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Disruption of Epithelial Barrier Integrity by *Salmonella enterica* Serovar Typhimurium Requires Geranylgeranylated Proteins

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Epithelial cells that line the human intestinal mucosa constitute the initial sites of host invasion by bacterial pathogens. A number of bacteria, such as *Salmonella* and *Yersinia* spp., have been shown to disrupt the integrity of the epithelial barrier, although little is known about the mechanisms underlying that effect. We found that polarized MDCK-1 epithelial cells infected with invasive *Salmonella enterica* serovar Typhimurium SL1344 exhibited marked changes in F-actin organization, an increase in the paracellular flux of dextran, and a rapid decrease in transepithelial electrical resistance (TER). In contrast, infection with an isogenic noninvasive mutant (*hilA*) increased the TER in these cells. Pretreating MDCK-1 cells with the inhibitors for tyrosine kinase (genistein) or phosphatidylinositol 3-kinase (wortmannin) did not affect invasion and subsequent perturbation of the epithelial barrier by serovar Typhimurium. Instead, the geranylgeranyltransferase 1 inhibitor GGTI-298, but not the farnesyltransferase inhibitor FTI-277, clearly reversed the capacity of serovar Typhimurium to disrupt the epithelial barrier. The substrates for GGTI-298 include Rho family GTPases, as indicated by inhibiting prenylation of Rac1 and Cdc42. Infection with wild-type serovar Typhimurium increased the level of activated Rac1 and Cdc42 and caused these proteins to accumulate apically in MDCK-1 cells. This *Salmonella*-induced accumulation of Rac1 and Cdc42 and alteration of the junction-associated proteins ZO-1, occludin, and E-cadherin in MDCK-1 cells were markedly inhibited by GGTI-298. These results suggest that activation of geranylgeranylated proteins, including Rac1 and Cdc42, is critical for disruption of barrier integrity by serovar Typhimurium in polarized MDCK-1 cells.

The gram-negative bacterium *Salmonella enterica* serovar Typhimurium is a leading cause of gastroenteritis in humans, and it induces a typhoid-like systemic disease in mice. This pathogen is acquired through ingestion of contaminated food or water and is assumed to cross the epithelial barrier at the level of the ileum or colon by invading enterocytes and M cells (19). It has been found that the ability to penetrate the intestinal mucosa correlates with the observed capacity of these bacteria to invade cultured, nonphagocytic cells, a process that is dependent on a bacterial type III secretion system (11, 12, 19). Activation of this secretion system directs the translocation of bacterial effector proteins into host cells, where they can modulate cell signal transduction pathways that ultimately induce a variety of responses. These responses include cytoskeletal rearrangements, bacterial internalization, and nuclear reactions leading to the production of proinflammatory cytokines, which are presumably essential for establishment of the *Salmonella* infection (9, 10, 16).

The tight junctions are located at contact sites between epithelial cells and between endothelial cells. It has been reported that formation and maintenance of tight junctions is regulated not only by the specific proteins of the junctions but also by the perijunctional actin cytoskeleton (4, 23). Tight junctions maintain the cellular polarity required for vectorial transport across the epithelium and serve as a paracellular barrier to restrict ion and solute diffusion. Accordingly, disruption of or interference with intestinal epithelial tight junctions may contribute to microbe-associated diarrhea. The permeability properties of tight junctions also depend on the integrity of the immediately adjacent adherens junctions. The basic constituent of an adherens junction is the transmembrane protein E-cadherin, which is associated with a number of intracellular proteins, called catenins, that link E-cadherin with some cytoskeletal components (4, 14). The injected toxins ExoS, YopE, and SptP from *Pseudomonas aeruginosa*, *Yersinia pestis*, and *Salmonella* spp., respectively, which are transferred into the eukaryotic target cells by the type III secretion system, inhibit Rho function by acting as Rho GAP proteins (2).

Although a variety of enteric pathogens perturb the epithelial barrier when they infect a host organism, the mechanisms underlying such a disturbance are probably distinct for each species of bacteria. For example, *Clostridium difficile* toxins and *Escherichia coli* cytotoxic necrotizing factor 1 enhance permeability by regulating the activity of Rho GTPases and disrupting actin microfilaments (13, 26, 27), and enteropathogenic *E. coli* induces tight junction dysfunction via phosphorylation of myosin light chains (38). NSP4 enterotoxin of rotavirus prevents transport of the ZO-1 protein to tight junctions during biogenesis and thereby impairs normal formation of these junctions (35).

Invasion of epithelial layers by serovar Typhimurium is known to increase tight junction permeability, and studies of MDCK cells infected with this species have suggested that such augmented penetrability involves modulation of the MDCK actin cytoskeleton but not direct interaction between the bacteria and tight junctions (17). It is known that contraction or disruption of perijunctional actin causes tight junction dysfunc-

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tion in epithelial cells. Moreover, it has been shown that serovar Typhimurium induces constriction of the perijunctional actin ring with kinetics similar to that of increased paracellular permeability (17). It was recently found, however, that the protein kinase inhibitor staurosporine prevented the perijunctional contraction but did not reverse the effects of serovar Typhimurium on the barrier function of tight junctions (18). Together, the cited results indicate that the epithelial response to *Salmonella* infection is multifactorial.

A number of serovar Typhimurium effector proteins or products have been shown to regulate various host cell signaling pathways, such as protein tyrosine phosphorylation, the small GTP-binding proteins Cdc42 and Rac, and phosphatidylinositol 3-kinase (PI3-kinase) (24, 31). All these effector substances influence key signaling events that control the actin cytoskeleton in a variety of systems, but their roles on modulations of epithelial barrier by serovar Typhimurium are largely unclear. For example, both Rac1 and Cdc42 are known to regulate tight junctions in MDCK cells (12, 13, 20). A recent study showed, for instance, that activation of Rac and Cdc42 by serovar Typhimurium had no effect on the barrier integrity in these cells (8). In addition, conflicting results have been reported about the role of protein tyrosine phosphorylation on the invasion of serovar Typhimurium into epithelial cells (25, 33). Therefore, the aim of the present study was to determine the signaling mechanisms underlying the disruption of epithelial barrier by serovar Typhimurium.

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MATERIALS AND METHODS

Reagents and Abs. The antibodies (Abs), reagents, and other materials and their sources were as follows: anti-Rac1 and anti-Cdc42 monoclonal Abs (BD Transduction Laboratories, San Diego, Calif.); rat anti-ZO-1 (Chemicon Inc., Temecula, Calif.) and rabbit antioccludin (Zymed, San Francisco, Calif.); goat anti-rat and anti-mouse Alexa 488, goat anti-rabbit Alexa 594, and rhodamine-labeled phalloidin (Molecular Probes); glutathione-Sepharose beads and electrophoresis reagents (Pharmacia Biotech, Uppsala, Sweden); and an enhanced chemiluminescence (ECL) kit (Amersham, Little Chalfont, United Kingdom); genistein (RBI, Natick, Mass.). GGTI-298 and FTI-277 were generously provided by Said Sebti (H. Lee Moffitt Cancer Center, Tampa, Fla.) (36); all other reagents were from Sigma (St. Louis, Mo.), unless otherwise indicated in the text.

Bacterial strains and growth conditions. The wild-type invasive serovar Typhimurium strain SL1344 and the isogenic mutant strain *hilA* (VV341) were kindly provided by C. A. Lee of Harvard Medical School (21, 22). The bacteria were grown in 2 ml of Luria-Bertani (LB) broth medium overnight at 37°C under agitation. These cultures were diluted 30 times with fresh LB medium in culture tubes with sealed caps and shaken for an additional 3.5 h at 37°C (optical density at 600 nm, about 0.30). Kanamycin (50 mg/liter) was added for culturing the *hilA* mutant. Aliquots of the bacterial culture were centrifuged at 2,000 × *g* for 5 min, washed twice with phosphate-buffered saline (PBS) (pH 7.4), and resuspended in Dulbecco's modified Eagle's medium (DMEM) without antibiotic.

Cell culture and treatment with inhibitors. MDCK-1 cells (passages 64 to 78) were grown for three days in DMEM supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 100 U of penicillin (GIBCO, BRL Life Technologies, Paisley, Scotland) per ml. The cells were cultured to confluence on Transwell filters (no. 3415; pore size, 3 μm; Costar, Badhoevedorp, The Netherlands) or in tissue culture plates at 37°C in a humidified atmosphere of 5% CO_2.

Cells were pretreated at 37°C for 30 min with 50 μM genistein to inhibit the activities of tyrosine kinase or with 100 nM wortmannin to inhibit PI3-kinase. Likewise, cells were pretreated at 37°C for 40 h with 5 μM GGTI-298 to inhibit protein geranylgeranylation or with 5 μM FTI-277 to block farnesylation. These inhibitors were present at the indicated concentrations throughout the infection period.

Bacterial infection. The confluent cell monolayers were washed free of penicillin and then stabilized by placing them in 24-well tissue culture plates covered with DMEM without antibiotic for 2 h at 37°C in a 5% CO_2 atmosphere. The cells were then infected for 1 h with different strains of serovar Typhimurium at a multiplicity of infection (MOI) of 50. The infected cells were washed with DMEM without antibiotic to remove nonadherent bacteria and then incubated with 50 μg of gentamicin/ml for 1 h to kill the extracellular bacteria. The monolayer was washed again, and the filters were placed in fresh medium without antibiotic.

Measurement of permeability of epithelial monolayers. The permeability of epithelial monolayers was assessed by measurement of transepithelial electrical resistance (TER) and paracellular flux of fluorescein isothiocyanate (FITC)-dextran (20 kDa). The TER of the cell monolayers was determined using an epithelial volt-ohm meter (EVOM; World Precision Instruments, Sarasota, Fla.) before and during the 1-to-3-h infection with different strains of serovar Typhimurium. The passage of 20-kDa FITC-dextran across the cell monolayers was determined as described previously (35).

Affinity precipitation of activated Rac1 and Cdc42. Activation of Rac1 and Cdc42 in MDCK cells was determined using a recently described method (5), with minor modifications. Briefly, the pGEX-4T3 construct encoding the GTPase-binding domain of human protein A kinase 1, kindly provided by G. M. Bokoch (Scripps Research Inst., La Jolla, Calif.), was expressed in *E. coli* as glutathione *S*-transferase (GST) fusion protein (GST-PBD). Expression of GST-PBD in transformed *E. coli* was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h, and after lysing the bacteria by sonication, the fusion proteins were purified on glutathione-Sepharose beads. MDCK cells in six-well tissue culture plates (10 cm^2) were serum starved for 16 h and then stimulated with serovar Typhimurium for the indicated periods of time. Thereafter, the cells were washed with ice-cold PBS, lysed in phosphorylation lysis buffer (3), and centrifuged to remove nuclei. The lysates were incubated with GST-PBD prebound to glutathione-Sepharose beads at 4°C for 40 min under rotation, and the beads were subsequently washed once with lysis buffer and three times with ice-cold buffer containing 1 mM dithiothreitol, 40 mM NaCl, 30 mM $MgCl_2$, and 0.5% NP-40 in 25 mM Tris-HCl (pH 7.5). Proteins on the beads were eluted with Laemmli sample buffer, separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE), and electrophoretically transferred onto nitrocellulose membranes. The presence of Rac1 and Cdc42 on the blots was detected with specific Abs and a commercial ECL kit. The specificity of this assay was confirmed by omitting GST-PBD (negative control) and by adding 100 μM GTP gamma subunit (positive control) during the precipitation step.

Determination of Rac1 and Cdc42 processing. MDCK cells were treated with either medium, 5 μM GGTI-298 or 5 μM FTI-277, for 40 h and lysed as described above. Equal amounts of cellular proteins were separated by SDS–12.5% PAGE and analyzed by Western blotting with Abs specific to Rac1 and Cdc42 as described above.

Invasion assay. The ability of *Salmonella* to invade MDCK cells was determined using the gentamicin-protective assay, as described elsewhere (21). The cells were seeded on 24-well tissue culture plates or a Transwell filter, grown for 3 days, and then infected with an initial inoculum of 1×10^7 to 2×10^7 CFU/well. To synchronize the infection of monolayers, the infected tissue culture plates were centrifuged at 200 × *g* for 5 min. The cells were then incubated for 60 min at 37°C, after which the overlying medium was replaced with 1 ml of tissue culture medium supplemented with 50 μg of gentamicin/ml. The monolayers were subsequently incubated with gentamicin for 60 min at 37°C and then washed with PBS and lysed with 0.2 ml of 1% Triton X-100 for 10 min. Samples were vigorously mixed with 0.8 ml of LB broth, and the viable intracellular bacteria were quantified by plating for CFU on LB agar plates after serial dilutions. Where indicated, the cells were pretreated with inhibitors as described above.

Fluorescence microscopy. Monolayers of MDCK-1 cells were grown to confluence on a Transwell filter and then incubated with bacteria for various amounts of time. Thereafter, the specimens were washed twice in PBS and fixed with 2.5% paraformaldehyde for 45 min on ice, washed in PBS, and incubated in $NaBH_4$ (0.5 mg/ml) for 10 min to reduce autofluorescence. The cells were subsequently permeabilized with 0.3% Triton X-100 for 7 min at room temperature, and F-actin was stained with rhodamine-labeled phalloidin for 45 min at 37°C in the dark. We used mouse anti-Cdc42/Rac1 and, as secondary Abs, goat anti-mouse Alexa 488 Abs. Rabbit antioccludin, rat anti-ZO-1, and mouse anti-E-cadherin Abs were used to localize the respective proteins by detecting the following secondary Abs: Alexa 594-tagged goat anti-rabbit, Alexa 488 TM goat

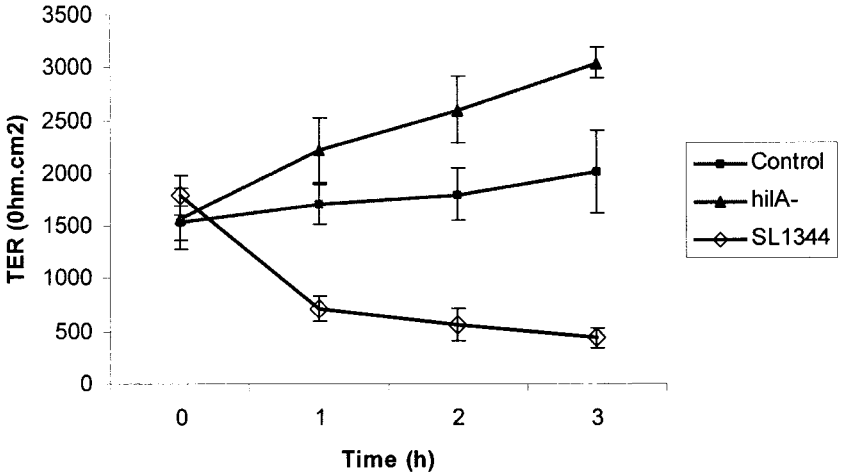

FIG. 1. Invasive serovar Typhimurium decreases the TER in polarized MDCK-1 cell monolayer. The cell monolayers were infected with SL1344 (wild type) or *hilA* mutant or treated with medium (Control) for 1 h, and this was followed by determination of TER at different times. The results are the means ± SD (error bars) of four experiments, each performed in triplicate.

anti-rat, and Alexa 488-labeled goat anti-mouse Abs. Finally, coverslips were washed in PBS and mounted on glass microscope slides in ultimate mounting medium containing antifade agent (CITIFLUOR Ltd., London, United Kingdom). The specimens were examined in a confocal microscope (Sarastro 2000; Molecular Dynamics, Sunnyvale, Calif.) using a 60× oil immersion objective (numerical aperture = 1.4). For fluorescence activation, either all lines or the 514-nm line of the argon laser was used in combination with a laser power of 10 mW maximum and a 535-nm primary beam splitter. For dual-stained samples, a second beam splitter (595 DRLP) was matched with a barrier filter, 600 EFLP (red channel), and an interference filter, 540 DF30 (green channel).

Scanning electron microscopy (SEM). MDCK-1 cells were grown to confluence on glass coverslips in 24-well tissue culture plates and then incubated with serovar Typhimurium SL1344 (wild type) and a noninvasive *hilA* mutant for 1 h. The infected cells were washed twice with PBS and then fixed with 1 ml of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C overnight. The cells were postfixed in 1% OsO_4 in 0.1 M sodium cacodylate buffer for 2 h. The fixed monolayers were dehydrated in ethanol and then dried from liquid CO_2 in a Polaron E3000 critical point apparatus and subsequently mounted with conducing carbon paint on metal stubs. The mounted specimens were sputter coated with a 10-nm thickness of platinum using a planar magnetron sputter coater installed in an Edwards E12E vacuum evaporator and then examined in a JEOL JSM-840 scanning electron microscope operated at 20 kV.

RESULTS

Invasive serovar Typhimurium causes reorganization of F-actin and disruption of barrier integrity in MDCK-1 cells. Invasion of MDCK-1 cells by serovar Typhimurium was determined by the gentamicin-protective assay. In agreement with results reported by other investigators (2, 32), the *hilA* mutant was severely deficient in its ability to invade MDCK-1 cells, and about 500-fold fewer gentamicin-protected bacteria than wild-type SL1344 strain bacteria were observed within the cells after 1 h of infection (data not shown). During apical infection with different serovar Typhimurium strains, we monitored changes in tight-junction integrity in MDCK-1 monolayers by measuring the TER and the paracellular flux of FITC-dextran. The results show that the TER was significantly decreased by the wild-type SL1344 strain but was markedly increased by the noninvasive mutant (Fig. 1).

Invasion of serovar Typhimurium also increased the transport of dextran from the apical domain to the basolateral chamber, with a kinetic that corresponds well to that of reduction in TER. After 3 h of incubation, the percentage of dextran flux (means ± standard deviations [SD]) in uninfected cells and the cells infected with *hilA* mutant were 0.1 ± 0.12 and 0 ± 0, respectively, whereas the wild-type SL1344 significantly increased the dextran flux to 0.5 ± 0.1, 5.2 ± 0.7, and 12. 6 ± 0.9, determined at 1, 2, and 3 h postinfection ($n = 4$).

Using confocal microscopy and SEM to examine the morphological changes in *Salmonella*-infected MDCK cells, we found that the *hilA* mutant adhered to the surface of the host cells and aggregated to form long filaments (Fig. 2a). In contrast, the wild-type bacteria formed both long and short filaments, and they disrupted the microvilli of the MDCK cells and induced membrane ruffles. During the later stages of bacterial internalization, these ruffles became larger and more elaborate and occasionally appeared as lamellipodial sheets (Fig. 2c). Few wild-type bacteria were visible on the apical surface of monolayers after incubation for 1 h, indicating that most of the attached bacteria had been internalized in the MDCK cells. In parallel, F-actin was markedly reorganized in the cells infected with wild-type bacteria (Fig. 2d) but not in those infected with the *hilA* mutant (Fig. 2b). Treatment of MDCK cells with 2 μM cytochalasin D completely blocked the invasion of wild-type serovar Typhimurium. However, such treatment also induced a dramatic decrease in TER in these cells. These results are in agreement with the general concept that actin cytoskeleton is essential for the formation and maintenance of tight junctions of host cells as well as for the invasion of serovar Typhimurium (28, 34).

Geranylgeranylated proteins are involved in the disruption of the epithelial barrier by serovar Typhimurium. Although *Salmonella* can activate several key regulators of the actin cytoskeleton, it has been shown that activation of PI3-kinase is not required for the invasion of bacteria, and conflicting results have been reported regarding the role of tyrosine kinase and Rho GTPases in this process (8, 11, 12, 25, 33). To address which of these signals is involved in the disruption of epithelial barrier observed in the present study, MDCK cells were pretreated with inhibitors of various signaling pathways and then exposed them to serovar Typhimurium. We found that neither

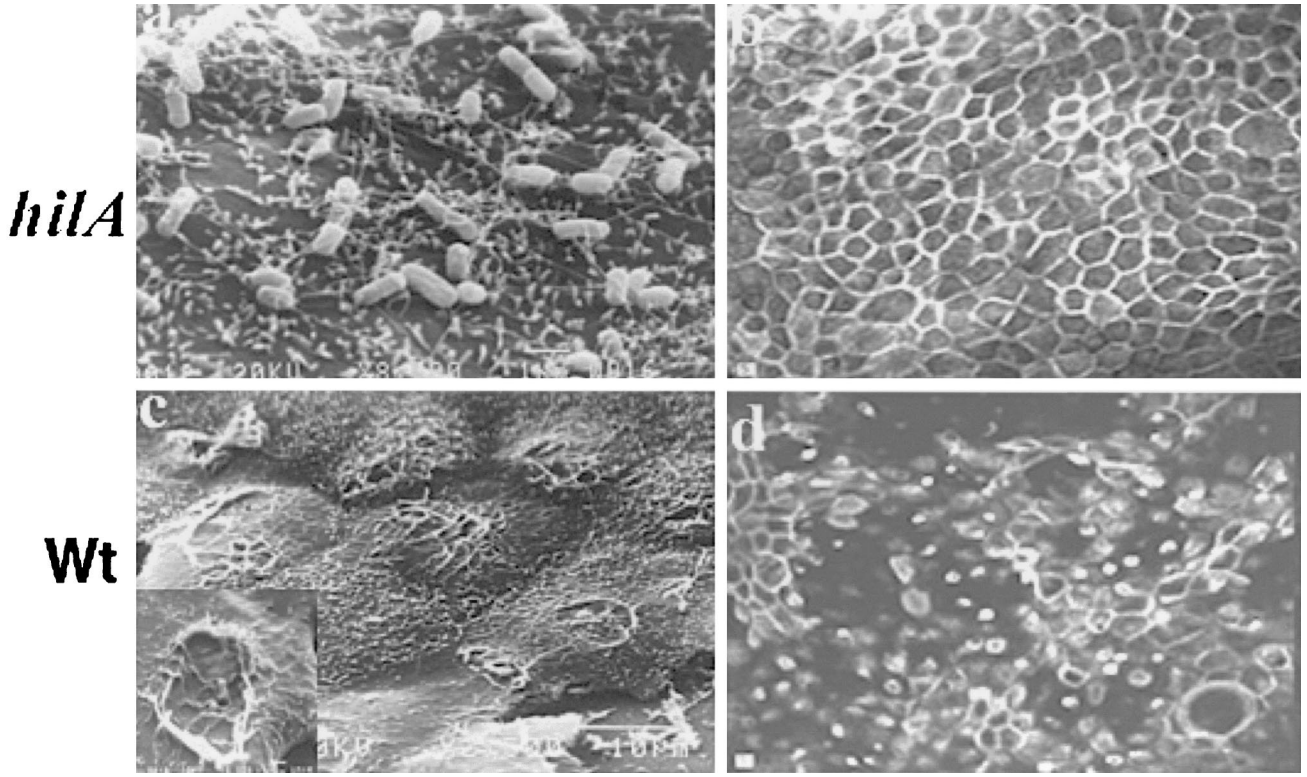

FIG. 2. Invasive serovar Typhimurium induces reorganization of the actin cytoskeleton in MDCK-1 cells. Monolayers of MDCK-1 cells were infected with wild-type (Wt) or *hilA* mutant serovar Typhimurium and subsequently stained for F-actin with tetramethyl rhodamine isothiocyanate-labeled phalloidin and examined in a laser scanning confocal microscope (b and d). More-detailed analysis by SEM (a and c) revealed disruption of the MDCK cell microvilli and "splash-like" focal aggregation and partial internalization of the wild-type serovar Typhimurium. The *hilA* mutant bacteria can be seen proliferating on the cell surface close to the microvilli. Bar = 10 μm.

genistein nor wortmannin, which, respectively, inhibit tyrosine kinase and PI3-kinase, affected the decreased TER in MDCK monolayers infected with the SL1344 strain (Fig. 3). However, pretreatment with GGTI-298, which inhibits protein geranylgeranylation, markedly blocked the capacity of invasive serovar Typhimurium to disrupt the MDCK-1 epithelial barrier; by contrast, inhibition of protein farnesylation by FTI-277 had no such effect (Fig. 3). Similarly, invasion of the cells by serovar

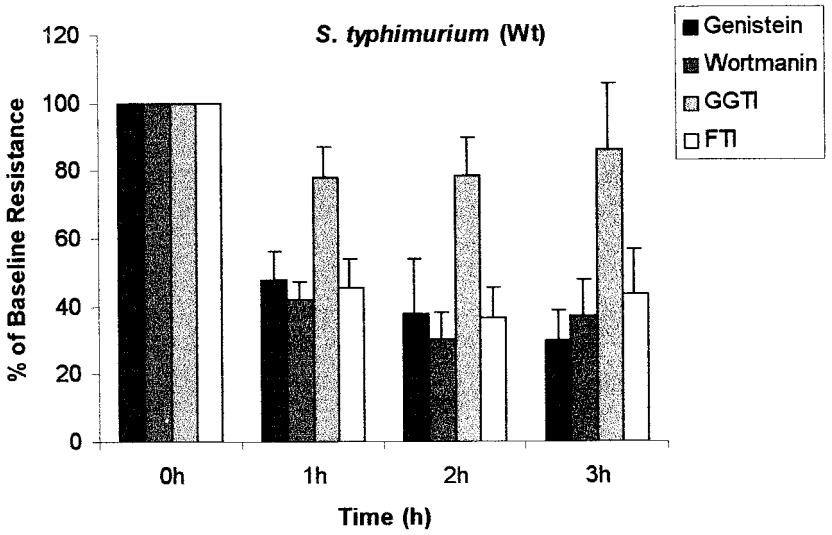

FIG. 3. Geranylgeranylated proteins, but not tyrosine kinase or PI3-kinase, must be activated to allow disruption of the epithelial barrier by serovar Typhimurium. MDCK-1 cells were pretreated with 100 nM wortmannin, 50 μM genistein, 5 μM GGTI-298, 5 μM FTI-277, or medium alone and then apically infected with SL1344. The TER was measured before and 1, 2 and 3 h after infection. Values are expressed as means + SD (error bars) of four separate experiments, each performed in triplicate. The starting TER values (100%) of the control are 1,503 ± 269 $\Omega \cdot cm^2$(mean 1,503 ± SD from three independent experiments, each performed in triplicate).

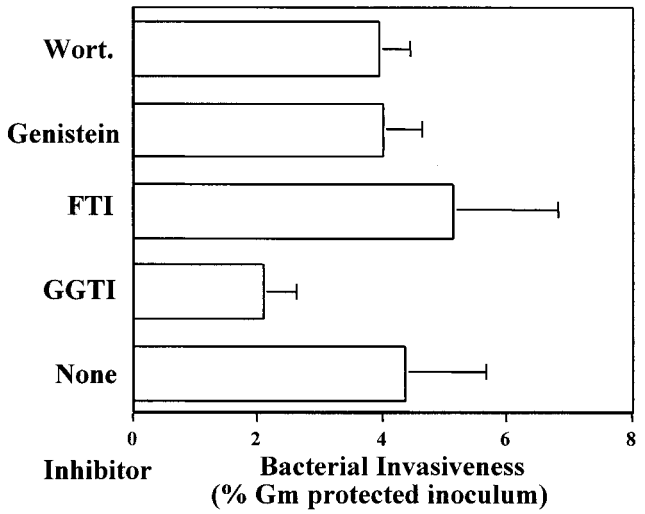

FIG. 4. Effect of various inhibitors on invasion of polarized MDCK-1 cells by *Salmonella*. MDCK-1 cells were pretreated with 100 nM wortmannin (Wort.), 50 μM genistein, 5 μM GGTI-298, 5 μM FTI-277, or medium alone (None) and then apically infected with serovar Typhimurium SL1344 for 1 h. The gentamicin-protected CFU were counted, and the results are presented as the means + SD (error bars) of three independent experiments, each performed in triplicate.

Typhimurium was impaired by GGTI-298, but not by FTI-277, genistein, or wortmannin (Fig. 4). Note that the same concentrations of wortmannin and genistein were found to completely block phagocytosis of serum-opsonized bacteria in U937 cells (our unpublished data), which indicates the effectiveness of those inhibitors. Similar results were obtained for the cells grown on a Transwell filter (data not shown). These results suggest that geranylgeranylated proteins must be activated if serovar Typhimurium is to be able to invade the MDCK-1 cells and subsequently open the epithelial barrier.

The most well known substrates for GGTase I are the Rho GTPases (e.g., Rac, Rho, and Cdc42) and the γ-subunits of trimeric G proteins (7). Also, it has been found that activation of Rac and Cdc42 by SopE and SopB are essential for *Salmonella* to enter the host cells (11, 12). Therefore, we tested the effect of GGTI-298 and FTI-277 on the prenylation of Rac and Cdc42. Figure 5 shows that GGTI-298, but not FTI-277, inhibits the processing of both Rac and Cdc42 in MDCK-1 cells, as indicated by the band shift. These results indicate that Rac1 and Cdc42 are the potential geranylgeranylated proteins regulating the disruption of epithelial barrier by serovar Typhimurium observed in the present study.

Activation of Rac and Cdc42 in MDCK cells infected with serovar Typhimurium. We next examined both the activation states and intracellular redistribution of Rac and Cdc42 in MDCK-1 cells infected with serovar Typhimurium. We measured the activation states by carrying out affinity precipitation, using the PBD of protein A kinase fused to GST, which binds only to the activated forms of these Rac and Cdc42, and subsequently performing Western blot analysis (5). Apical infection of MDCK-1 cells with wild-type serovar Typhimurium increased the level of GTP-bound Rac1 and Cdc42; this was detectable after 10 min of infection and reached a maximum after 30 min (Fig. 6). This activation was not due to the stress of the bacteria load, since infection with the noninvasive *hilA* mutant did not affect the level of GTP-bound Rac1 and Cdc42 (shown for wild-type and *hilA* strains in Fig. 6).

Confocal microscopy revealed that both Rac1 and Cdc42 are in a diffuse form in resting cells, visualized as lower protein intensity throughout the cytoplasm (Fig. 7a and b). Infection with wild-type serovar Typhimurium induced massive accumulation of both Rac1 and Cdc42 apically in the MDCK cells, whereas the noninvasive *hilA* mutant did not effect on these proteins (Fig. 7c and d). Furthermore, treatment with GGTI-298 prevented the altered distribution of Rac1 and Cdc42 in MDCK cells infected with the wild-type strain (Fig. 7g and h). These results indicate that Rac1 and Cdc42 activation are associated with internalization of *Salmonella* and disruption of the tight and adherens junctions in polarized MDCK cells.

***Salmonella*-induced alteration of host-cell junctional proteins requires the activation of geranylgeranylated proteins.** In uninfected (Fig. 8a to c) and *hilA* mutant-infected (Fig. 8d to f) MDCK monolayers, labeling of the tight junction markers ZO-1 and occludin and of the adherent junction protein E-cadherin was seen as a string-like pattern, indicating that both types of junctions were well established (Fig. 8a to c). In contrast, horizontal-sectioning confocal microscopy revealed that these structures were clearly rearranged and diminished in the cells infected with wild-type serovar Typhimurium (Fig. 8g to i): ZO-1 was not degraded but was instead moved from its perijunctional site, the perijunctional arrangement of occludin was essentially lost after 1 h of infection, and the E-cadherin pattern was altered to punctuate fluorescence. By comparison, in the cells pretreated with GGTI-298, the proteins ZO-1, occludin, and E-cadherin were still found to be associated with the tight and adherens junctions after entry of the bacteria (Fig. 8j to l). It should be noted that GGTI-298 did not affect TER as well as the intracellular localization of these junction-associated proteins in uninfected cells (data not shown).

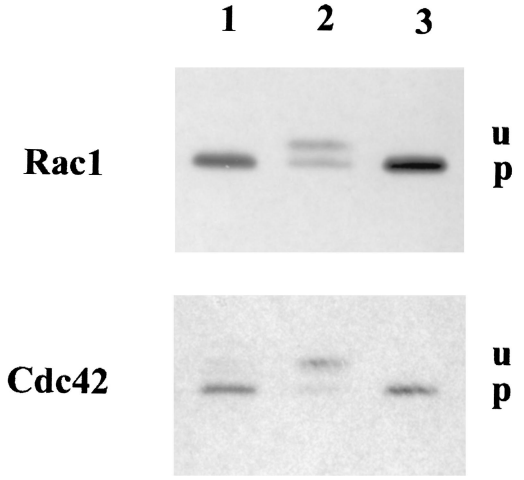

FIG. 5. Effect of GGTI-298 and FTI-277 on processing of Rac1 and Cdc42 in MDCK-1 cells. The cells were pretreated with medium alone (lane 1), 5 μM GGTI-298 (lane 2), or 5 μM FTI-277 (lane 3) for 40 h and lysed. Cellular proteins were subjected to Western blot analysis for Rac1 or Cdc42 to demonstrate inhibition of processing by a band shift from the processed (p) to the unprocessed (u) proteins. The blots shown are representative of five separated experiments.

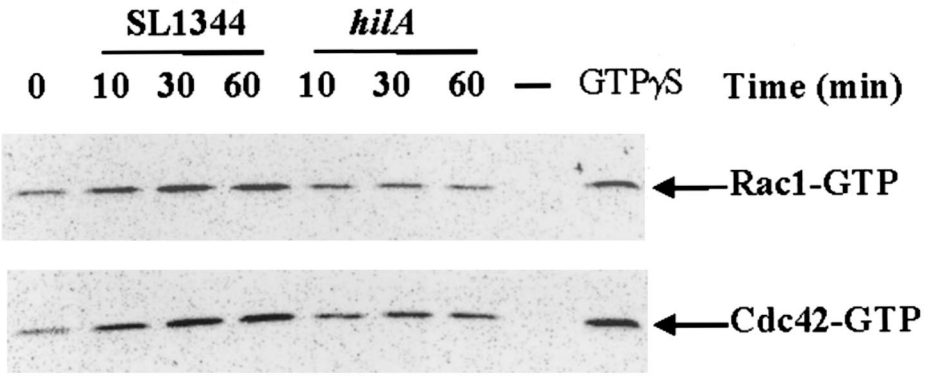

FIG. 6. Activation of Rac1 and Cdc42 in MDCK-1 cells by invasive serovar Typhimurium. MDCK-1 cells in six-well tissue culture plates were serum starved for 16 h and then infected with SL 1344 or *hilA* for the indicated periods of time. Thereafter, the cells were lysed, and the activated Rac1 and Cdc42 in the lysates were collected by affinity precipitation using GST-PBD prebound to glutathione-Sepharose beads. Proteins on the beads were separated by SDS-PAGE, transferred onto nitrocellulose membranes, blotted for Rac1 or Cdc42, and then subjected to ECL detection. The specificity of this assay was confirmed by omitting GST-PBD (lane —) or by adding 100 mM GTP gamma subunit (GTPγS) (lane 9) during the precipitation step. The blots shown are representative of four separate experiments.

The number of intracellular bacteria is dissociated from the decreased TER in GGTI-treated cells. Theoretically, the ability of pathogens to disrupt epithelial barrier could be due to either the stimulation of cell signaling by their virulence factors or merely to the increased number of bacteria within the cells. Since GGTI-298 reduced the invasion of bacteria by about 50%, we tested whether this reduced number of intracellular bacteria could account for the ability of GGTI-298 to block the reduction in TER caused by serovar Typhimurium. First, TER was measured in MDCK monolayers infected with the SL1344 strain at different MOI rates (20:1 and 50:1). When monolayers were infected at a lower MOI (20:1), giving about 50% internalized bacteria, the decreased TER in these cells was not significantly affected (data not shown). Second, we performed experiments to determine the number of intracellular bacteria at various time points after infection with the SL1344 strain. Rapid intracellular growth of bacteria was observed in MDCK cells pretreated with or without GGTI-298 (Fig. 9). However, although there were more bacteria within the GGTI-treated cells at 3 h postinfection than in the control cells at 2 h (Fig. 9), the number of intracellular bacteria did not correlate with their effect on TER (Fig. 3). These results indicate that the effects of GGTI-298 are most likely due to its inhibition of cell signaling events, preceding bacterial invasion.

DISCUSSION

Tight junctions hold cells together and play an integral role in maintaining cellular architecture (4, 14). Many enteric pathogens are known to perturb the intestinal epithelial barrier, but the underlying mechanisms are probably distinct for each species. *C. difficile* toxins enhance permeability by disrupting actin microfilaments within the perijunctional ring (9, 27), and enteropathogenic *E. coli* disrupts the epithelial barrier (30) via phosphorylation of myosin light chains (34). The NSP4 enterotoxin of rotavirus impairs normal formation of epithelial tight junctions by preventing transport of the ZO-1 protein needed for biogenesis of these junctions (35). *Salmonella* and *Shigella* spp. can also disrupt the epithelial barrier (10, 29). Moreover, our results suggest that invasive serovar Typhimurium alters the tight and adherens junction proteins in polarized MDCK cells by activating geranylgeranylated proteins. Many peripheral membrane proteins are concentrated at the tight junctions and collaborate to cross-link tight junction strands with the underlying actin-based cytoskeleton. A number of key signals that regulate actin, for instance tyrosine kinase, PI3-kinase, and the Rho family of small GTPases, have also been implicated in the function of tight junctions.

Although *Salmonella* has been shown to activate all the mentioned signals, our findings indicate that activation of tyrosine kinase or PI3-kinase is not required for *Salmonella*-effected disruption of the epithelial barrier. Furthermore, we noted that entry of these bacteria into host cells does not depend on the activity of these protein kinases, which confirms results reported by other investigators (6). Instead, we found that GGTI-298, an inhibitor of protein geranylgeranyltransferase I, markedly suppressed both invasion and disruption of the MDCK epithelial barrier by *Salmonella*.

The best-known protein substrates for GGTase-I include the Rho family of small G-proteins (Cdc42, Rac, and Rho), which are, as mentioned above, key regulators of the structure of the actin cytoskeleton (15). Activation of Rac and Cdc42, but not Rho, by SopE and SopB are critical for *Salmonella* to enter the host cells (12). In this context, we found that Rac1 and Cdc42 are directly targeted by *Salmonella*. This was indicated by the results of affinity precipitation and confocal microscopy showing that apical infection of polarized MDCK cells by invasive *Salmonella* led to activation of Cdc42 and Rac1, which correlated with a decrease in TER. Furthermore, GGTI-298 inhibited the processing of both Rac1 and Cdc42 which coincided with its ability to reverse the effect of *Salmonella* on the TER in these cells. GGTI-298 is known to inhibit different members of Rho family of the gamma subunit of trimeric G proteins (7, 37). Together, these results imply that both Rac1 and Cdc42 are critical regulators for this *Salmonella*-mediated process. Notwithstanding, we also found that treatment with GGTI-298 partly inhibited internalization of *Salmonella* in MDCK cells but that the rapid intracellular growth of the bacteria did not

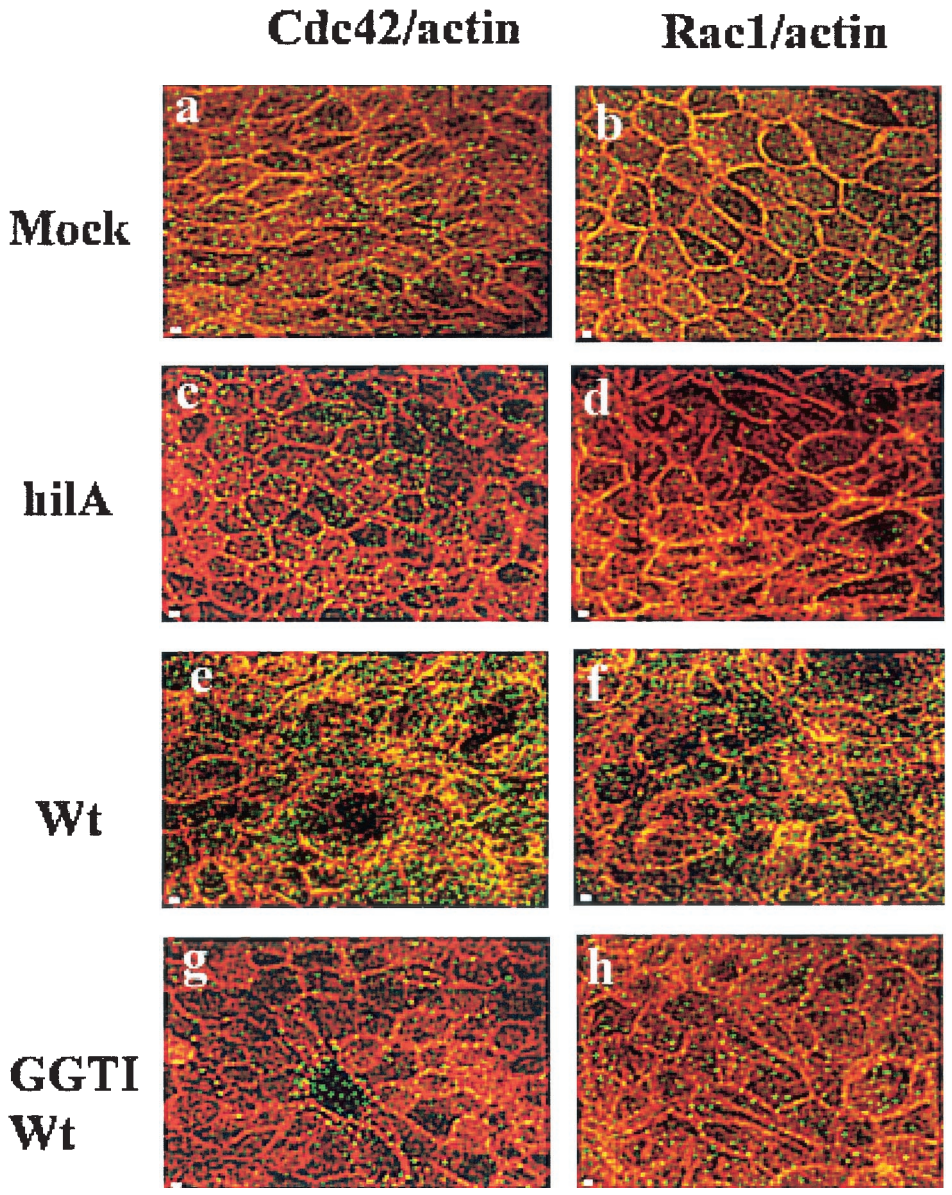

FIG. 7. Intracellular redistribution of Rac1 and Cdc42 in MDCK-1 cells infected with serovar Typhimurium. The cells were either mock infected (a and b) or infected with wild-type (Wt) SL1344 (e and f) or the *hilA* mutant (c and d) for 1 h and then fixed and labeled with anti-Cdc42 (a, c, e, and g) or anti-Rac1 (b, d, f, and h) Ab and rhodamine-labeled phalloidin against F-actin. The intracellular localization of these proteins was visualized using conjugated goat anti-mouse Ab (Alexa 488) and confocal laser scanning microscopy. (g and h) The cells shown were pretreated with GGTI-298 for 40 h before infection. Bar = 2 μm.

decrease the TER. This indicates that disruption of the epithelial barrier depends not only on the number of bacteria entering the host cells but also on the ability of *Salmonella* to activate host-cell signaling. Previous studies by other groups (13, 26, 27) have shown that *C. difficile* toxins A and B and *Clostridium botulinum* C3, which inactivate Rho GTPase, decrease the TER in T84 and Caco-2 cell monolayers. We used *C. difficile* toxin B and *C. botulinum* C3 as a control of Rho protein in these experiments. The MDCK-1 cell monolayers treated from the apical compartment with a final concentration (100 ng/ml) of toxin B and toxin C3 and assessed by measuring the electrical resistance. Note that these toxins did not change transepithelial resistance on tightly MDCK-1 cells during 2 h of incubation.

Our finding that apical *Salmonella* infection activated Rac1 confirms the results of Criss et al. (8). However, in contrast to our observation, they reported that bacteria entering MDCK-II cells at the apical pole did not activate Cdc42. This discrepancy may be due to differences in the cell clones and the experimental procedures used. They (8) employed low-resistance MDCK-II cells, which uniformly express tetracycline-repressible transactivator, where the infection with *Salmonella* had no effect on the integrity of the cultured epithelial monolayers.

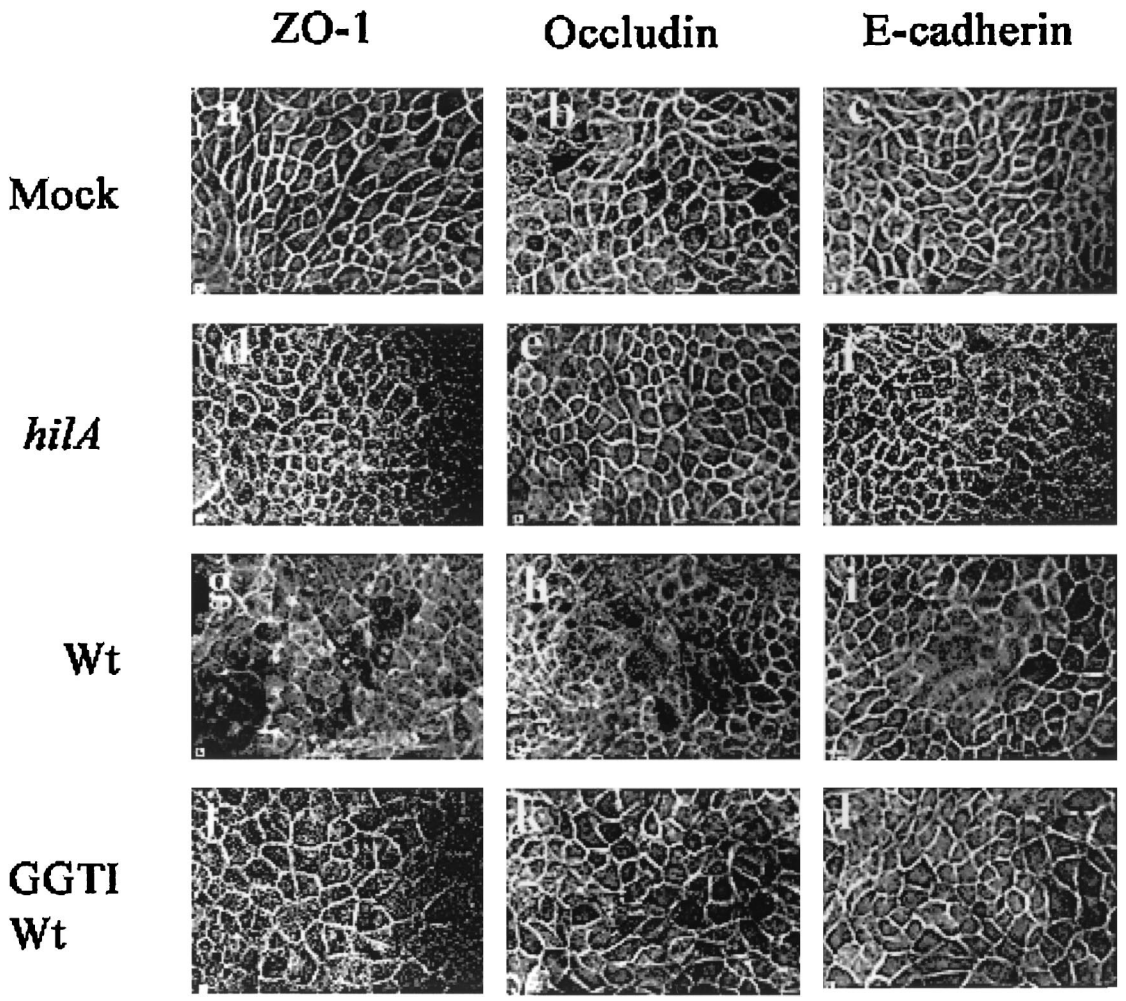

FIG. 8. The junction proteins in MDCK-1 cells are altered by interaction with serovar Typhimurium. The cells were either mock infected (a to c) or infected with the wild-type (Wt) SL1344 (g to i) or the *hilA* mutant strain (d to f) for 1 h and then fixed and labeled with anti-ZO-1 (a, d, g, and j), antioccludin (b, e, h, and k), or anti-E-cadherin (c, f, i, and l) Ab. The intracellular localization of these proteins was visualized using conjugated goat anti-rat, anti-rabbit, and anti-mouse (Alexa 488 and Alexa 594) Abs and confocal laser scanning microscopy. The wild-type cells in j, k, and i were pretreated with GGTI-298 for 40 h and then exposed to the bacteria and thereafter, respectively, were labeled with the ZO-1, occludin, and E-cadherin Abs. The characteristic arrangements of the tight and adherens junctions were lost after 1-h of infection with the wild-type bacteria. MDCK-1 cells infected with the *hilA* mutant were similar to the control cells (a, b, and c). Bar = 2 μm.

We also found that invasive *Salmonella* altered localization of the tight- and adherens junction-associated proteins ZO-1, occludin, and E-cadherin in epithelial monolayers and that treatment with GGTI-298 prevented the reorganization of these proteins. The mechanisms by which geranylgeranylated proteins, including Rac1 and Cdc42, regulate these junctional proteins have not been fully disclosed, although they seem to control cell-cell adhesion by reorganizing the actin cytoskeleton. There is now evidence that Rac1 and Cdc42 are directly involved in events at sites of intercellular contact. For example, Akhtar and coworker (1) have reported that a constitutively active form of Rac1 induces formation of large intracellular vesicles, around which Rac1 and E-cadherin become tightly colocalized. These investigators also found that Rac1 depletes levels of E-cadherin at sites of cell-cell contact by inducing clathrin-independent internalization of E-cadherin at the cell surface. Hence, they proposed that Rac1 participate in the dynamic rearrangement of E-cadherin-mediated cell-cell adhesion.

In summary, we found that the geranylgeranylated proteins Cdc42 and Rac1 were activated in polarized MDCK-1 cells during apical infection with invasive serovar Typhimurium. Activation of these proteins resulted in reorganization of the actin cytoskeleton this led not only to internalization of the bacteria, but also altered localization of tight and adherens junction proteins, which in turn disrupted the barrier function of these epithelial cells. Opening of a paracellular pathway may increase the invasion of serovar Typhimurium via lateral and basolateral pathways, which would explain the increased number of intracellular bacteria we observed within the MDCK cells after short-term (1-h) incubation with the bacteria. Interestingly, compared to uninfected and wild-type serovar Typhimurium-infected monolayers, TER increased with time in MDCK cells containing the *hilA* mutant strain. Together, our

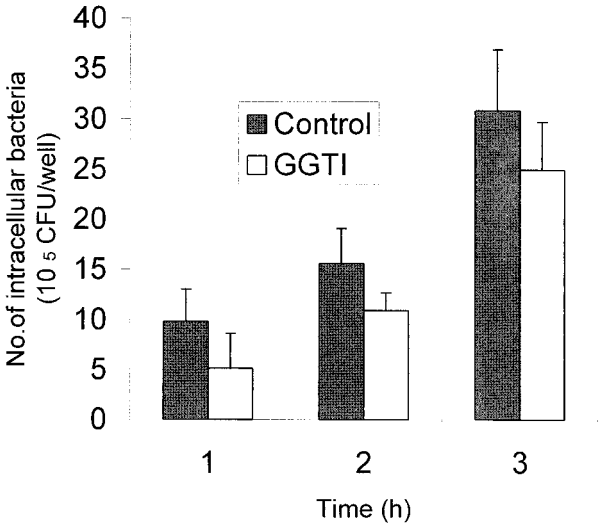

FIG. 9. Replication of serovar Typhimurium in MDCK-1 cells. The cells were pretreated with GGTI-298 or medium alone (controls) and then infected with serovar Typhimurium SL1344 for 1 h. Thereafter, the cells were further incubated for 1, 2, and 3 h in the presence of 50 μg of gentamicin/ml, and the internalized bacteria were counted. The results represent mean + SD (error bars) of three independent experiments, each performed in triplicate.

results suggest that activation of geranylgeranylated proteins constitutes a signal that affects the tight and adherens junctions so as to weaken and eventually disrupt the integrity of the epithelial barrier.

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