The GTPase Rac1 selectively regulates *Salmonella* invasion at the apical plasma membrane of polarized epithelial cells

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SUMMARY

The bacterial pathogen *Salmonella typhimurium* colonizes its animal hosts by inducing its internalization into intestinal epithelial cells. This process requires reorganization of the actin cytoskeleton of the apical plasma membrane into elaborate membrane ruffles that engulf the bacteria. Members of the Rho family of small GTPases are critical regulators of actin structure, and in nonpolarized cells, the GTPase Cdc42 has been shown to modulate *Salmonella* entry. Because the actin architecture of epithelial cells is organized differently from that of nonpolarized cells, we examined the role of two Rho family GTPases, Cdc42 and Rac1, in invasion of polarized monolayers of MDCK cells by *S. typhimurium*. Surprisingly, we found that endogenous Rac1, but not Cdc42, was activated during bacterial entry at the apical pole, and that this activation required the bacterial effector protein SopE. Furthermore, expression of dominant inhibitory Rac1 but not Cdc42 significantly inhibited apical internalization of *Salmonella*, indicating that Rac1 activation is integral to the bacterial entry process. In contrast, during basolateral internalization, both Cdc42 and Rac1 were activated; however, neither GTPase was required for entry. These findings, which differ significantly from previous observations in nonpolarized cells, indicate that the host cell signaling pathways activated by bacterial pathogens may vary with cell type, and in epithelial tissues may further differ between plasma membrane domains.

Key words: *Salmonella typhimurium*, Actin, Epithelial cell, Rac1, Cdc42

INTRODUCTION

The bacterial pathogen *Salmonella typhimurium* invades its animal hosts by entering and traversing the epithelial monolayer lining the intestine. *Salmonella* achieve this end by inducing epithelial cells, which are normally non-phagocytic, to internalize them in a process resembling phagocytosis (Francis et al., 1993; Takeuchi, 1967). Bacterial adhesion to the tips of apical microvilli activates a type III, contact-dependent secretory apparatus, through which bacterial gene products essential to the invasion process are released into the host cell cytosol (Collazo and Galan, 1997; Hueck et al., 1995; Kaniga et al., 1995; Miller et al., 1989). Localized disassembly of microvilli at these adhesion sites is followed by formation of actin-rich membrane ruffles, which are ultimately responsible for bacterial internalization (Finlay et al., 1991; Francis et al., 1992). Since ruffle formation is essential to the invasion process, understanding the development of these structures is critical to understanding *Salmonella* pathogenesis as a whole.

The regulation of cortical actin dynamics in response to extracellular stimuli has been ascribed to members of the Rho family of small GTPases, which includes RhoA, Rac1, and Cdc42 (Hall, 1998; Van Aelst and D'Souza-Schorey, 1997). As with other GTPases, Rho family members cycle between active (GTP-bound) and inactive (GDP-bound) conformations. Accessory factors modulate the nucleotide state of the GTPases. Guanine nucleotide exchange factors (GEFs) activate GTPases by facilitating the exchange of bound GDP for GTP. GTPase activating proteins (GAPs) enhance the intrinsic hydrolysis rate of the GTPases to promote their inactivation. In fibroblasts, activation of RhoA promotes formation of stress fibers and focal contacts; Rac1 yields lamellipodia and dorsal ruffles; and Cdc42 leads to the extension of filopodia (Kozma et al., 1995; Nobes and Hall, 1995; Ridley and Hall, 1992; Ridley et al., 1992). During cell spreading, Rho family members function sequentially, with initial activation of Cdc42, followed by Rac1 and RhoA (Nobes and Hall, 1995; Ridley et al., 1992). In other actin-dependent processes, distinct subsets of Rho GTPases become activated, often in a cell-type specific manner.

The involvement of Rho GTPases in *Salmonella typhimurium* invasion was initially examined in nonpolarized cell lines of both epithelioid (HeLa, COS-1) and fibroblastic (Rat-1) lineages. In these cells, Chen et al. demonstrated that invasion of *Salmonella* was primarily dependent on Cdc42 (Chen et al., 1996). Expression of a point mutant of Cdc42 unable to bind GTP, which acts in a dominant-inhibitory manner (Cdc42HsN17), prevented bacterial entry. Expression

of dominant-negative Rac1 (Rac1N17) partially inhibited internalization, but not as effectively as the Cdc42 mutant. In addition, Cdc42 mediated the *Salmonella*-induced activation of the signaling protein Jun kinase (JNK). It was subsequently determined that this modulation of Rho GTPase activity was dependent on two *Salmonella typhimurium* secreted proteins, SopE and SptP. SopE was characterized as a GEF for Rho GTPases by its ability to stimulate in vitro nucleotide exchange on Cdc42, Rac1, and RhoA, and is required for entry. Ectopic expression of SopE in mammalian cells was sufficient to induce actin-dependent membrane ruffling (Hardt et al., 1998; Rudolph et al., 1999; Wood et al., 1996). The recently described effector SopE2, which has 69% sequence similarity to SopE, possesses similar nucleotide exchange activity (Bakshi et al., 2000; Stender et al., 2000). SptP has GAP activity on Rho GTPases in vitro, and may aid in reassembly of the actin cytoskeleton after bacterial entry (Fu and Galan, 1999).

Given the unique structure of the enterocyte brush border, we hypothesized that the cytoskeletal regulatory factors co-opted by *Salmonella* during invasion would differ from those identified in studies with nonpolarized cells. We therefore investigated the requirement for the GTPases Cdc42 and Rac1 during *Salmonella* entry into the polarizing epithelial MDCK cell line. Surprisingly, dominant-negative Rac1, but not Cdc42, significantly inhibited bacterial entry at the apical aspect of these cells. Further, bacterial invasion was associated with an increase in endogenous Rac1 activation, an effect attributable to the *Salmonella* type III secretion system and SopE. These results suggest that the Rac1 GTPase is primarily responsible for regulation of *Salmonella* entry at this physiologically relevant site.

MATERIALS AND METHODS

Cell culture

The T23 clone of low-resistance MDCK strain II cells, which stably express the tetracycline-repressible transactivator, was a gift from Y. Altschuler and K. Mostov (Barth et al., 1997). T23-derived cell lines expressing activated (G12V) and dominant-negative (T17N) Rac1 and Cdc42 constructs under control of a tetracycline-responsive promoter (Gossen and Bujard, 1992) were first described by Jou et al. (Jou and Nelson, 1998). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, 10% FBS, and antibiotics in a 37°C, 5% CO_2 incubator. Transfectants were additionally maintained in 20 ng/ml doxycycline (Sigma, St Louis, MO) to repress expression of the transgene.

Induction of gene expression in the mutant Rac1 and Cdc42 T23 cells was achieved in two ways. For invasion assays and biochemical experiments, cells maintained in the presence of 20 ng/ml doxycycline were seeded on Transwell filter supports (Costar, Cambridge, MA) coated with rat tail collagen (Upstate Biotech, Lake Placid, NY). 48-72 hours later, cells were gently washed in Dulbecco's PBS and replaced in complete DMEM lacking doxycycline to induce expression. Monolayers were used for experimentation 48 hours after washout. Cells maintained in doxycycline for the duration of the experiment were used as controls. Under these conditions, V12Rac was expressed approximately 3- to 4-fold over endogenous Rac1 levels, and N17Rac was expressed approximately 1- to 2-fold over endogenous Rac1 (data not shown). Similar results were obtained for the V12Cdc42 and N17Cdc42 mutants (data not shown). Transmission electron microscopy confirmed that parental and transfected T23 cells formed a continuous monolayer on the filter supports without penetration of the filter pores. Monolayer integrity was assessed prior to each experiment by measuring transepithelial resistance with an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL); resistances for all cell lines were customarily > 200 $\Omega \times cm^2$, as previously reported for the parental T23 cells (Jou et al., 1998). Infection with *Salmonella* had no effect on the integrity of the monolayers over the duration of the experiments.

For immunofluorescence experiments, where higher levels of transgene expression were desired, polarized monolayers were established using a calcium-switch protocol (adapted from Jou et al., 1998). Briefly, cells were plated at subconfluency in the absence of doxycycline to induce expression of the transgene. 36 hours later, cells were trypsinized and plated at confluent density on collagen-coated filter supports (Costar) in low-calcium MEM lacking doxycycline (Gibco BRL, Grand Island, NY). After 3 hours, cells were rinsed in low calcium medium, and then placed in doxycycline-free DMEM for 48 hours longer to induce polarization. Cells maintained in the presence of doxycycline for the entire procedure were used as controls.

Bacterial strains

The wild-type *S. typhimurium* strain SL1344 and its isogenic derivative VV341, which is rendered entry-deficient by deletion of the *hilA* locus (Hueck et al., 1995), were used as positive and negative controls, respectively. AJK61 is an SL1344 derivative that contains an in-frame, non-polar *invGΔ1-1* mutation, introduced by homologous recombination with a plasmid (p65A2) containing a 513 bp deletion in the *invG* open-reading frame (ORF) (a kind gift from A. Kelly and C. Lee, Harvard Medical School). GG5, a kind gift from L. Guogas and C. Lee (HMS), was constructed by integrating an ampicillin-resistance suicide plasmid (pMG1: pIVET8 containing a fragment of *sopE*) into the *sopE* locus of SL1344 (Cherayil et al., 2000). This simultaneously disrupts the *sopE* ORF and generates a *lacZ* transcriptional fusion to the *sopE* gene. Immunoblotting with SopE and SopE2-specific antibodies, kindly provided by Dr Edouard Galyov (Institute for Animal Health, Compton, UK), confirmed that only the *sopE* locus was disrupted in the GG5 strain. Analysis of TCA-precipitated growth medium from SL1344 and GG5 strains demonstrated that disruption of *sopE* did not affect synthesis or secretion of bacterial proteins (data not shown).

To produce invasion-competent *Salmonella*, bacteria were grown under O_2-limited conditions as originally described (Lee and Falkow, 1990). Under these conditions, bacteria were in a late logarithmic phase of growth, corresponding regularly to $5\text{-}7\times10^8$ colony forming units (CFU)/ml. For each experiment, CFU were determined by plating serial dilutions of the overnight culture on MacConkey agar (Difco, Detroit, MI).

Confocal fluorescence microscopy

Parental and transfected T23 cells grown on 12 mm filter supports (Costar) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 10 minutes, followed by permeabilization in PBS/0.1% Triton X-100 for 5 minutes. Filters were removed from the supports and blocked in PBS containing 10% normal goat serum and 0.2% saponin. Exogenous GTPases were detected using anti-myc antibody 9E10 followed by Cy2-labeled donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, PA). The F-actin cytoskeleton was visualized using rhodamine-labeled or fluorescein-labeled phalloidin (Molecular Probes, Eugene, OR). For invasion experiments, monolayers (approximately 1.2×10^6 cells) were infected with SL1344 at a multiplicity of infection (MOI)=100 at 37°C for various times, then washed extensively prior to fixation to remove unbound bacteria. Bacteria were detected using a rabbit polyclonal anti-*Salmonella* lipopolysaccharide (LPS) antibody (H antigen, poly A-Z) (1:500, Difco) followed by Texas Red-labeled donkey anti-rabbit IgG (Jackson Immunoresearch). Images were viewed with a Zeiss

LSM 410 confocal laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY).

Scanning electron microscopy

Parental and N17Rac1-expressing T23 monolayers were infected with strain SL1344 at an MOI=100 for 20 minutes. Cells were washed to remove unbound bacteria, fixed in 4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and post-fixed in 2% OsO_4. Cells were dehydrated in a graded series of ethanols, dried in a critical point dryer, and coated with a gold/palladium mix. Samples were examined using a JEOL 6400 scanning electron microscope.

Assay of *S. typhimurium* invasion into polarized MDCK monolayers

Monolayers of parental and transfected T23 cells (approximately 3.5×10^5 cells on a 6.5 mm, 3 μm pore filter support; Costar) were washed with HBSS containing calcium and magnesium, buffered with 10 mM HEPES, pH 7.4 ($HBSS^+$; Sigma). For apical invasion, $HBSS^+$-washed bacteria were added at an MOI=30 to the upper (apical) well, and $HBSS^+$ placed in the lower (basolateral) well. For basolateral invasion, filter supports were placed with their basal surface facing up in a humidified chamber, and bacteria added at an MOI=30 to this surface. After 1 hour of invasion at 37°C, monolayers were assayed for their cell associated and internalized bacterial populations. Cell associated bacteria (equaling the number attached plus the number internalized) were released from the cells in 0.1 ml of 1% Triton X-100 at 4°C. To quantify internalized bacteria, infected cells were treated with 480 μg/ml gentamicin in $HBSS^+$ for 90 minutes at 37°C, washed extensively, and lysed. Lysates were resuspended in 0.9 ml Luria broth (LB) and CFU/ml determined as described above. Internalization is expressed as a percentage of the initial bacterial inoculum; an index of internalization is defined relative to the percent internalization of cells maintained in the presence of 20 ng/ml doxycycline (=1).

GTPase activation assays

GTP-bound Rac1 and Cdc42 were detected in T23 cell lysates essentially as described (Sander et al., 1998). A plasmid encoding the p21-binding domain (PBD) of p21-activated kinase 1 (PAK1) as a fusion protein with glutathione-*S*-transferase (GST-PBD) was a kind gift from B. Arthur and K. Burridge (University of North Carolina). T23 cells seeded on 24 mm, 3 μm pore filter supports (Costar) (approximately 5×10^6 cells per monolayer) were infected with *S. typhimurium* at MOI=100 for 30 minutes at 37°C. Cells were washed extensively at 4°C with $HBSS^+$, then lysed at 4°C in 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.1 M NaCl, 1% NP-40, 10% glycerol with 0.1 mM PMSF and 1 μg/ml each pepstatin, leupeptin, and antipain. Lysates were clarified with Sepharose CL-4B beads (Amersham Pharmacia Biotech, Piscataway, NJ) and then incubated for 45 minutes at 4°C with 40 μg GST (as control) or GST-PBD coupled to glutathione-Sepharose beads (AP Biotech). Proteins were separated on 13% acrylamide gels and transferred to nitrocellulose for Rac1 experiments, or polyvinylidene difluoride for Cdc42 experiments. To detect Rac1, blots were incubated with a monoclonal antibody (clone 102) (1:1,000, BD Transduction Laboratories, San Diego) followed by HRP-conjugated sheep anti-mouse IgG (1:2,000, AP Biotech). Cdc42 was detected with a monoclonal antibody (clone 44) (1:250, BD Transduction Laboratories) followed by HRP-conjugated sheep anti-mouse IgG (AP Biotech). 1/100 of each lysate was immunoblotted for the corresponding GTPase to confirm the presence of equal concentrations of protein. Signals within the linear range of the enhanced chemiluminescence detection method were scanned and quantified with IP Lab Gel (Signal Analytics, Vienna, VA). For each experimental condition, the ratio of GTP-bound to total GTPase was expressed relative to the uninfected control (=1).

RESULTS

Invasion of *Salmonella* into model polarized epithelia induces extensive reorganization of the apical actin architecture

The morphological changes occurring at the level of the intestinal epithelium during *Salmonella* invasion were initially described in a guinea pig model by Takeuchi, who observed membranous extrusions in the vicinity of apically invading organisms (Takeuchi, 1967). Similar observations have been made in vitro with polarizing epithelial cell lines (Finlay and Falkow, 1990; Finlay et al., 1989; Finlay et al., 1988). Since recent studies investigating the molecular requirements for *Salmonella* entry have utilized nonpolarizing tissue culture cells, we chose to characterize early events in bacterial invasion in a system that more accurately models the initial physiologic site of interaction with the pathogen. For this purpose, we selected the MDCK (strain II) cell line, a well characterized

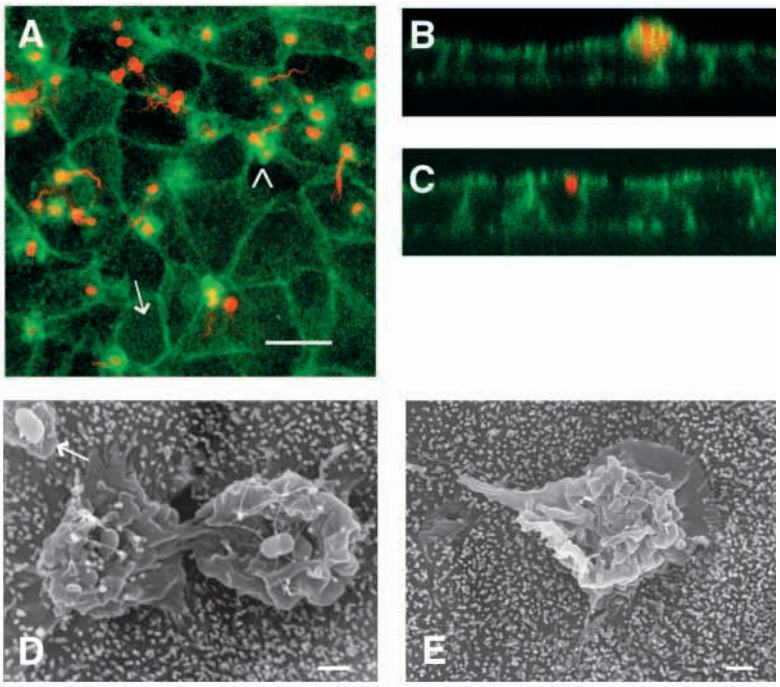

Fig. 1. Invasive *Salmonella* induce reorganization of the apical actin cytoskeleton of model polarized epithelia. (A-C) T23 cells cultured on filter supports were apically infected with SL1344 for 20 (A,B) or 60 (C) minutes. *Salmonella* were detected with a polyclonal antibody against the bacterial LPS followed by Texas Red-labeled donkey anti-rabbit IgG (red). Cells were costained with fluorescein-labeled phalloidin to visualize filamentous actin (green). Arrow indicates a cell with normal apical actin architecture. Arrowhead indicates actin rearrangement induced by invading *Salmonella*. Bar, 10 μm. (B,C) are confocal X-Z sections through representative monolayers. (D-E) T23 cells apically infected with SL1344 for 20 minutes were fixed and processed for scanning electron microscopy. Arrow in D indicates structure of a newly elicited membrane ruffle. Bars, 1 μm.

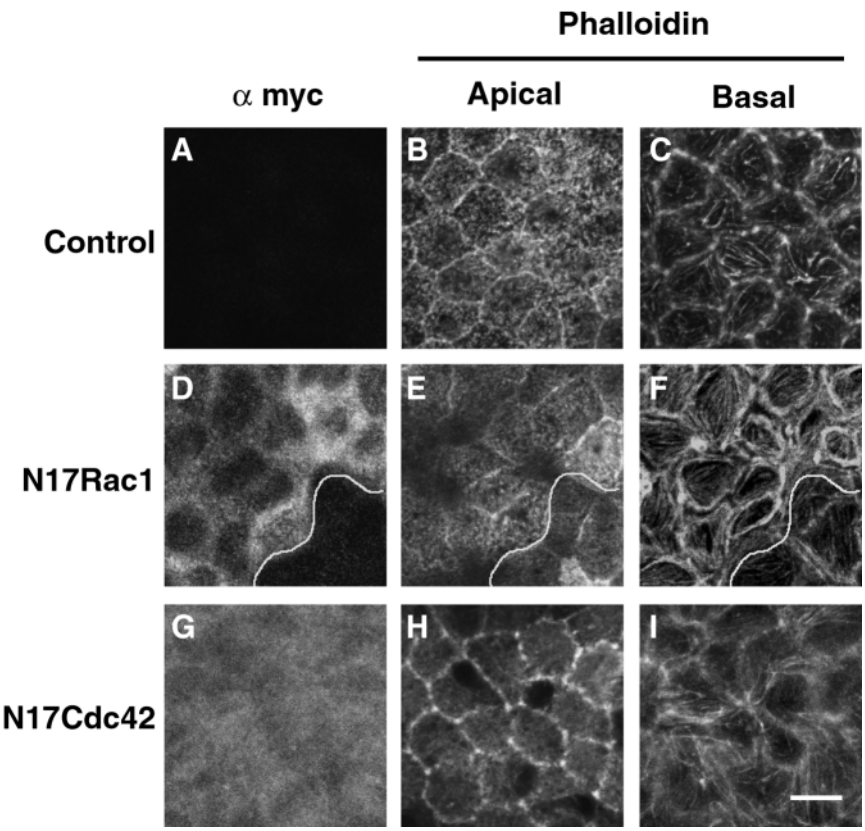

Fig. 2. Expression of dominant-negative mutants of Cdc42 and Rac1 in polarized T23 cells produces discernible effects on the actin cytoskeleton. Myc-tagged N17Rac1 (D-F) or N17Cdc42 (G-I) expressing cells were cultured on filter supports as described in Materials and Methods. N17Cdc42 cells maintained in the presence of doxycycline (A-C) were used as control. Expression of the transgenes was detected using a monoclonal anti-myc antibody (α myc) followed by Cy2-labeled donkey anti-mouse IgG. Filamentous actin was visualized with rhodamine-labeled phalloidin. Confocal images were taken through the apical and basal sections of each monolayer. White line on N17Rac1 panels indicates boundary between expressing and nonexpressing cells in the monolayer. Bar, 10 μm.

polarizing epithelial cell line of renal origin whose biology is similar in most respects to intestinal epithelial cells. This cell line has been used extensively for studies of epithelial polarity as it relates to membrane trafficking and cytoskeletal dynamics (Caplan and Matlin, 1989).

We first examined the apical actin cytoskeleton following *Salmonella* interaction with the MDCK epithelial cell surface (Fig. 1A-C). In uninfected cells, filamentous actin was organized into regularly spaced punctae, indicative of apical microvilli (Fig. 1A, arrow). This characteristic apical morphology was altered at sites where invasive *Salmonella* formed close contacts with the plasma membrane (Fig. 1A, arrowhead). During infection, polymerized actin was redistributed to bacterial adhesion sites. In X-Z confocal section, actin 'splashes' projected upwards from the apical surface of the monolayer to envelop the invading bacteria (Fig. 1B). Following *Salmonella* internalization, the apical filamentous actin regained its normal punctate appearance (Fig. 1C).

To visualize in greater detail the changes occurring to the apical plasma membrane during *Salmonella* invasion, infected

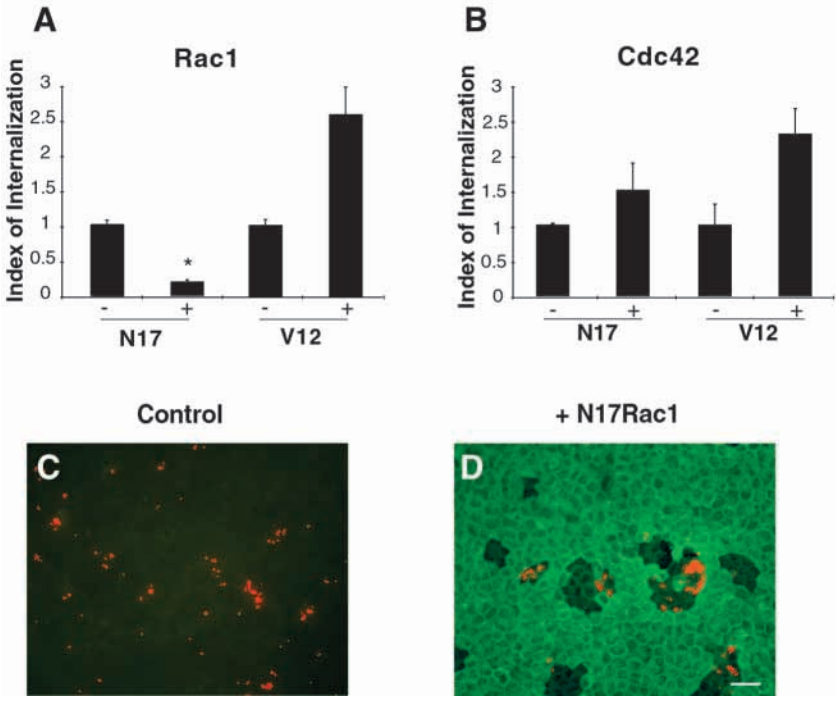

Fig. 3. Internalization of *Salmonella* at the apical pole of epithelial cells is inhibited by dominant-negative Rac1, but not dominant-negative Cdc42. (A,B) Monolayers of T23 cells expressing (+) activated (V12) or dominant-negative (N17) mutants of Rac1 (A) or Cdc42 (B) were infected from the apical pole with SL1344, and bacterial internalization was quantified as described in Materials and Methods. Cells maintained in doxycycline (–) to repress transgene expression were used as control. Data are the means (± s.d.) of 3 independent filters, and experiments were performed at least 3 times. *P<0.001 (Student's t-test). (C-D) Polarized T23 cells expressing (D) or not expressing (C) N17Rac1 were infected apically with SL1344, washed extensively, and fixed in 4% paraformaldehyde. Bacteria were detected using a polyclonal anti-*Salmonella* LPS antibody followed by Texas Red-labeled donkey anti-rabbit IgG (red). An antibody against the myc epitope tag was used to detect expression of the transgene, followed by Cy2-labeled donkey anti-mouse IgG (green). Bar, 30 μm.

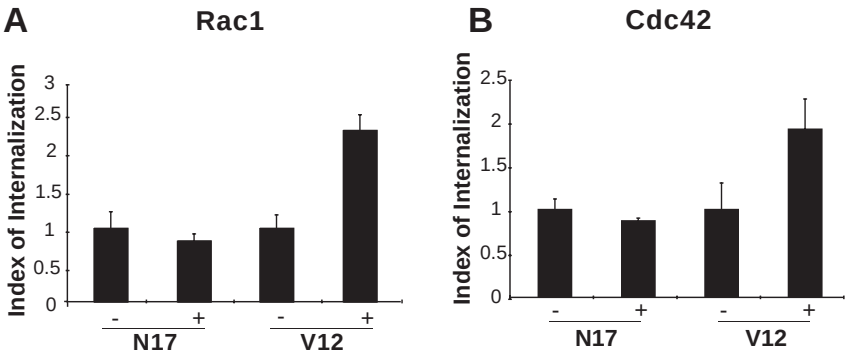

Fig. 4. Internalization of *Salmonella* at the basolateral pole of epithelia is not inhibited by dominant-negative Rac1 or Cdc42. Monolayers of T23 cells expressing (+) or not expressing (–) Rac1 (A) and Cdc42 (B) mutants were infected from the basolateral pole with SL1344 and internalization quantified as described in Materials and Methods. Data are the means (± s.d.) of 3 independent filters, and experiments were performed at least 4 times.

MDCK monolayers were observed by scanning electron microscopy. At sites where bacteria formed tight appositions with the apical plasma membrane, microvilli were disassembled, resulting in localized membrane ruffling (Fig. 1D, arrow). These ruffles became larger and more elaborate (Fig. 1D), occasionally appearing as lamellipodial sheets that covered the surface of neighboring cells (Fig. 1E). During the final stages of bacterial internalization, the ruffles transformed into more protrusive structures (Fig. 1D). Both the immunofluorescence and electron microscopy data are consistent with previous observations from other cultured epithelial cell lines (Finlay et al., 1989; Finlay et al., 1991).

Expression of dominant-negative GTPases in MDCK monolayers

A previous report by Chen et al. demonstrated that members of the Rho family of GTPases, specifically Cdc42 and (less prominently) Rac1, regulate the cytoskeletal changes necessary for *Salmonella* entry into nonpolarized cells (Chen et al., 1996). To investigate this question in model polarized epithelia, we utilized MDCK cell lines that inducibly express mutant Rac1 or Cdc42 constructs via the tetracycline repressible transactivator (Jou and Nelson, 1998). In this system, the presence of doxycycline in growth medium prevents expression of the transgene, while its removal induces protein synthesis. The concentration of doxycycline required for the repression of gene expression has no effect on *Salmonella* viability over the course of these experiments (data not shown).

We initially confirmed that the dominant-negative Rac1 and Cdc42 constructs in the T23 cell lines were expressed and functional by their ability to affect actin organization. N17Rac1- and N17Cdc42-expressing MDCK cells were plated on collagen-coated Transwell filter supports using a calcium-switch method to allow for maximal gene expression (Jou and Nelson, 1998). Confocal laser scanning microscopy was used to visualize filamentous actin in these cells, indicated by positive staining for rhodamine-labeled phalloidin, in both apical and basal focal planes. In the presence of doxycycline, no expression of the transgene was detected in either the N17Cdc42 (Fig. 2A-C) or N17Rac1 cell lines (not shown). In the apical plane of doxycycline-repressed N17Cdc42 cells, punctate microvillar staining was evident (Fig. 2B). At the basal surface, filamentous actin was organized into stress fibers, as well as bundles of cortical actin outlining the margins of each cell (Fig. 2C). The actin cytoskeleton appeared essentially the same in N17Rac1 cells maintained in the presence of doxycycline (data not shown).

Approximately 90% of the N17Rac1-transfected cells expressed the transgene after doxycycline washout (Fig. 2D-F). Nonexpressing cells tended to remain adhered to one another, forming isolated islands. This may be attributed to the weakening of cell-cell contacts in the presence of N17Rac1, as observed previously for both this cell line (Jou et al., 1998) and for other polarized cells expressing this mutant (Braga et al., 1997). The distribution of filamentous actin was strikingly modified in the N17Rac1 expressing cells. While actin organization in the apical domain was not overtly altered, at the basal surface, N17Rac1 expression produced an increase in both the number and thickness of stress fibers in each cell. In addition, rings of filamentous actin were prominently observed encircling the stress fibers. These elaborate structures were not previously reported for this clonal cell line (Jou and Nelson, 1998), which is most likely due to the relative maturity of our

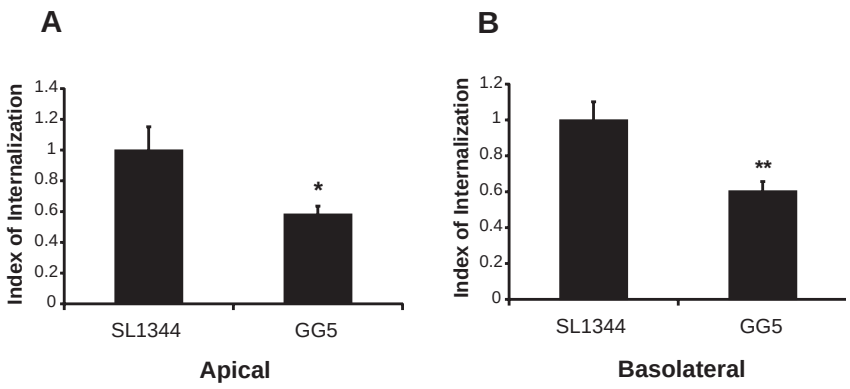

Fig. 5. SopE is required for *Salmonella* internalization at both apical and basolateral poles of MDCK monolayers. T23 cells were infected at the apical (A) or basolateral (B) pole with SL1344 or an isogenic mutant disrupted in the *sopE* locus (GG5). Bacterial internalization was quantified as described in Materials and Methods. Experiments were repeated at least 4 times. **P*<0.001; ***P*<0.0003 (Student's *t*-test).

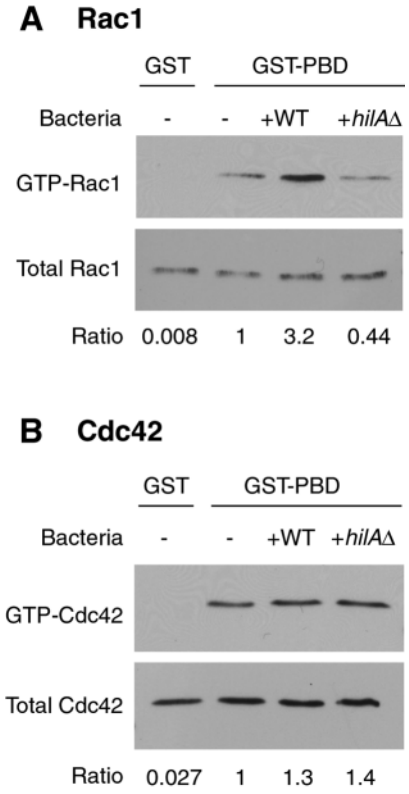

Fig. 6. Endogenous Rac1, but not Cdc42, is activated during apical invasion of *Salmonella*. (A) T23 cells were apically infected with SL1344 (lane 3) or the entry-deficient *hilA* deletion strain VV341 (lane 4), or maintained in $HBSS^+$ (lanes 1-2). GTP-bound Rac1 was isolated from cell lysates by affinity precipitation with GST alone as negative control (lane1) or GST-PBD (lanes 2-4) as described in Materials and Methods. Pulldowns (upper panel), as well as a fraction of the lysates (lower panel), were simultaneously blotted for Rac1. The ratio of GTP-bound to total Rac1 is expressed relative to uninfected cells (lane 2) (=1). (B) An identical experiment to A was conducted, except samples were immunoblotted for Cdc42. Data are representative of results from at least 5 experiments.

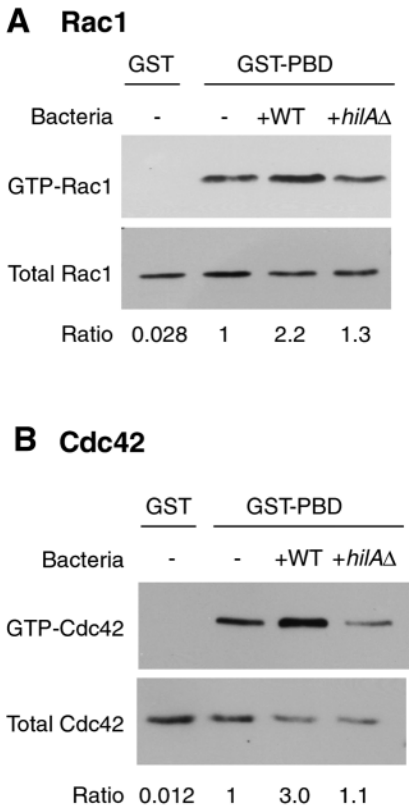

Fig. 7. Endogenous Rac1 and Cdc42 are both activated during basolateral invasion of *Salmonella.* (A) T23 cells were basolaterally infected with SL1344 (lane 3) or *hilA* deletion strain VV341 (lane 4), or maintained in $HBSS^+$ (lanes 1-2). GTP-bound Rac1 was isolated from cell lysates by affinity precipitation with GST alone as control (lane 1) or GST-PBD (lanes 2-4), and immunoblotted for Rac1, as in Fig. 6A. The ratio of GTP-bound to total Rac1 is expressed as in Fig. 6. (B) Cells were infected as described above and immunoblotted for Cdc42. Data are representative of results from at least 4 experiments.

monolayers at the time of fixation (16 hours of polarization vs 48 hours in our studies).

We then examined the distribution of filamentous actin in N17Cdc42-expressing monolayers, since the actin organization in this cell line has not been previously characterized. Almost every cell in the N17Cdc42 population expressed the transgene in the absence of doxycycline, and expression of the dominant-negative construct produced a filamentous actin staining pattern very different from that in control or N17Rac1-expressing monolayers (Fig. 2G-I). At the apical pole of N17Cdc42-expressing cells, microvillar staining was less prominent. Accumulations of polymerized actin were observed at the lateral margins of these cells as well as at discrete sites on the basal surface. In a subset of expressors, circular actin structures, similar to those observed in N17Rac1-expressing cells, were detected at the basal pole. Stress fibers otherwise appeared unaltered from what had been observed in nonexpressing cells. Taken together, these findings indicate that expression of dominant-negative Rac1 and Cdc42 induce distinct, phenotypic effects on the morphology of the actin cytoskeleton in polarized MDCK cells.

Dominant-negative Rac1 inhibits apical, but not basolateral, invasion of *Salmonella*

We next examined whether Rac1 and/or Cdc42 function were necessary for mediating *Salmonella* invasion into polarized epithelia. We were especially interested in whether internalization was regulated differently at the apical vs basolateral poles of these cells. Although initial exposure to *Salmonella* occurs at the lumenal surface of the epithelium, bacteria can rapidly gain access to the basolateral surface due to transit through M (microfold) cells (Jones et al., 1994). M cells are an important component of the mucosal immune system, as they specialize in the sampling and transport of lumenal contents to lymphoid cells of the Peyer's patches (Niedergang and Kraehenbuhl, 2000). In preliminary experiments, *Salmonella* were found to attach to and enter the apical and basolateral plasma membrane domains of T23 cells with equal efficiency, with approximately 3-5% of the bacterial inoculum internalized after one hour of infection (data not shown). These results are in agreement with reports of *Salmonella* invasion into the polarized intestinal T84 cell line (Gewirtz et al., 1999). The ability of our invasion assay to distinguish between internal and external bacteria was

confirmed by the observation that treatment of the monolayers with the actin depolymerizing agent cytochalasin D reduced the number of internalized bacteria at both poles to <10% of control levels (data not shown).

We first investigated the effects of Cdc42 and Rac1 mutants on bacterial invasion at the apical plasma membrane. The invasion profile for each mutant cell line was compared to the same cells maintained in the presence of doxycycline in order to exclude effects due to clonal variation. In contrast to previously reported results in nonpolarized cells, N17Cdc42 had no effect on apical entry of *Salmonella* (Fig. 3B). However, N17Rac1, which was expressed at levels similar to N17Cdc42 (data not shown), potently inhibited invasion of *Salmonella* at the apical pole. Expression of this dominant-negative construct reduced the number of internalized bacteria by 80%, compared to cells maintained in the presence of doxycycline ($P<0.001$) (Fig. 3A). The presence of N17Rac1 did not impair bacterial attachment to the apical cell surface, verifying that factors essential to *Salmonella* interaction with the brush border membrane were not altered by expression of the mutant protein (data not shown). Interestingly, activated mutants of both Rac1 and Cdc42 (V12 +) stimulated apical bacterial entry. The V12Cdc42-mediated increase may be due in part to indirect activation of Rac1 (see Discussion).

To further confirm the apical invasion defect of N17Rac1-expressing epithelial cells, we observed infected monolayers of these cells by immunofluorescence microscopy. Cells were washed extensively to remove the majority of the cell surface-bound bacteria; thus, essentially all the bacteria in these images reflect the internalized population. In the absence of N17Rac1 expression, *Salmonella* were detected within multiple cells per field (Fig. 3C). By contrast, *Salmonella* were rarely found internalized into N17Rac1-expressing cells. Rather, the majority of the bacteria were associated with the nonexpressing cells in the monolayer (Fig. 3D). This observation suggests that the invasion assays underestimated the severity of the N17Rac1-dependent block in internalization, since the defect was analyzed as a function of the entire population of cells, including nonexpressors. As expected, internalized bacteria were prominently observed in N17Cdc42-expressing T23 cells (data not shown). These data strongly suggest that Rac1, but not Cdc42, is required for *Salmonella* invasion at the apical plasma membrane of polarized epithelia.

While the apical brush border of epithelial cells is considered a specialized domain designed for specific functions like absorption, the basal and lateral membranes more closely resemble the surface of nonpolarized cells, due to their similar lipid and protein compositions (Caplan and Matlin, 1989; Handler, 1989; Simons and Wandinger-Ness, 1990). Therefore, we anticipated that Cdc42 function would be required for invasion of *Salmonella* at the basolateral membrane, as found for nonpolarized cells. Instead, we observed that neither Cdc42 nor Rac1 inhibited bacterial entry at the basolateral pole (Fig. 4). As with apical invasion, activated mutants of both Rac1 and Cdc42 stimulated basolateral *Salmonella* entry (Fig. 4). Taken together, these results indicate that the GTPases required for entry into the apical pole of epithelial cells differ from those that modulate entry at the basolateral surface, even though invasion occurs equally efficiently at both membrane domains.

Internalization of *Salmonella* at both apical and basolateral surfaces requires the activity of the SopE effector protein

Activation of Rho family GTPases by invasive *Salmonella* has been attributed to the bacterial effector protein SopE, a secreted product of the SPI1-encoded type III secretion system. SopE catalyzes nucleotide exchange on these GTPases in vitro and produces rearrangements in cortical actin architecture when overexpressed in mammalian cells (Hardt et al., 1998; Rudolph et al., 1999). While SopE is only expressed by a subpopulation of pathogenic *Salmonella* strains, a related protein, SopE2, is believed to be present in most strains of *Salmonella* (Bakshi et al., 2000; Stender et al., 2000). To investigate the requirement for SopE in bacterial invasion of polarized epithelia, MDCK monolayers were infected apically or basolaterally with wild-type *S. typhimurium* or an isogenic mutant containing a disruption of the *sopE* locus (GG5). The *sopE* mutant retained only 60% of the invasion capacity of wild-type bacteria at both epithelial membrane domains (Fig. 5A-B), a value comparable to that reported for *sopE* mutant internalization into nonpolarized cells (Stender et al., 2000). These results indicate that, despite differences in the involvement of Rac1 and Cdc42 during apical and basolateral entry, invasion at both poles requires the activity of the bacterial exchange factor SopE.

Apical invasion of *Salmonella* activates endogenous Rac1

To further confirm the role of Rac1 in apical bacterial entry, we examined the activation (i.e. GTP binding) state of the GTPase during *Salmonella* invasion, using a previously described affinity precipitation assay. This procedure is based on the interaction of GTP-bound, but not GDP-bound, Rac and Cdc42 with p21-activated kinase (PAK), a shared downstream effector (Benard et al., 1999; Sander et al., 1998). Apical exposure of T23 cells to *Salmonella* produced a substantial increase in levels of endogenous GTP-bound Rac1 as compared to uninfected cells (Fig. 6A). GTP-Rac1 formation was stimulated at as little as 5 minutes following exposure to the bacteria and reached a maximal level at 30 minutes, which

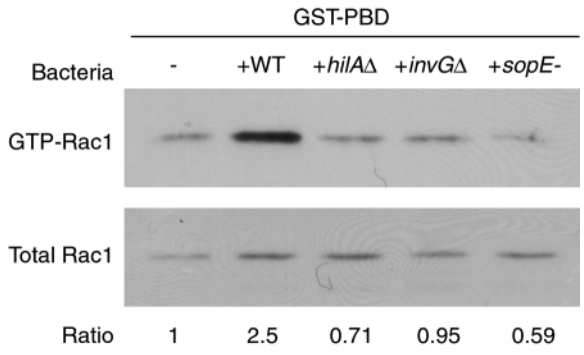

Fig. 8. Activation of Rac1 by apically invading *S. typhimurium* is dependent on the bacterial type III secretion apparatus and the SopE effector protein. T23 cells were apically infected with SL1344 (lane 2), *hilA* deletion strain VV341 (lane 3), *invG* deletion strain AJK61 (lane 4), *sopE* deletion strain GG5 (lane 5), or maintained in $HBSS^+$ as control (lane 1). GTP-Rac1 levels in each lysate were determined by affinity precipitation with GST-PBD as described in Fig. 6. Data are representative of results from 4 experiments.

was maintained after one hour of infection (data not shown). Infection with wild-type *Salmonella* for 30 minutes led to a 3- to 5-fold increase in GTP-Rac1 in the cells (lane 3). We ruled out that this effect was simply due to the stress of bacterial load by demonstrating that GTP-Rac1 levels were not increased when cells were infected with an entry-deficient *S. typhimurium* strain (*hilAΔ*; lane 4) (Hueck et al., 1995). As expected, expression of N17Rac1 inhibited the *Salmonella*-mediated increase in GTP-Rac1 levels (data not shown). In contrast, *Salmonella* invasion at the apical plasma membrane did not lead to the activation of endogenous Cdc42 (Fig. 6B). This observation is in accordance with the inability of the dominant-negative mutant of this GTPase to block internalization at the apical pole.

We then investigated the activation state of Rac1 and Cdc42 during *Salmonella* invasion at the basolateral pole. Remarkably, basolateral application of invasive bacteria led to a 3- to 4-fold increase in levels of both GTP-bound Rac1 (Fig. 7A) and Cdc42 (Fig. 7B), as compared to uninfected or *hilAΔ*-infected controls. This is in direct contrast to the inability of dominant-negative mutants of these GTPases to block bacterial entry at the basolateral surface and suggests that these GTPases may be involved in other aspects of *Salmonella* pathogenesis, such as initiation of host signal transduction cascades (see Discussion). In conjunction with the internalization assay data in Figs 3 and 4, these results indicate that *Salmonella* interaction with the apical vs the basolateral plasma membrane activates different subsets of GTPases, which then differentially affect bacterial entry.

To investigate whether apical Rac1 activation was dependent on the Rho GTPase exchange factor SopE, we compared GTP-bound Rac1 levels in MDCK monolayers infected with wild-type *Salmonella* to those infected with one of three mutant bacterial strains. One strain is a deletion in the gene for *hilA*, a transcriptional regulator of the type III secretion apparatus (Hueck et al., 1995). Strain AJK61 is deleted in the *invG* gene, which encodes a structural component of the type III secretion apparatus (Crago and Koronakis, 1998; Daefler and Russel, 1998); this strain produces, but cannot export, type III-regulated effector proteins like SopE. The final mutant utilized, GG5, is a disruption of the *sopE* gene itself. None of these mutant strains were able to elicit the increase in GTP-Rac1 levels observed in the presence of wild-type *Salmonella* (Fig. 8). Despite the fact that the GG5 mutant expresses SopE2, we were unable to detect any activation of Rac1 by that strain, suggesting that SopE2 alone is not sufficient to activate Rac1 in this assay system. Notably, we also found that GTPase activation during basolateral invasion was dependent on SopE function (data not shown). These results provide strong evidence that the SopE protein possesses GEF activity on Rho GTPases in vivo as well as in vitro, and furthermore that SopE is the primary activator of Rac1 during apical invasion of *S. typhimurium*.

N17Rac1 expression inhibits *Salmonella*-induced ruffle formation

Our results thus far indicate that Rac1 is the key regulator of *Salmonella* internalization at the apical plasma membrane. In order to determine how interfering with Rac1 function compromised the bacterial invasion process, we observed monolayers of N17Rac1 cells, grown in the presence or absence of doxycycline, by scanning electron microscopy. Uninfected monolayers of both cell populations had similar apical morphology, consistent with the immunofluorescence data in Fig. 2 (data not shown). In monolayers of infected, doxycycline-repressed cells, multiple ruffles, indicating sites of active invasion, were clearly detected in a given field (Fig. 9A). In the presence of N17Rac1, the formation of bacteria-induced membrane ruffles was dramatically decreased (Fig. 9B). However, no differences were noted in the architecture or size of these invasion structures. These observations were quantified by averaging the number of ruffles per unit surface area in the micrographs, using the same magnification and the same areas of the filters of control and N17Rac1-expressing cells. The number of entry foci on the N17Rac1-expressing cells was reduced by 70% (Fig. 9C), a value comparable to the reduction in internalization that was calculated from the invasion assay in Fig. 3. These results support our contention that N17Rac1 expression in epithelial cells prevents the invasion of *Salmonella* at the apical plasma membrane, and further indicate that the invasion defect in these cells is specifically due to a failure to generate membrane ruffles at bacterial adhesion sites.

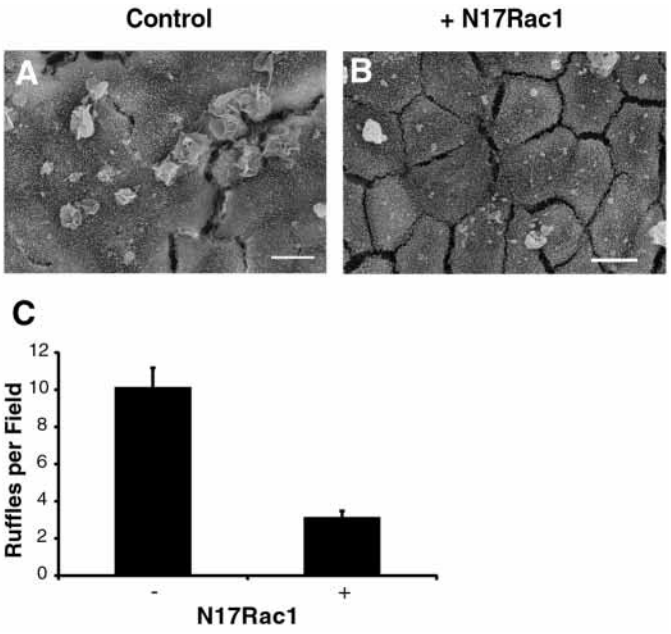

Fig. 9. Expression of N17Rac1 in T23 monolayers inhibits *Salmonella*-induced ruffle formation. (A-C) N17Rac1 cells in the presence (A) or absence (B) of doxycycline were infected with SL1344, then fixed and processed for scanning electron microscopy. Bar, 10 μm. For C, 12 images from the same regions of each filter were collected at ×2000 magnification (field size=3360 μm^2). *Salmonella*-induced ruffles in each field of control cells were counted and compared to the number observed in each field of N17Rac1 expressors. $P<0.0002$ (Student's *t*-test).

DISCUSSION

Invasive *Salmonella* first interact directly with their animal host at the apical surface of the intestinal epithelium. The successful breaching of this barrier is essential to bacterial replication and

colonization, and understanding the means by which these pathogens manipulate the host to internalize them is integral to understanding how they cause disease. In the present study, we tested the hypothesis that the cytoskeletal regulatory factors appropriated by *Salmonella* during entry into polarized epithelia would differ from those that direct invasion into nonpolarized cells. Our data reveal two important findings. First, we have determined that the GTPase Rac1 is required for bacterial entry at the apical plasma membrane of polarized epithelial cells. Invasion occurs concomitantly with activation of endogenous Rac1, and dominant-negative inhibition of Rac1 prevents both ruffle formation and *Salmonella* internalization. Second, the related GTPase Cdc42, which had been implicated as the key regulator of *Salmonella* entry in nonpolarized cells, is dispensable for apical entry and is not activated during invasion at this site. By contrast, we have found that neither Rac1 nor Cdc42 are required for invasion at the basolateral pole, although both GTPases are activated at the time of entry. These results imply that *Salmonella* invasion into polarized epithelia has different molecular requirements than entry into nonpolarized cells, which may be relevant to future studies examining host cell responses to bacterial infection.

In our in vitro model of *Salmonella*-enterocyte interaction, the bacteria elicit actin reorganization and membrane ruffling at the apical surface of polarized MDCK cells in a manner that is morphologically indistinguishable from ruffling in nonpolarized cell lines (see Fig. 1). However, during entry at the apical pole of epithelial cells, *Salmonella* encounter a complex, highly organized actin cytoskeleton unlike any other cell surface that they invade (see Fig. 2B-C). At the apical domain, polymerized actin is organized into rigid microvilli and the underlying terminal web, a crosslinked meshwork of actin filaments, which attaches to intercellular junctional complexes (Fath et al., 1993). Accordingly, the ability of *Salmonella* to reorganize the apical plasma membrane and its underlying actin architecture may require the mobilization of a unique set of cellular regulatory factors.

In this study we have identified the Rac1 GTPase as a factor that is obligatory for *Salmonella* invasion into the apical pole of polarized epithelia. Our observations are consistent with previous work detailing the effects of Rac1 on the actin cytoskeleton. In nonpolarized cells, Rac1 promotes the formation of lamellipodia, which are essential for dynamic cellular processes such as matrix-dependent spreading and chemotaxis (reviewed in (Hall, 1998)). Rac1-dependent membrane ruffles are derived from lamellipodia that fold back upon themselves, and are critical to actin-dependent events such as phagocytosis (see below). Normally, these actin rearrangements arise from Rac1 activation downstream of signals from a subset of plasma membrane receptors. GTP-bound Rac1 then interacts with effector proteins that directly modify the actin cytoskeleton. However, during bacterial invasion, *S. typhimurium* utilize the secreted proteins SopE and SopE2 to directly activate Rac1, thereby co-opting the host cell signaling pathways that direct ruffle formation. We are currently investigating which effectors of Rac1 participate in the invasion event.

Since the *S. typhimurium* protein SopE can catalyze nucleotide exchange on both Rac1 and Cdc42 in vitro (Hardt et al., 1998), it is surprising that Cdc42 is not activated during apical invasion. It is also surprising that a dominant-negative Cdc42 mutant does not block invasion, since inhibitory mutants of both Rac1 and Cdc42 would be expected to sequester SopE, blocking nucleotide exchange and thereby preventing entry. The most likely explanation for these findings is that SopE functions locally, at sites directly underlying bacterial-epithelial cell contact (Stender et al., 2000), and that during apical invasion Rac1 is more abundant at these sites than Cdc42. This may be because most of the Cdc42 in epithelial cells partitions to, and functions at, domains other than the brush border (Kroschewski et al., 1999). Another possibility is that Rac1, but not Cdc42, is specifically recruited to SopE-rich microdomains during apical invasion. In this scenario, N17Cdc42 would not block apical *Salmonella* internalization, because like its endogenous, wild-type counterpart, the dominant-negative protein cannot localize to SopE concentrations on the apical surface. It has been demonstrated in vitro that SopE prefers Rac1 to Cdc42 as a substrate (Hardt et al., 1998), and it is also possible that Cdc42 and SopE encounter each other at the apical plasma membrane, but that productive activation of Cdc42 does not subsequently occur in vivo. Future studies pinpointing the subcellular distribution of endogenous Rho GTPases in polarized epithelial cells should help to clarify this issue.

We confirmed that the exchange factor SopE plays a prominent role in bacterial invasion at both plasma membrane domains of epithelial cells, as evidenced by the decreased internalization efficiency of a *sopE* mutant (Fig. 5), and by the inability of this mutant to activate endogenous GTPases (Fig. 8). While not all pathogenic strains of *Salmonella* encode SopE, it has recently been reported by two independent groups that most strains possess SopE2, a protein with high sequence similarity to SopE (Bakshi et al., 2000; Stender et al., 2000). In the *S. typhimurium* strain we have used in these studies, SL1344, SopE2 has been shown to be less potent than SopE, as judged by the minimal invasion defect of a *sopE2* deletion strain, and by the decreased ability of SopE2 to elicit membrane ruffles when ectopically expressed in mammalian cells (Stender et al., 2000). These findings substantiate our results demonstrating that SopE, independently of SopE2, can mediate both GTPase activation and bacterial entry. Although we cannot rule out a potential role for SopE2, our data strongly suggest that SopE acts as the main regulator of Rho GTPase activation during epithelial cell invasion.

The events culminating in *Salmonella* entry into nonphagocytic cells mimic those of macrophage phagocytosis, a process that requires both Cdc42 and Rac1 activity (Benard et al., 1999; Caron and Hall, 1998; Cox et al., 1997; Lee et al., 2000). While bacterial invasion of nonpolarized cells resembles Fc receptor-mediated phagocytosis in its utilization of both these GTPases (Chen et al., 1996), *Salmonella* internalization at the apical plasma membrane is unique in its requirement for only Rac1. In this circumstance, Cdc42 activity may be dispensable because factors unique to this epithelial domain functionally substitute for this GTPase. Alternatively, the brush border actin may already be in a conformation that makes Cdc42 activity unnecessary. Cdc42 has been shown to induce the nucleation of new actin filaments through WASP and the Arp 2/3 complex (Rohatgi et al., 1999; Symons et al., 1996), and in nonpolarized cells the p41Arc subunit of Arp 2/3 localizes to SopE- and SopE2-induced membrane ruffles (Stender et al., 2000). Cdc42-dependent

nucleation of new actin filaments may provide the driving force for *Salmonella* entry into nonpolarized cells. However, at the apical pole of polarized epithelial cells, actin filaments in each microvillus are arranged such that the fast growing (barbed) ends face towards the lumen. Growth from the tips of these filaments could thereby provide the requisite actin polymerization for bacterial entry, without the need for de novo nucleation by Cdc42. Interestingly, we observed that expression of an activated mutant of Cdc42 was sufficient to promote entry at the apical pole (Fig. 3B). We attribute this effect to the ability of V12Cdc42 to indirectly activate Rac1. In Fc receptor-mediated phagocytosis, as one example, Cdc42 activation is necessary for downstream activation of Rac1 (Caron and Hall, 1998; Massol et al., 1998). In epithelial cells, expression of V12Cdc42 may initiate signals that result in increased GTP-Rac1 levels. The combined effect of this ectopic Rac1 activation with SopE-mediated activation would produce the observed increase in apical bacterial internalization.

Given the contention that the basolateral membrane of epithelia is organized similarly to the plasma membrane of nonpolarized cells (Caplan and Matlin, 1989; Handler, 1989; Simons and Wandinger-Ness, 1990), we anticipated that *Salmonella* invasion at this pole would require Cdc42 and perhaps Rac1 activity. Instead, we found that, although both Cdc42 and Rac1 were activated at the time of basolateral entry, neither was required for this process. These findings are in agreement with the results of Jones et al. who reported that *Salmonella* entry into unpolarized MDCK cells was not inhibited by expression of N17Rac1 (Jones et al., 1993). Interestingly, we also observed that the expression of constitutively active mutants of both Cdc42 and Rac1 stimulated basolateral bacterial internalization, perhaps by ectopically activating proteins that directly regulate the entry process. One candidate for such a factor is the GTPase RhoA, which, like Cdc42 and Rac1, produces discernible effects on the actin cytoskeleton. RhoA has been shown to regulate invasion by the bacterial pathogen *Shigella*, which enters cells through a process analogous to *Salmonella* invasion (Adam et al., 1996). Importantly, *Shigella* invades exclusively at the basolateral membrane of polarized epithelia (Mounier et al., 1992). Accordingly, investigations are currently underway to investigate the role of RhoA in basolateral invasion by *Salmonella*.

While Cdc42 and Rac1 are not necessary for *Salmonella* internalization at the basolateral plasma membrane, these GTPases may serve to modulate signal transduction cascades that are activated during bacterial invasion. For instance, activation of Jun kinase (JNK) and PAK1 during *Salmonella* invasion of nonpolarized cells requires functional Cdc42 (Chen et al., 1996; Chen et al., 1999). This raises the interesting prospect that differences in activation of these GTPases during apical vs. basolateral invasion might not only affect bacterial entry, but also these signaling cascades. One example of such polarized signaling is the transit of neutrophils across epithelial T84 monolayers, a hallmark of *Salmonella*-mediated gastroenteritis, which occurs following apical, but not basolateral, application of the bacteria (Gewirtz et al., 1999). Therefore, the differential activation of Rho family GTPases in polarized epithelia may induce additional changes in signal transduction events that have not yet been characterized.

In summary, our results strongly support a role for Rac1 in invasion of the pathogen *Salmonella typhimurium* at the apical plasma membrane of epithelial cells. As far as we are aware, this is the first time that the regulation of *Salmonella* invasion has been investigated in such detail in polarized epithelial cells, a model for the physiologic interactions between pathogenic bacteria and intestinal enterocytes. These findings, which differ significantly from previous observations in nonpolarized cells, suggest that the regulatory factors mediating bacterial-mediated endocytosis are cell type-specific. Furthermore, our data imply that the regulation of cortical actin organization, as well as the regulation of signal transduction cascades, occurs differently at the apical and basal aspects of epithelia. Understanding how subsets of Rho family GTPases modulate the invasion of *Salmonella* at each membrane domain of polarized epithelia can therefore provide insight into the roles of these GTPases in the dynamics of epithelial development.

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