracentrifuge. Gradients were fractionated (250-μl aliquots), and EspD was precipitated with 0.1% deoxycholate and 12% TCA. Protein pellets were dissolved in 500 μl of 0.2% Triton X-100 and the LUV pellet was sedimented by centrifugation at 50,000 rpm for 40 min at 4 °C. The LUV pellet was sequentially extracted with 1.0 ml of 500 mM NaCl, 100 mM Na2CO3, pH 11.5, and 8.0 M urea for 30 min at 25 °C. After each treatment the sample was separated into a supernatant and pellet fraction by centrifugation at 50,000 rpm for 40 min at 4 °C. Proteins were precipitated with 12% TCA and the distribution of EspD in the pellet was assessed by Western blot analysis.

Pore Formation by EHEC EspD

To verify that recombinant EspD spontaneously inserted into mammalian cell plasma membrane, HeLa cells grown in a 24-well microculture plate to ~75% confluency were incubated with 26 μg of recombinant EspD for 3 h in 1.0 ml of serum-free DMEM. The cells were washed with warm PBS to remove unbound protein, then scraped in 1.0 ml of ice-cold PBS containing a protease inhibitor mixture (Roche Applied Science), and lysed with a Dounce homogenizer, and crude membrane and cytosolic fractions were isolated by centrifugation at 14,000 rpm for 30 min at 4 °C. The membrane pellet was washed with 1.0 ml of cold PBS and sequentially extracted with 500 mM NaCl, 100 mM Na2CO3, pH 11.5, and 8.0 M urea. Samples were fractionated at 50,000 rpm for 40 min at 4 °C, and the distribution of EspD was determined by Western blot.

LUVs with SM/DOPC/DOPS/Chl were loaded with EspD at pH 4.5 or 7.2 and incubated for 15 min at 20 °C. The pH in both samples was adjusted to 7.2 and then placed on ice for 15 min prior to extraction with 1% Triton X-100 for 30 min at 0 °C prior to ultracentrifugation at 50,000 rpm for 40 min at 4 °C in a Beckman-Coulter tabletop centrifuge equipped with a TLA100.3 rotor. The distribution of EspD in the supernatant and pellet fraction was assessed by Western blot analysis.

Dye Leakage Assays—Lipid thin films were resuspended 50 mM sulforhodamine B (SRB) in PBS and then extruded through a 200-nm polycarbonate filter membrane. LUVs loaded with SRB were purified by gel filtration chromatography (Sephadex G50, GE Healthcare). For leakage assays, LUVs (20 μg of total phospholipid) diluted in SAS or PBS buffer were treated with EspD (0.2 μg), and the increase in fluorescence intensity was measured at 585 nm (at an excitation of 565 nm). The total amount of entrapped SRB was determined with the addition of 0.2% Triton X-100. To determine the effect of electrostatic interactions on pore formation, dye leakage assays were performed in SAS and PBS buffer containing 0.5 or 1.0 M NaCl. All leakage assays were performed at 25 °C.

Diameter of EspD Pore—The diameter of the EspD pores was assessed by leakage assays using carboxyfluorescein and fluorescein isothiocyanate (FITC)-conjugated dextrans with molecular masses of 4, 10, 40, or 70 kDa (Sigma) and Stokes radii of 1.8, 2.3, 3.2, 4.4 nm, respectively. Lipid films composed of SM/DOPC/DOPS/Chl were resuspended in PBS containing FITC dextran (5 mg/ml), and LUVs were prepared by extruding the multilamellar vesicle suspension through polycarbonate filters (200 nm). Lipid films composed of SM/DOPC/DOPS/Chl were resuspended in PBS containing FITC dextran (5 mg/ml), and LUVs were prepared by extruding the multilamellar vesicle suspension through polycarbonate filters (200 nm). FITC dextran-containing vesicles were purified by centrifugation at 50,000 rpm for 40 min at 4 °C in a Beckman-Coulter tabletop ultracentrifuge using a TLA100.3 rotor, and LUVs were washed three times with 1.0 ml of PBS to remove free FITC dextran.

FITC dextran-loaded LUVs (200 μg of phospholipid) were incubated with or without EspD (1 μg) in 500 μl 20 mM sodium acetate, pH 4.5, 150 mM NaCl for 10 min, and the LUVs were sedimented by centrifugation at 50,000 rpm for 40 min at 4 °C. Following centrifugation, the supernatant was removed, and the LUV pellet was dissolved in 500 μl 0.2% Triton X-100 in PBS. The fluorescence intensity of the pellet fraction was determined at an excitation of 492 nm and emission of 520 nm.

\[
\text{% leakage} = \left( \frac{F_{\text{con}} - F_{\text{EspD}}}{F_{\text{con}}} \right) \times 100
\]

where \( F_{\text{con}} \) is total fluorescence in the untreated control LUVs, and \( F_{\text{EspD}} \) is the total fluorescence in LUVs treatment with EspD (Equation 1).

Dual Quenching and Q Ratio—The interaction of EspD with lipid bilayers was examined by changes in the intrinsic fluorescence of espD mutants containing a single tryptophan (Trp) residue at positions Trp-47, Trp-178, or Trp-195. EspD (5 μg) was mixed with SM/DOPC/DOPS/Chl (44:24:12:20) SUVs (50 μg) in 100 μl of 20 mM sodium acetate, pH 4.5, or PBS and incubated at 25 °C for 5 min prior to recording the emission spectra from 300 to 420 nm. The solvent accessibility of the single Trp residue was measured by fluorescence quenching experiments using 0 to 0.25 M acrylamide and recording emission spectra.

For 10-doxyl nonadecane (10-DN) quenching experiments, 5.0 μg of mutant espD (Trp-47, Trp-178, or Trp-195) was
Pore Formation by EHEC EspD

added to (SM/DOPC/DOPS/Chl (44:24:12:20) SUVs (50 μg) or SUVs containing 10 mol % 10-DN (SM/DOPC/DOPS/Chl/10-DN (34:24:12:20:10) in 100 μl of 20 mM sodium acetate, 150 mM NaCl, pH 4.5, or PBS, pH 7.2. Samples were incubated at 25 °C for 5 min prior to recording the emission spectra. Fluorescence experiments were performed on a Varian Cary Eclipse spectrofluorometer at an excitation wavelength of 295 nm with excitation and emission slit widths of 5 nm. Emission spectra were recorded from 300 to 420 nm. All spectra were background corrected by subtracting spectra obtained for SUVs alone.

\[ Q_{\text{ratio}} = \frac{(F_0/F_{\text{acry}}) - 1)}{(F_0/F_{10-\text{DN}}) - 1) } \quad \text{(Eq. 2)} \]

where \(F_0\) is the fluorescence intensity in the absence of quencher; \(F_{\text{acry}}\) is the corrected fluorescence intensity in the presence 0.25 m acrylamide, and \(F_{10-\text{DN}}\) is the corrected fluorescence intensity in the presence of 10 mol % 10-DN (Equation 2).

Cross-linking Experiments—Full-length EspD (10 μg) or clostripain digested of EspD (10 μg) was added to SM/DOPC/DOPS/Chl (44:24:12:20) LUVs (1000 μg) in PBS, pH 7.2, or 20 mM sodium acetate, 150 mm NaCl, pH 4.5, and incubated at 25 °C for 15 min. Prior to the addition of 0.4 mM glutaraldehyde, the pH of both samples was adjusted to 7.2 with a 1.0 M sucrose density

\[ 500 \, \text{mM NaCl.} \]

DOPS/Chl (44:24:12:20) LUVs in 20 mM sodium acetate, pH 4.5, in SAS buffer and incubated for 15 min at 25 °C, and the reactions were terminated by acidification with 12% TCA. Precipitated proteins were analyzed by Western blot analysis.

Rate Zonal Centrifugation—EspD (5 μg) was loaded onto SM/DOPC/DOPS/Chl (44:24:12:20) LUVs (500 μg) by incubating at 25 °C for 15 min in 40 mM sodium acetate, pH 4.5, 150 mM NaCl. LUVs were sedimented by centrifugation at 50,000 rpm for 1 h in a Beckman-Coulter tabletop ultracentrifuge. The supernatant was removed, and the pellet was resuspended in 150 μl of PBS containing 1% sodium taurodeoxycholate and incubated at 25 °C for 30 min to solubilize the lipid bilayer. The LUV extracts or extracts prepared from HeLa cells infected with EPEC E2348/69 bacteria were layered on a 10–50% linear sucrose gradient prepared in PBS containing 0.2% sodium taurodeoxycholate, and complexes were resolved by centrifugation at 41,000 rpm at 6 °C for 18 h using an SW55 rotor. The mass of the EspD complexes was determined using a standard protein mixture containing bovine serum albumin (20 μg, 66 kDa), hemoglobin (50 μg, 64 kDa), catalase (100 μg, 240 kDa), and bovine IgM (50 μg, 950 kDa). Gradients were fractionated, and proteins were precipitated with TCA and then analyzed by Western blot using anti-EspD antibodies or by Coomassie Blue-stained SDS-PAGE (standard protein).

Bioinformatic Analysis of EHEC EspD—The transmembrane helices, coiled-coil motifs, and amphipathic helices in EspD were predicted using the HMMTOP, COILS, and Helixquest algorithms. Isoelectric points and net charge on EspD was estimated using the Protein Calculator version 3.4 program. All dye experiments were performed at least in triplicate using three independent preparation of EspD.

Results

Structural Analysis of EspD—Bioinformatic analysis of the E. coli 0157/H7 EDL933 EspD revealed that this protein contains two putative transmembrane helices spanning residues 176–200 (TMDI) and 227–251 (TMDII), the latter of which contains a number of serine residues (27, 30, 33). In addition, EspD contains two coiled-coil motifs situated between residues 138–171 (COIL I) and 334–370 (COIL II) (Fig. 1) as predicted using the COILS algorithm (34). More importantly, fragments encompassing residues 1–171 or 330–370 have been experimentally confirmed to form homodimers in solution (29, 30). It was postulated that following membrane binding these two EspD domains remain exposed to the aqueous environment and form contacts that facilitate oligomerization. Collectively, these analyses suggest that the N- and C-terminal segments are important for pore formation by contributing to EspD oligomerization. EspD also contains two regions (residues 24–40 and 66–83) with a high propensity to forming amphipathic helices, elements important for EspD function on HeLa cells (29).

Purification of EspD—Initial attempts to produce large amounts of recombinant E. coli EHEC EspD containing either an N- or C-terminal hexahistidine affinity in E. coli were confounded by very low expression levels that were only detected by Western blot analysis using an anti-EspD-specific polyclonal antibody (29). To improve production, EspD was expressed as
an N-terminal fusion protein linked to a CBD by a protein-splicing intein motif. Although an ~95-kDa band corresponding to the EspD-CBD fusion protein was not apparent on Coomassie Blue-stained gels of *E. coli* C41 whole cell lysates following isopropyl β-D-thiogalactopyranoside induction (Fig. 2A), Western blot analysis confirmed the presence of two immunoreactive proteins of ~40 and 95 kDa, which corresponded to the cleaved EspD protein and the intact EspD-CBD fusion construct (Fig. 2B). These results indicated that a portion of the fusion protein was undergoing protein splicing in vivo to generate the mature EspD that lacked N- or C-terminal affinity tags. The EspD-CBD fusion protein in the *E. coli* cytosolic fraction was purified on a chitin column, and the tagless form of recombinant EspD was recovered in the column eluate following a 48–72-h treatment with DTT to promote protein splicing (Fig. 2). The recombinant EspD was further purified by phase separation using the detergent Triton X-114 (31). EspD was recovered in the detergent phase, and the contaminating proteins remained in the aqueous phase. Coomassie-stained SDS-polyacrylamide gels showed that this approach produced EspD preparations with an apparent purity of ~90% (Fig. 2).

**Lipid Bilayer Binding Activity of EspD**—A hallmark feature of native EspD is the insertion of this protein into the host cell membrane (19, 27). To examine this event and to evaluate the phospholipids required for membrane binding, recombinant EspD was incubated with SUV composed of single phospholipids or lipid mixtures containing sphingomyelin, phosphatidylcholine, and cholesterol, which were selected to approximate the composition of the outer leaflet of the mammalian cell plasma membrane (35). SUVs were used for these initial experiments because multiple lipid bilayer composition could be rapidly generated by sonication. Sucrose density centrifugation experiments showed that at 25 °C in pH 7.2 buffer EspD bound quantitatively to DOPC, DOPS, and SM SUVs and was recovered at the top of the gradient. In contrast, no EspD binding was observed with DOPE SUVs. In the absence of liposomes recombinant EspD remained at the bottom of the sucrose gradient (Fig. 3A). To assess the effect of lipid mixtures, EspD was mixed with SUVs composed of SM/DOPC/DOPE, SM/DOPC/Chl, SM/DOPC/DOPS/Chl, or SM/DOPC/DOPE/DOPS, and in all cases, near quantitative binding of EspD was detected (Fig. 3B). This suggested that membrane binding was not impacted by the presence of DOPE. Addition of 500 mM NaCl, an ionic strength that disrupts electrostatic membrane–protein interactions, was not sufficient to dissociate EspD from SUVs (data not shown), suggesting that this interaction was stabilized by non-polar contacts with the hydrophobic core of the lipid bilayer. In contrast, no binding was observed with DOPE/Chl SUVs (Fig. 3B). To assess the effect of extravascular pH on membrane binding, EspD was incubated with SM/DOPC/DOPS/Chl LUVs at pH 4.5 and 7.2 prior to flotation centrifugation. Under both conditions, comparable levels of EspD were recovered from the top of the gradient (Fig. 3C, fractions 1–3), indicating that the pH did not influence the membrane binding event.

To investigate the nature of the EspD-membrane interaction, SM/DOPC/DOPS/Chl LUVs loaded with EspD at pH 4.5 or 7.2 were sequentially extracted with 500 mM NaCl, alkaline carbonate, and 8.0 M urea. Regardless of the pH, treatment of LUVs with 500 mM NaCl or 100 mM sodium carbonate, pH 11.5, conditions that remove extrinsic or peripheral membrane proteins (36), showed that the bulk of EspD was recovered in the membrane pellet, which indicated that EspD binding was stabilized by hydrophobic rather than electrostatic contacts with the lipid bilayer (Fig. 4). Following each treatment, the bulk of EspD partitioned with the membrane pellet, a behavior consistent with EspD inserting into the lipid bilayer and forming contacts with the hydrophobic core. Extraction of EspD-loaded LUVs with 8.0 M urea showed that recombinant EspD predominantly partitioned with the membrane pellet further verifying that the EspD-membrane interaction was mediated by nonpolar contacts with the core of lipid bilayer (Fig. 4).

To ensure that recombinant EspD exhibited a similar interaction with mammalian cell membranes, HeLa cells were
loaded with EspD, and a crude membrane preparation was sequentially extracted with 500 mM NaCl, alkaline carbonate, and 8.0 M urea. For all extraction conditions, EspD was quantitatively recovered with the membrane fraction and recapitulated the interactions observed with the LUVs.

Interestingly, SM/DOPC/DOPS/Chl LUVs loaded with EspD at pH 4.5 or 7.2, treated with Triton X-100 at 0 °C, and then subjected to high speed centrifugation revealed that EspD partitioned near quantitatively with a Triton X-100-insoluble pellet that likely contained detergent-resistant lipid microdomains due to the high concentration of sphingomyelin and cholesterol in the LUVs (Fig. 4B) (37). This is consistent with previous reports showing that EspD inserted into HeLa cell membranes was resistant to Triton X-100 extraction (27).

Dye Leakage Induced by EspD—The pore-forming activity of EspD was assessed by treating SM/DOPC/DOPS/Chl (44:24:12:20) LUVs loaded with the pH-insensitive self-quenching fluorescence dye SRB. Addition of EspD to LUVs at pH 7.2 at an EspD/phospholipid (EspD/PL) ratio of 1:1250 triggered only a modest dye release of 13% (Fig. 5A). However, shifting the extravesicular pH to 4.5 induced a more robust leakage of 58%. These data suggested that an acidic pH was required to facilitate membrane penetration or oligomerization of EspD (Fig. 5A).

Leakages assays performed using extravesicular buffers with pH 3.5 to 7.2 (EspD/PL, 1:1250) showed that the dye release was minimal between pH 5.5 and 7.2 (Fig. 5B). However, at a more acidic pH, dye leakage increased rapidly and reached a plateau of ~70%, with the mid-point occurring at pH 4.7 (Fig. 5B). It should be noted that EspD has a predicted pI of 5.4, and consequently this protein would carry ~11 positive charges at pH 4.7. Because negatively charged membranes have been shown to be important for pore formation in other systems (38–40), we examined the requirement of anionic phospholipid for EspD pore formation. Leakage experiments with SM/DOPC/DOPE/Chl (44:24:12:20) LUVs showed that replacement of DOPS with DOPE abolished dye leakage (Fig. 5B) and confirmed a role for anionic phospholipids in EspD pore formation. Flotation experiments showed that EspD efficiently bound to SM/DOPC/DOPE/Chl LUVs at neutral and acidic pH. The requirement for negatively charged phospholipids was further sub-

![Figure 3. Phospholipid specificity for EspD binding.](image-url)

![Figure 4. Interaction of EspD with lipid bilayers.](image-url)
stantiated by the finding that vigorous dye leakage, as a function of pH, was also observed when DOPS was substituted with DOPG (Fig. 5A). These data suggest a two-step mechanism consisting of a membrane binding event followed by a membrane penetration event that requires an anionic phospholipid and an acidic pH.

The effect of phosphatidylserine on EspD pore-forming activity was further assessed with SRB-loaded LUVs SM/DOPC/DOPS/Chl (44:24:12:20) or SM:POPC:POPS:Chl (44:24:12:20) and increasing EspD to phospholipid mole ratios of 1:2500. F, effect of fatty acid composition on pore formation was examined by dye leakage assays at pH 4.5 and pH 7.2 using LUVs composed of SM/DOPC/DOPS/Chl (44:24:12:20) (DO PL) or SM:POPC:POPS:Chl (44:24:12:20) (PO PL) and increasing EspD to phospholipid mole ratios.

FIGURE 5. Dye leakage activity of EspD. A, pore formation was evaluated by addition of EspD to SRB-loaded SM/DOPC/DOPS/Chl LUVs at pH 7.2, and dye release was monitored prior to shifting the pH to 4.5. B, effect of pH and phospholipid composition on pore formation was examined by adding EspD (EspD ratio 1:1250) to SRB-loaded SM/DOPC/DOPS/Chl or SM/DOPC/DOPE:Chl LUVs at various pH values and monitoring dye release. C, dependence of DOPS on EspD pore formation at pH 4.5 was assessed using SM/DOPC/DOPS/Chl (45:Y:X:20) membranes with increasing DOPS levels. The dependence of cholesterol on pore formation at pH 4.5 was evaluated using SM/DOPC/DOPS/Chl (45:Y:20:X) where the cholesterol mol % was varied. D, interaction affinity of EspD with lipid bilayers of various composition was determined by incubating a fixed amount of SUV with increasing concentrations of EspD and separating the free and SUV-associated EspD by sucrose density flotation and quantifying the protein in the bound fraction by ELISA. E, to assess the importance of electrostatic interactions on pore formation, leakage assays were performed in the presence of increasing NaCl concentrations using a constant EspD/phospholipid mole ratio of 1:2500. F, effect of fatty acid composition on pore formation was examined by dye leakage assays at pH 4.5 and pH 7.2 using LUVs composed of SM/DOPC/DOPS/Chl (44:24:12:20) (DO PL) or SM:POPC:POPS:Chl (44:24:12:20) (PO PL) and increasing EspD to phospholipid mole ratios.
DOPS increased dye leakage to 32%. Interestingly, incorporation of addition of DOPS into the membranes did not significantly increase SRB leakage from LUVs.

Previous studies implicated cholesterol as an important membrane component in EPEC and EHEC virulence (41, 42). Consequently, we evaluated the requirement of cholesterol in EspD pore formation by replacing a portion of the DOPC (1) in SM/DOPC/DOPS/Chl (45:26:20) with increasing amounts of cholesterol (X). Interestingly, LUVs lacking cholesterol, but containing 20 mol % DOPS, showed ~30% dye leakage, Fig. 5C. Augmenting the level of cholesterol in membranes to 20%, an amount typically found in mammalian cell plasma membranes, did not significantly increase dye release at pH 4.5 or 7.2. This result indicated that the presence of cholesterol in lipid bilayers was not a prerequisite for EspD pore formation.

To assess whether phosphatidylycerine was necessary for the initial contact of EspD with the membrane surface or for penetration of EspD through the lipid bilayer, binding experiments and dye leakage assays were performed in the presence of high NaCl concentrations. Addition of EspD to LUVs diluted in buffer containing either 0.5 or 1.0 M NaCl followed by sucrose density centrifugation to resolve LUV bound from unbound EspD revealed that in the presence or absence of high salt EspD (at an EspD/PL mol ratio of >1:4000) was quantitatively recovered with LUVs at the top of the sucrose gradient at pH 4.5 or 7.2 (data not shown). These data suggest that electrostatic interactions are not essential for promoting the initial contact of EspD with the membrane. This was not unexpected because previous studies suggested that an N-terminal amphipathic helix facilitated initial integration into the outer leaflet of the bilayer (29).

To evaluate the impact of phospholipid composition on the EspD-membrane interaction affinity, binding studies were performed by titrating SM/DOPC/DOPS, SM/DOPC/DOPS/Chl, or SM/DOPC/DOPS/Chl SUVs with increasing concentrations of EspD at pH 4.5 or pH 7.2 and the level of EspD bound to SUV quantified by ELISA following sucrose density centrifugation to separate free EspD. Saturation kinetics was observed for all three types of SUV, which suggested that the presence or absence of DOPS, DOPE, or cholesterol did not significantly alter the initial binding of EspD to these lipid bilayers. Similar binding kinetics were observed when the initial binding reaction was performed at pH 4.5 or 7.2 (Fig. 5D). It is interesting to note, however, that a slightly lower \( B_{max} \) value was obtained when the binding was performed at pH 7.2. Curve fitting of the data revealed a single binding isotherm with apparent dissociation constants ranging from ~67 to 132 nm for the EspD-membrane interaction. No dramatic difference in EspD binding affinity was observed with membranes lacking cholesterol (Table 1).

The importance of the anionic membrane for pore formation was next examined by performing dye leakage assays in the presence of increasing concentrations of NaCl. Leakage assays performed at pH 4.5 with SM/DOPC/DOPS/Chl LUVs revealed that NaCl above ~0.4 M NaCl caused a rapid diminishment of SRB release to ~17%, levels that were comparable with the leakage induced by EspD at pH 7.2 in the absence or presence of NaCl (Fig. 5E). Control experiments performed with the addition of NaCl alone to SRB-loaded LUVs showed no significant dye release. These data, together with the flotation assays, suggest that the presence of anionic lipids in the bilayer is important for EspD penetration through the membrane or in driving formation of a functional pore.

Initial dye leakage assays were performed with phospholipids containing dioleoyl fatty acids. To more closely approximate the fatty acid composition of phospholipids in the mammalian cell plasma membrane, assays were repeated with LUV containing POPC and POPS. Addition of increasing concentrations of EspD to LUV composed of dioleoyl phospholipids at pH 4.5 resulted in robust release of SRB, which reached a plateau of ~60% at an EspD/PL mole ratio of 1:500 (Fig. 5F). Similar experiments performed with LUV composed of POPC and POPS resulted in a dye leakage of ~57% at a EspD/PL ratio of 1:500. The rate of SRB leakage was notably lower and suggested that the reduced fluidity of the membrane containing POPC and POPS lipids impact the kinetics of pore formation (Fig. 5F).

Parallel experiments performed with DOPC/DOPS and POPC/POPS LUVs at pH 7.2 showed a linear response that suggested that the accumulation of high levels of this protein on the lipid bilayer likely caused a disruption of the membrane integrity that may not involve pore assembly (Fig. 5F).

**Orientation of EspD in Membranes—**To investigate the topology of the EHEC EspD in SM/DOPC/DOPS/Chl LUV at pH 4.5 and 7.2, we exploited the three Trp residues located on amphipathic helix I (Trp-47) (29) or within the first transmembrane helix (Trp-178 and Trp-195) as intrinsic fluorescence probe to evaluate the interaction of these secondary structural elements with the lipid bilayer (Fig. 1). To dissect these protein-membrane interactions, three espD variants containing only Trp-47, Trp-178, or Trp-195 were generated by introducing the mutations W178F, W195F, W47F, W195F, or W47F, W178F, respectively. Sucrose density flotation centrifugation experiments showed that at pH 7.2 all three espD mutants exhibited membrane binding activity similar to wild type EspD (Fig. 6A).

Dye leakage assays demonstrated that at pH 7.2, like the wild type EspD, none of the espD mutants triggered notable dye leakage. However, at pH 4.5, all three espD mutants induced dye release comparable with the wild type EspD (Fig. 6B).

Fluorescence spectroscopy analysis of the espD Trp-47, Trp-178, or Trp-195 mutants in the absence of SUVs at pH 4.5 or 7.2 showed that espD mutants exhibited an emission \( \lambda_{max} \) centered at ~346 nm (Fig. 6, C–E). The membrane binding of espD Trp-47 to SM/DOPC/DOPS/Chl SUVs at pH 4.5 and 7.2 resulted in a 4-nm blue shift in the emission \( \lambda_{max} \) consistent with this residue inserting into a more nonpolar environment (Fig. 6C). A similar 3-nm blue shift in the emission \( \lambda_{max} \) was observed when espD Trp-195 bound to SUVs at pH 4.5 (Fig. 6E). However, no notable change in the emission \( \lambda_{max} \) of the
Trp-178 mutant was detected in the presence of SUVs (Fig. 6D), which was diagnostic of Trp-178 localizing near the polar/nonpolar interface of the lipid bilayer. The binding of the three espD mutants to SUV membranes was accompanied by a notable decrease in the fluorescence intensity at pH 4.5 and 7.2. The only exception was espD Trp-47, which exhibited a slight increase in fluorescence intensity on binding SUVs at pH 4.5.

The espD mutants were used in dual quenching experiments that employed the quenching agents 10-DN and acrylamide (43) to evaluate the depth to which the Trp residues inserted into the lipid bilayer. Acrylamide preferentially quenches solvent-exposed or Trp residues located at the polar/nonpolar interface of the lipid bilayer. In contrast, 10-DN, a probe that embeds in the lipid bilayer core, quenches Trp residues that insert near the hydrophobic core of the membrane. The espD Trp-47, espD Trp-178, and espD Trp-195 mutants bound to SUVs at pH 4.5 showed a fluorescence quenching with acrylamide ranging from 0.82 to 1.22 (Table 2), values that are diagnostic of these Trp residues penetrating into the bilayer and becoming partially protected from acrylamide. Parallel experiments performed with SUVs containing 10-DN revealed that espD Trp-178 and espD Trp-195 were quenched to a greater extent than espD Trp-47 at pH 4.5 (Table 2). The Q ratios calculated for espD Trp-47, espD Trp-178, and espD Trp-195 were 2.49, 1.1, and 0.65, respectively (Table 2). The lower the value for the Q ratio, the deeper the Trp penetrates into the membrane. These values suggested that at pH 4.5, Trp-47 inserts into the membrane and localizes to the region near the polar/nonpolar interface, whereas the transmembrane helix containing amino acids Trp-178 and Trp-195 is predicted to insert perpendicularly into the membrane positioning these residues at different depths within the lipid bilayer.

FIGURE 6. Orientation of EspD in the lipid bilayer. A, capacity of the EspD (WT) and espD Trp-47 (W47), espD Trp-178 (W178), and espD Trp-195 (W195) mutants to bind SM/DOPC/DOPS/Chl LUVs was assessed by Western blot analysis of sucrose density centrifugation fractions. B, pore-forming activity of the espD mutants was assessed by monitoring SRB release from SM/DOPC/DOPS/Chl LUVs at pH 4.5. Insertion of espD Trp-47 (C), espD W Trp-178 (D), or espD Trp-195 (E) into SM/DOPC/DOPS/Chl SUVs membranes at pH 4.5 or 7.2 was examined by intrinsic fluorescence spectroscopy at an excitation wavelength of 295 nm. Spectra were corrected by subtracting traces with SUVs alone. F, depth of tryptophan insertion into the lipid bilayer at pH 4.5 or 7.2 was assessed by acrylamide-induced fluorescence quenching. For fluorescence spectroscopy experiments, an EspD/PL ratio of 1:500 was used.
At pH 7.2, acrylamide caused only a modest quenching of espD Trp-47 and espD Trp-195 bound to SUV. With 10-DN-containing SUVs, espD Trp-47 showed minimal quenching, which was indicative of this Trp inserting near the phospholipid headgroup interface. The espD Trp-195, however, exhibited a higher degree of quenching with 10-DN (Table 2), consistent with Trp-195 penetrating deeply into the hydrophobic region of the outer leaflet of the membrane. This association with the outer leaflet was supported by the observation that EspD failed to form a functional pore at pH 7.2. In contrast, espD Trp-178 exhibited a significant degree of quenching with 10-DN, which implied that Trp-178 had considerable conformational flexibility that allowed this residue to shift in the membrane between a phospholipid headgroup interface and the nonpolar alkyl chain region of the outer leaflet (44). The large Q ratio obtained with espD Trp-47 at pH 7.2 was consistent with this residue penetrating deeply into the outer leaflet of the membrane. In contrast, Q ratios calculated for Trp-178 and Trp-195 predict that the transmembrane domain containing these aromatic residues inserts into the bilayer and positioned both residues at similar depths in the hydrophobic core (Table 2).

To further assess the orientation of the Trp residues when EspD is bound to the membrane, Stern-Volmer experiments were performed by titrating espD mutant-loaded SUVs loaded with increasing concentrations of acrylamide. Comparable Stern-Volmer constants ($K_{SV}$) obtained for espD Trp-47 at pH 4.5 and 7.2 (Fig. 6F and Table 3) suggest that regardless of the environmental pH, Trp-47 inserts into the membrane near the phospholipid headgroups. At pH 4.5, espD Trp-178 and espD Trp-195 had $K_{SV}$ of $\sim 5.4$ m$^{-1}$ (Fig. 6F and Table 3), values that were notably higher than $K_{SV}$ values for espD Trp-47. These differences suggest that Trp-178 and Trp-195 residues are less solvent-accessible and likely insert near the polar/nonpolar interface of the membrane. It is possible, however, that the espD oligomerization accompanying pore formation may shroud these aromatic residues from acrylamide quenching. However, at pH 7.2, the $K_{SV}$ values for Trp-178 and Trp-195 were dramatically higher (Table 3); this response indicated that the helix spanning residues 176–200 inserted into the membrane in an orientation that was parallel to the lipid bilayer. This positions these Trp residues near the hydrophobic core and protected them from acrylamide quenching.

**Membrane Topology of EspD**—Studies with N- and C-terminal fragments of EspD showed that amphipathic domains and coiled-coil motifs in these regions (Fig. 1) were important for membrane binding and oligomerization on the host cell membrane (29, 30). To evaluate the requirement of these regions for pore formation, EspD was treated with clostripain, an arginine-specific endoproteinase that selectively removed residues 1–94 and 296–373. Western blot analysis of digests showed a progressive truncation of EspD from the N and C termini resulting in a predicted protease-resistant fragment spanning residues 95–295 (Fig. 7A). Dye leakage assays performed at pH 4.5 revealed that this core fragment containing the transmembrane helix hairpin and COIL I (Fig. 7B), although capable of binding to LUV membranes (Fig. 7C), induced a dye leakage of $\sim 14\%$ compared with the 35% leakage obtained with the full-length protein. It is tempting to speculate that removal of the COIL II motif impairs the capacity of the espD(95–295) fragment to oligomerize and form a pore.

To further examine the EspD membrane topology, EspD-loaded SM/DOPC/DOPS/Chl LUVs were treated with trypsin in the absence or presence of 0.5% Triton X-100. LUVs loaded at pH 4.5 and then digested with trypsin at pH 7.5 exhibited a notable resistance to proteolysis and required $\sim 60$ min for $\sim 80\%$ degradation of the parent protein (Fig. 7D). This resistance was attributed to EspD oligomerization and membrane insertion. Surprisingly, addition of Triton X-100 to LUVs loaded at pH 4.5 did not alter the degradation kinetics of EspD and suggested that the quaternary structure associated with pore formation contributed to the diminished rate of proteolysis (Fig. 7D). This conjecture is partially supported by the finding that EspD incubated in the presence of 0.5% Triton X-100 at pH 4.5 prior to shifting the pH to 7.5, for trypsin digestion, exhibited increased resistance to proteolytic cleavage and required $\sim 15–30$ min to achieve $\sim 60\%$ degradation. Alternatively, the association of the EspD complex with detergent-resistant microdomains at 0 °C may protect EspD from tryptic proteolysis (37). In contrast, LUVs loaded with EspD at pH 7.2 was highly susceptible to proteolysis and was completely degraded within 5 min, indicating that EspD was associated with the outer surface of the membrane and exposed to the protease (Fig. 7D). Solubilization of the latter LUVs with Triton X-100 did not alter this susceptibility to trypsin proteolysis.

**Pore Formation by EspD**—The effect of EspD concentration on pore formation was investigated by titrating SM/DOPC/DOPS/Chl LUVs with increasing concentrations of EspD and monitoring SRB release. At pH 5.5 and 7.2, SRB release increased linearly to $\sim 70\%$ leakage at an EspD/PL ratio of

### Table 2

**Quenching and Q ratio values**

| Parameter  | pH 4.5 | pH 7.2 |
|------------|--------|--------|
| Acrylamide$^a$ | 1.21   | 0.83   |
| 10-DN$^b$    | 0.49   | 0.20   |
| Q ratio      | 2.49   | 4.10   |

$^a$ Acrylamide quenching = $(F_0/F_{acryl}) - 1$.

$^b$ 10-DN quenching = $(F_0/F_{10-DN}) - 1$.

### Table 3

**Acrylamide quenching Stern-Volmer constants**

| Mutant  | pH 4.5 | pH 7.2 |
|---------|--------|--------|
|         | $K_{SV}$ | $K_{SV}$ |
| Trp-47  | 2.3 ± 0.3 | 2.8 ± 0.03 |
| Trp-178 | 5.4 ± 0.7 | 10.7 ± 1.0 |
| Trp-195 | 5.5 ± 0.4 | 18.2 ± 1.8 |
Oligomerization of EspD on model membranes at pH 4.5 or 7.2 was examined by treating SM/DOPC/DOPS/Chl LUVs loaded with EspD at pH 4.5 or 7.2 with 0.4 mM glutaraldehyde to pH 7.2 was examined by treating SM/DOPC/DOPS/Chl LUVs loaded with EspD at pH 4.5 then shifted to pH 7.2, to facilitate the cross-linking reaction, showed that a dimeric structure was rapidly trapped. Under these conditions, additional high order species migrating as a smear of ~245 kDa were also observed (Fig. 8C, upper panel). EspD oligomerization on membranes is further supported by the disappearance of the EspD monomer as a function of cross-linking reaction time (Fig. 8C, lower panel).

COIL I and II motifs (Fig. 1) have been proposed to mediate EspD-EspD interactions (29, 30). To assess the role of the COIL I in EspD oligomerization, cross-linking experiments were performed with the EspD fragment encompassing residues 95–295 generated by clostripain digest (Fig. 7B). Glutaraldehyde treatment of this espD(95–295) loaded onto SM/DOPC/DOPS/Chl LUVs at pH 4.5 or 7.2 resulted in a rapid the entrapment of an ~40-kDa dimer. Prolonging the reaction time failed to trap additional higher order species (Fig. 8D).

Solubilization of LUVs loaded with recombinant EspD at pH 4.5 with the nondenaturing detergent tauradeoxycholate and then analyzing the complexes from EspD by rate zonal centrifugation on a linear sucrose gradient showed that the bulk of EspD was detected in fractions 1–3 where the monomeric form of recombinant EspD would migrate. However, a small portion of the EspD migrated with a mass of ~280–320 kDa. It is unclear whether the abundance of EspD observed in the top fraction of the sucrose gradient is due to EspD that failed to oligomerize or a result of dissociation of the EspD complex following detergent extraction (Fig. 8E). Similar rate zonal centrifugation analysis of the EspD quaternary structure on LUVs loaded at pH 7.2 showed that EspD migrated nearly quantitatively as a monomeric species (Fig. 8E). These data suggest that membrane insertion is likely required for the assembly or stabilization of the EspD oligomeric structure.

To determine whether similar oligomeric structures were formed on host cell membranes during EPEC infection, membranes from HeLa cells infected with E. coli E2348/69 were extracted, and complexes formed by native EspD were examined. As with the recombinant EspD, both monomeric and oligomeric complexes with a mass of ~280–320 kDa were also detected in the mammalian cell membrane (Fig. 8E). For both the recombinant and native EspD, it is unclear whether the monomeric species detected in these samples is due to membrane-associated EspD that failed to oligomerize or a result of complex dissociations following membrane extraction. The presence of the 280–320-kDa complex, however, suggests that in membranes EspD assembles into structures containing 6–7 subunits.

Discussion

In this study, we used recombinant EHEC EspD to examine the biochemical events associated with membrane binding, insertion, and pore formation in model membranes. Nascent EspD is predicted to associate CesD and CesD2, chaperones that are important for maintaining EspD in an unfolded state and preventing EspD from aggregation to the E. coli cytosolic compartment (45, 46). On contact with epithelial cells, EspD is injected through the EspA needle complex (47) and is postulated to make an initial contact with the host membrane via an N-terminal amphipathic helix. This membrane-associated protein is thought to undergo oligomerization and subsequently
insert into the membrane to form a translocation pore. To mimic this process in vitro, we used unfolded recombinant EspD to investigate the interaction of EspD with model LUV membranes.

A number of studies have suggested that cholesterol may be an important membrane component for EPEC and EHEC adherence, effector protein translocation, and pedestal formation (48–51). However, in these studies using LUVs with increasing concentrations of cholesterol, we demonstrated that this sterol was not required for EspD binding or pore formation. This contrasts previous observations with the T3SS proteins SipB and IpaB that not only exhibited a high binding affinity for cholesterol but this sterol was also required for efficient injection of T3SS effector proteins into mammalian cells (42, 49, 52).

Consequently, it is tempting to speculate that for EPEC and EHEC infections, cholesterol may be required for the function of T3SS effector proteins that interact with membranes (41, 51).

Membrane binding revealed that, with the exception of phosphatidylethanolamine, EspD spontaneously bound to unilamellar vesicles containing single dioleoyl or palmitoyl-oleoyl phospholipids or lipid mixtures containing sphingomyelin. Biophysical analysis confirmed that EspD-membrane interaction is stabilized by contacts with the hydrophobic core of the lipid bilayer. Surprisingly, although EspD bound to SM/DOPC/Chol vesicles, a composition resembling the outer leaflet of the mammalian cell plasma membrane (35), it failed to induce pore formation unless the anionic lipids phosphatidylserine or phosphatidylycerol were incorporated into the

FIGURE 8. Characterization of the EspD pore. A, effect of pH and EspD concentration on pore formation was examined by titrating SRB SM/DOPC/DOPS/Chl LUVs with increasing concentrations of EspD at pH 4.0, 5.5, and 7.2, and the percent dye release was plotted as a function of the EspD/phospholipid ratio. B, diameter of the EspD pore formed at pH 4.5 was determined with LUVs loaded with carboxyfluorescein or FITC labeled 4-70 kDa dextrans. The oligomeric state of EspD on LUVs at pH 4.5 or 7.2 was assessed by glutaraldehyde cross-linking, and complexes were examined by Western blot on a 6% (upper panel) and 10% (lower panel) SDS-polyacrylamide gel (C). D, clostripain fragment of EspD encompassing residues 95–295 were glutaraldehyde cross-linked after loading protein onto LUVs at pH 4.5 or pH 7.2. E, complexes formed by recombinant and native EspD on LUV and HeLa cell membranes were examined by rate zonal centrifugation on a linear sucrose gradient following detergent extraction of complexes.
bilayer. This prerequisite suggests that phospholipid head groups may contribute to the folding or penetration of EspD across the membrane (38, 53, 54). A similar requirement for negatively charged phospholipids has been noted for the action of the bacterial toxin colicin Ia (55) and the T3SS proteins PopB/D, IpaB, and SipB (38, 39, 52, 54, 56). Under homeostatic conditions, the plasma membrane exhibits an asymmetric phospholipid distribution with phosphatidylserine and phosphatidylethanolamine being located predominantly on inner leaflet of the membrane. However, contact of EPEC or EHEC with the epithelial cell mediated by the bundle forming pili causes an increased translocation of phosphatidylserine to the host cell surface (41, 57–59). In this study, we show that a modest amount of phosphatidylserine (5 mol %) was sufficient to facilitate insertion of the transmembrane helix hairpin resulting in pore formation by EHEC EspD.

The N-terminal amphipathic regions of EspD were shown to be important for infectivity and pedestal formation (29). Intrinsin fluorescence techniques confirmed that the helix containing Trp-47 (Fig. 1, Amph I) inserts into the polar/nonpolar membrane interface providing an initial contact that anchors EspD to the host membrane as it emerges from the T3SS needle complex. Fluorescence dual quenching and proteolytic mapping studies further support a model where at pH 7.2 the amphipathic and transmembrane helices bind the membrane by inserting in a parallel configuration into the outer leaflet of the lipid bilayer (Fig. 9A). However, this association is not sufficient to promote pore formation as indicated by dye and FITC dextran release assays. A similar activity has been reported for SipB and IpaB that spontaneously insert into host membranes but failed to induce hemolysis or the assembly of a functional pore (42, 52, 56). In contrast, glutaraldehyde cross-linking and rate zonal centrifugation experiments showed that EspD, which formed functional pores on LUVs at pH 4.5 or were isolated from infected HeLa cell membranes, formed an oligomeric complex of ~280–320 kDa estimated to contain 6–7 EspD subunits. The arrangement of 12–14 transmembrane helices in a barrel configuration (60) would be sufficient to form a pore with an inner diameter of ~2.5 nm as measured for EspD, a diameter comparable with the size of the central channel of the needle apparatus formed by EspA (61). Surprisingly quaternary structure analysis revealed that EspD bound to LUV membranes at pH 7.2 was monomeric. However, it is not possible to discount the possibility that at the membrane surface EspD may form transient higher order structures that, following penetration of the lipid bilayer, would be stabilized by the packing of the transmembrane domains.

EspD that inserts into membranes at neutral pH forms oligomeric structures that are stabilized by COIL I-COIL I and COIL II-COIL II contacts (29, 30, 62) that are formed between adjacent EspD subunits. These oligomeric complexes form a pre-pore-like structure (Fig. 9A). This model is supported by cross-linking showing that espD95–295 formed predominantly dimers stabilized by COIL I-COIL I contacts (29) and despite retaining the transmembrane helix hairpin had impaired dye release activity likely due to the compromised capacity for this fragment to stable oligomers as a result of the loss of the COIL II domain. Formation of a functional pore was dependent on the incorporation of an anionic phospholipid into membranes and an acidic pH, conditions that drive the penetration of helices of channel-forming toxins and antimicrobial peptides into the lipid bilayer (39, 40, 54, 63–66). It is possible the insertion of EspD proceeds via a toroidal pore-forming mechanism that is known to be influenced by lipid composition (67) and may explain the requirement of anionic phospholipids for pore formation. It is conjectured that under acidic conditions the key amino acids on EspD become protonated, which lowers the thermodynamic barrier associated with insertion of charged residues through the hydrophobic core of the lipid bilayer (68, 69). It is interesting to note that in the presence of high salt the dye release from LUVs was dramatically diminished, which
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suggests that an electrostatic interaction between EspD and negatively charged lipids may be important for pore formation. This is further supported by binding studies suggesting that the interaction affinity of EspD with membranes was not significantly altered by the presence of phosphatidylycerine or cholesterol in the lipid bilayer. The absence of the former phospholipid did impair pore formation. Because the N and C termini of EspD are predicted to remain on the extracellular surface of the plasma membrane, the conversion of the EspD pre-pore to pore structure requires the translocation of the transmembrane hairpin loop spanning residues 200–227 (Fig. 9B) into the cytosolic compartment of mammalian cells. Dye leakage assays revealed that EspD pore formation was dramatically enhanced at a pH below 4.7, a value that approximates the $pK_a$ value for glutamate and aspartate ionization. Interestingly, the EHEC EspD hairpin loop contains two acidic amino acids, Asp-211 and Glu-215 (Fig. 9C), that would require protonation for penetration through the membrane that results in the re-orientation of the transmembrane helices from a parallel to perpendicular configuration with the membrane and packing of the helices from 6 to 7 EspD subunits to form a functional pore (Fig. 9B). Despite notable variations in length, the hairpin loops connecting the transmembrane domains of YopB, PopB, SipB, and IpaB all contain a number of acidic residues (Fig. 9C). Taken together with the observation that these translocator proteins display increased pore-forming activity at low pH, it is tempting to speculate that these charged loop residues play a pivotal role in pore assembly. However, this hypothesis needs to be validated by site-directed mutagenesis studies.

In the intestinal tract, it is conceivable that a localized acidic environment required for EspD membrane insertion may be generated by the following: (i) the metabolism of pathogenic E. coli microcolonies attached to epithelial cells (70); (ii) up-regulating the activity of the sodium-hydrogen exchanger 2 antiporter, which may cause a decrease in the cell surface pH (70, 71); and (iii) down-regulation of the monocarboxylate transporter 1 activity, which would result in the extracellular accumulation of short chain fatty acid metabolites and a decreased pH at the apical surface of the gut epithelial cells (72). It is also possible that contacts with EspA and EspB may be instrumental in driving EspD membrane insertion and pore assembly (28, 30).

Author Contributions—A. J. conceived, coordinated, designed experiments, and wrote the manuscript. A. C. designed and performed the bulk of experiments and assisted in data analysis. C. C.-F. was involved in generating immunological reagents and design of experiments in Fig. 3. D. B. and S. T. were involved with the preliminary EspD expression studies and HeLa cell experiments.

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