Salmonella enterica serovar Typhimurium effectors SopB, SopE, SopE2 and SipA disrupt tight junction structure and function

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Summary
Salmonella enterica serovar Typhimurium is a major cause of human gastroenteritis. Infection of epithelial monolayers by S. Typhimurium disrupts tight junctions that normally maintain the intestinal barrier and regulate cell polarity. Tight junction disruption is dependent upon the Salmonella pathogenicity island-1 (SPI-1) type 3 secretion system but the specific effectors involved have not been identified. In this study we demonstrate that SopB, SopE, SopE2 and SipA are the SPI-1-secreted effectors responsible for disruption of tight junction structure and function. Tight junction disruption by S. Typhimurium was prevented by inhibiting host protein geranylgeranylation but was not dependent on host protein synthesis or secretion of host-derived products. Unlike wild-type S. Typhimurium, ΔsopB, ΔsopE/E2, Δsipa, or ΔsipA/sopB mutants, ΔsopB/E2 and ΔsipA/sopE/E2 mutants were unable to increase the permeability of polarized epithelial monolayers, did not disrupt the distribution or levels of ZO-1 and occludin, and did not alter cell polarity. These data suggest that SPI-1-secreted effectors utilize their ability to stimulate Rho family GTPases to disrupt tight junction structure and function.

Introduction
Salmonella enterica serovar Typhimurium (S. Typhimurium) is a facultative intracellular pathogen that gains entry into the host through oral ingestion of contaminated food or water. It is a major cause of human gastroenteritis and infections result in significant morbidity and mortality worldwide. Critical for S. Typhimurium virulence is a type 3 secretion system (T3SS) encoded by the Salmonella pathogenicity island-1 (SPI-1) (Mills et al., 1995; Galan, 1999). T3SSs are needle-like protein complexes that mediate the translocation of specific proteins (effectors) from the bacteria directly into the host cell cytoplasm (Hueck, 1998; Ghosh, 2004). The SPI-1-encoded T3SS is activated upon contact with the host cell surface whereby it injects at least 12 effectors that mediate host cell signalling (Galan, 2001; Ehrbar et al., 2002). In vitro and in vivo model systems have been developed to examine the function of specific SPI-1 effectors. In the bovine model of Salmonella-induced gastroenteritis, SPI-1 effectors enable penetration of the ileal mucosa; induce polymorphonuclear neutrophil transmigration and fluid accumulation; and facilitate the induction of diarrhoea (Zhang et al., 2003). SPI-1 effectors are also responsible for early inflammation in the recently characterized mouse model for S. Typhimurium-induced enterocolitis (Barthel et al., 2003; Hapfelmeier et al., 2004). In cultured cell lines, SPI-1 effectors mediate membrane ruffling (Hardt et al., 1998a), bacterial invasion (Galan, 1996), chloride secretion (Bertelsen et al., 2004), apoptosis (Guiney, 2005) and tight junction disruption (Jepson et al., 1996; Tafazoli et al., 2003).

Located at the interface between epithelial cells, tight junctions are highly regulated protein complexes that are intimately linked to the actin cytoskeleton. Transmembrane proteins such as occludin and claudins establish homophilic interactions between adjacent cells, while plaque proteins such as ZO-1 act as adapters at the cytoplasmic surface of tight junctions, linking junctions to the actin cytoskeleton and various signalling pathways (Miyoshi and Takai, 2005). Tight junctions maintain a semi-permeable barrier, restricting paracellular movement of harmful immunogenic materials while controlling the movement of water, solutes and immune cells. Tight junctions also serve to create and maintain cell polarity, preventing diffusion of apical and basolateral membrane components. Many diarrhoeal pathogens are capable of overcoming the epithelial barrier by disrupting cell–cell junctions (Sousa et al., 2005). For example, type 3-
secreted effectors of enteropathogenic *Escherichia coli* disrupt the barrier by causing dissociation of occludin from tight junctions (Simonovic et al., 2000) while *Vibrio cholera* produces a metalloprotease that directly degrades the extracellular domain of occludin (Wu et al., 2000). In terms of disease, the breakdown of tight junctions is thought to contribute to diarrhoea (Hecht, 2001; Fasano, 2002; Sandle, 2005).

Rho family GTPases are key regulators of tight junctions and activation of either Rac, Cdc42, or Rho can disrupt tight junction structure and function (Braga, 2002). Interestingly, *S. Typhimurium* possesses several SPI-1 T3SS-delivered effectors that activate Rho family GTPases. SopE and the closely related protein SopE2, act as G-nucleotide exchange factors (GEFs) for Cdc42 GTPases. SopE and the closely related protein SopE2, act as G-nucleotide exchange factors (GEFs) for Cdc42 and Rac1 (Hardt et al., 1998a; Stender et al., 2000; Friel et al., 2001; Zhou et al., 2001; Zhou and Galan, 2001). SopB (also called SigD) is a potent inositol phosphatase that indirectly stimulates Cdc42-dependent reorganization of the actin cytoskeleton presumably via phosphoinositide fluxes (Zhou et al., 2001).

Infection of polarized epithelial cell monolayers by *S. Typhimurium* disrupts tight junction structure and function (Finlay and Falkow, 1990; Jepson et al., 1995; 1996; 2000; Tafazoli et al., 2003) yet the specific bacterial effector(s) responsible has/have not been identified. Using polarized epithelial monolayers, we have identified the specific SPI-1-secreted effectors responsible for *S. Typhimurium*’s ability to compromise the epithelial barrier. Our data suggest that these effectors utilize their ability to stimulate Rho family GTases and modulate the actin cytoskeleton in order to disrupt the permeability, tight junction structure and polarity of epithelial cells.

**Results**

*Inhibition of protein geranylgeranylation prevents barrier disruption by S. Typhimurium*

*Salmonella enterica* serovar Typhimurium possesses several effectors that activate Rho family GTases. Because Rho family GTases regulate cellular junctions, we determined whether they were involved in *S. Typhimurium*-induced disruption of tight junctions using the well-characterized human intestinal epithelial cell line, Caco-2/TC7. Prior to infection by invasive *S. Typhimurium*, polarized monolayers were treated with GTTI-298, an inhibitor of geranylgeranyltransferase I (GGTase-I), which is required for Rho GTase activation (Casey and Seabra, 1996). Transepithelial resistance (TER), a measure of the integrity of the epithelial barrier, was monitored over the course of a 4 h infection. Both 15 and 50 μM GTTI-298 alone had no effect on the barrier (Fig. 1) and did not influence bacterial adherence or bacterial viability (data not shown).

At 4 h post infection, wild-type *S. Typhimurium* decreased TER to 57.84 ± 2.36% of the TER at time zero. Pretreatment of cells with either concentration of GTTI-298 significantly reduced the disruption of the epithelial barrier by *S. Typhimurium* (*P* < 0.001; Fig. 1). Accordingly, these results demonstrate that protein geranylgeranylation, and likely Rho GTase activation, is involved in barrier disruption by *S. Typhimurium*.

**Barrier disruption by S. Typhimurium does not require host protein synthesis or secretion of host-derived products**

Activation of Rho family GTases can trigger reorganization of the actin cytoskeleton as well as initiate gene transcription (Benitah et al., 2004; Sorokina and Chernoff, 2005). To determine which function of Rho family GTases was required for barrier disruption by *S. Typhimurium*, cells were pretreated with 5 μM cycloheximide, an inhibitor of eukaryotic protein synthesis. To confirm this concentration of cycloheximide-blocked protein synthesis during infection, IL-8 production was assayed in the presence and absence of the inhibitor. As expected, infection resulted in a considerable increase in IL-8 production as compared with uninfected cells (Fig. 2A; Hobbie et al., 1997; Mynott et al., 2002). Pre-
treatment of Caco-2/TC7 cells with 5 μM cycloheximide significantly inhibited IL-8 production upon infection (P < 0.05) indicating that this concentration of cycloheximide effectively blocked protein synthesis during a 4 h infection (Fig. 2A). Cycloheximide alone had no effect on TER and pretreatment of cells prior to infection did not prevent disruption of the barrier by S. Typhimurium over the course of a 4 h infection (Fig. 2B). Therefore, host protein synthesis is not required for barrier disruption by S. Typhimurium. To test whether soluble epithelial-derived products produced upon infection were acting in an autocrine or paracrine fashion on the barrier, basolateral supernatants from cells infected for 4 h were filter-sterilized and placed on the basolateral side of polarized Caco-2/TC7 monolayers. No change in TER was detected when monitored for 10 h (data not shown). Therefore, S. Typhimurium-induced barrier disruption is not dependent on host protein synthesis or secretion of host-derived products. These data provide evidence that barrier disruption by S. Typhimurium is a result of the effect of Rho GTPases on the actin cytoskeleton.

SopE, SopE2, SopB and SipA disrupt the barrier function of polarized epithelial cell monolayers

Disruption of the epithelial barrier by S. Typhimurium is SPI-1-dependent (Jepson et al., 1996; Tafazoli et al., 2003) yet the specific SPI-1-secreted effectors responsible have not been identified. We focused on three SPI-1-secreted effectors that activate Rho family GTPases (i.e. SopB, SopE and SopE2) and another that directly interacts with actin (i.e. SipA). Polarized Caco-2/TC7 monolayers were infected with various bacterial mutants and the barrier function was assessed by measuring TER. To confirm the role of SPI-1 in barrier disruption, cells were infected with an ΔinvA mutant that lacks a functional SPI-1 T3SS (Galan and Curtiss, 1991). Over the course of 4 h, TER remained unchanged in uninfected and ΔinvA-infected cells (Fig. 3), confirming that the drop in TER is indeed SPI-1-dependent. In order to evaluate the role of the SPI-2 T3SS, cells were infected with an ΔssaR mutant that is defective for secretion of SPI-2 effectors (Brumell et al., 2001). After 4 h, the TER of cells infected with wild-
type bacteria or the ΔssaR mutant decreased to 41.02 ± 2.479% and 45.37 ± 5.338% of the TER at time zero respectively (Fig. 3). Thus, SPI-2-secreted effectors are not involved in barrier disruption. Next, we investigated the role of specific SPI-1 effectors. All of the effector mutants adhered to an equal extent to Caco-2/TC7 monolayers (data not shown). The ΔsopB and ΔsopE/E2 mutants were able to decrease the TER to a similar extent as wild-type S. Typhimurium, while TER was similar to the uninfected control cells upon infection with the ΔsopB/E/E2 mutant (Fig. 3). Therefore, SopB, SopE and SopE2 are collectively required for barrier disruption by S. Typhimurium. Infection by a ΔsipA mutant had the same effect on the barrier as wild-type bacteria, as did a ΔsipA/ΔsopB mutant (Fig. 3). However, a role for SipA in barrier disruption was revealed as the ΔsipA/sopE/E2 mutant strain was unable to decrease the TER (Fig. 3). Collectively, these data indicate that SopB, SopE, SopE2 and SipA are the effectors responsible for decreasing the TER of polarized epithelial cells, and while SipA and SopB are involved, they are insufficient for barrier disruption.

\section*{Complementation analysis verifies that SopB, SopE, SopE2 and SipA disrupt the barrier function of polarized epithelial cell monolayers}

In order to confirm that the inability of ΔsipA/sopE/E2 and ΔsopB/E/E2 mutants to disrupt the barrier was attributable to specific deletion of the genes encoding SipA, SopB, SopE and SopE2, we performed a complementation analysis. Polarized Caco-2/TC7 cells were infected, as above, with the complemented strains and TER was measured over the course of a 4 h infection. Transformation of the ΔsopB/E/E2 mutant with the low-copy-number vector expressing either SopB, SopE, or SopE2 significantly restored the strain’s ability to decrease the TER when compared with the empty vector control (P < 0.05 for SopB; P < 0.001 for SopE and SopE2; Fig. 4). Similarly, transformation of the ΔsipA/sopE/E2 mutant with plasmids expressing SopE or SopE2 significantly restored this strain’s ability to disrupt TER when compared with the empty vector control (P < 0.05 for SopE; P < 0.01 for SopE2; Fig. 4). Of note, while ΔsopE or ΔsopE2 mutants were not tested, ectopic expression of either SopE or SopE2 restored the mutant strains’ ability to decrease TER. This confirms that both SopE and SopE2 can disrupt tight junctions. Expression of SipA from a high-copy-number plasmid within the ΔsipA/sopE/E2 strain also significantly complemented this strain’s ability to decrease the TER when compared with the empty vector control (P < 0.001; Fig. 4). These results verify that the SPI-1-secreted effectors SipA, SopB, SopE and SopE2 are indeed responsible for the decrease in TER upon infection by S. Typhimurium.

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mutants (P < 0.01; Fig. 5). Together with the TER data, these data confirm the role of SopB, SopE, SopE2 and SipA in the disruption of epithelial barrier function.

**SopE, SopE2, SopB and SipA alter the localization and expression levels of ZO-1 and occludin**

Disruption of the localization of tight junction proteins ZO-1 (cytoplasmic) and occludin (transmembrane) is SPI-1-dependent (Tafazoli et al., 2003). Because SopB, SopE, SopE2 and SipA were involved in the disruption of epithelial barrier, we examined whether these effectors were also responsible for changes in the localization or expression levels of tight junction proteins. Polarized Caco-2/TC7 monolayers were infected at the apical pole with various strains of *S*. Typhimurium for 4 h, and the localization of ZO-1 and occludin was examined by confocal microscopy. In uninfected and ΔinvA-infected cells, ZO-1 and occludin localized to the lateral cell membrane and formed a characteristic, contiguous web-like pattern (Fig. 6). In contrast, ZO-1 and occludin were highly disrupted in cells infected with wild-type *S*. Typhimurium, appearing in a discontinuous bead-like pattern, with increased levels appearing in the cytoplasm (Fig. 6). An ΔssaR mutant strain disrupted ZO-1 and occludin to the same extent as wild type, further supporting the above data that tight junction disruption occurs independently of SPI-2. The ΔsopB and ΔsopE/E2 mutant strains were able to displace ZO-1 and occludin; however, when all three effectors were absent, *S*. Typhimurium was no longer able to disrupt tight junction structure (Fig. 6) indicating that SopB, SopE and SopE2 are collectively required for disruption of tight junction structure. The ΔsipA and ΔsipA/sopB strains disrupted ZO-1 and occludin; however, a role for SipA in tight junction disruption was identified as the ΔsipA/sopE/E2 mutant was unable to disrupt localization of these two proteins (Fig. 6). These data demonstrate that together SopB, SopE, SopE2 and SipA are collectively responsible for the disruption ZO-1 and occludin localization and that, while involved, both SopB and SipA are insufficient to disrupt tight junction structure.

Detergent solubility of tight junction proteins can be used as an indicator of their membrane and cytoskeletal association (Stuart and Nigam, 1995; Nusrat et al., 2000). Accordingly, we assessed the Triton-X solubility properties of ZO-1 and occludin during an infection by *S*. Typhimurium. Western blot analysis of Triton-X-soluble and -insoluble fractions from Caco-2/TC7 revealed that ZO-1 was detected exclusively in the Triton-X-insoluble protein fraction (Fig. 7). Infection by wild-type *S*. Typhimurium decreased ZO-1 in the Triton-X-insoluble fraction ~66% of basal levels after 4 h (Fig. 7). Interestingly, over the course of an infection, ZO-1 remained undetectable in the Triton-X-soluble protein fraction, indicating perhaps that ZO-1 was not redistributed to cellular compartments accessible by the Triton-X lysis buffer or that soluble ZO-1 was rapidly degraded, making increased soluble levels difficult to observe. In the same protein extractions, occludin was localized primarily in the Triton-X-insoluble fraction with a minimal amount appearing in the Triton-X-soluble fraction (Fig. 7). Upon infection with wild-type *S*. Typhimurium, occludin levels in the Triton-X-insoluble protein fraction decreased to ~46% of basal levels after 4 h (Fig. 7). Although expression of occludin decreased over time, the ratio of Triton-X-soluble and -insoluble was maintained. Over the course of 4 h, infection by ΔsipA, ΔsopB, ΔsipA/sopB, ΔsopE/E2 mutants decreased ZO-1 and occludin expression to a similar extent as wild-type bacteria (Fig. 7). Conversely, infection with either a ΔsopB/E/E2 or ΔsipA/sopE/E2 mutant did not appreciably perturb the expression level of ZO-1 or occludin (Fig. 7). In accordance with our other measurements of tight junction structure and function, SopB, SopE, SopE2 and SipA are the bacterial effectors modulating disruption of tight junctions.

**SopB, SopE, SopE2 and SipA disrupt epithelial cell polarity**

Next we determined whether infection by *S*. Typhimurium altered the polar distribution of proteins. For these experiments, we used the canine epithelial cell line MDCK as
it is arguably the best-characterized polarized cell line and because many reagents for cell polarity studies are canine-specific. Polarized MDCK cells were infected with S. Typhimurium and monitored for redistribution of apical and basolateral markers. In uninfected cells, GP135 and E-cadherin were restricted to apical and basolateral surfaces respectively (Fig. 8). However, 4 h after infection with wild-type bacteria, E-cadherin and GP135 staining was apparent on both the apical and basolateral surfaces respectively (Fig. 8). Therefore, S. Typhimurium infection results in the intermixing of apical and basolateral membrane components causing loss of cell polarity. Next, we investigated which bacterial effectors were responsible for the disruption of cell polarity. The $\Delta$sipA, $\Delta$sopB, $\Delta$sopE/E2, $\Delta$sipA/sopB mutants all altered the polar distribution of GP135 and E-cadherin (Fig. 8). In contrast, and in agreement with our other measures of tight junction function, infection of polarized monolayers with $\Delta$invA, $\Delta$sopB/E/E2, $\Delta$sipA/sopE/E2 mutants did not affect the fence function of the monolayer (Fig. 8). Accordingly, S. Typhimurium disrupts cell polarity via SopB, SopE, SopE2 and SipA.

**Discussion**

The intestinal epithelium functions as a physical barrier. Tight junctions seal epithelial cell layers, performing a ‘gate’ function that restricts paracellular passage of ions, water, solutes and immune cells. Tight junctions also perform a ‘fence’ function in that they regulate cell polarity by acting as diffusion barriers that physically separate apical and basolateral membrane components. Infection by

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**Fig. 6.** SipA, SopB, SopE and SopE2 disrupt ZO-1 and occludin localization. Caco-2/TC7 cells were grown on Transwells for 18–21 days. Polarized Caco-2/TC7 monolayers were apically infected with wild-type S. Typhimurium and mutant derivatives for 4 h whereby cells were immunostained for occludin and ZO-1. En face Z-projection of confocal slices. Scale bar = 5 μm.
Geranylgeranylation in tight junction disruption by Typhimurium has been identified in a study performed in 1997; Schmidt et al. (1997). The constitutive activation of Rho GTPases (Flatau et al., 1997) increases the permeability of epithelial monolayers via the actin cytoskeleton (Ciesla and Bobak, 1998). Disruption of cellular junctions through disorganization of the diarrhea-gene pathogen Salmonella enterica serovar Typhimurium (Sousa et al., 2002; Sandle, 2005). In this study, we have identified the specific bacterial effectors responsible for disrupting tight junction structure and function. Our results identify a novel role for SopB, SopE, SopE2 and SipA in the pathogenesis of S. Typhimurium.

Modulation of the actin cytoskeleton through activation or inhibition of Rac1, Cdc42, or Rho can perturb the gate and fence function of tight junctions (Braga, 2002). Co-opting host cell Rho GTPases in order to disrupt cell–cell junctions is a common strategy used among several diarrheal pathogens (Sousa et al., 2005). For example, toxins of the diarrheagenic pathogen Clostridium difficile monoglucosylate Rho family GTPases thereby inducing disruption of cellular junctions through disorganization of the actin cytoskeleton (Ciesla and Bobak, 1998). E. coli increases the permeability of epithelial monolayers via its cytotoxic necrotizing factor 1, which causes the constitutive activation of Rho GTPases (Flatau et al., 1997; Schmidt et al., 1997). While the role of protein geranylgeranylation in tight junction disruption by S. Typhimurium has been identified in a study performed using MDCK cells (Tafazoli et al., 2003), we thought it important to corroborate these findings using a cell line relevant to human disease. Accordingly, for these studies we used the well-characterized human intestinal epithelial cell line, Caco-2/TC7. These monolayers have defined apical and basolateral surfaces, well-developed brush borders and functional tight junctions. Dominant-negative or constitutively active constructs of Cdc42, Rac1, or Rho cannot be used to address the role of Rho GTPases in tight junction disruption by S. Typhimurium as their expression alone will perturb the barrier function of the epithelium (Braga, 2002). Our studies utilized a pharmacological inhibitor of GGTPase-1-dependent protein geranylgeranylation that, at the concentrations utilized, blocks Rho GTPase activity without altering tight junctions. Inhibition of protein geranylgeranylation effectively prevented tight junction disruption by S. Typhimurium. Inhibition was significant yet incomplete and therefore we recognize there may be another Rho GTPase-independent mechanism of tight junction disruption. We are unable to investigate this at this time given the limitations of the inhibitors currently available.

**Fig. 7.** SipA, SopB, SopE and SopE2 modulate expression of ZO-1 and occludin. Western blot analysis of Triton-X-soluble (S) and -insoluble (I) fractions at the indicated time points post infection. Equal amounts of protein (15 μg) were loaded in each lane. Results shown are representative of at least two independent experiments.

**Table 1.** SipA, SopB, SopE and SopE2 modulate expression of ZO-1 and occludin. Western blot analysis of Triton-X-soluble (S) and -insoluble (I) fractions at the indicated time points post infection. Equal amounts of protein (15 μg) were loaded in each lane. Results shown are representative of at least two independent experiments.

**Fig. 8.** SipA, SopB, SopE and SopE2 are responsible for disrupting cell polarity. MDCK cells were grown on Transwells for 4 days. Polarized monolayers were apically infected with wild-type S. Typhimurium or mutant strains for 4 h. Cells were immunostained for the basolateral marker, E-cadherin, or the apical marker, GP135. XZ-slice using confocal microscopy. Arrows indicate areas where cell polarity has been lost. Scale bar = 5 μm.

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that higher concentrations of the inhibitor perturbs the integrity of the barrier (data not shown). However, the results presented here strongly suggest that in human intestinal cells, Rho family GTPases are involved in tight junction disruption by \textit{S. Typhimurium}.

Rho family GTPases mediate diverse cellular activities that include not only reorganization of the actin cytoskeleton but also modulation of gene transcription, primarily through their effects on mitogen-activated protein kinase and NF-xB signalling cascades (Benitah et al., 2004; Sorokin and Chernoff, 2005). Therefore, it was possible that \textit{S. Typhimurium}-induced barrier disruption was caused by Rho GTPase-dependent epithelial-derived products. Using a eukaryotic-specific inhibitor of protein synthesis, we establish that \textit{de novo} protein synthesis is not required for loss of barrier function by \textit{S. Typhimurium}. Soluble products such as IFN\textgamma, TNF\textalpha, IL-1\beta, IL-4, IL-8 and IL-13 have been reported to disrupt tight junctions (Clayburgh et al., 2004). In polarized endothelial cells infected with dengue virus, secreted IL-8 acts in an autocrine manner and contributes to tight junction disruption in these cell monolayers (Talavera et al., 2004). In this study, we demonstrate that secreted soluble factors from \textit{S. Typhimurium}-infected epithelial cells do not play a role in intercellular disruption. By discounting the role of epithelial-derived products, our findings support the idea that tight junction disruption by \textit{S. Typhimurium} is a result of the effect of Rho GTPases on the actin cytoskeleton.

The effects of \textit{S. Typhimurium} on tight junctions are dependent upon SPI-1 (Jepson et al., 1996; Tafazoli et al., 2003) yet the contribution of specific SPI-1-secreted effectors to these events had not previously been addressed. Accordingly, using various SPI-1-secreted effector mutants, we investigated the mechanism of \textit{S. Typhimurium}-induced intercellular disruption. Because of the involvement of Rho family GTPases in tight junction disruption by \textit{S. Typhimurium}, the Rho GTPase activators SopB, SopE and SopE2 were considered likely candidate effectors to be involved. In the absence of SopB or SopE/E2, TER decreased to the same extent as wildtype-infected cells. However, compared with wildtype-infected cells, a \textita{sopB/E2} mutant did not disrupt TER. Because of partial functional overlap, studying the role of single effectors has proven to be difficult as disruption of a single effector often results in only a minor change in a cellular phenotype (Ehrbar et al., 2002). The synergistic activation of Rho family GTPases by SopB, SopE and SopE2 is known to contribute to invasion into host cells (Zhou et al., 2001; Ehrbar et al., 2002). Consequently, because of their complementary effects on Rho family GTPases, tight junction disruption by \textit{S. Typhimurium} was prevented only in the absence of all three effectors. Together, we have shown that tight junction disruption by \textit{S. Typhimurium} can be prevented by inhibiting Rho GTPases or \textit{S. Typhimurium}'s ability to activate Rho GTPases via SopB, SopE and SopE2.

SipA is another SPI-1-secreted effector that modulates the cytoskeleton by directly binding to actin, stabilizing F-actin filaments and increasing the bundling activity of the host protein, T-plastin (Zhou et al., 1999). In the absence of SipA, or SipA and SopB, TER decreases to the same extent as wildtype-infected cells. However, when the effect of a \textit{sopE2}sopE2 mutant (TER drops) is compared with that of a \textita{sipA}sopE2 mutant (TER remains undisturbed), an important role for SipA in tight junction disruption is revealed. Interestingly, the fact that the \textita{sipA}sopE2 mutant was unable to disrupt TER demonstrates that SopB alone is insufficient to disrupt tight junctions. Conversely, the lack of tight junction disruption by the \textita{sopB/E2} mutant reveals that SipA is also insufficient to alter tight junctions. Previous transfection studies have demonstrated that SopB and SipA expression is sufficient to modulate the actin cytoskeleton (Zhou et al., 2001; Higashide et al., 2002). Whether SipA and SopB cooperate or whether either functions with SopE or SopE2 in order to disrupt tight junctions is unknown; however, a recent study has provided evidence that cooperation exists between SipA and SopB during invasion into polarized and non-polarized epithelial cells (Raffatellu et al., 2005).

Tight junctions do not appear to be simple seals but rather allow selective passage of certain components through the paracellular pathway (Miyoshi and Takai, 2005). Therefore, the existence of selective channels within tight junctions had been proposed. Under some experimental conditions, there can be a functional dissociation of paracellular flux from TER in that TER can remain undisturbed while paracellular diffusion of small molecules increases over time (Baldet al., 1996). These observations suggest that TER and paracellular flux may not measure the same characteristics of epithelial permeability. TER is an instantaneous measurement of ionic conductivity that reflects epithelial integrity as well as tight junction ion selectivity, while paracellular flux reflects permeability over longer periods of time and allows the determination of the size selectivity of the paracellular diffusion barrier. Therefore, in order to definitively prove that the \textit{sopB/E2} and \textita{sipA}sopE2 mutants were not able to affect the epithelial permeability, we measured paracellular flux of FITC-dextran during a 4 h infection. Compared with wild-type \textit{S. Typhimurium}, paracellular flux was significantly reduced with both mutants thereby confirming that SipA, SopB, SopE and SopE2 are the effectors responsible for increasing the permeability of epithelial monolayers.

The formation of tight junctions is associated with an increase in the resistance of tight junction complexes to solubility in detergent-salt extractions. Conversely, disas-
In summary, we have identified SopB, SopE, SopE2 and SipA as the specific SPI-1-secreted effectors responsible for disruption of tight junction structure and function by S. Typhimurium. Our data support that these effectors are signalling through Rho family GTPases in order to exert their effect on epithelial barrier function. Our results identify a novel role for SopB, SopE, SopE2 and SipA in the pathogenesis of S. Typhimurium and these findings have important implications for Salmonella enteritis as disruption of tight junctions is likely to contribute to the production of diarrhea.

**Experimental procedures**

### Cell culture

The human intestinal epithelial cell line Caco-2/TC7 and the canine kidney epithelial cell line MDCK type II (American Type Culture Collection) were incubated at 37°C in a humidified atmosphere with 5% CO₂. Cells were grown in Dulbecco's minimal Eagle medium (DMEM) supplemented with 10% fetal calf serum and 1% non-essential amino acids. To obtain polarized monolayers, cells were grown on permeable Transwell filters (polyester; 6.5 or 24 mm diameter; 3 μm pore size; Corning, NY). Caco-2/TC7 cells were seeded onto Transwells at an initial density of 7.6 × 10⁵ cells cm⁻² and were grown for 18–22 days, until TER stabilized between 250 and 300 Ohms cm⁻². MDCK cells were initially seeded at 1.0 × 10⁵ cells cm⁻² and were grown for 4 days. Culture medium was replaced every 2–3 days.

### Generation of bacterial mutants and complemented strains

We introduced unmarked non-polar internal deletions of sopB and sipA by allelic exchange as previously described by Steele-Mortimer et al. (Steele-Mortimer et al., 2000) and Zhang et al. (Zhang et al., 2002) respectively. All chromosomal deletions were confirmed by polymerase chain reaction (PCR) and sequencing. All bacterial strains used in this study are described in Table 1.

To construct the psopE2 plasmid, sopE2 and its promoter region were PCR-amplified from SL1344 chromosomal DNA using Elongase (Invitrogen Life Technologies, Burlington, ON) and the oligonucleotide primers SOPE2FWD (5'-GACCATGGCTTTCGACGCCAGTATGTCGATG-3') and SOPE2REV (5'-GACGAAATCCATCGAGGGCATTCTGAAG-3') to generate a 1761 bp fragment. This fragment was cloned into pCR2.1 (Invitrogen) and its sequence confirmed by DNA sequencing. This plasmid was subsequently digested with EcoR1 and Nco1 and the insert was cloned into the corresponding restriction enzyme sites in pACYC184.

### Bacterial infection of polarized epithelial cells

GGTI-298 (Calbiochem, La Jolla, CA) was applied to Caco-2/TC7 cells 40 h prior to infection. Cycloheximide (Sigma Aldrich) was added 15 min prior to infection. S. Typhimurium was grown for 16 h in Luria broth (LB) supplemented with streptomycin (100 μg ml⁻¹), tetracycline (3 μg ml⁻¹), or kanamycin (25 μg ml⁻¹). Cultures were diluted 1:33 into LB without antibiotic and grown for 3 h prior to infection. Bacterial cultures were centrifuged at 8000 r.p.m. and resuspended (equal volume) in phosphate-buffered saline (PBS). Caco-2/TC7 cells were infected at an approximate multiplicity of infection of 50.

### Measurement of TER and paracellular flux

Transepithelial resistance was measured using an epithelial vol-
was measured with a fluorometer (excitation 492 nm, emission medium was collected and the concentration of FITC-dextran for tight junction disruption. After a 4 h infection, the basolateral cells incubated in P/EGTA buffer served as the positive control.

Immediately prior to infection, FITC-dextran was added to the 2 mM ethylene glycol-bis(N-hydroxyethyl)ether)-N,N'N'-tetraacetic acid (EGTA)]. To measure paracellular flux, 4 and 40 kDa fluorescein isothiocyanate (FITC)-dextran (Sigma, St Louis, MO) was dissolved in P buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4; 1 mM sodium pyruvate; 10 mM glucose; 145 mM NaCl; 3 mM CaCl₂; 145 mM NaCl) or P/EGTA buffer [10 mM HEPES, pH 7.4; 1 mM sodium pyruvate; 10 mM glucose; 145 mM NaCl; 2 mM ethylene glycol-bis(N-hydroxyethyl)ether)-N,N',N'-tetraacetic acid (EGTA)]. To measure paracellular flux, cells were allowed to equilibrate in P buffer 1 h prior to infection. Immediately prior to infection, FITC-dextran was added to the apical compartment to give a final concentration of 10 mg ml⁻¹.

Uninfected cells served as a negative control while uninfected cells incubated in P/EGTA buffer served as the positive control for tight junction disruption. After a 4 h infection, the basolateral medium was collected and the concentration of FITC-dextran was measured with a fluorometer (excitation 492 nm, emission 520 nm).

IL-8 enzyme-linked immunosorbant assay (ELISA)

The basolateral culture media was collected and IL-8 concentration was determined by ELISA (R&D Systems) as recommended by the manufacturer.

Immunofluorescence

For ZO-1, occludin and E-cadherin staining, cells were washed extensively with PBS and fixed with 20% paraformaldehyde for 20 min at room temperature. Cells were washed extensively after fixation and were permeabilized and blocked with 7% normal goat serum (NGS) and 0.1% Triton X-100 in PBS overnight at 4°C. For GP135 staining, cells were fixed in ice-cold methanol at −20°C for 20 min. Cells were rehydrated in PBS for 10 min and then blocked in 1% bovine serum albumin/10% NGS for 30 min. Antibodies were used at the following concentrations: anti-ZO-1 (1:200; Zymed, San Francisco, CA), anti-occludin (1:200; Zymed), mouse anti-GP135 (1:10; a kind gift from Dr George Ojakian), mouse anti-Salmonella lipopolysaccharide (LPS) (1:1000; Difco) and rabbit anti-Salmonella LPS (1:200; Difco).

Following incubation with primary antibodies, the cells were washed extensively with PBS and probed with 1:200 dilutions of anti-mouse 488, anti-rabbit 488, anti-mouse 668, and anti-rabbit 668 Alexa dye-conjugated antibodies (Molecular Probes). Membrane filters were cut out of Transwells and were mounted on glass slides in Mowiol (Aldrich). Samples were viewed on a Zeiss Axiosvert S100 TV microscope attached to a Bio-Rad Radiance Plus confocal scanhead. Confocal sections were projected using ImageJ v.1.36b and were imported into Adobe Photoshop and Illustrator CS2 (San Jose, CA).

Western blot analysis

Infected monolayers were washed three times with PBS. Extractions were performed by overlaying cells with ice-cold CSK buffer [10 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), pH 6.8; 50 mM NaCl; 300 mM sucrose; 3 mM MgCl₂; 0.5% Triton X-100] containing a Complete protease inhibitor tablet (Roche). Cells were extracted for 20 min on ice, collected and spun at 13 200 r.p.m. at 4°C for 15 min. The supernatant (soluble fraction) was collected. The pellet (insoluble fraction) was disrupted by adding SDS-1P buffer [1% sodium dodecyl sulphate (SDS); 10 mM Tris-Hcl, pH 7.5; 2 mM ethylenediaminetetraacetic acid (EDTA)] containing a Complete protease inhibitor tablet. The insoluble fraction was boiled for 5 min and sonicated until the pellet was resuspended. The protein concentration of each sample was quantified using a bicinechoninic acid assay (Sigma). Equal amount of total protein (15 μg) was loaded in each lane of the gel. Samples were electrophoresed through a 6% (for ZO-1) or 10% (for occludin) SDS polyacrylamide gel and transferred onto nitrocellulose membrane (Millipore, Bedford, MA). The membranes were incubated overnight at 4°C in blocking buffer [Tris-buffered saline, 0.1% Tween 20 (TBST), 5% skim milk] and then incubated for 3 h at room temperature with primary antibodies diluted in blocking buffer. After washing in TBST, the membranes were incubated with the appropriate secondary antibody diluted in blocking buffer for 1 h at room temperature. After washing in TBST, the bands were detected using an enhanced chemiluminescence (ECL) kit (Amersham), according to the manufacturer’s instructions.
Bands of interest were quantified using ImageJ v.1.36b software. All Western blots are representative of at least two experiments carried out.

**Statistical analysis**

Data were analysed using PRISM 4.0. One-way ANOVAs were performed using a 95% confidence interval. Bonferroni’s and Dunnett’s tests were applied post hoc.

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