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Enteropathogenic *E. coli* Effectors EspG1/G2 Disrupt Microtubules, Contribute to Tight Junction Perturbation and Inhibit Restoration

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Summary

Enteropathogenic *Escherichia coli* (EPEC) uses a type 3 secretion system to transfer effector proteins into the host intestinal epithelial cell. Several effector molecules contribute to tight junction disruption including EspG1 and its homolog EspG2 via a mechanism thought to involve microtubule destruction. The aim of this study was to investigate the contribution of EspG-mediated microtubule disruption to TJ perturbation. We demonstrate that wild type EPEC infection disassembles microtubules and induces the progressive movement of occludin away from the membrane and into the cytosol. Deletion of *espG1/G2* attenuates both of these phenotypes. In addition, EPEC infection impedes barrier recovery from calcium switch, suggesting that inhibition of TJ restoration, not merely disruption, prolongs barrier loss. TJs recover more rapidly following infection with *espG1/G2* than with wild type EPEC, demonstrating that EspG1/G2 perpetuate barrier loss. Although EspG regulates ADP-ribosylation factor (ARF) and p21-activated kinase (PAK), these activities are not necessary for microtubule destruction or perturbation of TJ structure and function. These data strongly support a role for EspG1/G2 and its associated effects on microtubules in delaying the recovery of damaged tight junctions caused by EPEC infection.

Introduction

Enteropathogenic *E. coli* (EPEC) is an enteric pathogen and a major cause of infantile diarrhea in the developing world (Hill *et al.*, 1991, Fagundes-Neto *et al.*, 1997). Like most

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of the Gram-negative enteric pathogens, EPEC uses a type-3 secretion system (TTSS) to deliver bacterial effector proteins directly into the host cell cytosol (Jarvis *et al.*, 1995, Jarvis *et al.*, 1996, Wolff *et al.*, 1998). One of the well-established phenotypes of EPEC infection is relocation of tight junction (TJ) proteins to the cytosol and the associated perturbation of barrier function (McNamara *et al.*, 2001, Muza-Moons *et al.*, 2004, Shifflett *et al.*, 2005). Similar phenomena occur in vivo (Savkovic *et al.*, 2005, Shifflett *et al.*, 2005, Zhang *et al.*, 2010).

The effector EspG1 and its homolog EspG2 contribute to EPEC pathogenesis. EspG2 is 42% identical and 62% similar to EspG1 (Elliott *et al.*, 2001). EspG1 was first described as a homolog of the *Shigella flexneri* effector VirA to which it is 21% identical and 40% similar (Elliott *et al.*, 2001). An EPEC EspG1/G2 double mutant can be complemented by EspG1, EspG2 or VirA indicating a high level of functional conservation (Tomson *et al.*, 2005, Smollett *et al.*, 2006). Like VirA, the first phenotype to be ascribed to EspG1/G2 was disruption of microtubule networks. Infection with wild type EPEC disrupts microtubules and degrades tubulin; these phenotypes are present in a comparable degree if either EspG1 or EspG2 is deleted, but are absent following infection with an EspG1/G2 double mutant (Shaw *et al.*, 2005, Tomson *et al.*, 2005). Transient expression of EspG1 in epithelial cells induces microtubule disruption in the absence of other EPEC effectors (Tomson *et al.*, 2005). In reductionist systems, purified EspG1 or EspG2 bind to tubulin and depolymerize microtubules in solution without additional proteins (Matsuzawa *et al.*, 2004, Hardwidge *et al.*, 2005). The mechanism by which EspG1/G2 effect microtubule disassembly remains unknown, but studies of VirA suggest that it is neither mediated directly nor via protease activity (Germane *et al.*, 2008). EspG1/G2 also contribute to loss of epithelial barrier function as infection with wild type EPEC decreases transepithelial electrical resistance (TER) greater than that induced by a *espG1/G2* mutant (Tomson *et al.*, 2005). In studies comparing EspG1 and EspG2, both effectors induced similar increases in paracellular permeability, further reinforcing their functional similarity (Matsuzawa *et al.*, 2005).

The attaching/effacing pathogen enterohemorrhagic *E. coli* (EHEC) expresses a single EspG which is 98% identical and similar to EPEC EspG1 (Elliott *et al.*, 2001). Recent work has identified a new role for EHEC EspG as a regulator of GTPase signaling (Germane *et al.*, 2011, Selyunin *et al.*, 2011) EspG binds to ADP-ribosylation factor (ARF) and p21-activated kinase (PAK) (Germane *et al.*, 2011, Selyunin *et al.*, 2011). EspG-bound ARF is sequestered in its active, GTP-bound conformation, disrupting the normal guanine nucleotide cycle. PAK interaction with EspG is dependent on ARF binding and once bound, PAK activity is increased 7.6-fold (Selyunin *et al.*, 2011). A separate study reported that EspG binds to the Rac/Cdc42-binding site of PAK1 (Germane *et al.*, 2011). Although not demonstrated, the EspG-ARF_{GTP} interaction may impede Golgi trafficking and induce dispersal, however we note that Golgi dispersal is also a hallmark of microtubule disruption (Wehland *et al.*, 1983, Cole *et al.*, 1996, Yang *et al.*, 1998, Thyberg *et al.*, 1999, Clements *et al.*, 2011, Selyunin *et al.*, 2011). The PAK family of proteins acts downstream from Rac1 and Cdc42 GTPases and regulate cytoskeletal dynamics and cell motility. By enabling PAK activation EspG may promote actin remodeling regardless of the status of native Rac/Cdc42 (Bokoch, 2003). In addition, EspG-ARF_{GTP} has been shown to form an inhibitory ternary

complex with Rab1, interrupting host cell secretion (Dong *et al.*, 2012). EspG has also been reported to have significant Rab1 GAP activity (Dong *et al.*, 2012).

Despite its role in loss of TER, the mechanism by which EspG1/G2 contribute to TJ disruption has not been determined. Although a role for microtubules in epithelial TJ barrier function has not been conclusively demonstrated, it was recently reported that microtubules fortify barrier integrity in epidermal sheets (Sumigra *et al.*, 2012). We speculate that EspG-induced microtubule loss plays a role in EPEC-induced TJ dysfunction as both microtubules and TJs recover if infection is eradicated (Simonovic *et al.*, 2000, Tomson *et al.*, 2005). TJs are highly dynamic with occludin, claudin-1 and ZO-1 constitutively recycling between the membrane and the cytosol (Morimoto *et al.*, 2005, Nishimura *et al.*, 2008, Shen *et al.*, 2008, Dukes *et al.*, 2011). While microtubules are clearly involved in apical protein recycling, it is not known if they participate in the recycling of TJ proteins. It has been demonstrated, however, that disruption of microtubules with nocodazole significantly restricts the movement of occludin (Subramanian *et al.*, 2007, Glottfelty *et al.*, 2014). It is possible therefore that disruption of microtubules by EPEC EspG interferes with host cell homeostatic mechanisms that maintain the TJ in the steady state. The hypothesis of this study is that EPEC EspG1 impedes TJ recovery by disrupting microtubules thus perpetuating the loss of barrier function. The aim of this study is to determine the role of EspG1 in EPEC-induced TJ disruption and recovery. Herein we provide data that strongly support this hypothesis.

Results

EPEC infection induces internalization of occludin

It is well established that TJ proteins recycle continually between the membrane and cytosol (Morimoto *et al.*, 2005, Shen *et al.*, 2008, Dukes *et al.*, 2011, Dukes *et al.*, 2012). Our lab and others have shown that EPEC infection increases cytosolic occludin however it is not known if this is due to the internalization of membrane-associated occludin or the accumulation of newly synthesized protein moving through the Golgi (Simonovic *et al.*, 2000, McNamara *et al.*, 2001, Shifflett *et al.*, 2005, Guttman *et al.*, 2006). We therefore determined whether the cytosolic accumulation of occludin induced by EPEC infection is due to the internalization of membrane-associated occludin (Simonovic *et al.*, 2000, McNamara *et al.*, 2001).

For these studies, T84 intestinal epithelial cells were biotinylated as previously described in order to externally label all plasma-membrane bound proteins. (Morimoto *et al.*, 2005, Nishimura *et al.*, 2008). One group of monolayers was immediately stripped of biotin to demonstrate the efficacy of this step. Over a time course of EPEC infection, remaining extracellular biotin was stripped, infected cells were lysed, and internalized, biotinylated (intracellular) proteins were pulled down with avidin beads and immunoblotted for occludin. As expected, no occludin was pulled down from cells that were biotinylated and immediately stripped. EPEC infection caused a significant and progressive increase in occludin internalization compared to uninfected control cells (Fig. 1 and Supplementary Fig. 1). This suggests that the enhancement of cytosolic occludin over the course of EPEC infection is at least in part due to protein internalization indicating that an aspect of bacterial

pathogenesis may include impeding host cell mechanisms that aim to recycle occludin back to the plasma membrane in the steady state. These data help to clarify the mechanism of a well-established phenotype of EPEC infection.

EPEC infection delays TJ recovery

Since EPEC effectors appear to collectively impair the homeostatic process of occludin recycling, we queried whether they might delay TJ recovery from injury. To address this question, T84 monolayers were infected with wild type EPEC until TER decreased by ~40%, indicating that effector proteins had been translocated to the host cell cytosol. Calcium switch assays were then performed to determine the effect of EPEC on TJ recovery (Cassidy *et al.*, 1967, Cereijido *et al.*, 1981, Stuart *et al.*, 1994, Denker *et al.*, 1998, Farshori *et al.*, 1999). Calcium was chelated, which nearly ablated TER as expected, then calcium was reintroduced using media that also contained gentamicin to kill EPEC but permit cytosolic effector proteins to persist. Recovery of TER was monitored and compared between uninfected and previously infected monolayers. Uninfected control monolayers reached $84 \pm 6.6\%$ of baseline TER within 4 hours (Fig. 2). Monolayers which had been infected with EPEC then treated with gentamicin at the time calcium was restored, recovered TER to only $46 \pm 4.8\%$ of baseline within the same time suggesting that EPEC effector proteins impede TJ restoration from insult (Fig. 2).

EspG1/G2 shift localization of TJ proteins to the cytosol

Limited published data suggest that microtubules participate in the maintenance of TJ structure and function (Sumigraay *et al.*, 2012). Given that EspG1/G2 have been shown to perturb microtubules, we turned our investigation to their impact on TJ disruption in the context of EPEC infection. Caco-2 cells were used for this assay instead of T84 cells as Caco-2 cells have a phenotype resembling small bowel enterocytes, the primary target of EPEC, while T84s more resemble colonic crypt cells (Dharmasathaphorn *et al.*, 1984, Hidalgo *et al.*, 1989).

EPEC infection of Caco-2 intestinal epithelial cells for 4 hours resulted in severely fragmented or absent microtubules compared to control monolayers while cells infected with a *espG1/G2* strain displayed largely intact microtubule networks (Fig. 3A). Infection with *espG1/G2* complemented with *espG1* induced microtubule disruption in a manner comparable to infection with wild type EPEC. These data are in accord with previously published results (Hardwidge *et al.*, 2005, Shaw *et al.*, 2005, Tomson *et al.*, 2005).

Wild type EPEC infection of intestinal epithelial cells for 4 hours induced occludin accumulation in the cytosol, as previously reported (Fig. 3B) (Simonovic *et al.*, 2000, McNamara *et al.*, 2001). In contrast, following infection with *espG1/G2*, a lesser amount of cytosolic occludin was seen. Infection with *espG1/G2/pespG1* induced TJ disruption similar to the wild type phenotype (Fig. 3B). These data suggest that EspG1/G2-induced microtubule loss contributes to TJ disruption, possibly by impairing the constitutive recycling of occludin back to the membrane resulting in cytosolic accumulation.

The impact of EspG1/G2 on TJ proteins was not restricted to occludin. Staining of Madin-Darby canine kidney epithelial cells revealed that the localization of ZO-1 and claudin 1 was also perturbed following infection with wild type EPEC (Fig. 4A). Interestingly, EPEC had a less significant impact on the localization of claudin 4. Infection with *espG1/G2*, however, induced minimal change in the localization of ZO-1 and claudin 1 indicating a role for these effectors in TJ perturbation.

Infection of Caco-2 cells with wild type EPEC induced significant perturbation of ZO-1 and claudin 1, with peripheral protein continuity lost. Claudin 2 exhibited mislocalization to a lesser degree while claudin 4 localization was unaffected. (Fig. 4B). Infection with *espG1/G2*, again had a much lesser effect on ZO-1 and claudin 1 and -2, with overall preservation TJ structure. These data underscore the important role of EspG1/G2 in EPEC-mediated TJ disruption.

Deletion of *espG1/G2* promotes TJ recovery after EPEC infection

In view of our finding that EPEC infection delays TJ restoration in calcium switch assays, we examined the impact of EspG1/G2 on TJ restoration from EPEC-induced injury. Caco-2 monolayers were infected with wild type EPEC and *espG1/G2* and after TER decreased by ~40%, monolayers were washed, media containing gentamicin was added and recovery was monitored. Eight hours after the addition of gentamicin, wild type-infected monolayers attained only $62 \pm 1.3\%$ of baseline TER values (Fig. 5). *espG1/G2*-infected monolayer, on the other hand, recovered significantly more barrier function, $78 \pm 2.3\%$, suggesting that intact MT networks promote TJ recovery after EPEC infection (Fig. 5). It should be noted that the significantly longer infection time with *espG1/G2* is likely associated with a greater concentration of translocated bacterial effectors into these cells compared with wild type. However a more rapid recovery of TER after *espG1/G2* infection is observed, underscoring the potency of EspG1/G2 in preventing barrier restoration.

Pharmacological induced microtubule destruction compensates for EspG during *espG1/G2* infection

EspG1/G2 contribute to loss of barrier function. Cold temperatures and nocodazole induce loss of microtubules (Turner *et al.*, 1989, Baas *et al.*, 1994, Breton *et al.*, 1998, Banan *et al.*, 2000). To help discern if EspG's effect on TER is mediated via its impact on microtubules, these structures were perturbed in Caco-BBE cells using cold temperature and nocodazole treatment prior to infection with *espG1/G2*. Caco-BBEs were employed for these experiments because their higher baseline TER allows for a more reliable and sensitive assay. Cold temperatures and nocodazole alone decreased TER by $\approx 20\%$ (Fig. 6). Wild type infection rapidly decreased TER and infection with *espG1/G2* induced a gradual loss of TER as previously reported (Fig. 6) (Simonovic *et al.*, 2000, McNamara *et al.*, 2001, Tomson *et al.*, 2005). Infection of monolayers with disrupted microtubule networks (cold +nocodazole) with *espG1/G2* decreased TER more rapidly than the mutant alone, reaching TER levels comparable to wild type infection by 5 hours (Fig. 6). These data demonstrate that pharmacologic microtubule disruption compensates for the absence of EspG during *espG1/G2* infection and suggest that the contribution of these effectors to TER loss is at least in part due to their impact on microtubules.

EspG destroys microtubules, disperses the Golgi and perturbs TJs

Despite substantial literature documenting the microtubule-loss phenotype, there is debate surrounding the impact of EspG1/G2 on microtubules. One report showed Golgi dispersal 20 minutes after microinjection of EHEC EspG into rat kidney cells, but did not observe microtubule destruction (Selyunin *et al.*, 2011). A separate study demonstrated Golgi dispersal after transfection of EPEC EspG1 and EHEC EspG, but reported alterations in the structure of microtubules rather than frank depolymerization (Clements *et al.*, 2011).

To determine the effects of EspG1/G2 on microtubules in our model system, EPEC EspG1 was transiently overexpressed in Madin-Darby canine kidney epithelial monolayers, selected for enhanced transfection efficiency. Given the established functional similarity between EspG1 and EspG2, we used EspG1 in these assays. Eight hours following EspG1 transfection, microtubule networks were disrupted (Fig. 7A). Overexpression of the effector EspF, which has no reported impact on microtubules, did not alter these structures (Fig. 7A). Expression of RFP-EspG1, but not GFP-EspF, also induced progressive tubulin degradation, a phenotype associated with wild type EPEC infection (Fig. 7B) (Tomson *et al.*, 2005). In control cells, the Golgi exhibit a perinuclear localization (Fig. 7C). EspG1 induced a diffuse cytoplasmic Golgi stain indicating dispersal of the organelle (Fig. 7C). To confirm these results in an infection model, intestinal epithelial cells were infected with wild type EPEC and *espG1/G2* and stained for giantin. Wild type infection induced Golgi dispersal, whereas *espG1/G2*-infected cells retained a predominantly perinuclear Golgi localization, although some dispersal was observed, consistent with the presence of NleA (Fig. 7D) (Kim *et al.*, 2007).

To investigate the impact of EspG on TJs, attached cells were transfected with EspG1, fixed after 8 hours and stained for occludin. EspG1 induced occludin relocation from the plasma membrane to the cytosol, suggesting that occludin regulation is perturbed (Fig. 7E). Taken together, these data suggest that EspG1 has significant effects on microtubules and TJs. Prior studies reported that ectopic expression of EspG1/G2 did not affect TER but led to increased paracellular permeability to 4 kDa dextran (Matsuzawa *et al.*, 2005). These functional data concur with the significant structural phenotypes induced by EspG1.

The effects of EspG on microtubules are not dependent on ARF- and PAK-binding

The mechanism of EspG-mediated microtubule destruction is still unknown. Recent work has proposed a new role for EHEC EspG as a GTPase regulator, binding to and modulating ARF and PAK (Germane *et al.*, 2011, Selyunin *et al.*, 2011). To determine if these specific interactions participated in EspG-mediated destruction of microtubules, the effect of ARF- and PAK- binding deficient EHEC EspG was tested. The significant structural and functional conservation between EPEC EspG1 and EHEC EspG, they are 98% identical, imply that similar results would be obtained using EPEC constructs (Tomson *et al.*, 2005, Smollett *et al.*, 2006, Clements *et al.*, 2011).

Transient expression of wild type EHEC ARF- and PAK-binding mutants in MDCK epithelial cells induced microtubule disruption after 8 hours (Fig. 8). Expression of EspF had

no effect on microtubules (Fig. 7A). These data suggest that the interaction of EspG with ARF and PAK does not contribute to microtubule disruption.

In order to confirm the results obtained in the transfection model, cells were also infected with *espG1/G2* complemented with either ARF binding deficient or PAK binding deficient EHEC EspG and the impact on microtubules was assessed. The ARF- and PAK-binding mutants were cloned into a bacterial expression vector and to complement the *espG1/G2* strain. Infection of Caco-BBE cells with both of these strains induced microtubule disruption (Fig. 9A) confirming the data obtained in the transfection model. Infection with *espG1/G2/espG ARF* and *espG1/G2/espG PAK* also induced mislocalization of occludin from the membrane to the cytosol, similar to the phenotype observed with wild type EPEC infection and infection with *espG1/G2/espG* (Fig. 9B). Taken together, these data suggest that the ability of EspG to interaction with ARF and PAK does not contribute to microtubule destruction and downstream effects on TJ structure.

Bacterial delivery of EspG binding mutants induces TER loss

EspG1/G2 contribute to loss of TJ barrier function (Tomson *et al.*, 2005) therefore the role of EspG interaction with ARF or PAK on loss of TJ function was determined. TER of Caco-BBE intestinal epithelial cells decreased rapidly following infection with wild type EPEC (Fig. 10A) while infection with *espG1/G2* induced a significantly slower decline in TER as previously described (Fig. 10A) (Tomson *et al.*, 2005). Complementation of *espG1/G2* with EspG ARF- or PAK-binding deficient EHEC EspG restored the kinetics of TER loss to that seen with wild type EPEC infection (Fig. 10A) and *espG1/G2* complemented with wild type *espG1*. Complementation with an empty bacterial vector reproduced the *espG1/G2* phenotype (Fig. 10B). Taken together, these data suggest that the interactions of EspG with ARF and PAK small GTPases do not contribute to EspG-induced loss of TJ function.

Discussion

TJ disruption is a major component of EPEC pathogenesis. Despite identifying several effectors responsible for EPEC-associated disease, the mechanisms remain unclear. We show here for the first time that microtubules play a vital role in TJ recovery from EPEC infection. Our data strongly support a role for *EspG1/G2* in TJ disruption due to microtubule destruction and the sequelae. TJ disruption is triggered by a number of stimuli. Interferon- γ (IFN- γ) induces internalization of TJ proteins that co-localize with actin and myosin II (Utech *et al.*, 2005). IFN- γ -dependent internalization of TJ proteins occurs via macropinocytosis (Bruewer *et al.*, 2005). Tumor necrosis factor, on the other hand, induces caveolin-dependent endocytosis of TJ proteins and perturbation of barrier function (Marchiando *et al.*, 2010). Although EPEC infection has been shown by several studies to induce accumulation of occludin in the cytosol and corresponding loss of barrier function, until the current study it was not known that internalization is at least partially responsible for these phenotypes (Simonovic *et al.*, 2000, McNamara *et al.*, 2001, Shifflett *et al.*, 2005).

Blocking Golgi-based protein secretion also induces intracellular accumulation. NleA and *EspG1/G2* disperse the Golgi via different mechanisms (Tomson *et al.*, 2005, Kim *et al.*,

2007, Thanabalasuriar *et al.*, 2010, Clements *et al.*, 2011). These effectors also contribute to loss of TER, implying that inhibiting protein secretion may contribute to loss of TJ integrity (Tomson *et al.*, 2005, Thanabalasuriar *et al.*, 2010). However, EspF and Map are also required for TJ disruption. They do not affect protein secretion, suggesting that alternative mechanisms, such as internalization, may be responsible for the initial loss of barrier function (McNamara *et al.*, 2001, Dean *et al.*, 2004, Shifflett *et al.*, 2005, Dean *et al.*, 2006). To determine whether intracellular occludin associated with EPEC infection originates from the membrane, we used a biotinylation/pull-down assay. Our data establish that internalization of membrane-bound occludin is partially responsible for cytosolic occludin accumulation. While we cannot rule out the possibility that Golgi-dispersal contributes to this phenotype, our findings establish that, like the epithelial response to cytokines, EPEC induces peripheral proteins to traffic to or be retained in the cytosol. We speculate that EPEC-induced internalization may be due to interruption of constitutive TJ protein recycling.

Previously published data imply that recycling of TJ proteins participates in barrier restoration. *E. coli* cytotoxic necrotizing factor-1 (CNF-1) induces occludin association with recycling endosomes, suggesting that internalized protein can traffic back to the membrane and hasten recovery (Hopkins *et al.*, 2003). Termination of EPEC infection permits restoration of TJ structure and function, implying that EPEC effectors interfere with TJ maintenance and thus prevent recovery (Simonovic *et al.*, 2000). To address this possibility, we infected monolayers with EPEC, performed a calcium switch assay and killed any attached bacteria with gentamicin. Our data demonstrate that EPEC effectors impede recovery of TJ function after insult. Prior studies have focused on disruption of TJs. Inhibition of restoration may be a hitherto undescribed aspect of EPEC pathogenesis.

The precise mechanism by which EPEC disrupts TJs remains unknown, and multiple effectors have been shown to contribute including EspG (Tomson *et al.*, 2005). While microtubule destruction was the first function identified for EspG, there has been recent controversy in the literature regarding this role. Initially, microtubule networks were found to be absent under EPEC microcolonies (Shaw *et al.*, 2005). Using deletion mutants, EspG1/G2 were identified as the required effectors for this phenotype and were found to localize directly under attached microcolonies in areas exhibiting microtubule loss (Shaw *et al.*, 2005). In a separate study, transfection of *espG1* induced microtubule loss in epithelial cells in the absence of other EPEC effectors (Tomson *et al.*, 2005). Further reductionist studies demonstrated purified EspG directly bound to tubulin heterodimers and could depolymerize purified microtubules in vitro (Matsuzawa *et al.*, 2004, Hardwidge *et al.*, 2005).

Recently, new investigations called into question the impact of EspG on microtubules. One study showed that microinjection of recombinant EspG into rat kidney cells did not perturb microtubules (Selyunin *et al.*, 2011). Notably, the same group found that EspG induced Golgi dispersal suggesting that EspG may have hitherto unexplored functions. A separate study determined that transiently expressed EHEC EspG significantly inhibited Golgi-based protein secretion, though the mechanism remains unclear (Clements *et al.*, 2011). Ectopic expression of EspG in this study, using plasmid transfection into HeLa cells, induced

thickening of microtubules rather than destruction. Differences between cell lines, method of protein expression, quantity of DNA used and time points examined may explain the variability between studies.

Our data contribute to the body of literature supporting a role for EspG in the context of microtubule destruction. We chose to then elucidate the role of EspG-induced microtubule disruption on TJ perturbation. Using the pharmacological agent, nocodazole, as an EspG substitute we demonstrated equivalent TER loss by *espG1/G2* compared to wild type EPEC. Low dose of nocodazole was used to minimize drug-induced effects on TER. Attempting to compensate the activity of a bacterial effector with a pharmaceutical presents unique challenges as nocodazole and EspG1/G2 are unlikely to disrupt microtubules with equivalent kinetics or mechanisms and it is impossible to equilibrate concentrations. Earlier times are likely less representative of true compensation, especially given the order in which EPEC effectors enter the host cell cytosol; in the hierarchy of effector translocation, EspG1/G2 follows that of Map and EspF, which also contribute to tight junction alteration (Mills *et al.*, 2013). Although earlier time points show only partial compensation, we attribute this to unequal effects of nocodazole and EspG1/G2 on microtubules. Later time points show an equivalent loss of TER compared to wild type infection. We therefore interpret the ability of pharmacologic microtubule obliteration to functionally compensate *espG1/G2* to suggest that EspG1/G2's contribution to TJ disruption is at least in part due to its effect on microtubules via a mechanism that is yet to be defined.

To further elucidate the role of EspG as a potential inhibitor of TJ restoration during EPEC infection, we treated monolayers with wild type EPEC or a *espG1/G2* strain, allowed TER to drop by ~40% and terminated the infection with gentamicin. Loss of TER after wild type infection was more rapid compared with *espG1/G2* infection as published previously (Tomson *et al.*, 2005). We speculate that intact microtubules enable continuous barrier restoration during infection with *espG1/G2* and thus forestall rapid TER loss. EspF, MAP and NleA are still expressed by *espG1/G2* thus barrier function is ultimately compromised. The more rapid recovery of *espG1/G2* infected monolayers compared to wild type further supports a role for EspG as an inhibitor of TJ restoration. Taken together, these data indicate that efficient TJ restoration requires microtubules and that the role of EspG1/G2 in EPEC pathogenesis includes inhibition of TJ recovery.

Additionally, infection with *espG1/G2* significantly attenuated the mislocalization of TJ proteins potentially because intact microtubules permitted trafficking back to the membrane. Transient expression of EspG1 alone induced occludin accumulation in the cytosol. These data underscore the importance of microtubules in TJ homeostasis and identify a potential mechanism by which EspG1/G2 contribute to TJ loss. To further define the relationship between EspG's impact on microtubules and its role in barrier disruption, this effector was ectopically expressed in epithelial cells. Our data support the finding that EspG1/G2 induce microtubule depolymerization in the absence of other bacterial proteins in this model system. Microtubule disruption was not a non-specific effect of protein overexpression as EspF did not induce microtubule loss or tubulin degradation. Additionally, our data demonstrate that EspG1 disperses the Golgi both in transfection and infection models but cannot conclude whether this is due to EspG1-induced microtubule loss or due to another

function of EspG. Prior work has shown occludin to be degraded during EPEC infection and the trafficking of newly synthesized occludin requires intact microtubules (Gut *et al.*, 1998, Tomson *et al.*, 2005). EspG1/G2 may thus retard TJ recovery in multiple ways, by inducing degradation of tubulin monomers, by inhibiting recycling of TJ proteins back to the plasma membrane and by preventing newly synthesized TJ proteins from reaching the membrane via Golgi dispersal.

Microtubule destruction is one role identified for EspG1/G2, but the mechanism is unknown. To determine if the interaction of EspG with ARF and PAK were linked to microtubule dysfunction, we transfected EHEC EspG ARF- and PAK-binding mutants and stained for tubulin. All constructs induced microtubule disassembly. Bacterial delivery of EHEC EspG and the ARF- and PAK-binding mutants also perturbed microtubules, confirming that in the absence of interaction with either protein, EspG still induces microtubule destruction. Additionally, complementation of *espG1/G2* with EHEC *espG*, the ARF- or PAK-binding mutant induced accumulation of occludin in the cytosol and a drop in TER comparable to wild type infection.

Taken together, these data suggest that interaction with ARF and PAK are not required for EspG-mediated perturbation of microtubules and downstream TJ disruption, but we cannot exclude GTPase activity as a potential mechanism contributing indirectly to these phenotypes. Recent work has identified *Tre-2/Bub2/Cdc16* (TBC)-like dual-finger motifs in both VirA and EPEC EspG, a homologous catalytic domain shared by GAPs for Rab GTPases (Dong *et al.*, 2012). EspG showed significant GAP activity toward Rab1, inducing relocalization from the intermediate compartment/*cis*-Golgi to a diffuse pattern. Resulting inactivation of Rab1 attenuated Golgi-mediated secretion of a reporter protein. The same group solved the crystal structure of the ARF6-EspG-Rab1 complex and reported that not only did ARF6 and Rab1 bind separate surfaces of EspG, but Arf6 binding had little impact on EspG's GAP activity. Thus the ARF- and PAK-binding mutants used in our study have intact GAP activity, which may contribute to the role of EspG in EPEC pathogenesis via an indirect mechanism that is yet to be described.

Both ARFs and Rabs have been shown to participate in microtubule-dependent trafficking. The intracellular pathogen *Salmonella* Typhimurium has been demonstrated to recruit Arl8B, an ARF family GTPase, to *Salmonella*-containing vacuoles (SCVs) and to tubular endosomes extending along microtubules (Kaniuk *et al.*, 2011). Arl8B is required for kinesin-1 recruitment to SCVs, a crucial step in bacterial pathogenesis as SCVs are trafficked in a microtubule-dependent manner to the host cell periphery to undergo cell-cell transfer (Szeto *et al.*, 2009, Kaniuk *et al.*, 2011). The microtubule motors kinesin and dynein are known Rab effector proteins. By interacting with motor complexes, specific Rabs direct trafficking of vesicular cargo along microtubules (Horgan *et al.*, 2010b, Horgan *et al.*, 2010a, Mukhopadhyay *et al.*, 2011, Kurowska *et al.*, 2012).

Although our data indicate that EspG's interactions with ARF do not directly participate in microtubule perturbation, sequestering active ARF and inactivating Rab1 may be additional mechanisms by which EspG interferes with microtubule-based trafficking. We speculate that EspG may thus contribute to pathogenicity in multiple ways. It destroys "molecular

highways” potentially interrupting constitutive TJ protein recycling. It induces Golgi dispersal impeding trafficking of newly synthesized proteins and disabling the host cell’s ability to replace those proteins degraded by EPEC infection such as tubulin and occludin. EspG may potentially further inhibit protein replacement by sequestering active ARF and inactivating Rabs, both of which are known to recruit/direct microtubule motors.

Multiple effectors have been shown to affect TJ perturbation and we anticipate that further work will reveal roles for other effectors in TJ restoration. For example, EPEC pathogenesis involves a balance of pro-inflammatory factors, such as flagellin, and anti-inflammatory factors secreted via the TTSS (Sharma *et al.*, 2006). NF- κ B-mediated signaling has been shown to downregulate claudin-1, -2, and -4 as well as occludin and lead to significant loss of TER (Al-Sadi *et al.*, 2007, Boivin *et al.*, 2009, Tang *et al.*, 2010, Fischer *et al.*, 2013). Additional data support a role for NF- κ B-mediated signaling in TJ protein regulation. Lipopolysaccharide (LPS) induces increased paracellular permeability and loss of TJ structure through redistribution of ZO-1 and occludin in endothelial cells (Zhang *et al.*, 2013). Attenuating expression of I κ B α with resolvin D1, a lipid mediator, led to reduced paracellular permeability and corresponding upregulation of TJ proteins compared to cells treated with LPS alone (Zhang *et al.*, 2013). We recently published data demonstrating that NleH1 and NleH2 dampen the host inflammatory response by inhibiting activation of NF- κ B (Royan *et al.*, 2010). This suggests that NleH1 and NleH2 may in fact promote TJ restoration by preventing downregulation of TJ proteins.

To take a broad view, we hypothesize that multiple EPEC effectors act in concert to affect the rate of TJ restoration. NleC degrades p65, c-Rel and p50, blocking NF- κ B-mediated signaling, possibly preventing downregulating TJ proteins and thereby promoting recovery (Pearson *et al.*, 2011). NleE inhibits IKK β and the subsequent degradation of I κ B and may have the same effect (Nadler *et al.*, 2010). NleB also contributes to impeding NF- κ B-mediated signaling during EPEC infection (Nadler *et al.*, 2010, Newton *et al.*, 2010). While these effectors might promote TJ restoration, NleA may work in concert with EspG1/G2 to prevent it. NleA has been shown to inhibit COPII-dependent anterograde protein trafficking by binding to the Sec24 subunit (Gruenheid *et al.*, 2004, Kim *et al.*, 2007, Thanabalasuriar *et al.*, 2010). Preventing proper trafficking of newly synthesized proteins may impede TJ restoration.

Our data support a two-armed model of EPEC-induced TJ disruption. We propose that the first arm is disruption of intact TJs, likely initiated by EspF and Map. The mechanism has not been described, but our data reveal that internalization of TJ proteins plays a role in TJ perturbation. We speculate that the second arm is inhibition of TJ restoration, which is mediated by EspG1/G2 via destruction of microtubule networks with consequent loss of constitutive protein recycling, and interruption of microtubule-dependent trafficking. NleA may play a role in impeding TJ recovery as well the effectors with anti-inflammatory properties potentially preventing complete TJ obliteration. Further work is needed to tease out in exquisite detail the ways in which these varied effectors collectively impact the rate of TJ restoration and contribute to EPEC pathogenesis.

Experimental Procedures

Cell culture

Caco-2 cells (ATCC, Manassas, VA) were maintained and propagated according to ATCC guidelines. Briefly, cells were passaged every 4 days in Dulbecco's Modified Eagle's Media (Life Technologies, Grand Island, NY) supplemented with 15 mM NaHCO₃ and 20% fetal bovine serum. Caco-2 BBE cells were grown in the same fashion. MDCK I cells were the generous gift of Dr. Jerrold Turner (University of Chicago). MDCK cells were passaged every 4 days in Dulbecco's Modified Eagle's Media (Life Technologies, Grand Island, NY) supplemented with 15 mM NaHCO₃, 15 mM HEPES and 10% fetal bovine serum. T84 cells (ATCC, Manassas, VA) were passaged every 10-14 days in DMEM supplemented with 30 mM HEPES, F-12 nutrients and 5% fetal bovine serum. All cell lines were used between passages 10 and 40.

Reagents and plasmids

Nocodazole (Sigma, St. Louis, MO) was used at a concentration of 5 µg/ml in electrophysiology studies. GFP-EspG, the ARF- and PAK-binding mutants were constructed as previously described (Selyunin *et al.*, 2011). The ADP-ribosylation factor binding mutant is a Glu-Arg mutation at position 392. The p21-activated kinase binding mutant is an Asn-Ala mutation at position 212. Both of these constructs have been extensively verified and published (Selyunin *et al.*, 2011). GFP-EspF was made by Dr. Andrew Weflen and has also been verified and published (Weflen *et al.*, 2010).

Bacterial culture

The EPEC strain E2348/69 was used in all experiments. *espG1/G2* and *espG1/G2* complemented with *espG1* have been previously described (Elliott *et al.*, 2001, Tomson *et al.*, 2005). Wild type and *espG1/G2* were inoculated into Luria-Bertani broth and grown overnight with shaking at 37°C. Cultures were inoculated 1:33 into cold, serum-free, antibiotic-free cell culture medium (see "Cell Culture") and grown with shaking at 37°C to the logarithmic growth phase, or OD₆₀₀ 0.4. Cultures were spun down and resuspended in an equal volume of serum-free medium. Caco-2 cells were infected at an MOI of 40, T84 cells at an MOI of 800.

Cloning

To create *espG* ARF- and PAK plasmids for *espG1/G2* complementation, inserts were cloned using PCR primers 5' ATA AAT GGA CTT AAT AAT GAC TCC GC (forward) and 5' AGT GTT TTG TAA GTA CGT TTC (reverse). PCR products were double digested using EcoRI and BamHI (New England Biolabs, Ipswich, MA) as was the bacterial vector pTrcHis2 (Life Technologies, Grand Island, NY). Digested inserts and vector were ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA), transformed into OneShot TOP10 competent cells (Life Technologies, Grand Island, NY) and spread on LB-agar plus 100 µg/ml ampicillin. Single colonies were picked, grown overnight with shaking at 37°C in LB broth plus 100 µg/ml ampicillin and DNA was miniprep. Correct insertion of *espG* and GTPase binding mutant sequences were verified using double digestion of plasmids.

Plasmid stock was made using the Qiagen Plasmid-Midi prep kit (Qiagen, Valencia, CA), eluted in DNA- and RNAase-free water and stored at -20°C .

Creation of competent cells and transformation

Competent *espG1/G2* was made by growing the strain in LB broth plus 50 $\mu\text{g}/\text{ml}$ kanamycin (Sigma, St. Louis, MO) to OD_{600} 0.6. Bacteria were pelleted by spinning 15 min at 5k RPM at 4°C . Pellets were resuspended in 3 volumes of ice-cold 15% glycerol (Sigma, St. Louis, MO) in 0.1M CaCl_2 and spun 15 min at 5k RPM at 4°C . Washing and spinning was repeated 3 times.

Final pellets were resuspended in 600 μl of the glycerol wash buffer and frozen immediately in liquid nitrogen. Cells were stored at -80°C .

For transformation, 100 μl cells were thawed on ice and mixed with 20 μg plasmid prep in an ice-cold GenePulser electroporation cuvette (Bio-Rad, Hercules, CA). Mixture was electroporated using the GenePulser XCell (Bio-Rad, Hercules, CA) and immediately 900 μl SOC media was added. Transformed bacteria were grown with shaking at 37°C for 1 hour and spread on LB-agar plates plus 100 $\mu\text{g}/\text{ml}$ ampicillin and 50 $\mu\text{g}/\text{ml}$ kanamycin. Colonies were selected after 18 hours of incubation at 37°C .

Immunofluorescence

Occludin staining—cells were fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature. Fixed cells were washed in 50 mM NH_4Cl for 25 min, permeabilized for 3×10 minutes in 0.05% saponin (Sigma, St. Louis, MO) in Membrane Blocking Solution (Life Technologies, Grand Island, NY) and blocked for 1 hour in Membrane Blocking Solution. Cells were stained using mouse anti-occludin antibody (product T5168, Sigma, St. Louis, MO) at a concentration of 1:200 overnight at 4°C and fluorophore-conjugated goat anti-mouse secondary antibody at a concentration of 1:500 for 1 hour (Molecular Probes) at room temperature. Cells were washed 5×5 min in PBS $\pm$ DAPI (product D9542, Sigma, St. Louis, MO), washed in distilled water and mounted using Prolong Gold anti-fade reagent (Life Technologies, Grand Island, NY).

ZO-1, claudin 1-2, claudin 4 staining—for MDCKs, cells were fixed 30 minutes in 10% TCA on ice, and permeabilized with 0.1% Triton-X 30 minutes. For Caco-2s, cells were fixed 1 hour in 1% PFA, treated with 50 mM ammonium chloride for 25 minutes, and permeabilized with 0.05% saponin for 30 minutes. Antibodies used (Life Technologies, Grand Island, NY) were ZO-1 (339100), claudin 11 (519000), claudin 2 (325600), claudin 4 (329400). All primary antibody incubations were 1:100 for 1 hour. Secondary antibodies were from Life Technologies (Carlsbad, CA) A11034 anti-rabbit and A11029 anti-mouse, 1:200 for 1 hour. Cells were mounted as above.

Tubulin staining—cells were fixed and stained following our previously published protocol (Tomson *et al.*, 2005). Three solutions were prepared: microtubule stabilizing buffer or MTSB (1mM EGTA, 4% PEG8000, 100mM PIPES, pH 6.9), TSP was prepared by dissolving 0.5% Triton X-100 (Life Technologies, Grand Island, NY) in MTSB and 20

mg of dithiobis succinimidylpropionate or DSP (Pierce Biotechnology, Rockford, IL) was dissolved in 1 ml sterile DMSO. Half (500 μ l) the DSP was dissolved in 50 ml HBSS (Life Technologies, Grand Island, NY), and the remaining 500 μ l DSP dissolved in TSB. 4% paraformaldehyde was prepared by dissolving 800 mg PFA (Fisher Scientific, Waltham, MA) in 20 ml MTSB at 60°C overnight. PFA was 0.2 μ m filtered and stored at -20°C. Cells were incubated in room temperature HBSS/DSP for 10 min, TSP/DSP for 10 min, TSP for 10 min and PFA for 15 minutes. Fixed cells were blocked for 30 min in Membrane Blocking Solution (Life Technologies, Grand Island, NY), and incubated with mouse anti-tubulin (product 3526, clone B-5-1-2, Sigma, St. Louis, MO) at a concentration of 1:200 overnight at 4°C. Cells were washed 5 $\times$ 5 min in PBS and incubated in fluorophore-conjugated goat anti-mouse secondary antibody at a concentration of 1:500 for 1 hour (Molecular Probes) at room temperature. Cells were washed 5 $\times$ 5 min in PBS plus DAPI (product D9542, Sigma, St. Louis, MO), rinsed in distilled water and mounted using Prolong Gold anti-fade reagent (Life Technologies, Grand Island, NY).

Giantin staining—cells were fixed in 3% paraformaldehyde in phosphate buffered saline (Life Technologies, Grand Island, NY) for 10 min at room temperature, permeabilized in 0.5% Triton X-100 in PBS, blocked in 3% bovine serum albumin (Life Technologies, Grand Island, NY) for 10 min, stained using rabbit anti-giantin antibody (ab80864, Abcam, Cambridge, MA) at 1:100 for 1 hour and washed 5 $\times$ 5 min in PBS. Cells were incubated with fluorophore-conjugated anti-rabbit secondary antibody at 1:400 (Molecular Probes, Carlsbad, CA) for 1 hour and washed 5 $\times$ 5 min in PBS plus DAPI. Slides were rinsed in distilled water and mounted using Prolong Gold (Life Technologies, Grand Island, NY)

Images of fixed cells were captured using a Leica DM4000B epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) and a Retiga Exi CCD camera (Qimaging, Surrey, BC, Canada). Images were captured using SlideBook 4.2 software (Intelligent Imaging Innovations, Denver, Co) and minimally processed using ImageJ.

Transfection

EspG and EspF constructs—For immunofluorescence, cells were plated on glass coverslips in 2 cm² wells and grown to 50-60% confluence. For each well, 0.8 μ g of each construct was added to 50 μ l of Opti-MEM reduced-serum media. For each well, 2 μ l Lipofectamine (Life Technologies, Grand Island, NY) was added to 50 μ l of Opti-MEM and incubated at room temperature for 5 min. The two were combined and incubated for 30 min at room temperature and 100 μ l was added to each well. An additional 400 μ l of Opti-MEM was added for a total volume of 500 μ l. Cells were incubated for indicated times and fixed/stained (see “Immunofluorescence”).

Electrophysiology studies

Caco-2 or T84 cells were grown to confluence (minimum of 7 days for Caco-2) on 0.33 cm² Transwell permeable supports of 0.4 μ m pore size. T84 cells were grown for 7-14 days. Transepithelial electrical resistance was measured using an epithelial voltohmmeter and STX2 manual electrodes (World Precision Instruments, Sarasota, FL). Average resistance for Caco-2 cells was 300 Ω ·cm², and for T84 cells 1-1500 Ω ·cm². Numerical baseline

resistance of each Transwell was set to 0 and all percent changes in resistance were calculated based on initial readings.

For assays done at 4°C, all Transwells were incubated for 30 min, then kept on ice and ice-cold medium with or without 5 µg/ml nocodazole was added to both the interior and exterior of the wells. Transwells were returned to 4°C for a further 30 min and then infected with indicated strains.

For infection studies, bacteria were added directly to Transwells at the indicated MOI without aspiration.

For the tight junction recovery assay, Caco-2 cells were grown as previously indicated and infected with either wild type EPEC or *espG1/G2*. TER was followed and when approximately 40% of initial TER was lost, cells were washed in warm medium and fresh medium containing 200 µg/ml gentamicin was added. Recovery of TER was monitored.

For pharmacological “complementation” of *espG1/G2*, Caco-2 cells were grown as previously indicated on Transwells and treated with cold/nocodazole as described above. After 1 hour of recovery at 37°C selected monolayers were infected with either EPEC strain grown to logarithmic growth phase (see “Bacterial culture”). TER was followed.

Statistical analyses were performed using Student’s t-test (unpaired).

Calcium switch assay

Cells grown on Transwell permeable supports were incubated in 1 mM EDTA in Ca²⁺/Mg²⁺-free PBS, pH 7.4 (Sigma, St. Louis, MO) for 10-20 min at 37°C. TER was measured at baseline and followed after chelation. Once resistance had fallen by ~85-90%, monolayers were washed with warm cell culture medium and medium was replaced with calcium-containing medium. Cells were incubated at 37°C and TER was measured every hour.

For T84 monolayers that were pre-infected with EPEC, 200 µg/ml gentamicin (Life Technologies, Grand Island, NY) was added to recovery medium.

Western blotting

Anti-tubulin—35 cm² of Caco-2 cells were harvested in 300 µl Laemmli buffer (Bio-rad, Hercules, CA) plus a 1:100 dilution of protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL). Lysates were sonicated on ice and boiled for 10 minutes. Samples, 10 µl each, were separated on a hand-cast 12% SDS-PAGE gel and transferred to nitrocellulose using a Mini-Protean Tetra Cell apparatus (Bio-rad, Hercules, CA). Membranes were blocked for 1 hour in 5% milk and blotted overnight using anti-tubulin antibody (product T5168, Sigma, St. Louis, MO) at a 1:1000 concentration. Membranes were washed 5 × 5 min in Tris-buffered saline tween-20 (50 mM Tris, 150 mM NaCl, 0.05% Tween 20). Membranes were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (Sigma, St. Louis, MO) at 1:5000 for 2 hours, washed 5 × 5 min in TBST and developed using the ECL Western Blotting Detection Kit (GE Life Sciences, Pittsburgh, PA).

Anti-occludin—membranes were blocked in 5% milk in TBST and incubated overnight in 1:1000 rabbit anti-occludin (product #71-1500, Life Technologies, Grand Island, NY). Membranes were washed 5 × 5 min in TBST and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Sigma, St. Louis, MO) at 1:5000 for 2 hours, washed 5 × 5 min in TBST and developed using the ECL Western Blotting Detection Kit (GE Life Sciences, Pittsburgh, PA). Anti-actin: membranes were treated as above but were incubated for 1 hour with 1:5000 rabbit anti-actin (product #A2066, Sigma, St. Louis, MO). Secondary antibody immunoblotting was performed as above.

Biotin pull-down assay

All solutions were made in phosphate buffered saline plus 0.9 mM calcium and 0.33 mM magnesium unless otherwise noted.

T84 cells grown to confluence on 10 cm² dishes were incubated with 3 mls 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce Biotechnology, Rockford, IL) on ice with shaking at 4°C for 1 hour. To quench biotin cells were washed 3 × 5 min in 50 mM NH₄Cl on ice with shaking at 4°C. Biotinylated cells were inoculated with logarithmic phase bacteria as described in “Bacterial culture” at an MOI of 40 or incubated with plain cell media (control). After indicated hours of infection at 37°C, cells were washed twice in warm PBS. Biotin was stripped for 3 × 10 min in 2.5 mM CaCl₂, 100 mM NaCl, 100 mM Tris-Cl and 50 mM sodium 2-mercaptoethanesulfonate (MESNA, Sigma, St. Louis, MO) on ice with shaking at 4°C. Strip solution was quenched by incubating cells for 3 × 5 min in 5 mg/ml iodoacetate (Sigma, St. Louis, MO) on ice with shaking at 4°C. Cells were lysed in 400 µl of modified RIPA buffer: 50 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 1.25% Triton X-100, 0.25% SDS plus 1:100 protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL).

Lysates were sonicated on ice for 1 min and centrifuged at 4500 rpm for 15 min at 4°C. All subsequent steps were performed on the supernatant only.

Protein concentration was assayed using a BCA protein assay reagent (Pierce Biotechnology, Rockford, IL). Lysates were diluted 1:10 in sterile PBS and pipetted in duplicate into a 96-well plate. Assay reagent was diluted 1:3 in sterile water as directed and 100 µl applied to diluted lysates. Absorbance at 562 nm was read using a plate reader and protein concentration extrapolated using a standard curve.

For each sample 800 µg of protein was added to 200 µl of immobilized avidin slurry (Pierce Biotechnology, Rockford, IL) and incubated with shaking at 4°C for 3 hours. Samples were spun down at 4500 rpm for 1 min to pellet avidin. Supernatant was decanted and 1 ml PBS was added to wash the avidin and any associated biotinylated proteins. Pelleting and washing was repeated 5 times. For each sample, 180 µl Laemmli buffer (Bio-rad, Hercules, CA) plus a 1:100 dilution of protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL) was added to the avidin and captured protein and boiled for 10 min. Supernatant was separated on a 12% hand-cast SDS-PAGE gel. Simultaneously, 20 µg of protein input was also separated and blotted. Proteins were transferred to nitrocellulose using the Protean XL system (Bio-rad, Hercules, CA) and blotted for occludin (see “Western Blotting: anti-occludin”).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. EPEC infection induces internalization of occludin

T84 intestinal epithelial cells were biotinylated and either treated with EDTA to induce internalization of occludin, or infected with EPEC. Cells were then stripped of any remaining extracellular biotin and lysed. Biotinylated (internalized) proteins were pulled down with avidin beads. Precipitated proteins were blotted for occludin. Lysate input was also blotted for occludin for comparison. Protein input (left) displays equal amounts of occludin. Pulled down proteins show no internalization after biotinylation/stripping. Calcium chelation induced occludin internalization. EPEC infection induced progressive internalization of occludin over 3 hours.

Figure 2. EPEC infection delays TJ recovery

T84 intestinal epithelial cells were infected with EPEC till TER decreased ~40%. A calcium switch assay was performed to completely disrupt TJs. Uninfected controls were also chelated. When TER decreased to ~90% of baseline, cells were washed in warm media and fresh media containing normal calcium concentrations was added. Recovery media also contained gentamicin to kill attached bacteria while allowing cytosolic EPEC effectors to persist. Pre-infected, chelated cells recovered significantly less barrier function compared to uninfected, chelated controls. ($n = 3$ separate experiments, each condition in triplicate, $*p < 0.05$)

Figure 3. EspG1/G2 promote relocalization of occludin to the cytosol

(A) Infection of Caco-2 intestinal epithelial cells with wild type EPEC (red) for 4 hours induces microtubule (green) destruction. Nuclei are shown in blue. Microtubules are largely intact after infection with *espG1/G2* (red). Complementation of *espG1/G2* with *espG1* restores the wild type phenotype and microtubules are lost. (B) Uninfected Caco-BBE controls show paracellular occludin (red). Four hours of wild type EPEC infection (lower panel, green) induces occludin relocalization to the cytosol. Infection with *espG1/G2* (green) does not perturb TJ structure, but infection with *espG1/G2* complemented with *espG1* induces the wild type phenotype.

Figure 4. EspG1/G2 induce mislocalization of TJ proteins during EPEC infection

(A) MDCK cells were treated with wild type EPEC or a *espG1/G2* for 4 hours then fixed, permeabilized and stained for ZO-1, claudin 1 (CI-1), claudin 4 (CI-4). All three TJ proteins were disrupted by wild type EPEC with minimal disruption with a *espG1/G2* mutant. ZO-1 and claudin 1 were reliably perturbed by EPEC while claudin 4 changes were somewhat more variable suggesting that claudin 4 is the least impacted TJ protein of those tested. (B) Caco-2 human intestinal epithelial cells were infected with either wild type EPEC or a *espG1/G2* mutant for four hours and were then fixed and stained for ZO-1, claudin 1 (CI-1),

claudin 2 (Cl-2) and claudin 4 (Cl-4). Wild type EPEC infection caused substantial disruption of ZO-1 and claudin-1 with a more minor disruption of claudin 2, the leaky claudin. Claudin 4 was not notably changed compared to uninfected controls. In every case deletion of *espG1/G2* helped preserve TJ structure compared with wild type infection.

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Figure 5. Restoration of TER after EPEC infection is enhanced by deletion of *espG1/G2*
Caco-2 cells infected with wild type EPEC lost barrier function more rapidly than *espG1/G2*-infected cells. When TER dropped by ~40%, cells were treated with gentamicin to terminate the infection and permit TJ recovery. *espG1/G2*-infected cells recovered barrier function more rapidly than wild type-infected cells. ($n = 3$ separate experiments, each condition in triplicate, \$ $p < 0.01$)

Figure 6. Microtubule destruction by nocodazole compensates for EspG with *espG1/G2* infection

Wild type EPEC infection precipitously dropped TER. Infection of Caco-BBE cells with *espG1/G2* induced a gradual drop in TER. Cold temperatures and nocodazole decreased TER by ~20%. Microtubule disruption with cold/nocodazole followed by *espG1/G2* infection induced a rapid loss of barrier function compared to *espG1/G2* infection alone. ($n = 3$ separate experiments, each condition in triplicate, \$ $p < 0.01$)

Figure 7. EspG destroys microtubules, disperses the Golgi and perturbs TJs

(A) Transient expression of EPEC RFP-EspG1 in MDCK cells induces microtubule fragmentation 8 hours post-transfection. Similar expression of control GFP vector alone or GFP-EspF does not affect microtubules. (B) EspG1 expression also induces progressive tubulin degradation. EspF has no effect on tubulin levels. (C) Control MDCK cells display a perinuclear Golgi shown by giantin staining (green). RFP-EspG1 expression disperses the Golgi apparatus. Nuclei are shown in blue. (D) Control Caco-2 cells display perinuclear Golgi localization (green), wild type EPEC infection (red) induces Golgi dispersal. Infection with *espG1/G2* (red) does not disperse the Golgi. (E) Occludin is paracellular in RFP-vector-transfected MDCK cells, but 8 hours after RFP-EspG1 transfection, it relocates to the cytosol. Nuclei are shown in blue.

Figure 8. EspG ARF- and PAK-binding mutants perturb microtubules

Transient expression of EHEC GFP-EspG that cannot bind to ARF or EHEC GFP-EspG that cannot bind to PAK all induce microtubule (red) destruction in MDCK cells. Nuclei are shown in blue.

Figure 9. Bacterial delivery of EspG binding mutants induces microtubule loss and cytosolic occludin accumulation

(A) EHEC EspG, EHEC EspG that cannot bind to ARF and EHEC EspG that cannot bind to PAK were cloned into a bacterial expression vector and transformed into *espG1/G2*. Infection of Caco-BBE cells with these complements (green) induced microtubule destruction (red). Nuclei are shown in blue. (B) Infection with these complements (green) also induces relocalization of occludin (red) to the cytosol, similar to the wild type phenotype (Fig. 3B).

Figure 10. Bacterial delivery of EspG binding mutants induces TER loss
 (A) Infection of Caco-BBe cells with wild type EPEC decreased TER rapidly. *espG1/G2* infection induced a gradual TER loss. Infection with *espG1/G2* + EspG-ARF-binding mutant or +EspG-PAK-binding mutant restored the wild type phenotype. ($n = 3$ separate experiments, each condition in triplicate, $p =$ non-significant comparing wild type infection and all complements) (B) Infection with *espG1/G2* complemented with empty vector does not alter the resistance phenotype of *espG1/G2*. Infection with *espG1/G2* complemented

with *espGI* induces a more precipitous loss of barrier function. ($n = 3$, separate experiments, each condition in triplicate)