Involvement of c-Src Tyrosine Kinase Upstream of Class I Phosphatidylinositol (PI) 3-Kinases in Salmonella Enteritidis Rck Protein-mediated Invasion

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The Salmonella outer membrane protein Rck mediates a Zipper entry mechanism controlled by tyrosine phosphorylation and class I phosphatidylinositol 3-kinase (PI 3-kinase). However, the underlying mechanism leading to this signaling cascade remains unclear. The present study showed that using Rck-coated beads or Rck-overexpressing Escherichia coli, Rck-mediated actin polymerization and invasion were blocked by PP2, a Src family tyrosine kinase inhibitor. In addition, phosphorylation of Src family kinases significantly increased after stimulation with Rck. The specific contribution of c-Src, one member of the Src family kinases, was demonstrated using c-Src-deficient fibroblasts or c-Src siRNA transfected HeLa cells. We also observed that Rck-mediated internalization led to the formation of a complex between c-Src and at least one tyrosine-phosphorylated protein. Furthermore, our results revealed that the c-Src signal molecule was upstream of PI 3-kinase during the Rck-mediated signaling pathway as Rck-mediated PI 3-kinase activation was blocked by PP2, and PI 3-kinase inhibitor had no effect on the Src phosphorylation. These results demonstrate the involvement of c-Src upstream of the PI 3-kinase in the Zipper entry process mediated by Rck.

Salmonella enterica are members of a Gram-negative enteropathogenic bacteria family that causes a wide range of food- and water-borne diseases, from gastroenteritis to typhoid fever (1). The mode of host contamination by Salmonella occurs mainly by the oral route. Ingested bacteria are able to cross the intestinal epithelium in different ways. They can be captured by dendritic cells and translocated through M cells, and they can also trigger their own internalization into enterocytes, allowing the bacteria to disseminate to extraintestinal sites and to colonize the liver and spleen. Salmonella have developed two morphologically distinct mechanisms to invade nonphagocytic host cells characterized by different types of membrane rearrangements: the “Trigger” mechanism and the “Zipper” mechanism (1, 2).

The Salmonella internalization process that has been studied the most is the Trigger mechanism, characterized by the appearance of large membrane ruffles at the bacterial entry site. This mechanism is induced by a series of bacterial effector proteins, which are injected into host cells by a type three secretion system (T3SS-1) encoded by the Salmonella pathogenicity island 1 (SPI-1) (3). The second mechanism involves an invasin Rck, which induces a Zipper mode of entry (4). Rck is an outer membrane protein that is necessary and sufficient to induce the entry of coated beads (4) and noninvasive Escherichia coli strains (5). Rck induces the activation of host cell signal transduction pathways, leading to the reorganization of the host actin cytoskeleton, the formation of a weak membrane rearrangement at the site of bacteria-cell contact, and the entry of the bacteria into the host cell (4). The study of Rck-mediated entry has recently enabled a model of the signaling cascade to be constructed, where interaction of Rck with its unknown cell receptor induces (p85-p110) PI 3-kinase activation through tyrosine phosphorylation of one or more unknown proteins (6). Until now, the mechanism induced by Rck, leading to tyrosine phosphorylation and class I PI 3-kinase activation, has not been characterized.

c-Src is a member of a large family of cytoplasmic protein-tyrosine kinases that includes c-Fyn, c-Yes, Lck, Blk, Lyn, Hck, Yrk, and Fgr (7). Most of the Src family kinases are restricted in their expression, often in cells of the neural and hematopoietic lineage, whereas some, especially c-Src, c-Yes and, c-Fyn, are more broadly expressed. All these proteins share a characteristic topology, including a myristoylation sequence at the amino terminus (8).
terminus required for their association with membranes. A catalytic domain and regulatory sequences are localized at the carboxyl terminal tail followed by Src homology (SH) domain 3 and 2. The catalytic activity of the Src family tyrosine kinases is regulated by selective tyrosine phosphorylation allowing a switch between an inactive “closed” conformation and a catalytically active “open” configuration. When there is intramolecular binding of the SH2 domain to the phosphorylated Tyr-527, Src family kinases are inactive. Dephosphorylation of this site releases the SH2 and SH3 from intramolecular interactions, which is accompanied by phosphorylation of Tyr-416, resulting in activation of its intrinsic kinase activity. This conformational change allows the SH2 and SH3 domains to bind to heterologous molecular partners and enables the tyrosine phosphorylation of kinase domain substrates (7). Activation of the Src family tyrosine kinases plays key roles in cellular physiological processes, and it is also hijacked by different pathogens to trigger their internalization. For example, during Shigella flexneri internalization, the translocon component IpaC, through its interaction with the soluble fraction on glutathione-Sepharose 4B beads, as described previously (4, 6). c-Src is also required for cell invasion initiated by Yersinia pseudotuberculosis or InlA from Listeria monocytogenes (9–11).

In this study, we investigated the role of c-Src in the Salmonella Rck-mediated signaling pathway, leading to bacterial internalization. We demonstrated that c-Src is required in this Zipper entry process at a stage prior to the activation of the PI 3-kinase-Akt-Rac1 signaling cascade.

EXPERIMENTAL PROCEDURES

Cell Lines—Human epithelial placental cells (Jeg-3, ATCC number: HTB-36) were grown in minimum Eagle’s medium containing GlutaMAX (Invitrogen) and supplemented with 1 mM nonessential amino acids, 1 mM sodium pyruvate, 10% inactivated fetal bovine serum, and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml) (Invitrogen). African green monkey kidney epithelial cells (MA104, Health Protection Agency Culture Collections (HPACC): 85102918) were cultured in Dulbecco’s modified eagle medium (DMEM), 25 mM glucose supplemented with 10% inactivated fetal bovine serum, 2 mM L-glutamine, and antibiotics. Fibroblasts derived from c-Src, c-Yes, c-Fyn triple knock-out mouse embryos (SYF cells) (12) and SYF+c-Src were kindly provided by Dr. C. Hauck (Zentrum für Infektionsforschung, Würzburg, Germany) and cultured in DMEM supplemented with 1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% inactivated fetal bovine serum on gelatin-coated (0.15% in phosphate-buffered saline (PBS)) cell dishes.

Reagents—All inhibitors were dissolved in dimethyl sulfoxide (DMSO) at the following stock concentration: wortmannin 10 mM (Cell Signaling); PP2 20 mM (Calbiochem). The maximum final concentration of DMSO never exceeded 0.1% (v/v) in drug-treated cells.

Bacterial Strains and Growth Conditions—The bacterial strains used in this study are listed in Table 1. Bacteria were routinely grown in Luria-Bertani (LB) broth with antibiotic selection at the following concentrations: 34 μg/ml chloramphenicol and 100 μg/ml carbenicillin, with shaking at 150 rpm at 37 °C overnight.

Purification of Fusion Proteins and Coating of Latex Beads—GST and GST-113–150 Rck proteins were induced in subcultures of E. coli BL21 pLysS expressing 113–159 in pGEX-4T-2 with 1 mM isopropyl-1-thio-β-d-galactopyranoside for 4 h at 37 °C. GST and GST-113–159 Rck were purified as described previously (4, 6). Bacteria were resuspended in lysis buffer (50 mM Tris pH 8, 40 mM EDTA, 25% sucrose, 100 mM MgCl₂, 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and cComplete protease inhibitor mixture (Roche Applied Science)) and sonicated. After clearing, fusion proteins were affinity-purified from the soluble fraction on glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer’s instructions. Purified GST alone or GST-113–159 Rck proteins were coated on latex beads (Sigma), as described previously (4, 6).

Adherence and Invasion Assay—Cell monolayers were grown in 24-well tissue culture plates (Falcon) for 3–4 days to obtain a confluent monolayer. Before infection, cells were incubated overnight in antibiotic-free medium. They were infected for 1 h at 37 °C at a bacterium-cell ratio of 5:1. For adherence assays, after bacteria-cell contact, cells were gently washed at least four times with PBS (pH 7.5) and then disrupted with 1 ml of cold distilled water. Viable bacteria (intra- and extracellular) were counted after plating serial dilutions on tryptic soy agar. Invasion was quantified by the gentamicin protection assay to kill any remaining extracellular bacteria, as described previously (4, 6). After a 90-min incubation with 100 μg/ml gentamicin (Invitrogen), cells were washed and then lysed by adding 1 ml of cold distilled water. The number of viable bacteria released from the cells was counted as for the adhesion assays.

Immunoprecipitation—Cells were grown in 100-mm dishes for 3–4 days to obtain a confluent monolayer. Cells were incubated overnight in antibiotics and serum-free medium and then with GST- or GST-113–159 Rck-coated beads as described above. In some experiments, cells were pretreated with DMSO or PP2 for 30 min prior to the addition of GST-113–159 Rck-coated beads. After a 10-min incubation, cells were rinsed with cold PBS and solubilized by the addition of 500 μl of ice-cold lysis buffer (1% Nonidet P-40, 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, and protease inhibitor mix-

### Table 1

| Strains         | Relevant characteristic(s)                           | Reference |
|-----------------|------------------------------------------------------|-----------|
| E. coli-GST     | An E. coli strain, which overexpressed GST           | 4         |
| E. coli-rck     | An E. coli strain, which overexpressed GST linked to rck sequence | 4         |
| E. coli-113–159 Rck | An E. coli strain, which overexpressed GST linked to 113–159 rck sequence | 4         |
Rck-mediated Entry Pathway

Immunoprecipitation was performed using anti-phosphotyrosine (4G10; Millipore) as described previously (6). In brief, after preclearance, protein concentrations of the lysates were determined, and equal quantities of total protein were used for immunoprecipitation. After 1 h of incubation at room temperature and washing steps, immunoprecipitates were denatured by boiling in sample buffer for 5 min and then stored at −20 °C. Proteins present in immunoprecipitates and lysates were analyzed by Western blotting using a rabbit polyclonal antibody against PI 3-kinase p85 (1:5000; Upstate Biotech Millipore) or a mouse monoclonal antibody against c-Src (clone GD11; 1:1000; Millipore), as described previously (6).

Western Blotting Analysis of Src Family Kinases—Jeg-3 cells were grown in 24-well plates for 3–4 days to obtain a confluent monolayer. Cells were incubated overnight in serum- and antibiotic-free medium and then incubated for 10 min with GST-coated or GST-113–159 Rck-coated beads. Cells were washed with ice-cold PBS and then lysed with 100 µl of SDS-PAGE sample buffer. Proteins were analyzed by SDS-PAGE and Western blotting using antibodies against tubulin (1:1000; Sigma) or phospho-Src family Tyr-416 (1:1000; Cell Signaling). The expression of c-Src was examined in SYF and SYF+Src cell extracts by Western blot analysis using anti-c-Src (clone GD11; diluted 1:1000) antibodies from Millipore.

Immunofluorescence Microscopy—MA104 cell monolayers on coverslips were incubated overnight in DMEM medium without antibiotics before the addition of beads (about 150 beads per cell). Cells were pretreated with DMSO or PP2 for 30 min prior to adding GST-coated or GST-113–159 Rck-coated beads. Cells were washed with ice-cold PBS and then fixed for 10 min in 4% paraformaldehyde solution. The cells were permeabilized for 5 min in 0.2% Triton X-100 in PBS. GST was stained with a polyclonal antibody (Sigma; diluted 1:100), and actin was stained with rhodamine-phalloidin (Sigma; diluted 1:100). Alexa Fluor 488-labeled goat anti-rabbit (Molecular Probes; diluted 1:200) was the secondary antibody used. Coverslips were finally mounted in fluorescence mounting medium (Dako) and analyzed with an Olympus Fluoview 500 confocal laser-scanning microscope (Olympus).

Small Inhibitory RNA (siRNA)—c-Src and control siRNA were purchased from Dharmacon. Jeg-3 cells plated on 24-well plates were transfected with 100 nm siRNA according to the manufacturer's instructions. Adhesion and invasion assays were performed 48 h after transfection. At the same time, siRNA was evaluated by probing extracts of the transfected cells with monoclonal antibodies against c-Src (clone GD11; Millipore; 1:1000) and against α-tubulin (Sigma; 1:1000) for loading control.

RESULTS

Blocking Src Family Tyrosine Kinases Reduces Rck-mediated Internalization—The Rck protein of Salmonella Enteritidis, expressed in E. coli BL21 (E. coli-rck), promotes adhesion to and invasion of fibroblasts and epithelial cells (4, 6). Previous studies revealed that the Rck-dependent entry process triggers tyrosine phosphorylation of cell proteins (6). It was therefore of interest to determine the role of tyrosine kinases in Salmonella Rck-mediated internalization of MA104 epithelial cells. Invasion and adhesion assays were performed in the presence of different concentrations of PP2, a selective Src family tyrosine kinase inhibitor (13, 14). Fig. 1B shows that inhibition of Src family tyrosine kinases by PP2 reduced dramatically and in a dose-dependent manner the Rck-mediated invasion. No change in the number of cell-associated bacteria was observed (Fig. 1A). These results suggested that the Src family tyrosine kinases are required for Rck-mediated internalization.

Src Family Tyrosine Kinases Are Involved in Rck-mediated Actin Polymerization—Rck-triggered internalization is preceded by formation of an actin-rich accumulation (2). To evaluate the role of Src family tyrosine kinases in actin polymerization induced during this process, MA104 cells were pretreated with PP2 and incubated with beads coated with the 113 to 159 peptide of Salmonella enteritidis Rck fused to glutathione S-transferase (GST-113–159 Rck). This peptide has been shown to be necessary and sufficient to promote adhesion, actin polymerization, and internalization (4). The actin cytoskeleton was stained with rhodamine-phalloidin. Stacks of confocal

FIGURE 1. Src family kinase is required for Rck-mediated internalization. MA104 cells were incubated with PP2 at the concentrations indicated above for 30 min prior to the addition of E. coli-rck (multiplicity of infection 1:5). After 1 h at 37 °C, the total cell-associated bacteria (A) and intracellular bacteria (B) were scored as described under “Experimental Procedures.” Each value represents the mean ± S.D. of at least three independent experiments, with two infected wells evaluated per experiment.
Src Family Tyrosine Kinases Are Activated during Rck-mediated Entry—Tyr-416 is a crucial step in reaching maximal Src family tyrosine kinase activity (7). To determine whether Rck-mediated entry was able to activate Src family kinases, Jeg-3 cells were incubated with GST-113–159 Rck-coated beads. Latex beads coated with GST alone were used as a control. After incubation, cells were lysed. Two antibodies were used, one recognizing Src family kinases when phosphorylated on Tyr-416 and the other recognizing α-tubulin as a control for protein loading. As shown in Fig. 3A, Tyr-416 phosphorylation of Src family tyrosine kinase was increased following stimulation with GST-113–159 Rck-coated beads when compared with GST-coated bead stimulation, whereas the Rck-induced Src phosphorylation was abolished by PP2 (Fig. 3B). These results indicate that Src family tyrosine kinases are activated during Rck-mediated invasion.

c-Src Kinase Plays a Key Role in the Rck-dependent Invasion Process—As the Src family tyrosine kinases include nine known members, we investigated the role of c-Src, one of these members, which has already been identified as being involved in different bacterial entry processes. To investigate the role of the c-Src tyrosine kinase, fibroblasts (SYF cells) derived from c-src−/− c-yes−/− c-fyn−/− deficient mouse embryos were used (12). SYF cells lack these three members of the Src family kinases (c-Src, c-Fyn, and c-Yes), which are widely expressed in mammalian cells (15). SYF cells expressing the c-Src protein (SYF+c-Src) were used as a control. First, the expression of c-Src in SYF and SYF+c-Src cells was verified with Western blotting using a specific antibody against c-Src (Fig. 4A). This figure confirms that SYF cells are c-Src-depleted and that c-Src is overexpressed in SYF+c-Src cells. We observed that Rck-mediated invasion was significantly higher in SYF cells expressing the c-Src protein than in SYF cells (Fig. 4C). The number of internalized bacteria increased 6-fold in SYF cells expressing c-Src protein, whereas no change was observed in the total number of cell-associated bacteria (Fig. 4B). These data demonstrate that c-Src is involved in Rck-mediated internalization.

To corroborate this finding, we used siRNA to inhibit c-Src tyrosine kinase and evaluate the entry level of E. coli–rck. siRNA was expressed in Jeg-3 cells to individually knock down the c-Src protein. Fig. 5A shows that c-Src siRNA inhibited the c-Src expression level in transfected cells obtained by Western blotting analysis using a specific antibody against c-Src, whereas there was no inhibition in control cells transfected with control siRNA. The entry of E. coli–rck was reduced by 85% in cells transfected with c-Src siRNA, whereas the number of adherent bacteria on c-Src siRNA cells was equivalent to

FIGURE 2. Src family kinases are necessary during Rck-mediated actin polymerization, leading to internalization. A–C, recruitment of actin during entry of GST-113–159 Rck-coated beads in MA104 cells. Actin (in red) is localized at the entry site of the beads (GST in green). D–F, effect of PP2 on actin polymerization required during internalization mediated by GST-113–159 Rck-coated beads. MA104 cells were pretreated with PP2 for 30 min prior to the addition of GST-113–159 Rck-coated beads. Confocal laser scanning microscopy shows horizontal (A and B and D and E; bar = 20 μm) and vertical (C and F; bar = 2 μm) sections of cells.

FIGURE 3. Src family kinases activity is correlated to Rck-mediated internalization. A, serum-starved MA104 cells were incubated with beads coated with GST-113–159 Rck or GST alone for 10 min at 37 °C. Cells were then lysed, and phosphorylated and total c-Src was detected using a polyclonal anti-phospho-Src family Tyr-416 and a mouse monoclonal anti-c-Src antibody respectively. B, serum-starved MA104 cells were pretreated with either DMSO or 10 nM PP2 and incubated with beads coated with GST-113–159 Rck or GST alone for 10 min at 37 °C. After cell lysis, anti-phospho-Src family Tyr-416 and total c-Src were detected, A and B, the figures show an immunoblotting analysis from one experiment, representative of three.
Overall, these results confirm that c-Src is necessary for Rck-mediated internalization. As c-Src is activated via an interaction of its SH2 domain with phosphotyrosine (16, 17), we also investigated whether this was the case during Rck-mediated internalization. To this end, we conducted co-immunoprecipitation with antibodies to phosphotyrosine. Incubation of epithelial cells with GST-113–159 Rck-coated beads increased the amount of c-Src co-immunoprecipitated with at least one tyrosine-phosphorylated protein (Fig. 6).

**FIGURE 4. c-Src kinase is necessary for Rck-mediated internalization.** A, SYF and SYF + c-Src cells were lysed and analyzed by Western blotting for c-Src expression using specific c-Src antibodies. B and C, SYF and SYF + c-Src cells were infected with E. coli-GST and E. coli-rck (multiplicity of infection 1:50) for 1 h at 37 °C. The percentage of total cell-associated bacteria (B) and internalized bacteria (C) was determined as described under “Experimental Procedures.” Each value represents the mean ± S.D. of at least three independent experiments, with two infected wells evaluated per experiment. Control cells (Fig. 5B). Overall, these results confirm that c-Src is necessary for Rck-mediated internalization.

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**FIGURE 5. c-Src kinase RNAi inhibit Rck-mediated internalization.** A, c-Src was knocked down in Jeg-3 cells, and protein levels were verified by Western blotting. CTL, control siRNA. B, adhesion and invasion assays in c-Src knockdown Jeg-3 cells were performed with E. coli-rck (multiplicity of infection 1:5) for 1 h at 37 °C. The numbers of adherent (white bars) and internalized bacteria (black bars) were determined and expressed relative to values obtained for cells treated with control RNAi arbitrarily set at 100%. Data are the mean ± S.D. of at least three independent experiments, with two infected wells evaluated by experiment.

**FIGURE 6. Rck-mediated internalization requires phosphorylation of tyrosines that interact with c-Src.** MA104 cell monolayers were incubated overnight in DMEM medium without serum and antibiotics. MA104 cells were incubated with beads coated with GST-113–159 Rck. Cells were then lysed, tyrosine-phosphorylated proteins were immunoprecipitated (IP) with anti-Tyr(P) antibodies, and c-Src kinase was detected by immunoblotting. The picture corresponds to a protein immunoblot from one experiment, representative of three.
membrane protein Rck induces internalization in a receptor-mediated mechanism, referred to as Zipper (1, 2). The main mechanistic difference between the Trigger and Zipper modes of entry is that the former is triggered from “inside” via the action of bacterial effectors delivered by secretion systems, whereas the latter is promoted from “outside” through activation of host cell receptors. The initial interaction between the bacterial protein and its cell receptor induces a cascade of signals, including protein phosphorylation and/or recruitment of adaptors and effectors. These signals induced by Rck involve the PI 3-kinase, Akt, and Rho GT-Pases and lead to actin polymerization and membrane extension that culminate in phagocytic cup formation and bacterial internalization (4, 6).

In this study, we demonstrated the essential role of c-Src in Rck-mediated internalization. Our results show that the specific Src family kinase inhibitor, PP2, blocked Rck-mediated invasion. Further results show that Src family kinase activation, which is mainly regulated by Tyr-416 phosphorylation (19), increased significantly in epithelial cells stimulated with Rck, and this increased phosphorylation was abolished by PP2, used at a concentration shown to inhibit Salmonella Rck-mediated invasion effectively. This result demonstrates that at least one member of the Src tyrosine kinase family is activated during the Rck signaling cascade. c-Src is a member of a large family of cytoplasmic protein-tyrosine kinases that includes c-Fyn, c-Yes, Lck, Blk, Lyn, Hck, Yrk, and Fgr (7). We show here that c-Src is necessary for the Rck-mediated internalization by demonstrating that the invasion level was lower in transfected cells with specific siRNA targeting c-Src than in transfected cells with control siRNA. Moreover, the invasion level increased in c-Src-deficient fibroblasts expressing the c-Src protein when compared with the c-Src-deficient fibroblasts. However, the mechanism by which c-Src is activated during the Rck-mediated signaling cascade remains unknown, as well as the role of the other members of the Src family kinases.

We have previously demonstrated that PI 3-kinase signaling is required during Rck-mediated internalization (6). Direct interaction of the p85 subunit of PI 3-kinase with the SH2 and SH3 domains of c-Src has also been demonstrated (20). In this study, our results show that the amount of c-Src/PI 3-kinase complex increased in epithelial cells stimulated with Rck. We also found that the pretreatment of epithelial cells with wortmannin, a PI 3-kinase-specific inhibitor, had no effect on the Rck-induced c-Src activation, whereas the pretreatment of cells with Src-specific inhibitor abolished the activation of PI 3-kinase stimulated by Rck. These results indicate that c-Src is an upstream signaling molecule of PI 3-kinase during the Rck-mediated invasion. However, how c-Src is activated during the entry process mediated by Rck remains to be determined.

c-Src could be activated downstream of integrin engagement during bacterial internalization as described previously for other pathogens. For Yersinia, c-Src activation is the result of the upstream involvement of β1-integrins (21), whereas Neisseria meningitidis interacts with α5β1-integrins on the host cell surface to activate c-Src and induce cell invasion (22). The need for β1-integrins during Rck-mediated internalization was thus investigated. However, no decrease in the level of Rck-mediated invasion was observed when specific antibodies were used to

**DISCUSSION**

*Salmonella* has developed different mechanisms to induce its internalization into host cells via a remodeling of the cellular cytoskeleton. The main entry mechanism of *Salmonella*, referred to as Trigger, requires the T3SS-1, whereas the outer
block β1-integrins or when β1-integrin knock-out cells were used. These data strongly suggest that β1-integrins are not the cell receptor of Rck (data not shown). However, other integrins could be involved in Salmonella entry, leading to tyrosine phosphorylation, which is required for Rck-mediated entry. An alternative hypothesis is the direct or indirect role of a tyrosine-kinase receptor. For example, the InlB protein of *L. monocytogenes* may bind to a protein that can itself act as a bridge between the bacterium and a transmembrane receptor, which mediates the entry process.

Activation of c-Src during bacterial internalization is a crucial step during other Zipper-like entry processes, as for the InlA-dependent uptake of *L. monocytogenes* and for *Yersinia* sp. (11, 21). However, c-Src activation does not indicate a Zipper entry process because it is not involved in the InlB-dependent uptake of *L. monocytogenes* and has been described in some Trigger entry processes. c-Src activation does not seem to be involved in the Trigger invasion mechanisms induced by *Salmonella*. Indeed, the pretreatment of cells with a Src family tyrosine kinase inhibitor or the use of knock-out c-Src-Fyn-Yes cells did not affect the T3SS-1 internalization process (24). However, the Trigger invasion mechanisms induced by *S. flexneri* do involve c-Src kinase. The translocon component IpaC, through its carboxyl terminus, participates in early signaling events by allowing the recruitment and activation of the c-Src tyrosine kinase at the bacterial entry site (8, 25).

In conclusion, our results have important implications for understanding the molecular mechanisms of the cell processes driving the Zipper entry of *Salmonella*. Indeed, new evidence has shown that *Salmonella* is able to use different pathways to enter phagocytic and nonphagocytic cells. As c-Src, like other cellular components such as Rho GTPase, is involved in both the Trigger and the Zipper entry processes, a challenging task will be to determine why the Trigger mechanism is related to the important membrane ruffling process, whereas the Zipper mechanism only induces weak membrane rearrangements.

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