Enteropathogenic *Escherichia coli* Type III Effectors EspG and EspG2 Disrupt the Microtubule Network of Intestinal Epithelial Cells

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Enteropathogenic *Escherichia coli* infection of intestinal epithelial cells leads to localized depletion of the microtubule cytoskeleton, an effect that is dependent on delivery of type III translocated effector proteins EspG and Orf3 (designated EspG2) to the site of depletion. Microtubule depletion involved disruption rather than displacement of microtubules.

Induction of an attaching and effacing (A/E) lesion on the intestinal mucosa is a pathogenic mechanism shared by a number of enteric human and animal pathogens, including enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) (reviewed in reference 15). A/E lesion formation is characterized by localized destruction (effacement) of brush border microvilli, intimate attachment of the bacillus to the host cell plasma membrane, and formation of an underlying pedestal-like structure in the host cell, a process that involves exploitation of both the host cell actin (16) and intermediate filament cytoskeletons (1).

The genes encoding the A/E phenotype are encoded on a pathogenicity island (PAI) termed the locus of enterocyte effacement (LEE) (9, 20). The LEE encodes a filamentous type III secretion system (FTTSS) (6, 17, 23) that delivers effector proteins which subvert host cell physiology for the benefit of the extracellular bacterium (10). Five translocated effector proteins (Map, Tir, EspF, EspG, and EspH) are encoded within the LEE (reviewed in reference 11). A number of type III EPEC and EHEC effector proteins have recently been shown to be carried on prophages (2, 12, 21).

The LEE-encoded effector EspG does not appear to be involved in A/E lesion formation (8, 24), but it does share homology with a region of the VirA effector of *Shigella* which interacts with tubulin heterodimers and causes microtubule instability (26). In some EPEC strains, an EspG-like protein (Orf3) is found on a different PAI termed EspC (8). This suggested microtubules as a likely target for EspG and Orf3. EPEC colonizes brush border cells of the human small intestine. The aim of this investigation was to determine the effect of EspG and Orf3 on the microtubule network following EPEC infection of intestinal brush border cells.

EspG and Orf3 are translocated by the LEE FTTSS into host cells. EspG has been shown to be translocated into host cells by the LEE FTTSS (8). Secretion of Orf3 by wild-type

EPEC but not by an FTTSS mutant similarly suggested that Orf3 is also a type III effector protein (19). To confirm this, we used a novel fluorescence method based on a translational fusion of the protein of interest with mature TEM-1 β -lactamase. Translocation can be detected directly within live host cells using the fluorescent β -lactamase substrate CCF2/AM (3). We performed this translocation assay infecting HeLa cells with wild-type E2348/69 (18) and, as control, FTTSS (*escF*) mutant EPEC strains carrying pICC305 and pICC306, plasmids which encode a translational fusion of EspG and Orf3 to TEM-1 β -lactamase under the control of an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter (Table 1). We verified the production of the fusion proteins in whole bacterial cell lysates by Western blot assay (data not shown) and analyzed their translocation into infected HeLa cells (Fig. 1). Uninfected HeLa cells or cells infected with EPEC expressing pCX340 (negative control, empty vector) appeared green, indicating the absence of TEM-1 activity (Fig. 1b). In contrast, cells infected with bacteria expressing EspG-TEM or Orf3-TEM appeared blue (Fig. 1c and d), indicating that TEM-1 was translocated into the host cells. Moreover, this translocation was fully dependent on an active type III secretion system, given that no translocation was observed when HeLa cells were infected with the *escF* mutant strain carrying pICC305 or pICC306 (Fig. 1e and f). These results confirm Orf3 as a type III translocated protein.

EPEC induces microtubule depletion in intestinal epithelial cells. We used polarized monolayers of Caco-2 intestinal brush border cells grown on permeable filters as a model of EPEC infection which more closely resembles the small intestinal mucosa than the commonly used undifferentiated epithelial cells of nonintestinal origin. Twelve-day-old fully differentiated Caco-2 cells were infected with a synchronized bacterial culture (primed bacteria) which, due to expression of bundle-forming pili (13), results in the formation of bacterial aggregates which rapidly adhere to Caco-2 cells. Priming also results in expression of the LEE FTTSS and intimin such that bacteria rapidly (<30 min) translocate effectors and produce A/E lesions on contact with host cells (5). In this study, to examine microtubules, cells were infected with primed bacteria for 1 h, washed to remove nonadhering bacteria, extracted in 0.5%

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TABLE 1. Strains and plasmids used in the study

Strain or plasmid	Description	Source or reference
Strains		
E2348/69	Wild-type EPEC O127:H6	18
UMD880	<i>espA</i> in <i>E. coli</i> E2348/69	4
ICC171	<i>escF</i> ::Km ^r in <i>E. coli</i> E2348/69	25
SE710	<i>espG</i> ::pJP5603 in <i>E. coli</i> E2348/69	8
SE1207	<i>espG</i> ::pJP5603 Δ <i>orf3</i> ::pJP5608 in <i>E. coli</i> E2348/69	8
ICC191	<i>orf3</i> ::Km ^r in <i>E. coli</i> E2348/69	This study
Plasmids		
pCX340	pBR322 derivative, cloning vector used to fuse genes to <i>blaM</i> , which encodes the mature form of TEM-1 β -lactamase	3
pICC305	pCX340 derivative, expressing EspG fused to TEM-1	This study
pICC306	pCX340 derivative, expressing Orf3 fused to TEM-1	This study
pCX327	pCX340 derivative, expressing residues 1–16 of Cif fused to TEM-1	3
pSA10	pKK177-3 derivative containing <i>lacI</i> ^a	22
pICC307	pSA10 derivative encoding C terminus HA-tagged EspG	This study

Triton X-100 for 30 s, and fixed in 0.3% glutaraldehyde in BRB80 (80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 6.8) buffer. Mouse monoclonal α - and β -tubulin (Sigma) or rat tubulin antibodies (AbCam) were used for microtubule staining in conjunction with Alexa₄₈₈ (green)- and Alexa₅₉₄ (red)-conjugated goat anti-mouse or anti-rat immunoglobulin G second antibody conjugates (Molecular Probes). Fluorescein- or rhodamine-conjugated phalloidin (Sigma) was used to stain cell actin, and bacteria were stained with propidium iodide (Molecular Probes). Fluorescence imaging was performed using a Leica TCS SPII Spectral Confocal Microscope, and confocal illustrations show either single-image sections or image projections through the cell.

In uninfected Caco-2 cells, microtubules were observed to be heavily concentrated in the apical region of the cell just below the brush border (Fig. 2). Following 1 h of infection with wild-type EPEC strain E2348/69, we observed localized areas

of microtubule depletion in the apical region of the cell which corresponded exactly to the site of adherent bacterial microcolonies (Fig. 2). This result shows that extracellular EPEC is able to disrupt or displace microtubules specifically from the cytosol immediately beneath adherent bacterial microcolonies. The lack of any observed effect on the microtubule cytoskeleton beneath microcolonies of E2348/69 mutant strain UMD880, which lacks an FTTSS (Fig. 2), shows that this effect on the microtubule cytoskeleton is dependent on a functional type III secretion system and effector protein translocation.

Caco-2 cell microtubule disruption is EspG/Orf3 dependent. Map, Tir, EspF, and EspH LEE effector protein mutants had no effect on the microtubule cytoskeleton of infected Caco-2 cells (data not shown). Wild-type EPEC E2348/69 possesses the EspC PAI and thus Orf3 (8). Based on homology with *Shigella* VirA and the fact that EspG can compensate for VirA in *Shigella*-induced microtubule disruption (26), EspG and Orf3 were the prime candidate effectors targeting the microtubule cytoskeleton. We therefore examined mutants deficient in *espG*, in *orf3*, and in both *espG* and *orf3*. An *orf3* mutant (ICC191) was constructed for the study in E2348/69 using the one-step λ Red recombinase method (7) and verified by PCR (Table 1). Following a 1-h infection, the *espG* mutant exhibited localized microtubule depletion similar to the wild type, as did the *orf3* mutant (Fig. 2). In contrast, the double *espG orf3* mutant did not induce microtubule depletion (Fig. 2). Complementation of this effect was assessed using the double-knock-out mutant possessing cloned *espG* (Table 1). Lack of efficient bundle-forming pilus expression by this strain meant that bacteria did not aggregate efficiently during priming. Consequently, complementation studies were carried out using unprimed bacteria and a 3-h infection. Although only small microcolonies of adherent bacteria were produced by this strain, it was clear, nevertheless, that the ability to disrupt the microtubule cytoskeleton was restored when the *espG orf3* double mutant was complemented with *espG* (Fig. 2). These results indicate that both EspG and Orf3 are effectors that can induce microtubule depletion and only when both effectors are absent is this effect eliminated. In view of this defined Orf3 phenotype, we renamed Orf3 EspG2.

EspG is localized to the region of microtubule depletion.

FIG. 1. Translocation of EPEC effector proteins EspG and Orf3 into live HeLa cells using TEM-1 fusion and fluorescence microscopy. HeLa cells were infected with E2348/69 expressing pCX327 (positive control) (a), pCX340 (negative control) (b), pICC305 (c), or pICC306 (d) or with EPEC *escF* expressing pICC305 (e) or pICC306 (f). Blue fluorescence demonstrates translocation of EspG and Orf3 when expressed in the E2348/69 background (c, d), whereas green fluorescence indicates lack of translocation when expressed from the type III secretion *escF* mutant (e, f).

FIG. 2. Confocal images showing uninfected Caco-2 cell monolayers (row 1); Caco-2 cells infected for 1 h with primed cultures of E2348/69 (row 2), type III secretion mutant UMD880 (row 3), the *espG* mutant (row 4), the *orf3* mutant (row 5), and the *espG orf3* double mutant (row 6); and cells infected for 3 h with an unprimed culture of the *espG orf3* double mutant complemented with cloned *espG*. Cells were stained for microtubules (MT; column 1) and bacteria (column 2). Columns 1 and 2 are maximum projections; column 3 shows transverse projections through cell monolayers that have also been stained for cellular actin (red). E2348/69-, *espG*-, and *orf3*-infected cells reveal microtubule depletion (column 1, asterisks) beneath adherent bacterial microcolonies (column 2, asterisks) but not in cells infected with UMD880 or the *espG orf3* double mutant. Microtubule depletion was restored with the *espG orf3* double mutant possessing cloned *espG*. Bars, 20 μ m.

FIG. 3. Confocal image projections through Caco-2 cell monolayers infected for 1 h with wild-type E2348/69 (a), E2348/69(pICC307) (b), *espG*(pICC307) (c), and *escF*(pICC307) (d) and stained for HA (green), actin (red), and bacteria (blue). EspG-HA is translocated into Caco-2 cells by E2348/69(pICC307) (b) and *espG*(pICC307) (c) and is localized beneath bacterial microcolonies. EspG-HA is not translocated by the type III mutant *escF*(pICC307) (d). Bar, 10 μ m.

Microtubule depletion could be due to depolymerization or displacement of microtubules, although, based on studies of *Shigella* VirA, EspG is likely to induce microtubule disruption as a direct result of binding to tubulin. We therefore localized EspG in infected Caco-2 cells and performed time course studies to determine the kinetics of EspG translocation and microtubule depletion. EspG was visualized using carboxy hemagglutinin (HA)-tagged EspG expressed from an IPTG-inducible promoter in wild-type E2348/69 and in the *espG* mutant with the FTTSS *escF* mutant used as control (Table 1). EspG-HA was detected using a monoclonal HA antibody (Covance). Following a 1-h infection with E2348/69(pICC307) or *espG*(pICC307), EspG was detected within Caco-2 cells and localized immediately beneath adherent bacterial microcolonies in the region of microtubule depletion (Fig. 3b and c). No labeling was seen in uninfected cells, in cells infected with wild-type E2348/69 lacking HA-tagged EspG (Fig. 3a), or in cells infected with the *escF*(pICC307) control strain, which was unable to translocate EspG-HA (Fig. 3d).

In a time course experiment (Fig. 4A), no translocated EspG was detected after 15 min, but it was present in cells after 30 min and subsequently up to 1 h. A/E lesion formation was also first detected after 30 min (data not shown), suggesting that

FIG. 4. Confocal images of Caco-2 cells (A, B) and HEP-2 cells (C) infected with E2348/69(pICC307). Panel A, cells infected for 15, 30, 45, and 60 min and stained for HA (green), actin (red), and bacteria (blue). Translocated EspG-HA is detectable in cells only after 30 min and then continuously up to 1 h. Panels B and C, cells infected for 30 min and stained for HA (green) and microtubules (MT; red). In Caco-2 cells, bacteria were stained blue. In HEP-2 cells, bacteria were visualized by phase contrast. Both panels show translocated EspG-HA beneath adherent bacterial microcolonies colocalized with areas of microtubule depletion. Microtubules appear to be disrupted rather than displaced. Bars: A and C, 10 μ m; B, 20 μ m.

FIG. 5. Confocal images showing uninfected Caco-2 cells (A) and Caco-2 cells infected for 1 h with wild-type E2348/69 (B). Cells were stained for actin (green) and bacteria (red), and image sections show the apical, basolateral, and basal regions of cells and z projections through the cell monolayer. Caco-2 cells possess an apical brush border rich in actin and membrane-associated basolateral and basal actin (A). E2348/69 infection results in bacterial adhesion and actin accretion at the apical brush border surface of Caco-2 cells but no detectable changes in basolateral and basal cell actin (B). Bars, 10 μ m.

EspG is translocated concurrently with Tir, which is essential to produce A/E lesions.

Since translocated EspG-HA was first detected after 30 min, we used this time point to examine colocalization of EspG-HA and microtubules. Unfortunately, under the conditions used for optimal microtubule staining, HA staining did not occur. We therefore used formalin-fixed cells to detect EspG-HA, which resulted in a less well-preserved microtubule cytoskeleton. Following a 30-min infection of Caco-2 cells, translocated EspG-HA colocalized with areas where microtubule depletion was just beginning to occur (Fig. 4B). A similar experiment using undifferentiated HEp-2 cells showed a similar colocalization of EspG-HA and areas of microtubule depletion, but in this case the resolution of the microtubule cytoskeleton clearly suggested disruption of microtubules (Fig. 4C). Evidence that microtubules were being displaced rather than depleted (i.e., increased fluorescence intensity adjacent to the site of microtubule depletion) was not observed. This is consistent with the recent report of Matsuzama et al. (19), who demonstrated direct interaction of EspG and Orf3(EspG2) with tubulin and the ability of these effectors to destabilize microtubules in vitro.

EspG involvement in actin microfilament rearrangements. The recent report by Matsuzama et al. (19) also showed that EspG and EspG2 modulate the host cell actin cytoskeleton. These effectors induced actin stress fiber formation in serum-starved HeLa epithelial and Swiss 3T3 fibroblast cells by a mechanism that involved release of microtubule-bound GEF-H1 and activation of the RhoA-ROCK signaling pathway (19). In Caco-2 cells, actin is concentrated in the apical brush border surface and also along the basolateral and basal cell surfaces (Fig. 5A). Following a 1-h infection, wild-type E2348/69 produced actin rearrangements and A/E lesions on the apical brush border surface of Caco-2 cells but we observed no alterations to the basal and basolateral actin cytoskeleton (Fig. 5B). The *espG*, *espG2*, and *espG espG2* mutants all pro-

duced A/E lesions on Caco-2 cells similar to the wild type and as with the wild type, there was no detectable alteration to the basal and basolateral actin cytoskeleton (data not shown). The different infection conditions used in this study compared to those of Matsuzama et al. might explain the observed differences in actin rearrangements, although this is unlikely since, in our experience, a 1-h infection with primed EPEC produces infection levels and cellular effects equivalent to a 3-h infection with unprimed bacteria. In polarized intestinal cells, we were unable to detect EspG/EspG2-dependent modulation of the actin cytoskeleton, which suggests that microtubule-dependent activation of signaling pathways promoted by these effectors in intestinal cells results in downstream effects different from those seen in nonpolarized epithelial cells and fibroblasts.

This and studies of other bacterial pathogens (14, 26) clearly show that bacterial pathogens have developed different strategies to modulate host cell microtubule networks. EPEC and shigellae destabilize the microtubule network, something that has been shown to be important for invasion by shigellae. The interesting questions that remain are what role microtubule destabilization might have in EPEC infection considering that the bacterium remains extracellular and whether EspG and EspG2 have identical functions, as suggested by this study, or have complementary activities that remain to be distinguished.

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