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Salmonella typhimurium induces membrane ruffling by a growth factor-receptor-independent mechanism

(GTPases/invasion)

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ABSTRACT Invasive *Salmonella typhimurium* induces dramatic actin rearrangements on the membrane surface of mammalian cells as part of its entry mechanism. These changes, which are best characterized as membranous ruffles, closely resemble the membrane changes that occur when a growth factor binds to its receptor. Recently, inhibition of the function of the small GTPases rac and rho in quiescent serum-starved fibroblasts was demonstrated to abolish growth factor-mediated ruffling and stress-fiber formation, respectively. In addition, actin changes induced by the oncogene *ras* were also shown to be regulated by rac and rho. Because *Salmonella*-induced actin rearrangements resemble those caused by growth factors, we investigated whether ras, rho, or rac regulates the membrane ruffling elicited by *S. typhimurium*. Surprisingly, inhibition of the functions of these GTPases had no effect on the ability of invasive *S. typhimurium* to induce membrane ruffles on a variety of tissue culture cells including Madin–Darby canine kidney cells, Swiss 3T3 fibroblasts, and Hep-2 cells. These results led us to examine the interactions of *S. typhimurium* with Henle-407 intestinal cells, which lack epidermal growth factor receptor on their membrane surface. We found no difference in the ability of invasive *S. typhimurium* to induce membrane ruffling and to enter Henle-407 cells with or without the epidermal growth factor receptor on the membrane surface. We, therefore, conclude that invasive *S. typhimurium* induces membrane ruffling and its own internalization by a rac-independent, growth factor-receptor-independent signaling pathway.

Salmonella infections range in severity from self-limiting gastroenteritis (food poisoning) to life-threatening systemic enteric fevers (typhoid fever). Although the severity of salmonellosis depends upon many bacterial–host cell interactions, the ability of *Salmonella* to initiate either type of infection depends upon its initial ability to penetrate the epithelial barrier of the bowel. The events leading to *Salmonella typhimurium* entry of intestinal enterocytes were first described in an EM study by Takeuchi (1). Interactions between bacteria and intestinal tissue were examined in starved, opium-treated guinea pigs several hours after oral challenge with 10^8 invasive *S. typhimurium*. Organisms in close contact with guinea pig enterocytes appeared to be in the process of causing the dissolution of the apical microvilli of the brush border cells. At later stages of the entry process, the cytoplasm of the host cell could be observed protruding from the cell and appeared to be engulfing the bacteria. Actin staining of *Salmonella*-infected tissue culture cells has demonstrated that membrane rearrangements, or ruffling, also occur *in vitro* (2–6). These cytoskeletal rearrangements seem to be an integral component of *Salmonella* entry, as the inhibitors of actin polymerization, cytochalasin B and D, block *Salmonella*-induced membrane rearrangements and internalization (6–10).

The actin cytoskeleton is integral to many eukaryotic cellular functions including motility, chemotaxis, cell division, and endocytosis (11–13). Control of actin-filament polymerization is required for each of these processes, yet little is known of the regulation of these mechanisms. Recently, however, the small GTPases rac and rho were found to be involved in the regulation of growth factor-induced membrane ruffling and stress-fiber formation (14, 15). The effect of growth factor addition to quiescent serum-starved fibroblasts was mimicked by introduction of purified proteins into the serum-starved cells. Microinjection of an activated mutant rac protein into serum-starved Swiss 3T3 cells induced an immediate accumulation of polymerized actin in membrane ruffles, whereas microinjection of activated rho protein stimulated focal adhesion and stress-fiber formation. Subsequently, inhibiting the function of these proteins was shown to block growth factor-induced actin rearrangements in serum-starved fibroblasts. As membrane ruffling seems an essential component of the *Salmonella* entry process, we have investigated whether the small GTPase rac, as well as ras and rho, regulate *Salmonella*-induced membrane ruffling and the bacterial entry process.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *S. typhimurium* strain SL1344 is an invasive mouse virulent strain (16). *S. typhimurium* strain BJ66 is an SL1344 derivative with an oxygen-regulated Tn*5lac* chromosomal insertion that confers a noninvasive phenotype upon the strain. The plasmid pBDJ127 carries chromosomal DNA from *S. typhimurium* SL1344, which complements the invasion defect of *S. typhimurium* BJ66.

Bacteria were grown in Luria–Bertani broth on a roller drum at 37°C to early exponential phase (aerobic culture) or statically overnight at 37°C to midexponential phase (oxygen-limited culture) as described (17). Antibiotics were added to the culture medium when necessary.

Cell Culture Conditions. Swiss 3T3 fibroblasts, Madin–Darby canine kidney cells (MDCK), and Hep-2 epithelial cells were grown in Dulbecco's modified Eagle's medium (GIBCO/BRL) with 10% fetal calf serum (GIBCO/BRL). Henle-407 human intestinal cells were grown in basal minimal medium with Hanks' balanced salts (GIBCO/BRL) and 10% fetal calf serum. To obtain quiescent serum-starved Swiss 3T3 cells, 5×10^4 cells were seeded onto coverslips in a 24-well tissue culture plate and allowed to grow for several

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Abbreviations: MDCK, Madin–Darby canine kidney; FITC, fluorescein isothiocyanate; BFA, brefeldin A; EGF, epidermal growth factor.

days. When the cells were for a microinjection experiment, coverslips were first marked with a diamond-tip pen to facilitate the localization and visualization of microinjected cells. After 8–12 days of growth, the medium in the wells was replaced 16 hr before use with serum-free Dulbecco's modified Eagle's medium/0.2% $NaHCO_3$. Confluent MDCK cell monolayers were prepared by seeding 5×10^4 cells into wells and allowing them to grow for 6–8 days. For experiments involving Hep-2 or Henle-407 tissue culture lines, 1×10^5 cells were seeded onto glass coverslips in wells of a 24-well tissue-culture plate ≈18 hr before use.

Tissue Culture Assays. Invasion assays were initiated by infecting wells containing tissue culture cells with $\approx 10^7$ bacteria. The bacteria were centrifuged onto the cell monolayers in microtiter plate adapters in a bench-top centrifuge at 185 × *g* for 15 min. The bacteria were allowed to interact with the tissue culture monolayer for an additional 15 min before unbound bacteria were removed by washing with phosphate-buffered saline five times. For immunofluorescence assays the cells were then fixed, permeabilized, and stained as described below. For quantitative assays, RPMI 1640 tissue culture medium containing gentamicin at 100 μg/ml was added to the monolayers and incubated for 90 min at 37°C in 5% carbon dioxide (18). After killing extracellular bacteria with gentamicin, the monolayers were washed three times with phosphate-buffered saline and lysed with 1% Triton X-100 (Sigma) for 10 min. After addition of Luria–Bertani broth, dilutions of each well were plated to count the bacteria within the host cells.

Immunofluorescence. Tissue culture cells grown on glass coverslips were prepared for fluorescent microscopy by fixing with 4% formaldehyde for 30 min, permeabilizing with 0.2% Triton X-100 for 15 min, and blocking with 10% fetal calf serum in phosphate-buffered saline. To visualize filamentous actin, cells were stained with rhodamine-labeled phalloidin as described (3). *S. typhimurium* was labeled directly with free fluorescein isothiocyanate (FITC), which covalently binds to free amine groups on bacterial membrane proteins without affecting viability or invasiveness (6). Microinjected cells were detected with FITC-conjugated-goat anti-rat IgG antibody (Pierce) used at a 1:500 dilution. Anti-epidermal growth factor (EGF)-receptor monoclonal antibody 108 (mAb108) ascites (19) were obtained from Suzanne Pfeffer (Stanford University School of Medicine, Stanford, CA) and was used at 1:1000 dilution. FITC-conjugated-rabbit anti-mouse IgG antibody was used at 1:500 dilution.

Microinjection. Swiss 3T3 fibroblasts, MDCK cells, and Hep-2 cells were used for microinjection experiments. As needed, anti-*ras* neutralizing monoclonal antibody Y12-259, the negative dominant protein V12N17rac1, or exoenzyme C3 transferase was injected into the cytoplasm of cells ≈1 hr before use. Rat immunoglobulin G (IgG) was coinjected with the proteins to serve as a marker for microinjected cells. V12N17rac1 protein was purified as described from a rac1 cDNA clone (15).

For experiments involving brefeldin A (BFA) and EGF, Henle-407 cells were pretreated with BFA at 5 μg/ml (Epicentre Technologies, Madison, WI) for 30 min. Saturating amounts of EGF (20 nM) (Sigma) were then added to the cells in the presence of 5 μg of BFA per ml and allowed to bind and internalize EGF receptor for 1 hr. The EGF was then washed away, and invasive *S. typhimurium* was added to the cells in the presence of BFA at 5 μg/ml, and the protocol for the immunofluorescence or quantitative assay was followed.

RESULTS

Effect of *S. typhimurium* on Swiss 3T3 Fibroblasts. Although it has been noted that invasive strains of *Salmonella* induce cytoskeletal rearrangements on cultured mammalian cells as an integral component of the internalization process (3), the effect of *Salmonella* on Swiss 3T3 fibroblasts is unknown. We examined the ability of *S. typhimurium* strain SL1344, grown in the oxygen-limited conditions that induce the invasive phenotype, to cause actin changes at the membrane surface of confluent monolayers of quiescent Swiss 3T3 cells serum-starved for 15 hr. As described by Ridley and Hall (14), serum starvation of Swiss 3T3 fibroblasts caused the actin structures of these cells to dissociate (Fig. 1*A*). When invasive *S. typhimurium* was added to serum-starved fibroblasts, a dramatic accumulation of polymerized actin was observed (Fig. 1*B*). These actin structures, which are less localized than *Salmonella*-induced actin rearrangements on cultured epithelial cells, appear identical to ruffles induced by growth factors on serum-starved Swiss 3T3 cells (15). The induction of membrane ruffling depended on the invasiveness of the *Salmonella* strain, as the mutant strain BJ66, which lacks the ability to enter tissue culture cells (unpublished data), was unable to induce the kind of actin rearrangements seen with parental strain SL1344 (Fig. 1*C*). Plasmid pBDJ127, which complements the invasion defect of strain BJ66, restored the ability of strain BJ66 to induce membrane ruffles in quiescent Swiss 3T3 cells to the same levels observed with the parent strain (Fig. 1*D*). As noted above, *Salmonella*-induced membrane ruffling of Swiss 3T3 fibroblasts is virtually indistinguishable from ruffling induced by serum and growth factors in these same cells. However, although we found that invasive *S. typhimurium* induced extensive membrane ruffling, no stimulation of stress-fiber formation was seen. In contrast, addition of growth factors (i.e., platelet-derived growth factor, EGF) to quiescent serum-starved Swiss 3T3 cells first stimulated membrane ruffling, followed several minutes later by the formation of focal adhesions and the appearance of actin stress fibers (15).

Effect of Inhibiting Small GTPases on the Ability of *S. typhimurium* to Ruffle Membrane. Growth factor-mediated

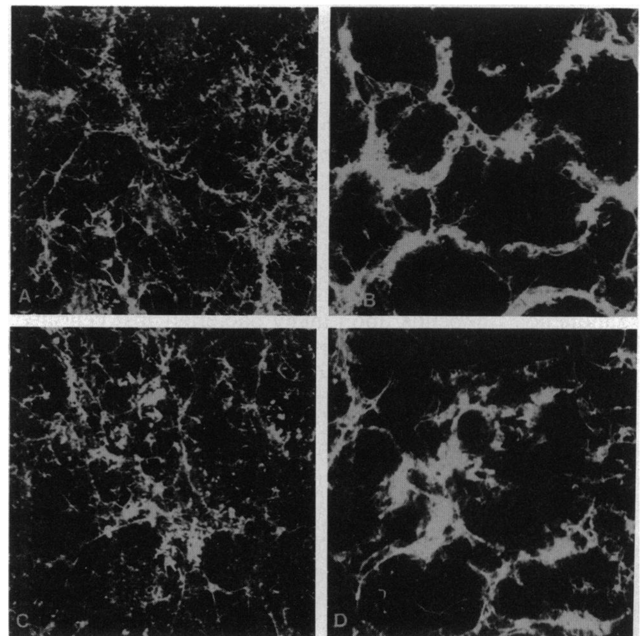

FIG. 1. Invasive *S. typhimurium* induces formation of actin rearrangements in the form of membrane ruffles on quiescent serum-starved Swiss 3T3 fibroblasts. Invasive *S. typhimurium* SL1344 (*B*), noninvasive *S. typhimurium* BJ66 (*C*), and invasive *S. typhimurium* BJ66 pBDJ127 (*D*) were incubated with confluent serum-starved cells for 30 min. Uninfected serum-starved quiescent Swiss 3T3 cells are shown in *A*. Cells were fixed, permeabilized, and stained with rhodamine–phalloidin to visualize filamentous actin filaments. (×290.)

membrane ruffling is regulated by the GTPase rac1 (15). Two other GTPases, rho and ras, have also been implicated in regulating actin rearrangements in fibroblasts (14, 15). We studied whether inhibiting the function of these proteins has any effect upon *Salmonella*-induced membrane ruffling in a variety of cell types, including quiescent serum-starved Swiss 3T3 cells, MDCK cells, and Hep-2 cells. The ras function was specifically inhibited by microinjecting the ras-neutralizing antibody Y13-259 into cells at 10 mg/ml. Actin staining of these microinjected cells revealed that invasive *S. typhimurium* strain SL1344 elicited membrane ruffles on the surface of cells despite disruption of ras function (Fig. 2 *A* and *B*). The rho function was inhibited in cells by microinjection of exoenzyme C3 transferase (3–30 μg/ml), a concentration that blocks EGF-induced focal adhesion and stress-fiber formation (data not shown; ref. 14). As seen in Fig. 2 *C* and *D*, the ability of *S. typhimurium* to cause cytoskeletal rearrangements was unaffected by inactivation of the rho protein. This result was not entirely unexpected, as the rho protein regulates actin rearrangements (focal adhesion and stress-fiber formation) that occur after the initial membrane-ruffling events. We next inhibited the function of endogenous rac1 protein by microinjection of dominant negative mutant V12N17rac at 400 μg/ml, a concentration that blocks all actin polymerization induced by growth factors. To our surprise, inhibition of the function of this regulatory GTPase had no inhibitory effect on the ability of *S. typhimurium* to induce actin polymerization and membrane ruffling on tissue culture cells (Fig. 2 *E* and *F*). We repeated the experiment with V12N17rac1 at 800 μg/ml, but again *Salmonella*-induced changes were unaffected. Control experiments, using EGF as stimulus, insured that these levels of microinjected rac1 protein completely blocked growth factor-induced cellular changes in Swiss 3T3 cells (data not shown) as described (15).

***S. typhimurium* Ruffling and Entry of Henle-407 Intestinal Cells Pretreated with EGF and BFA.** Inhibition of the small GTPases that regulate the signaling pathways of growth factors has no detectable effect upon *S. typhimurium*-induced membrane rearrangements in MDCK cells, Hep-2 cells, and Swiss 3T3 fibroblasts. As a recent report has implicated involvement of the EGF receptor in *Salmonella* entry of Henle-407 cells (20), we investigated the ability of *S. typhimurium* to interact with Henle-407 cells that lacked EGF receptor on their membrane surface. Henle-407 intestinal cells were pretreated with BFA at 5 μg/ml for 30 min at 37°C to block the transport of newly synthesized EGF receptor to the membrane surface. BFA specifically blocks protein transport from the endoplasmic reticulum to the Golgi apparatus by disrupting Golgi membranes (21, 22). After preincubation with BFA, the cells were incubated with 20 nM EGF for 1 hr in the presence of BFA at 5 μg/ml to internalize preexisting surface-localized EGF receptor. After EGF binding to its receptor, the ligand–receptor complex is internalized via coated pit endocytosis, and the EGF receptor is subsequently degraded in a lysosomal compartment (23). This procedure was used to dramatically reduce EGF-receptor localization on the membrane surface of Henle cells. As a control, we stained for the presence of the EGF receptor on untreated and BFA-treated cells. Fig. 3*A* shows that untreated Henle-407 cells stain heavily for the EGF receptor, whereas cells incubated with 20 nM EGF and BFA had undetectable levels of EGF receptor when using immunofluorescence detection (Fig. 3*C*). Preincubation of the tissue culture cells with only BFA for 90 min did not reduce the receptor levels on the cellular membrane (data not shown). We then examined the ability of *S. typhimurium* strain SL1344 to ruffle membrane and to enter Henle-407 cells that were untreated, BFA-treated, or treated with BFA and EGF. Rhodamine–phalloidin staining of cellular filamentous actin clearly showed no difference in the ability of invasive *S. typhimurium* to induce membrane ruffling on BFA- and EGF-treated Henle-407 cells as compared with untreated cells (Fig. 3 *B* and *D*). No membrane ruffling was seen on the cells without addition of invasive bacteria. The ability of invasive *S. typhimurium* to enter Henle-407 cells, treated as described above, was then measured. Table 1 shows that invasive *Salmonella* entered BFA-treated and BFA- and EGF-treated Henle cells equally well as the bacteria entered untreated cells. As a control, a gentamicin assay was done, in which the bacteria were allowed to interact with the cell monolayer

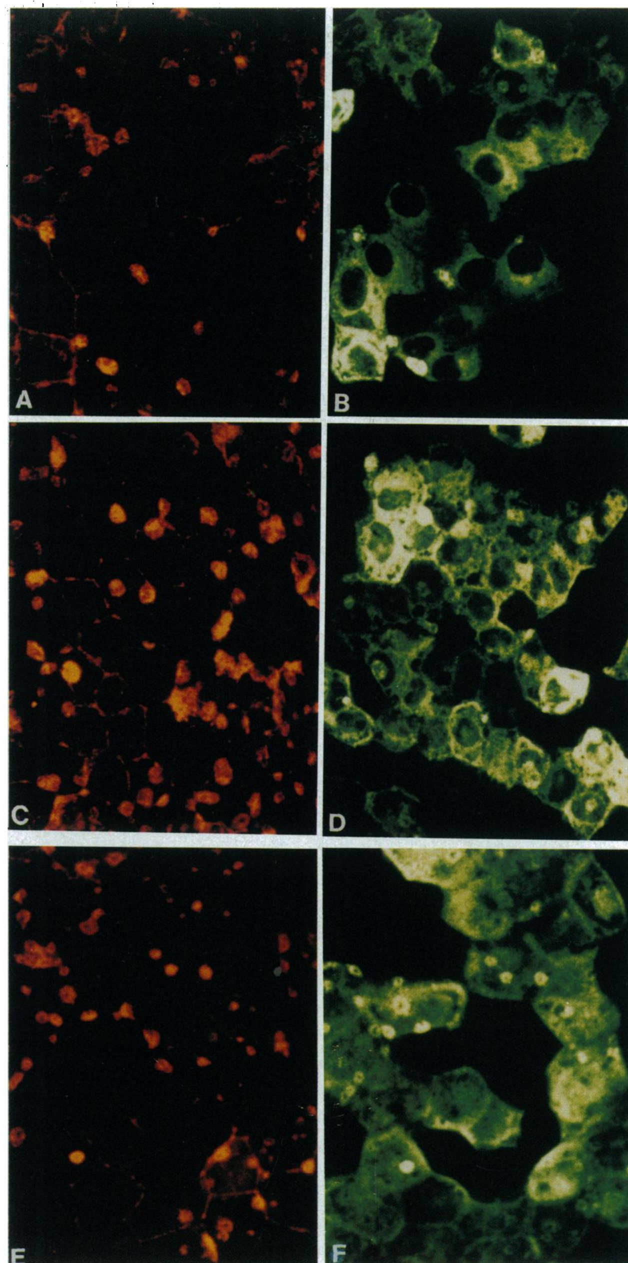

FIG. 2. Inhibition of ras, rho, and rac function has no effect on *S. typhimurium*-induced membrane ruffling of MDCK cells. After infection with invasive *S. typhimurium* strain SL1344, cells were stained with rhodamine–phalloidin to visualize cellular actin (*A*, *C*, and *E*) or with FITC-labeled anti-rat IgG to detect cells that had been microinjected (*B*, *D*, and *F*). Cells were microinjected with ras neutralizing antibody at 10 mg/ml (*A*, *B*), C3 transferase at 3–30 μg/ml (*C*, *D*), or mutant V12N17rac at 400 μg/ml (*E*, *F*). Comparison of the fields reveals that actin ruffles occur without bias on injected and uninjected cells. (×200.)

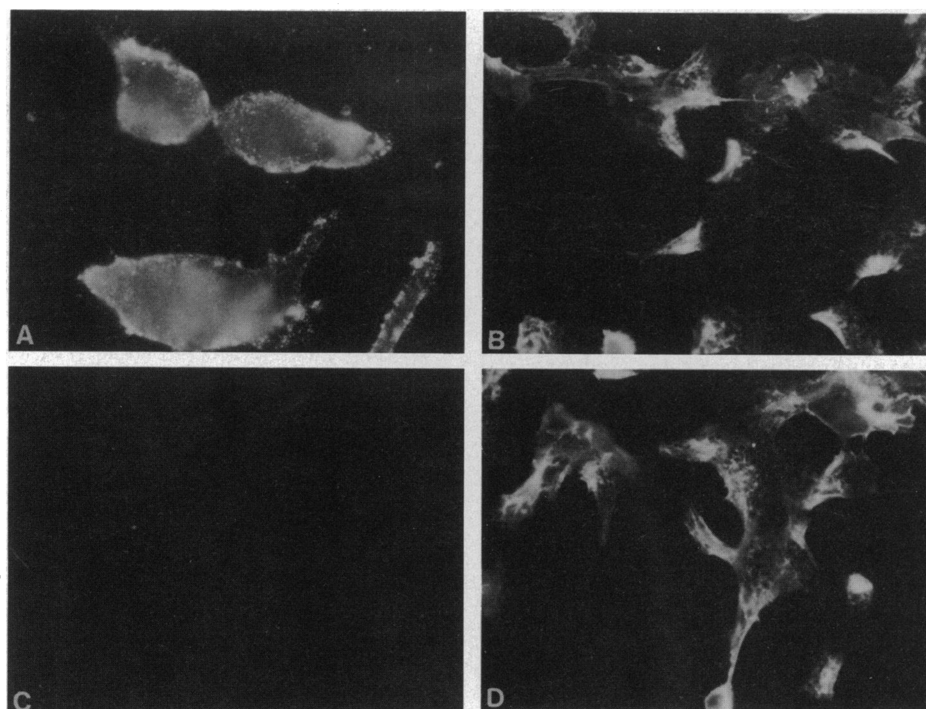

FIG. 3. *S. typhimurium*-induced membrane ruffling of Henle-407 intestinal cells that lack EGF receptor localized to the membrane surface. Henle-407 cells were untreated (*A, B*) or BFA- and EGF-treated (*C, D*) before addition of invasive *S. typhimurium* strain SL1344. EGF receptor was detected by mouse anti-EGF-receptor mAb108 and FITC-conjugated rabbit anti-mouse antibody (*A, C*), and filamentous actin was detected with rhodamine–phalloidin after incubation of the cells with invasive *S. typhimurium* (*B, D*). In *C*, four cells were visible by phase-contrast microscopy. (×320.)

without centrifugation to demonstrate that centrifugation was not inducing the organisms to enter the cells in the absence of EGF receptor. As seen in Table 1, the same results were obtained when the centrifugation step was omitted.

DISCUSSION

A variety of extracellular stimuli, including invasive bacteria, induce cytoskeletal rearrangements of mammalian cells. However, relatively little is understood about how the signals that these stimuli generate cause actin polymerization at the cell surface. Recently the ras-related small GTPase rac1 was shown to regulate the formation of actin ruffles (15). Increasing the intracellular concentration of rac1 protein by microinjection increased macropinocytosis in subconfluent Rat2 cells and increased membrane ruffling in confluent monolayers of Rat2 cells and serum-starved Swiss 3T3 cells. These effects were similar to cellular responses to mitogens, suggesting that rac1 played a role in growth factor-signaling events. Further experiments revealed that inhibition of rac1 activity in Swiss 3T3 cells prevented the ruffling that was normally induced by serum, platelet-derived growth factor, EGF, insulin, bombesin, and phorbol 12-myristate 13-acetate. It seems apparent, therefore, that the GTPase, rac1, is a primary regulator of growth factor-induced membrane ruffling.

Table 1. Ability of *S. typhimurium* strain SL1344 to enter Henle-407 intestinal cells that lack EGF receptors on the membrane surface

Treatment	Centrifugation	Inoculum surviving gentamicin treatment, % ± SD
None	+	0.90 ± 0.16
BFA	+	1.19 ± 0.43
BFA + EGF	+	0.98 ± 0.16
None	−	1.46 ± 0.13
BFA	−	1.52 ± 0.13
BFA + EGF	−	1.53 ± 0.01

Invasive *S. typhimurium* strain SL1344 (10^7) was added to Henle-407 cells (10^5) treated as indicated (BFA, 5 μg/ml; 20 nM EGF); bacteria were allowed to interact with the monolayer for 30 min before gentamicin addition.

We have shown here that invasive salmonellae induce cytoskeletal changes in quiescent serum-starved Swiss 3T3 cells that appear identical to the changes induced by growth factors. These similarities suggested that *Salmonella* bacteria could be using one or more growth factor-signaling pathways to induce cellular changes that lead to bacterial uptake. However, when we blocked endogenous rac1 activity by microinjection of a negative dominant protein, V12N17rac1, the ability of *S. typhimurium* to elicit cytoskeletal rearrangements in quiescent serum-starved Swiss 3T3 fibroblasts was unaffected. Similar results were obtained when the experiment was duplicated with either MDCK or Hep-2 epithelial cells. Our observation that invasive *S. typhimurium* does not elicit the formation of focal adhesions and stress fibers in Swiss 3T3 cells also suggests that the GTPase rac is not activated during bacterial entry because growth factor stimulation of rac has been shown to lead to stress-fiber formation (15). These data lead us to suggest that invasive *S. typhimurium* initiates a membrane-ruffling signal in mammalian cells by a rac-independent pathway.

Substantial efforts are being made to understand the cellular signals generated when invasive salmonellae interact with mammalian cells. Work was recently published which showed that phosphorylation of the EGF receptor on Henle-407 cells could be detected 3–4 hr after exposure to invasive *S. typhimurium* (20). However, our finding that invasive *S. typhimurium* induces membrane ruffling in cells with inhibited rac activity is inconsistent with the idea that bacterial internalization requires the EGF receptor directly. More-

over, we have shown here that *S. typhimurium* is fully capable of inducing membrane rearrangements and entering Henle-407 cells that lack surface expression of the EGF receptor. It has also been shown that the protein kinase inhibitors staurosporine, genistein, and tyrphostin, which block EGF-receptor activation, inhibit entry by a pathogenic *Yersinia* strain but have no effect on *S. typhimurium* invasion (24). Furthermore, binding studies with radiolabeled growth factor have demonstrated that EGF binds efficiently to highly purified basolateral membranes from intestinal enterocytes, but binding to purified apical membranes is undetectable (25). These observations suggest either that EGF-receptor phosphorylation is an indirect effect of *Salmonella*-induced membrane ruffling or that multiple pathways for *Salmonella* invasion exist. Galán and coworkers (20) also found that addition of EGF to tissue culture cells specifically allowed a noninvasive *S. typhimurium* strain to be internalized but did not allow a noninvasive *E. coli* RDEC strain to be internalized. In contrast, recent work (6) has shown that ruffling membranes, regardless of the stimulus, can take up even higher numbers of invasive *Salmonella*, in addition to allowing the passive uptake of noninvasive *Salmonella, Yersinia*, and *Escherichia* bacteria and even of latex beads. Collectively, these data indicate that the EGF-receptor pathway, as well as growth factor-receptor pathways controlled by rac, are not directly required for *Salmonella*-induced membrane rearrangements and entry.

To our knowledge, this is the only reported instance of stimulation of membrane ruffling by a rac-independent mechanism to date. Unless invasive *Salmonella* pathogens have evolved another mechanism for activating membrane ruffling, the bacteria seem to induce their own uptake by stimulating components of the membrane-ruffling pathway downstream of rac1 and independently of growth factor signaling. A membrane protein is thought to be the primary downstream target of rac (26), and we suggest that this protein may also be the receptor for *Salmonella* species. Identification of this receptor and its signaling pathway will be essential in understanding the cellular signals generated by invasive *Salmonella* species. In addition, identification and characterization of the components of this pathway will greatly increase our understanding of fundamental cellular mechanisms and functions that lead to membrane movement and motility.

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