INFECTION AND IMMUNITY, Nov. 1994, p. 4969–4974
0019-9567/94/$04.00+0

Vol. 62, No. 11

Salmonella typhimurium Invasion of Epithelial Cells: Role of Induced Host Cell Tyrosine Protein Phosphorylation

ILAN ROSENSHINE,[1] SHARON RUSCHKOWSKI,[1] VIDA FOUBISTER,[1,2] AND B. BRETT FINLAY[1,2,3]*

Biotechnology Laboratory,[1] Department of Biochemistry and Molecular Biology,[2] and Department of Microbiology and Immunology,[3] University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

Received 11 March 1994/Returned for modification 9 May 1994/Accepted 9 August 1994

***Salmonella typhimurium* invades nonphagocytic epithelial and fibroblast cells via a process resembling phagocytosis. We have compared some phenotypes that are involved in *S. typhimurium* invasion by using different host cell lines, including HeLa, Henle-407, and A431. Infection with either wild-type *S. typhimurium*, bacterial culture supernatant, or the noninvasive *invA* mutant was associated with induction of tyrosine phosphorylation of host cell mitogenic activating protein kinase. However, we did not detect induction of tyrosine phosphorylation of the epidermal growth factor receptor in *S. typhimurium*-infected cells. Treatment with the tyrosine protein kinase inhibitor genistein did not reduce *S. typhimurium* invasion into any of these cell lines. These results suggest that *S. typhimurium* invasion is independent of host cell epidermal growth factor receptor activation.**

During infection, *Salmonella* species invade the host intestinal epithelium. This ability is thought to contribute to its pathogenicity. The process of intestinal invasion can be mimicked in vitro by infecting tissue culture cells of epithelial origin. By using this in vitro system, it was found that the host cell plays an active role in bacterial uptake. Membrane ruffling is frequently observed on the surface of infected cells at the site of bacterial entry (6, 7). Cytochalasin D, which inhibits actin polymerization, also blocks *Salmonella typhimurium* uptake (6). In addition, invading *S. typhimurium* induces extensive rearrangement of host actin filaments and other cytoskeleton-associated proteins, including alpha-actinin, tropomyosin, ezrin, and talin (6). Presumably, when *Salmonella* species bind to the host cell surface, they transduce an uptake signal in the host cell. In response, the epithelial cell rearranges its cytoskeletal elements, causing membrane ruffling and internalization of the associated bacteria.

Protein tyrosine phosphorylation appears to function as an uptake signal in several processes involved in particle internalization by eukaryotic cells. These include Fc receptor-mediated phagocytosis (reference 10 and references within) and uptake of some invasive pathogens by nonphagocytic cells (16, 17). Membrane ruffling, similar to that observed during *Salmonella* invasion, is also induced by several mitogenic factors including epidermal growth factor (EGF) (7, 15). It was recently reported that infection of Henle-407 cells with *S. typhimurium* is accompanied by induction of tyrosine phosphorylation of the host EGF receptor (EGFR) and that exogenously added EGF specifically restored the invasiveness of a noninvasive *S. typhimurium* mutant but no other adherent bacterium (9). It was further suggested that *S. typhimurium* activates the EGFR to trigger an elaborate biochemical cascade, including activation of mitogenic activating protein kinase (MAPK), which leads to Ca^{2+} influx-dependent internalization of the attached bacterium (14).

In contrast to these findings, Francis et al. found that EGF treatment appears to induce nonspecific uptake of bacteria and latex beads by epithelial cells (7). In addition, tyrosine protein kinase (TPK) inhibitors or anti-EGFR antibodies do not reduce the invasion efficiency of *S. typhimurium* (9, 17). Moreover, *S. typhimurium* also invades 3T3 cells that do not have EGFR (7, 9) and HeLa cells in Ca^{2+}-free medium (18). Bliska et al. (2) suggested that these discrepancies originated from usage of different cell lines by different researchers. According to this suggestion, *S. typhimurium* may use alternative uptake signals when invading different cell lines. For example, when invading Henle-407 cells, *S. typhimurium* activates EGFR to induce uptake, but the bacteria might use other signaling strategies with HeLa cells. Another possible explanation for these differences is that *S. typhimurium* simultaneously induces several uptake signals which are equally effective in inducing bacterial uptake.

To examine these hypotheses, we compared the abilities of invading *S. typhimurium* strains to induce host protein tyrosine phosphorylation by using several different host cells, including HeLa and Henle-407 epithelial cell lines and A431, an epidermal cell line which overexpresses EGFR (4). We also used these cell lines to compare the sensitivities of the invasion processes of the strains to the TPK inhibitor genistein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cells lines. The following *Salmonella* strains were used: *S. typhi* 205aTy (3), *S. choleraesuis* SL2428 (13), and *S. typhimurium* SL1344 (6), SR11 (8), and SB111, an *invA* mutant derived from SR11 (8). *Escherichia coli* DH5α and HB101, enteropathogenic *E. coli* E2348/69 (5), and *Yersinia enterocolitica* 8081c (22) were also used. The plasmid pVM101 (22) was used for transformation with the *Y. enterocolitica inv* gene. For infection, the bacteria were grown at 37°C without shaking to a density of about 1.8×10^8 bacteria per ml. Host cells were infected at multiplicity of infection of 100.

The epithelial cell lines Henle-407 (ATCC CCL6), HeLa (ATCC CCL2), and A431 (ATCC CRL1555) were grown and assayed at 37°C, under 5% CO_2, in minimal essential medium (MEM; for HeLa cells) or Dulbecco MEM (DMEM; for A431 and Henle-407 cells), supplemented with 10% fetal calf serum. In some experiments, the cells were serum starved by preincubating with MEM containing only 1% fetal calf serum for 18 h.

* Corresponding author. Phone: (604) 822-2210. Fax: (604) 822-9830. Electronic mail address: bfinlay@unixg.ubc.ca.

This treatment upregulated many cell surface receptors (including EGFR) but had no effect on the efficiency of *S. typhimurium* invasion (data not shown).

Invasion assay. Assays in regular or Ca^{2+}-free medium were performed as described elsewhere (18).

Protein extraction for total protein analysis. Cultured cells were seeded at a density of 2×10^6 per 100-mm-diameter petri dish. The next day, cells were infected as described in the figure legends or treated for 10 min with 500 nM EGF (UBI Inc.) or 20 min with 0.8 nM EGF (see Fig. 6). With all treatments, ruffling was confirmed by phase-contrast microscopy. The infected or EGF-treated cells were placed on ice, washed three times with 10 ml of cold phosphate-buffered saline (PBS), scraped into 1.5 ml of PBS, pelleted, and lysed in 150 μl of lysis solution (1% Triton X-100, 50 mM Tris-HCl [pH 7.6], 0.4 mM $NaVO_4$, 1 mM NaF, 0.1 mg of phenylmethylsulfonyl fluoride per ml, and 10 μg of leupeptin per ml). The lysate was centrifuged, and the supernatant (1% Triton X-100-soluble fraction) was mixed with 40 μl of 5× loading buffer. The insoluble pellet was dissolved in 80 μl of 2.5× loading buffer. The lysates were boiled for 7 min and cleared prior to resolution by gel electrophoresis.

Immunoprecipitation. To immunoprecipitate EGFR, cells were grown and infected as described previously (9) and then immunoprecipitated by one of two methods. The infected or EGF-treated cells were placed on ice, washed three times with 10 ml of cold PBS, and lysed in 1.5 ml of lysis solution (see above). The lysate was cleared, incubated with mouse anti-EGFR antibodies (ICN clone c11; 3 μg/ml) for 16 h, preabsorbed with beads coated with goat anti-rabbit antibodies (Bio-Rad Immunobeads), and absorbed with the same beads which had been precoated with rabbit anti-mouse immunoglobulin G (IgG). The immunocomplexes were washed, boiled for 7 min in 100 μl of 2× sodium dodecyl sulfate (SDS) buffer, and cleared prior to resolution by gel electrophoresis. Alternatively, the method described by Galan et al. (9) was followed precisely, including washes with Hanks balanced salt solution and lysis with the buffer described previously (9). EGF was added to a final concentration of 5 ng/ml (0.8 nM) and incubated for 20 min as described previously (9). Sepharose-protein A beads (Pharmacia) coupled to rabbit anti-mouse IgG Fc antibodies (Jackson Laboratories) were also used for immunoprecipitations. Tyrosine-phosphorylated proteins were immunoprecipited as described previously (20).

Western blotting (immunoblotting). Western blotting was carried out as described previously (16). Murine monoclonal anti-phosphotyrosine (UBI Inc.; 4G10; 0.5 μg/ml) or anti-EGFR (ICN Inc.; c11; 0.5 μg/ml) and rabbit polyclonal anti-MAPK (UBI Inc.; erk1-CT; diluted 1:500) were used. For detection, we used either anti-mouse IgG alkaline phosphatase-conjugated antibodies (Calbiochem; 0.25 μg/ml) or anti-mouse IgG peroxidase-conjugated antibodies. To detect peroxidase blots, we used the ECL (Enhanced Chemiluminescence System; Amersham) detection system and either film (Kodak X-OMAT) or a chemiluminometer (Siemens).

Detection of MAPK. MAPK detection was done as described elsewhere (20). Supernatant from *S. typhimurium* was obtained by filtering a standing overnight culture (10^8 bacteria) through a 0.2-μm-pore-size cellulose acetate filter.

RESULTS

The ability of invading *S. typhimurium* bacteria to induce tyrosine phosphorylation of host proteins was examined. The cells were infected with *S. typhimurium* at a multiplicity of infection of 100 bacteria per host cell. At increasing times after infection, the cells were lysed with a buffer containing 1% Triton X-100. Both the Triton X-100-soluble fraction (which contains the host cytosolic and membrane proteins) and the Triton-X-100-insoluble fraction (which contains the host cytoskeletal and nuclear proteins as well as the bacterial proteins) were analyzed by immunoblotting with anti-phosphotyrosine or anti-EGFR antibodies. In addition, the number of intracellular *S. typhimurium* bacteria at increasing times after infection was determined.

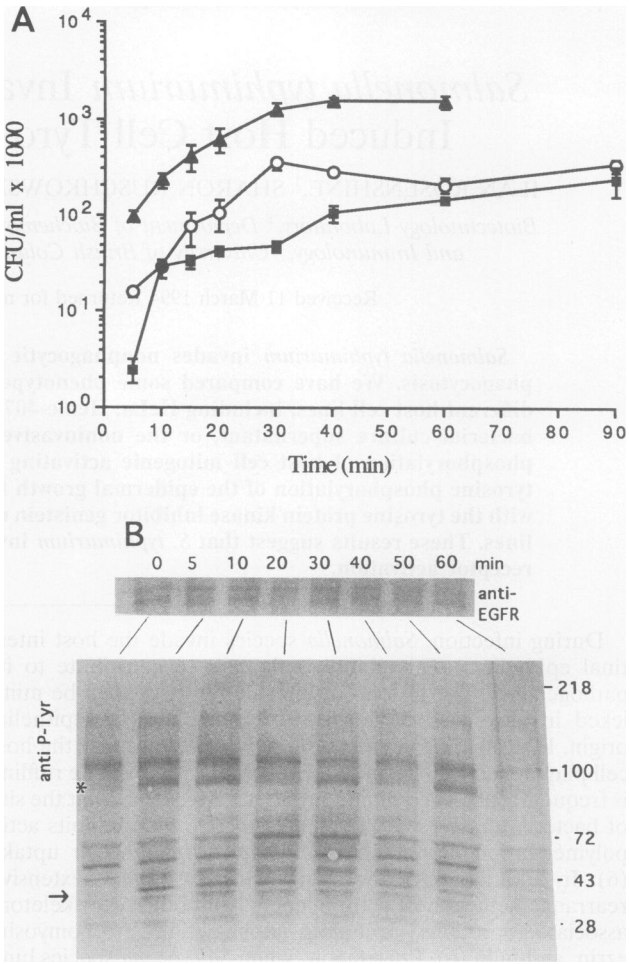

FIG. 1. (A) Kinetics of *S. typhimurium* SL1344 invasion into HeLa (○), Henle-407 (▲), and A431 (■) cells; (B) profile of tyrosine-phosphorylated proteins in the 1% Triton X-100 extract of HeLa cells infected with *S. typhimurium*. Cells were infected and incubated at 37°C, under 5% CO_2, for increasing times. Proteins were extracted and resolved by SDS-PAGE (with a 4 to 15% gradient polyacrylamide gel) and subjected to immunoblotting analysis with anti-phosphotyrosine (anti-P-Tyr) or anti-EGFR antibodies. *S. typhimurium* induced tyrosine phosphorylation of a host protein approximately 40 kDa in size (indicated by arrow). Bands showing irreproducible differences in intensity are marked with an asterisk. Molecular mass markers (in kilodaltons) are shown on the right.

S. typhimurium invaded all examined cell lines efficiently and rapidly (Fig. 1A). Induction of tyrosine phosphorylation of a host protein that migrated upon SDS–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) at approximately 40 kDa was apparent 5 min after HeLa cells were infected (Fig. 1B). Similar results were also obtained with Henle-407 and A431 cells (data not shown). We identified this tyrosine-phosphory-

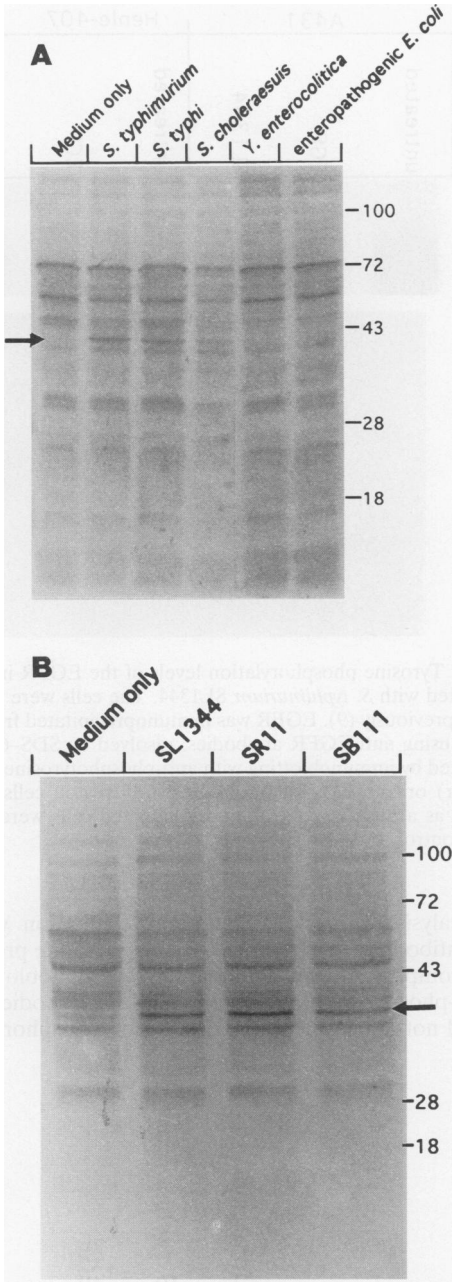

FIG. 2. Induction of tyrosine phosphorylation of MAPK (arrow) in Henle-407 cells infected with different enteropathogens (A) and *S. typhimurium* strains (B). Bacteria were grown in DMEM at 37°C, under 5% CO_2. Cells were infected with *Salmonella* strains for 10 min and with *Y. enterocolitica* and enteropathogenic *E. coli* for 30 min. Proteins were then extracted from the infected cells, resolved by SDS–12% PAGE, and analyzed by immunoblotting with anti-phosphotyrosine antibodies. Molecular mass markers (in kilodaltons) are indicated on the right of each panel.

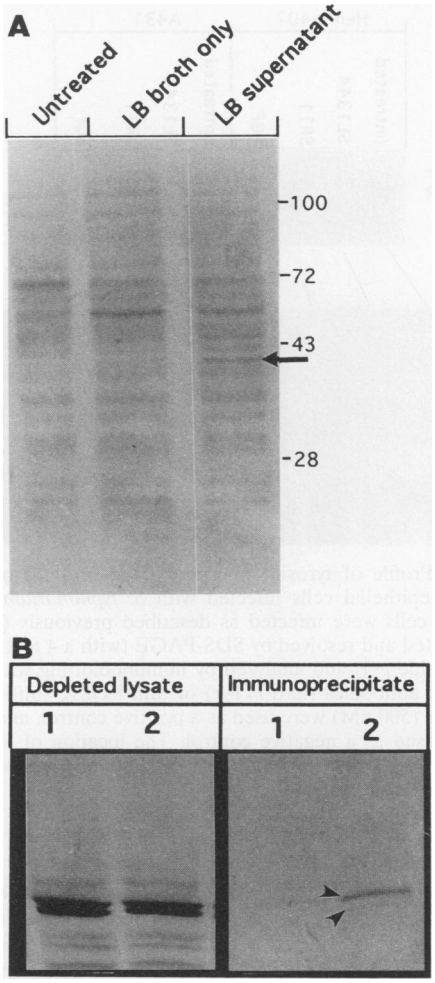

FIG. 3. Induction of tyrosine phosphorylation of p42 and p44 MAPK in Henle-407 cells treated with filtrate of *S. typhimurium* SL1344 culture (LB supernatant). Bacteria were grown in Luria-Bertani broth at 37°C for 16 h to a density of 1×10^8 to 2×10^8 bacteria per ml without shaking. The culture was cleared by centrifugation and filtered through a 0.2-μm-pore-size cellulose acetate filter. Henle-407 cells (3×10^6 cells per petri dish) were treated with 5 ml of culture supernatant or fresh Luria-Bertani broth for 10 min at 37°C. (A) The proteins were extracted from the infected cells, resolved by using SDS–12% PAGE, and analyzed by immunoblotting with anti-phosphotyrosine antibodies. Molecular mass markers (in kilodaltons) are shown on the right. (B) The tyrosine-phosphorylated proteins were immunoprecipitated. The immunoprecipitate and the depleted lysate proteins were resolved by SDS–12% PAGE and analyzed by immunoblotting with anti-MAPK antibodies (20). Proteins from cells treated with fresh medium (lanes 1) or with culture supernatant (lanes 2) are shown. The MAPK isoforms p44 and p42 are indicated by arrowheads.

lated protein as p44 MAPK as described below, confirming the reported activation of host MAPK during *S. typhimurium* invasion (14).

A 10-min infection of Henle-407 cells with *S. typhi* and *S. choleraesuis* resulted in induction of protein tyrosine phosphorylation similar to that seen with *S. typhimurium* (Fig. 2A). In contrast, induction of MAPK tyrosine phosphorylation was not evident in cells infected for up to 30 min with other pathogens, including *Y. enterocolitica* and enteropathogenic *E. coli* (Fig. 2A).

MAPK tyrosine phosphorylation was also induced in cells infected with the noninvasive *invA S. typhimurium* mutant SB111 (Fig. 2B). This indicated that the induction of tyrosine phosphorylation of MAPK is not mediated by this invasion locus. Similar induction of MAPK tyrosine phosphorylation was apparent in cells treated with filtered supernatants of *S. typhimurium* cultures (Fig. 3A). This suggests that the factor

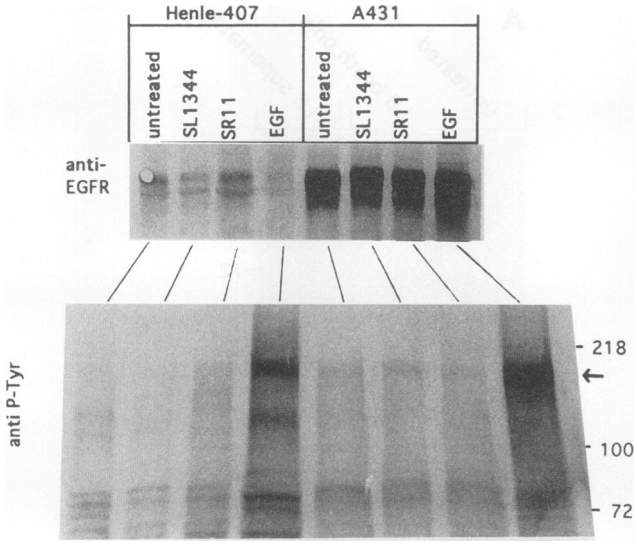

FIG. 4. Profile of tyrosine-phosphorylated proteins of A431 and Henle-407 epithelial cells infected with *S. typhimurium* SL1344 or SR11. The cells were infected as described previously (9). Proteins were extracted and resolved by SDS-PAGE (with a 4 to 15% gradient polyacrylamide gel) and analyzed by immunoblotting with anti-phosphotyrosine antibodies (anti-P-Tyr) or anti-EGFR antibodies. EGF-treated cells (500 nM) were used as a positive control, and uninfected cells were used as a negative control. The location of the EGFR is indicated by an arrow. Molecular mass markers (in kilodaltons) are shown on the right.

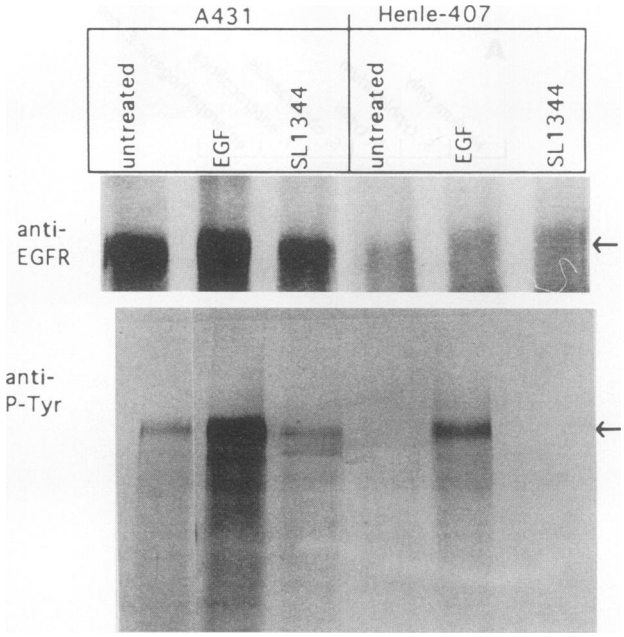

FIG. 5. Tyrosine phosphorylation levels of the EGFR in epithelial cells infected with *S. typhimurium* SL1344. The cells were infected as described previously (9). EGFR was immunoprecipitated from the cell lysates by using anti-EGFR antibodies, resolved by SDS–6% PAGE, and analyzed by immunoblotting with anti-phosphotyrosine antibodies (anti-P-Tyr) or anti-EGFR antibodies. EGF-treated cells (500 nM) were used as a positive control, and uninfected cells were used as a negative control.

involved in the induction of tyrosine phosphorylation of host MAPK is released by *S. typhimurium* into the medium.

Lysates of cells treated or untreated with filtered supernatants of *S. typhimurium* cultures were examined by immunoprecipitation with anti-phosphotyrosine antibodies followed by immunoblot analysis with anti-p44 and -p42 MAPK antibodies (20). Clear induction of tyrosine phosphorylation of p44 MAPK and weak induction of p42 MAPK were apparent in the treated cells (Fig. 3B). These results demonstrate that the approximately 40-kDa protein is MAPK. However, in both cases, only a small fraction of the total cellular p42 and p44 MAPK was phosphorylated (Fig. 3B).

Other than MAPK, we did not detect induction of tyrosine phosphorylation of any other host protein (including the 170-kDa EGFR) in either the Triton-soluble fraction (Fig. 1B) or the insoluble fraction (data not shown). Occasionally, irreproducible differences in the intensity of some bands were observed (Fig. 1B), but we did not consider these as induction. Similar amounts of EGFR in the different samples were confirmed (Fig. 1B). As a positive control, we activated the EGFR by adding EGF (0.8 to 500 nM). In this case, tyrosine phosphorylation of EGFR and other proteins was strongly induced in all cell lines tested (Fig. 4 and 5 and data not shown). As with HeLa cells, EGFR tyrosine phosphorylation was also not detected in *S. typhimurium*-infected Henle-407, MDCK, or A431 cells (Fig. 4 to 6 and data not shown).

These results are in contrast to the described induction of EGFR tyrosine phosphorylation in Henle-407 cells infected with *S. typhimurium* (9). Therefore, we attempted to reproduce the results that were obtained by Galan et al. (9). Henle-407 cells were infected as described previously (9) and extracted, and the extracts were either denatured immediately (by mixing with loading buffer containing SDS and boiling) for whole-cell lysate analysis or used for immunoprecipitation with anti-EGFR antibodies. The whole-cell extracts and the precipitated immunocomplexes were examined by immunoblot analysis with anti-phosphotyrosine and anti-EGFR antibodies.

We did not detect induction of tyrosine phosphorylation of

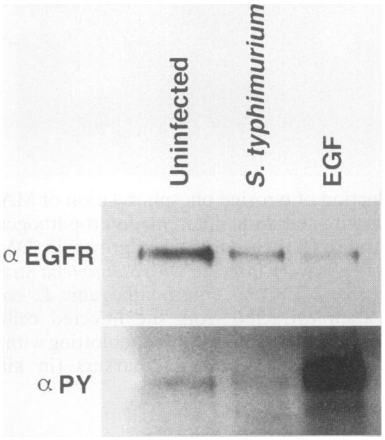

FIG. 6. Detection of EGFR tyrosine phosphorylation by treatment with 0.8 nM EGF or *S. typhimurium*. Henle-407 cells were infected as described previously (9) with *S. typhimurium* SL1344 or 0.8 nM EGF for 20 min prior to lysis, immunoprecipitation with EGFR antibodies (αEGFR), and Western blot analysis with anti-phosphotyrosine antibodies (αPY) by using a chemiluminescence detection system. After exposure, the same blot was stripped and reprobed with anti-EGFR antibodies.

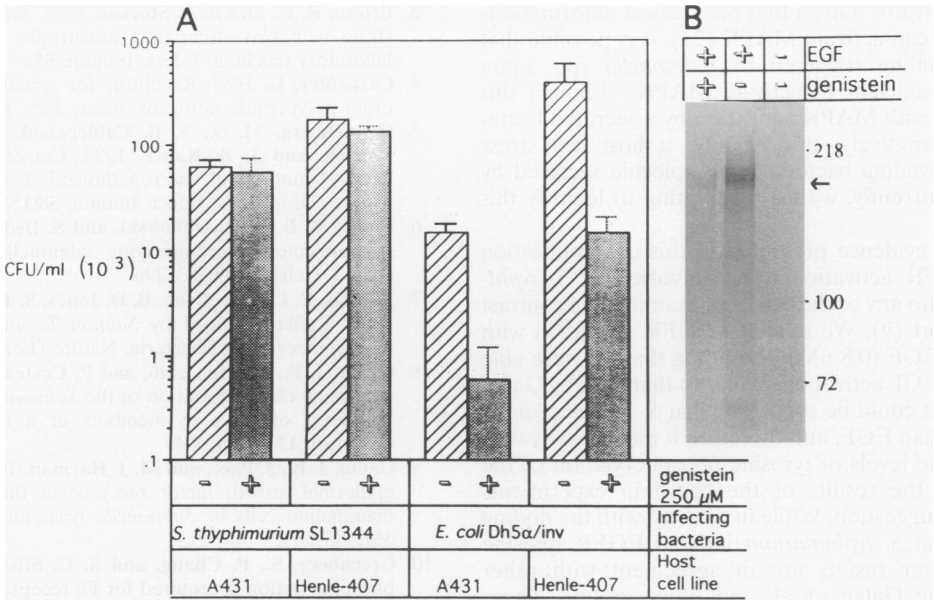

FIG. 7. (A) Bacterial uptake into Henle-407 and A431 cells treated or untreated with genistein. (B) Inhibition, by genistein, of EGFR kinase activity in A431 cells. Genistein or carrier (dimethyl sulfoxide) was added to the cells 15 min prior to infection with *S. typhimurium* SL1344 or *E. coli* DH5α/*inv* or before addition of EGF. The number of intracellular bacteria was determined, or alternatively, cell lysates were analyzed by SDS–6% PAGE and immunoblotting with anti-phosphotyrosine antibodies.

the EGFR in the Triton-soluble (Fig. 4) or -insoluble fractions (data not shown) of whole-cell lysates or in the immunoprecipitated EGFR samples from cells infected with *S. typhimurium* (Fig. 5 and 6). Similar amounts of EGFR were present in the different samples (Fig. 4 to 6). Again, EGFR was tyrosine phosphorylated in EGF-treated cells (Fig. 4 to 6). When the same experimental procedures were used, EGFR tyrosine phosphorylation was also not detected in *S. typhimurium*-infected HeLa cells (data not shown).

To ensure that this assay was sufficiently sensitive, we treated Henle-407 cells with *S. typhimurium* or 0.8 nM EGF for 20 min as described previously (9). Ruffles were seen with both *S. typhimurium*-infected and EGF-treated samples. Samples were washed, lysed, and immunoprecipitated with mouse anti-EGFR antibody with Sepharose beads conjugated to rabbit anti-mouse antibodies. Western blots were then analyzed with anti-phosphotyrosine antibodies, stripped, and redeveloped with anti-EGFR antibodies to determine the concentration of EGFR in each sample. Two detection methods were used for the Western blots. EGFR tyrosine phosphorylation was detected at this low concentration of EGF by using chemiluminescence followed by film detection (Fig. 6) or detection with a chemiluminometer (not shown). However, no EGFR phosphorylation was detected with either method in samples infected with *S. typhimurium* (Fig. 6 and data not shown) even though ruffles were seen in the samples prior to processing.

We examined the effect of the TPK competitive inhibitor genistein on the activity of EGFR kinase and on the efficiency of *S. typhimurium* invasion by using the different cell lines. We used HeLa, Henle-407, and A413 cell lines to confirm previous reports (1) that genistein inhibits EGFR tyrosine phosphorylation. Genistein, at a concentration of 250 μM, inhibited approximately 90% of the EGFR kinase activity even in A431 cells that overexpress EGFR (Fig. 7B). In contrast, genistein did not reduce the efficiency of *S. typhimurium* invasion into any of the cell lines (Fig. 7A) (17). However, genistein inhibited invasion mediated by cloned *Yersinia* invasin (Fig. 7A) (17) and the TPK-dependent uptake of enteropathogenic *E. coli* (16). These results suggested that genistein-sensitive TPK activity, including EGFR kinase, is not needed for *S. typhimurium* invasion of epithelial cells.

DISCUSSION

In this report, we examined the effect of *S. typhimurium* invasion on host cell tyrosine phosphorylation. Induction of tyrosine phosphorylation of a host protein upon infection with *S. typhimurium* was seen in all cell lines examined, including HeLa, Henle-407, and A431. By using antibodies that specifically recognized the MAPK isoforms p44 and p42 (20), we identified this protein as p44 MAPK and found that tyrosine phosphorylation of p42 MAPK is also weakly induced. This activation also occurred when supernatants from overnight cultures were added to these cells. MAPK activation also occurred in samples that were infected with a noninvasive *S. typhimurium* mutant (*invA*), although activation was not triggered by infection with *Y. enterocolitica* or enteropathogenic *E. coli*. We have previously shown that *Listeria monocytogenes* activates MAPK in cultured cells (20), and recent data demonstrated that this activation is mediated by listeriolysin O and is not mediated by bacterial invasion (19). We also showed that the detergent saponin, which punctures membranes by a mechanism similar to that of listeriolysin O, activates MAPK (19). The mechanism by which *Salmonella* spp. activate MAPK remains unclear. Since the noninvasive *invA* mutant still activated MAPK, it would suggest that this activation is independent of the *invA*-mediated invasion pathway. Moreover, it appears to be a secreted product. Given that *L. monocytogenes* secretes hemolysin, we considered whether this activation could be due to a similar mechanism in *Salmonella* spp. A cytolysin, salmolysin, is produced by *S. typhimurium* (12). We tested isogenic mutants in this cytolysin and found that noncytolytic mutants of *S. typhimurium* and their isogenic parental strains both activated MAPK, indicating that this molecule is

not the MAPK activator. Given that mechanical deformations in eukaryotic cells can activate MAPK (21), it is possible that the membrane ruffling triggered by *Salmonella* spp. upon invasion may be sufficient to activate MAPK, although this does not correlate with MAPK activation by a secreted bacterial product. Alternatively, this may be a host cell stress response to the invading bacteria or a molecule secreted by *Salmonella* spp. Currently, we are attempting to identify this factor.

Several lines of evidence presented in this communication indicate that EGFR activation is not involved in *S. typhimurium* invasion into any of the cell lines examined, in contrast to a previous report (9). We detected EGFR activation with small amounts of EGF (0.8 nM), indicating that we were able to detect levels of EGF activation similar to that used by Galan et al. (1 nM) (9). It could be suggested that *S. typhimurium* is much less potent than EGF, and therefore it induces only very low or undetectable levels of tyrosine phosphorylation of the EGFR. However, the results of the genistein experiments argue against this suggestion. While in contrast with the finding of Galan et al. that *S. typhimurium* induces EGFR tyrosine phosphorylation, our results are in agreement with other results obtained by Galan et al. and other groups. These include the inability to block *S. typhimurium* invasion with anti-EGFR antibodies (9), the efficient *S. typhimurium* invasion into Henle-407 cells that were stripped of their EGFR (11), and the efficient *S. typhimurium* invasion into 3T3 fibroblast cells that do not possess the EGFR (7).

Although the signals that are utilized by *S. typhimurium* to invade cultured cells are still under investigation, a general picture is emerging. It appears that the mechanisms used by this organism are conserved throughout various cell types. Upon bacterial addition, host membrane ruffling is rapidly triggered, and transient cytoskeletal rearrangement occurs. MAPK is activated, although this may not be linked directly to invasion, and the EGF receptor does not appear to become tyrosine phosphorylated. The uptake signals are insensitive to genistein and staurosporin (two potent kinase inhibitors), and bacterial invasion is insensitive to depletion of extracellular calcium, although intracellular calcium may be needed for the process. The only detectable differences between cell lines relate to the invasion kinetics and efficiency of bacterial uptake into different cells.

ACKNOWLEDGMENTS

We thank J. Galan, S. Libby, and V. Miller for bacterial strains and plasmids.

V.F. was supported by a Natural Sciences and Engineering Research Council of Canada studentship, and I.R. was supported by a Canadian Gastroenterology Fellowship. This work was supported by operating grants to B.B.F. from the Medical Research Council of Canada and the Howard Hughes International Research Scholar Program.

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