

# Interferon $\alpha$ Inhibits a Src-mediated Pathway Necessary for *Shigella*-induced Cytoskeletal Rearrangements in Epithelial Cells

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**Abstract.** *Shigella flexneri*, the causative agent of bacillary dysentery, has the ability to enter nonphagocytic cells. The interferon (IFN) family of cytokines was found to inhibit *Shigella* invasion of cultured epithelial cells. We show here that IFN- $\alpha$  inhibits a Src-dependent signaling cascade triggered by *Shigella* that leads to the reorganization of the host cell cytoskeleton. Immunofluorescence studies showed that IFN- $\alpha$  inhibits *Shigella*-induced actin polymerization required for bacterial entry into cells. Phosphorylation of cortactin, a Src-substrate specifically tyrosyl-phosphorylated during *Shigella* entry, was inhibited by IFN- $\alpha$ . Overexpression

of a dominant interfering form of pp60c-src led to inhibition of *Shigella*-induced cytoskeletal rearrangements and decreased cortactin phosphorylation indicating a role for Src in *Shigella* entry. Also, *Shigella* uptake in cells that expressed constitutively active Src was unaffected by IFN- $\alpha$  treatment. We conclude that Src kinase activity is necessary for *Shigella* invasion of epithelial cells and that IFN- $\alpha$  inhibits this Src-dependent signaling pathway.

**Key words:** *Shigella* • interferon • actin • cytoskeleton • Src

**S**HIGELLA *flexneri*, a gram negative bacterium, causes bacillary dysentery in humans by invading the colonic epithelium and eliciting an intense inflammatory reaction that leads to the destruction of the epithelium. During the course of infection, several cytokines are produced in the colon and rectum, including IL1, IL8, tumor necrosis (TNF)<sup>1</sup>  $\alpha$ , and interferon (IFN)  $\gamma$  (Raqib et al., 1995b). Among these, IFN- $\gamma$  is of particular interest because it was shown to have a protective effect in mice challenged with enteropathogens. For example, mice deficient for IFN- $\gamma$  or for IFN- $\gamma$  receptor exhibit an increased susceptibility to *Listeria monocytogenes*, as well as *Shigella*, whereas administration of recombinant IFN- $\gamma$  improves mice resistance against these pathogens (Buchmeier and Schreiber, 1985; Huang et al., 1993; Way et al., 1998). Orally administered IFN- $\gamma$  was also shown to have a pro-

TECTIVE role in mice infected with *Salmonella* (Degré and Bukholm, 1995). In most instances, IFN is thought to exert its bactericidal effects by activating the immune system, in particular by stimulating the intracellular killing properties of macrophages. Interestingly, it was shown that  $\alpha$  and  $\gamma$  IFNs could also inhibit *Shigella* invasion of epithelial cells (Niesel et al., 1986). Although the specific step(s) of *Shigella* infection impaired by IFNs (i.e., cell entry, intracellular multiplication, cell to cell spread) have not been characterized, this inhibitory effect of IFNs on specific invasive properties of *Shigella*, crucial for its virulence, could affect the establishment of infection (Sansonetti, 1993). In healthy individuals, the colonic epithelial layer expresses high levels of the IFN- $\gamma$  receptor and the onset of the disease correlates with its downregulation suggesting that ligand and receptor interacted with each other during disease (Raqib et al., 1995a).

After inducing its internalization by host cells, *Shigella* escapes from the phagocytic vacuole and multiplies within the cytosol. *Shigella* can then spread from cell-to-cell using actin-based motility (Bernardini et al., 1989). *Shigella* does not establish intimate contact with the cell surface, but it induces massive cytoskeletal rearrangements at the site of entry. The *Shigella* Ipa invasins are essential for the entry process (Sasakawa et al., 1988; Ménard et al., 1993; Tran

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1. *Abbreviations used in this paper:* DAPI, 4,6-diamidino-2-phenylindole; FAK, focal adhesion kinase; IFN, interferon; mAb, monoclonal antibody; MAP kinase, mitogen activated kinase; TNF, tumor necrosis.

Van Nhieu et al., 1997). They are released by the Mxi-spa type III secretion system upon contact between *Shigella* and the cell surface (Allaoui et al., 1992). During entry, *Shigella* triggers the formation of protrusions that are sustained by long actin filaments in the area proximal to its site of interaction with the cell membrane to form an "entry structure" (Adam et al., 1995). Actin polymerization is likely to provide the force responsible for the formation of cellular protrusions that engulf the bacteria in a macropinocytotic-like process. In this entry structure, actin-associated proteins such as plastin,  $\alpha$ -actinin, and cortactin, as well as focal adhesion components such as vinculin and talin, accumulate (Jockush et al., 1995; Nobes and Hall, 1995; Tran Van Nhieu et al., 1997).

The signaling pathways involved in *Shigella* entry are still poorly understood, but small GTPases as well as tyrosine kinases have been shown to be involved in this process. The use of the Rho inhibitor C3 demonstrated that *Shigella*-induced actin rearrangements are dependent upon Rho (Adam et al., 1996). Also, during entry, *Shigella* triggers a tyrosine kinase cascade leading to massive phosphorylation of cortactin (Dehio et al., 1995). Since cortactin is a substrate of the tyrosine kinase pp60c-src, activation of the kinase Src during the *Shigella* entry process was suggested (Wu et al., 1991; Wong et al., 1992). Interestingly, both Rho and pp60c-src have been implicated in the formation of focal adhesions (Jockush et al., 1995). This, together with the recruitment of focal components such as vinculin and talin at the site of *Shigella* entry, argues for the notion that *Shigella* utilizes cellular processes involved in focal adhesion formation during cell entry. It has also been proposed that *Shigella* invasins could induce the formation of a focal adhesion-like structure by binding to the  $\alpha 5 \beta 1$  integrin (Watarai et al., 1996).

We show here that IFN- $\alpha$  blocks the early steps of *Shigella* invasion of epithelial cells. IFN- $\alpha$  inhibits *Shigella*-induced actin polymerization as well as recruitment of other cytoskeletal proteins. We also show that the effect is due to inhibition of a Src-dependent signaling pathway.

## Materials and Methods

### Bacterial Strains and Growth Conditions

M90T is a wild-type invasive strain of *S. flexneri* serotype 5. BS176 is a noninvasive mutant of M90T cured of the 220-kb virulence plasmid. The plasmid p1018 is a pUC derivative that encodes the AfaE adhesin from uropathogenic *Escherichia coli*, as well as a spectinomycin resistance marker (Labigne-Roussel et al., 1984). *Shigella mxiD* mutant strain is M90T in which the *mxiD* gene has been inactivated (Allaoui et al., 1992) and which is deficient for cell contact-dependent secretion. The wild-type strain S58 of *Salmonella typhimurium* was used (Hermant et al., 1995).

Bacterial strains were grown in tryptic soy broth at 37°C with agitation. To prepare bacteria for invasion assay, overnight cultures of *S. flexneri* or *S. typhimurium* were diluted 100-fold and grown to midexponential phase ( $OD_{550} = 0.3$ ). Bacteria were recovered by centrifugation at 5,000 g for 10 min, washed in PBS and resuspended in DME and 50 mM Hepes, pH 7.3.

### Antibodies, Plasmids, and Reagents

The anti-phosphotyrosine 4G10 and anti-cortactin 4F11 mAbs were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The anti-ezrin rabbit polyclonal antibody was a gift from Paul Mangeat (Université de Montpellier, France). The anti-PKR mAb 71/10 was a gift from Eliane Meurs (Institut Pasteur, Paris). *Shigella* was detected with a rabbit polyclonal anti-LPS serum (Mounier et al., 1997). The anti-Src mAb clone

327 was from Oncogene Science Inc. (Uniondale, NY). The anti-mouse and anti-rabbit IgG antibody coupled to rhodamine were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Bodipy-labeled phalloidin, anti-mouse, and anti-rabbit IgG antibody coupled to Cascade blue were purchased from Molecular Probes, Inc. (Eugene, OR). Purified recombinant IFN- $\alpha 2b$  was a gift from D. Gewert (Wellcome Foundation, Ltd, Beckenham, Kent, UK).

Wild-type, inactive and constitutively active forms of chicken Src were cloned into the vector pSG5 that had been modified to include a different polylinker region (from Thorsten Erpel, European Molecular Biology Laboratory) to generate, pSG5/srcK+, pSG5/srcK- and pSG5/srcF plasmids, respectively. The kinase inactivation mutation in Src was Lys295 to Met (Twamley-Stein et al., 1993). The constitutively activating mutation in Src was tyrosine 527 to phenylalanine (Cartwright et al., 1987). The three constructs were a gift from Sarah Courtneidge (EMBL, Heidelberg, Germany).

### Cells and Cell Transfection

HeLa S3 cells were grown at 37°C in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS in a 10% CO<sub>2</sub> incubator. Transfections were performed by electroporation using a Gene Pulser apparatus (Bio Rad Laboratories, Hercules, CA). About  $5 \times 10^6$  HeLa cells were resuspended in 200  $\mu$ l DMEM containing 10% FBS with 5  $\mu$ g of pSG5/c-src (wild-type, constitutively active or inactive form) and the plasmid pSV2neo. A 960  $\mu$ F/300 V/200W pulse was then applied. Stable transfectants were selected with 0.8 mg/ml geneticin (GIBCO BRL).

### Gentamicin Protection Assays

Infection of HeLa cells was essentially as described (Isberg and Falkow, 1985). The bacterial suspension was added to cells (multiplicity of infection = 100:1), samples were centrifuged at 780 g for 10 min at room temperature and incubated at 37°C for 30 min. Extracellular bacteria were killed by 30 min incubation in MEM containing 10% FBS and gentamicin at a final concentration of 50  $\mu$ g/ml. The number of intracellular bacteria was determined after lysis of the cells with PBS containing 0.5% (wt/vol) sodium deoxycholate, and colony-forming units counting on trypticase soya agar plates.

### Immunofluorescence Techniques

HeLa cells were incubated with bacteria expressing AfaE adhesin (Labigne-Roussel et al., 1984) at OD = 0.05 (multiplicity of infection = 10:1), for 10 min at room temperature to allow for bacterial adhesion. Samples were then transferred to 37°C to allow bacterial entry. After various time periods, cells were fixed for 20 min in PBS containing 3.7% (wt/vol) paraformaldehyde and washed three times with PBS. Cells were permeabilized with 0.1% Triton X-100 for 5 min before antibody staining. For inside/outside staining of bacteria, samples were incubated before permeabilization with the monoclonal anti-LPS antibody IgC20 (Mounier et al., 1997), and secondary anti-mouse IgG antibody linked to Cascade blue to label extracellular bacteria. Samples were subsequently permeabilized and total bacteria were visualized by staining with an anti-LPS polyclonal antibody against *S. flexneri* serotype 5a at a 1:500 dilution. Samples were then incubated with anti-rabbit Ig antibody coupled to rhodamine. Bodipy-phalloidin was used to label F-actin. To determine the number of cells per field, cell nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI) at a concentration of 0.1  $\mu$ g/ml. Preparations were analyzed using a conventional fluorescence microscope (BH2-RFCA; Olympus Optical Co., Ltd) or a confocal laser scanning microscope (Wild Leitz Instruments GmbH, Heidelberg, Germany). Pictures from the latter were recorded on a flat screen monitor with high linearity.

### Image Analysis

Images were acquired from a fluorescence microscope equipped with a CCD camera (Sony, Tokyo, Japan) connected to an AG-5 frame grabber (Scion Corp., Frederick, MD). Typically, five images per field were recorded: one image for the fluorophore labeling the foci and, for each of the two fluorophores labeling the bacteria, one image focused on the apical side and one image focused on the basal side of the sample. A dedicated semiautomatic program was developed to detect, count and classify bacteria and foci from the fluorescence images. This program runs on a SPARC station Ultra1 (Sun Microsystems, Mountain View, CA) to which a Series 151/40 digital image processor (Imaging Technology, Bedford,

MA) is connected. First, a min-max algorithm is used to combine the images from the two focal planes into a single image where spots are well in focus. The spot detection is then performed automatically by a multiresolution algorithm based upon selectively filtering an undecimated wavelet decomposition of the image through the use of wavelet coefficient thresholding and correlation (Olivo, 1996). The resulting binary masks are then used to compute geometrical parameters such as centroid, surface, principal axes and roundness with a central moment algorithm on the basis of which the algorithm is able to discern bacteria or foci from background fluorescent structures. To count the number of internal bacteria, a logical XOR operation is applied between the images showing the total bacteria and the external bacteria. Results are presented as the mean  $\pm$  SEM of the percentage of internalized bacteria relative to the total number of bacteria. The program yields data tables for each time point which were used for generating values in the figures. Statistical significance was assessed by Student's *t* test (Press et al., 1992).

### Immunoblots

HeLa cells were plated at a density of  $2 \times 10^5$  cells/35-mm dish the day before and incubated with bacteria expressing the AfaE adhesin at OD = 0.3, for 10 min at room temperature to allow for bacterial adhesion. Samples were then transferred to 37°C to allow bacterial entry. After various time periods, samples were put on ice and washed three times in cold PBS. Samples were scraped in 200  $\mu$ l of lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, and 1 mM ortho-vanadate) and transferred to microfuge tubes. After 15 min incubation, lysates were cleared by centrifugation and the protein concentration was measured by Bradford assay.

Equal amounts of protein were analyzed by Western blotting, after SDS-PAGE in gels containing either 8% or 5% polyacrylamide. The filters were blocked in PBS containing 0.1% Tween-20 and 1% gelatin for 30 min. Filters were incubated with the primary antibody for 1 h in PBS containing 0.1% Tween and 0.1% gelatin with the following antibody concentration: 0.5  $\mu$ g/ml for anti-phosphotyrosine mAb 4G10 and anti-Src mAb 327; 0.1  $\mu$ g/ml for anti-PKR mAb 72/10. After washing in PBS containing 0.1% Tween, blots were incubated with a secondary antibody coupled to peroxidase or alkaline phosphatase (Nycomed Amersham, Buckinghamshire, UK). After washing with PBS containing 0.1% Tween-20, blots were processed with the ECL detection kit (Nycomed Amersham). For quantitative measurements, blots were processed with the ECF detection kit (Nycomed Amersham) and fluorescence signals were analyzed using a fluorimager (Molecular Dynamics, Inc., Sunnyvale, CA).

## Results

### Effect of IFN- $\alpha$ on *Shigella* Uptake by HeLa Cells

To analyze more specifically the effect of IFN- $\alpha$  on the entry process, we first tested the capacity of HeLa cells to internalize *Shigella* in a gentamicin protection assay carried out after a short incubation time. Cells were treated with IFN- $\alpha$  for 20 h, then challenged with *Shigella* for 30 min before adding gentamicin (Materials and Methods). As shown in Fig. 1 *a*, IFN- $\alpha$  treatment at a concentration of 500 U/ml led to a 70% inhibition of *Shigella* entry compared with untreated cells. A slightly reduced effect was seen with 50 U/ml IFN- $\alpha$  (Fig. 1 *a*, 50). Under comparable conditions, *Salmonella* entry into cells showed little inhibition, with no significant differences between the 50 and 500 U/ml treatments (Fig. 1 *b*). These results indicate that IFN- $\alpha$  specifically impairs the ability of cells to internalize *Shigella*.

To confirm these observations and further analyze the kinetics of the *Shigella* entry process in cells treated with IFN- $\alpha$ , we measured *Shigella* uptake using immunofluorescence and computer-assisted analysis. To specifically analyze initial steps of the entry process, *Shigella* strains expressing the AfaE adhesin (Materials and Methods)

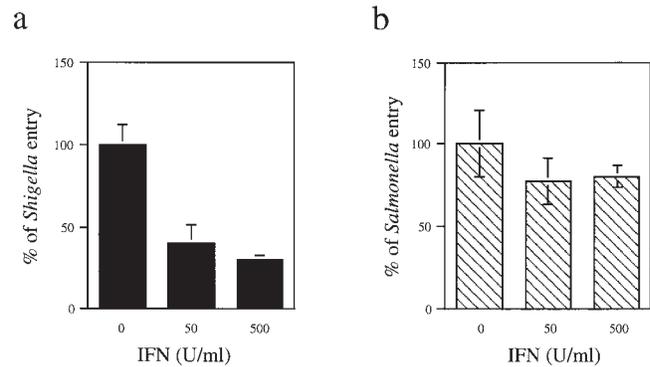
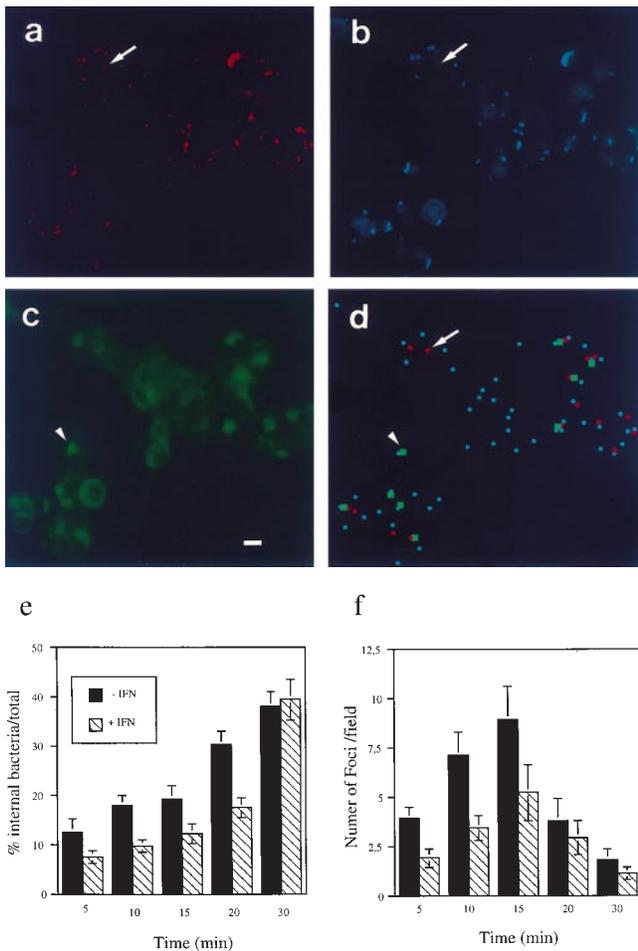


Figure 1. IFN- $\alpha$  inhibits *Shigella* uptake. HeLa cells were treated with 0, 50, and 500 U/ml of IFN- $\alpha$ , challenged with *Shigella* (*a*) or *Salmonella* (*b*) for 30 min, and the percentage of intracellular bacteria was determined by the gentamicin assay (Isberg and Falkow, 1985).

were used to synchronize the infection. After allowing bacterial attachment at room temperature, samples were incubated at 37°C for different periods of time and the cells were fixed. Extracellular bacteria were labeled before permeabilizing the cells (Fig. 2 *b*), whereas total bacteria were labeled after permeabilization with a different fluorochrome (Fig. 2 *a*). Images were analyzed automatically by a program that allows determination of the number of intracellular bacteria labeled with one fluorochrome (Fig. 2 *d*, red spots) or extracellular bacteria colabeled with both fluorochromes (Fig. 2 *d*, blue spots). The total number of cell-associated bacteria did not show significant variations between IFN- $\alpha$ -treated and untreated cells, corresponding to about three bacteria per cell. Fig. 2 *e* shows the ratio of internal over total bacteria for each time point. For cells that were not treated with IFN- $\alpha$ , the percentage of intracellular bacteria increased steadily, with values ranging from 12% at 5 min to 37% at 30 min (Fig. 2 *e*, filled bars, 5–30). In contrast, *Shigella* entered cells that were pretreated with IFN- $\alpha$  less efficiently, with a reduction of 50% compared with untreated cells at early time points (Fig. 2 *e*, hatched bars, 5–20). At the 30-min time point, however, in both treated and untreated cells, bacterial internalization was similar ( $\sim$ 40% of total bacteria), suggesting that expression of the AfaE adhesin masked the IFN- $\alpha$  inhibitory effect after prolonged incubation. Similar effects linked to the AfaE adhesin were previously observed for entry defective *Shigella* mutant strains (Tran Van Nhieu et al., 1997), and the use of the AfaE adhesin probably led to underestimation of the IFN inhibition. These results are consistent with data obtained in gentamicin assays and argue for an inhibitory role of IFN- $\alpha$  in early events during *Shigella* entry.

### Analysis of the Effect of IFN- $\alpha$ on *Shigella*-induced Foci

To analyze the effect of IFN- $\alpha$  on *Shigella*-induced actin rearrangements during entry, foci were visualized by labeling F-actin. Foci formation is a dynamic, continuous process and strict criteria of definition must be adopted to



**Figure 2.** IFN- $\alpha$  affects early events in *Shigella* entry and inhibits foci formation. Cells (Untreated or treated with 500 U/ml of IFN- $\alpha$ ) were challenged with *Shigella* expressing the AfaE adhesin for various time points (5–30 min). *Shigella* entry was measured using specific inside/outside stains. (a) Staining of total bacteria after permeabilization (red). (b) Staining of extracellular bacteria before cell permeabilization (blue). (c) Foci were visualized by labeling F-actin with Bodipy-phalloidin. (d) Bacteria and foci were counted automatically using dedicated computer programs (Materials and Methods), the analysis was performed for ten microscope fields per time points; bacteria labeled in a but not in b were scored as internal and represented as red spots (arrows indicate such an example); bacteria labeled with both fluorochromes were scored as external (blue spots); scored foci were represented as green squares (arrowheads indicate an example). (e) Percentage of internal/total bacteria. (f) Average number of foci per field. Filled bars, untreated cells; hatched bars, cells treated by IFN- $\alpha$ . Bar, 10  $\mu$ m.

obtain reproducible results. To count *Shigella*-induced foci, a computer-assisted analysis of fluorescence images was developed (see Materials and Methods).

F-actin labeled foci were analyzed automatically, according to their shape and peak of fluorescence intensity. An example in Fig. 2 c shows *Shigella*-induced foci stained with Bodipy-labeled phalloidin. Fig. 2 d shows automatic analysis of the foci in the same field (green squares). *Shigella*-induced foci localized either at the periphery or at the apical surface of the cell, the latter being most abun-

dant. For technical reasons, only apical foci were automatically scored. The bar chart in Fig. 2 f shows the number of foci as a function of time in IFN- $\alpha$ -treated (hatched bars) or untreated cells (filled bars). For untreated cells, *Shigella*-induced foci appeared as early as 5 min and a maximum value of nine foci per field was observed at 15 min (Fig. 2 f, filled bars). A sharp decrease was observed at 20 min, and by 30 min very few foci were detected. When cells were pretreated with IFN- $\alpha$ , similar kinetics were observed with a maximum scored at 15 min (Fig. 2 f, hatched bars). However, IFN- $\alpha$  treatment resulted in  $\sim$ 50% inhibition in the number of foci at time points 5 and 10 min and a 40% inhibition at 15 min (Fig. 2 f, hatched bars, 5–15,  $P < 0.05$ ). As incubation time increased, the IFN- $\alpha$  inhibition effect on *Shigella* induced foci formation was less pronounced, with no significant difference at 20 and 30 min (Fig. 2 f, hatched bars, 20 and 30). IFN- $\alpha$  treatment did not lead to any gross modifications of the actin cytoskeleton.

Confocal microscopy analysis was used to determine if, in addition to the reduction in number of foci, actin polymerization at the site of *Shigella* entry was qualitatively affected. 10 foci were chosen randomly from IFN- $\alpha$ -treated (Fig. 3, bottom) and untreated cells (top) and analyzed using the same parameters. Despite some variations in the level of F-actin labeling among the different foci, a weaker signal was generally observed when cells were pretreated with IFN- $\alpha$ . Confocal analysis indicated that recruitment of other cytoskeletal proteins such as ezrin and  $\alpha$ -actinin at the entry foci was also affected by IFN- $\alpha$  treatment (not shown). These data further support the notion that IFN- $\alpha$  interferes with early events during *Shigella* entry, by impairing ability of the bacterium to reorganize the actin-cytoskeleton into entry structures.

### Analysis of *Shigella*-induced Tyrosine Phosphorylation

Cortactin, a Src-substrate recruited at the site of *Shigella* entry is specifically phosphorylated on tyrosine residues (Dehio et al., 1995). Therefore, we analyzed whether phosphorylation of cortactin induced by *Shigella* upon entry into HeLa cells was affected by IFN- $\alpha$ . HeLa cells were challenged with *Shigella* and at different time points, cells were lysed and their content in tyrosine-phosphorylated proteins was analyzed by Western blot with anti-phosphotyrosine mAb. As previously described, phosphorylation of cortactin was induced upon *Shigella* entry as early as 5 min, peaking at 20 min and decreasing at 30 min (Fig. 4 a, arrowhead), with kinetics similar to the induction of F-actin foci. IFN- $\alpha$  pretreatment reduced overall cortactin phosphorylation (Fig. 4 a, compare + with -) and this effect was detectable at every time point, but most significantly at early time points (Fig. 4 a, 5 and 10). Also, IFN- $\alpha$  had an inhibitory effect on tyrosine-phosphorylation of some unidentified 68-kD species, whereas phosphorylation of other proteins was unaffected by IFN- $\alpha$  treatment (Fig. 4 a). Quantification of the IFN- $\alpha$  inhibitory effect by scanning densitometry of similar gels after detection with a fluorescent substrate (Materials and Methods) indicated that, at 5 and 10 min after infection, the levels of tyrosine-phosphorylated cortactin were reduced by  $\sim$ 50% in IFN- $\alpha$ -treated cells.

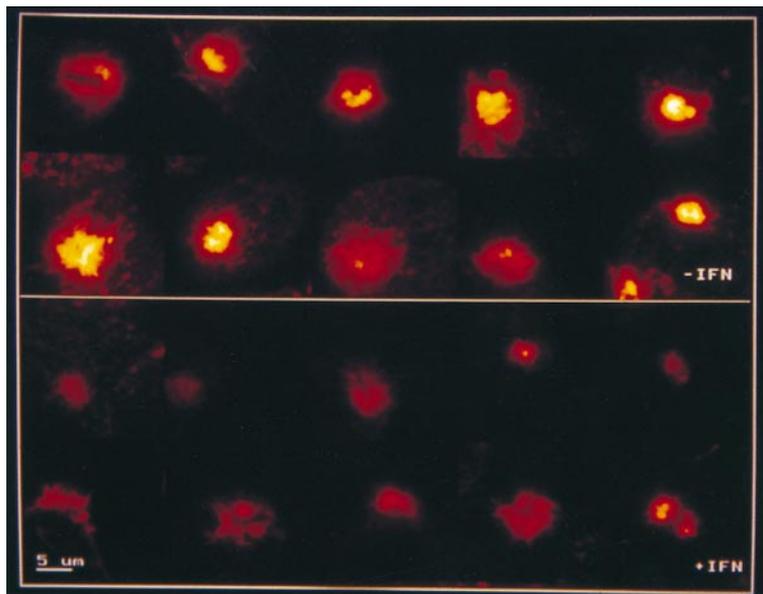


Figure 3. IFN- $\alpha$  inhibits *Shigella*-induced actin polymerization at the site of entry. Cells were challenged with *Shigella* expressing the AfaE adhesin, fixed after 10 min incubation at 37°C and stained for F-actin. 10 foci of entry were chosen randomly and analyzed by confocal microscopy. Cells were treated with 500 U/ml of IFN- $\alpha$  (bottom) or untreated (top) before *Shigella* challenge.

In addition to cortactin, we identified two other proteins that were phosphorylated upon *Shigella* entry (Fig. 4, b and c). A 260-kD protein (p260) was phosphorylated with the same kinetics as cortactin. IFN- $\alpha$  inhibited *Shigella*-

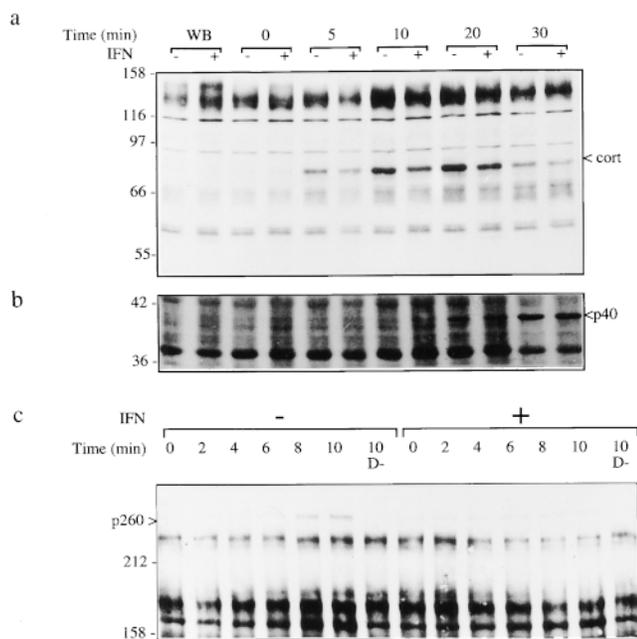


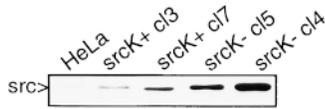
Figure 4. *Shigella* induces the phosphorylation of cortactin, p40 and p260. Untreated cells (–) or cells treated with 500 U/ml of IFN- $\alpha$  (+) were challenged with *Shigella* expressing the AfaE adhesin for various time points, lysates were analyzed by Western blot using anti-phosphotyrosine mAb. Samples were fractionated on gels containing 8% (a and b) and 5% (c) polyacrylamide. 0, 5, 10, 15, 20, 30, cells challenged with *Shigella* for 0, 5, 10, 15, 20, 30 min, respectively. WB, no bacteria; D–, cells challenged with *Shigella mxiD* mutant. IFN- $\alpha$  inhibits p260 and cortactin (cort) phosphorylation.

induced p260 phosphorylation to the same extent as cortactin, with maximal inhibitory effects at early time points (Fig. 4 c) Also, a 40-kD protein (p40) was specifically phosphorylated during entry but with different kinetics as p40 phosphorylation was detectable at 20 min and increased thereafter (Fig. 4 b). In contrast to p260 and cortactin, phosphorylation of p40 was unaffected by IFN- $\alpha$  treatment. This protein was identified as the MAP kinase family member ERK2 (Duménil, G., unpublished data). When cells were challenged with a non-invasive *Shigella* strain, no change in the levels of p260 or p40 phosphorylation could be detected (Fig. 4 c, D– and data not shown) indicating that phosphorylation of these proteins was specific for *Shigella* invasion. No significant change in the phosphorylation of FAK was detected although it is possible that high basal levels of FAK phosphorylation in HeLa cells may have prevented detection of minor variations (not shown).

Thus, *Shigella* entry into cells triggers the phosphorylation of several proteins with at least two different kinetics. A subset of proteins, including cortactin and p260, is phosphorylated with kinetics that correlate with entry foci formation, and is affected by IFN- $\alpha$  pretreatment.

#### Overexpression of a Dominant Interfering Mutant of pp60c-src Inhibits *Shigella*-induced Cortactin Phosphorylation and Cytoskeletal Rearrangements

To further define the role of pp60c-src on *Shigella* entry, we analyzed the characteristics of *Shigella* entry in HeLa transfectants overexpressing a dominant interfering form of pp60c-src. Stable clones expressing a catalytically inactive form of pp60c-src (srcK–; Twamley-Stein et al., 1993), the wild-type form of pp60c-src (srcK+) or the vector alone were selected (Materials and Methods), and the level of Src protein was measured by anti-Src Western blot analysis. Src was only slightly overexpressed in the srcK+ clones, whereas the levels of expression of Src in the srcK– clones could reach <10-fold the level in parental



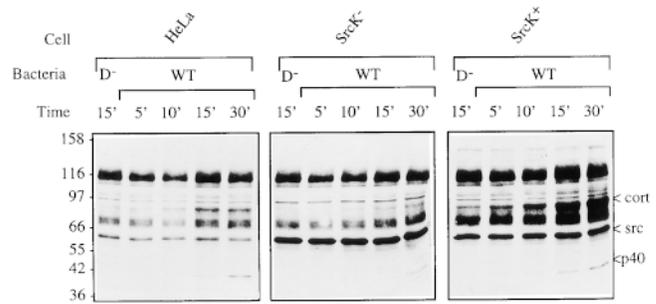
**Figure 5.** Pp60c-src expression levels in stable transfectants. HeLa cells were transfected with a plasmid construct mediating expression of either wild-type chicken Src (*srcK+*) or a kinase inactive mutant of Src (*srcK-*) and stable clones were selected. Lysates of two representative clones were analyzed by Western blot analysis using anti-Src mAb. *HeLa*, parental HeLa cells; *srcK+* *cl3* and *srcK+* *cl7*, HeLa cells transfected with wild-type Src, clones 3 and 7, respectively; *srcK-* *cl4* and *srcK+* *cl5*, HeLa cells transfected with wild-type Src, clones 4 and 5, respectively.

cells. Two independent clones for each construct were used in this study (Fig. 5).

*Shigella*-induced protein phosphorylation was first investigated in these transfectants. Cells transfected with the vector only exhibited the usual kinetics of phosphorylation, with cortactin phosphorylation peaking at 15 min (Fig. 6, *HeLa*). In contrast, cells overexpressing the dominant interfering form of Src exhibited reduced levels of cortactin phosphorylation that was hardly detectable at early time points and increased slightly at 30 min (Fig. 6, *srcK-*). Thus, cortactin phosphorylation was markedly reduced in *srcK-* cells as compared with control cells. The induction of p40 phosphorylation was reduced, although to a lesser extent than that of cortactin, upon overexpression of the dominant interfering form of Src.

Overexpression of wild-type pp60c-src led to the opposite effects: cortactin was hyper-phosphorylated and an increase in the phosphorylation levels of cortactin could be observed as soon as 5 min after bacterial challenge (Fig. 6, *srcK+*). P40 and p260 phosphorylation were increased in *srcK+* cells and could be detected earlier in comparison with control cells (Fig. 6, *srcK+* and not shown). These results suggest that pp60c-src, or a member of the Src family, is responsible for *Shigella*-induced cortactin phosphorylation.

The role of pp60c-src on the induction of actin rearrangements at the site of *Shigella* entry was investigated using immunofluorescence techniques. Expression of the Src constructs in HeLa cells did not result in significant changes in cell morphology (not shown). *srcK-* cells (Fig. 7 c), however, presented more actin cables when compared with parental cells (Fig. 7 a). Transfectants were challenged with *Shigella*, and at different time points, samples were fixed and double-stained for F-actin (Fig. 7, d-f, green) and bacterial LPS (Fig. 7, d-f, red). *Shigella*-induced foci in *srcK+* cells appeared similar to foci induced in parental cells, although moderately less abundant. In *srcK-* cells, however, the number of foci was drastically reduced. The average number of bacteria associated per cell was not significantly different among the different transfectants. Fig. 7 g presents the kinetics of foci formation, scored as the number of foci per cell. As expected, the number of foci increased steadily in control cells between 5 and 15 min, <0.12 foci per cell, and decreased sharply at 20 min (Fig. 7 g, squares). *srcK-* cells showed a marked reduction in the number of foci at all time points, peaking at 0.026 foci per cell (Fig. 7 g, triangles). *Shigella* uptake was



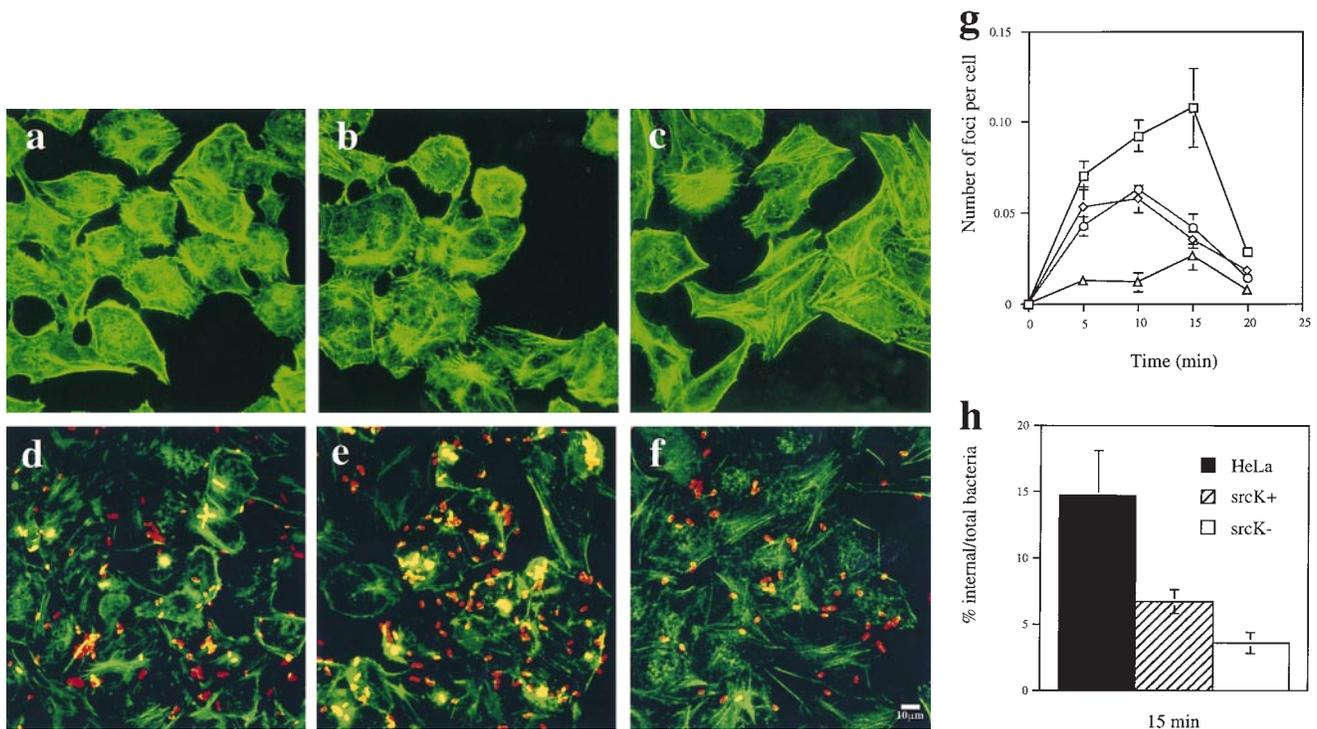
**Figure 6.** Src is responsible for *Shigella*-induced cortactin phosphorylation. *srcK-*, *srcK+* and control cells (*HeLa*) were challenged with *mxiD-* or wild-type (WT) *Shigella* and their content in phosphotyrosyl proteins was analyzed by Western blot using anti-phosphotyrosine mAb. 5, 10, 15, 30, cells challenged with *Shigella* for 5, 10, 15, 30 min, respectively; *D-*, cells challenged with the *Shigella mxiD* mutant. Approximately 10  $\mu$ g of protein was loaded per lane.

then quantitated by inside/outside immunofluorescence staining. Consistent with the reduction of foci number, the amounts of internalized bacteria in the *srcK-* cells decreased by 75% after 15 min of infection when compared with the control cells (Fig. 7 h, empty bar). Taken together, these results indicate that pp60c-src activity is required for the formation of *Shigella* actin foci and for *Shigella* entry.

Interestingly, overexpression of wild-type Src also led to a decrease in the ratio of internalized bacteria (Fig. 7 h, hatched bar). *srcK+* cells only internalized about half the amounts of bacteria compared with wild-type cells. *srcK+* cells also consistently exhibited less foci than wild-type cells, with a significant difference at 15 min where only 0.04 foci per cell were scored. Also, the kinetics of appearance of foci in *srcK+* cells were altered. The number of foci peaked at 0.063 foci per cell after 10 min infection and decreased rapidly thereafter. The kinetics of foci formation in *srcK+* cells suggested that overexpression of Src resulted in a more rapid downmodulation of the *Shigella* entry foci. These results suggest that Src activity is required both for foci induction, as well as for the downregulation of actin foci induced by *Shigella* upon entry.

### ***IFN- $\alpha$ Does Not Inhibit *Shigella* Entry into Cells That Express a Constitutively Active Form of pp60c-src***

As shown above, IFN- $\alpha$  treatment induces a decrease in *Shigella*-induced cortactin phosphorylation suggesting that IFN- $\alpha$  inhibits *Shigella* entry by affecting Src activation. To further define the relationship between IFN- $\alpha$  inhibition of *Shigella* entry and Src, we investigated the effects of IFN- $\alpha$  on cells that express a constitutively active form of Src (Cartwright et al., 1987). HeLa cells were transfected with an allele of *src* bearing a mutation at position 527 that substituted a tyrosine into a phenyl alanine (Y527F). Stable clones were selected (*srcF*) and their relative levels of Src was analyzed by Western blot. Overexpression of Src was about two- to threefold relative to the amounts in the parental cells (Fig. 8 b, *Src*). When challenged with *Shigella*, *srcF* cells shared characteristics similar to *srcK+* cells. The kinetics of foci formation (Fig. 7 g,



**Figure 7.** Expression of a kinase negative form of Src leads to inhibition of foci formation and of bacterial uptake. (a–c) Uninfected cells labeled for F-actin. (d–f) Cells were challenged for 15 min with wild-type *Shigella*. Samples were fixed and processed for immunolabeling of F-actin (green) and bacteria (red). A typical microscope field is shown for control cells (a and d), srcK+ cells (b and e) and for srcK– cells (c and f). (g) Foci of entry were scored 5, 10, 15, and 20 min after infection (squares, parental HeLa; diamonds, srcF; circles, srcK+; triangles, srcK–). (h) The percentage of internal/total bacteria after 15 min of infection was determined by inside/outside immunofluorescence staining scored automatically (filled bars, srcK+ cells; hatched bars, parental HeLa cells; empty bars, srcK– cells).

diamonds), the efficiency of *Shigella* entry and the phosphorylation pattern (not shown) after *Shigella* invasion were indistinguishable in srcF cells and in srcK+ cells.

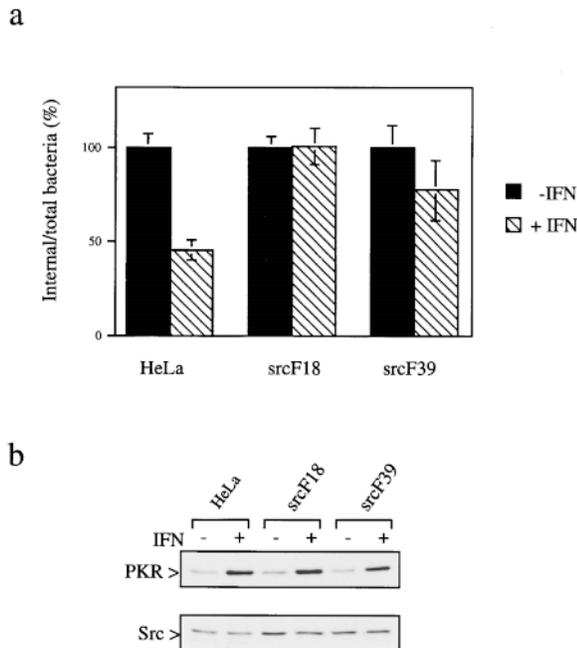
The effect of IFN- $\alpha$  on srcF cells was evaluated by comparing the efficiency of *Shigella* internalization in treated and untreated cells. Cells were challenged for 10 min with *Shigella* expressing the AfaE adhesin, and internalized bacteria were visualized by immunostaining with and without permeabilization as described previously. As expected, IFN- $\alpha$  inhibited *Shigella* internalization in HeLa cells by 50% (Fig. 8 a, HeLa, compare filled with hatched bars). In contrast, IFN- $\alpha$  had no significant effect on bacterial internalization in either of the srcF clones (Fig. 8 a). No inhibition was found for the srcF18 clone, and the srcF39 showed modest levels of inhibition reaching at best 20% of bacterial entry. Also, when *Shigella*-induced foci of F-actin were scored, no difference could be detected between untreated SrcF cells and SrcF cells pretreated with IFN- $\alpha$  (not shown). To control that srcF cells were responsive to IFN- $\alpha$ , we analyzed the levels of expression of PKR, a serine/threonine kinase upregulated by IFN- $\alpha$  that is involved in the antiviral effects of this cytokine (Meurs et al., 1990). As expected, the amount of PKR increased in HeLa cells in response to the IFN- $\alpha$  treatment (Fig. 8 b, PKR). A comparable increase in PKR protein was detected in both SrcF clones (Fig. 8 b, PKR), suggesting that IFN- $\alpha$  signaling still occurred in srcF cells. Western blot

analysis of whole cell lysates indicated that the amounts of Src did not change upon IFN- $\alpha$  treatment (Fig. 8 b, Src). Therefore, IFN- $\alpha$  still induces gene transcription, but is unable to inhibit *Shigella* entry in srcF cells. These results suggest that IFN- $\alpha$  affects *Shigella* entry by specifically inhibiting a Src-dependent pathway.

## Discussion

### IFN- $\alpha$ Inhibits *Shigella*-induced Foci Formation

We report here that IFN- $\alpha$  inhibits *Shigella* entry into epithelial cells by interfering with actin rearrangements and recruitment of cytoskeletal proteins, such as ezrin and  $\alpha$ -actinin, involved in formation of the entry structure of this bacterial pathogen. Interestingly, the inhibitory effect appeared rather specific for *Shigella* entry since IFN- $\alpha$  treatment per se did not lead to any appreciable changes in the overall aspect of the actin cytoskeleton and *Salmonella* invasion was not inhibited. The different effects of IFN- $\alpha$  on the entry of the two pathogens is surprising since *Salmonella* induces cellular extensions that are similar to those induced by *Shigella* during invasion of epithelial cells (Francis et al., 1993). Moreover, *Salmonella* and *Shigella* express factors for entry that share significant homologies (Hermant et al., 1995). Despite these analogies, the two pathogens may have selected distinct in-



**Figure 8.** Effect of IFN- $\alpha$  on cells expressing constitutively activated Src. (a) Internalization of *Shigella* into HeLa, srcF18 and srcF39 cells. The three cell lines were treated with IFN- $\alpha$  and challenged with *Shigella* for 15 min. Extracellular bacteria were labeled before permeabilizing the cells, whereas total bacteria were labeled after permeabilization with a different fluorochrome. The bar chart present the ratio of internal/total bacteria as a percentage of the untreated cells (filled bars, untreated cells; hatched bars, cells treated by IFN- $\alpha$ ). (b) Induction of PKR by IFN- $\alpha$  in HeLa, srcF18 and srcF39 cells. Cells were treated with IFN- $\alpha$ , lysed, and an equal amount of protein was submitted to Western blot analysis with anti-PKR (PKR) and anti-Src (Src) antibodies.

vasion strategies. Consistent with this, *Salmonella* and *Shigella* induce different patterns of tyrosine phosphorylation of host proteins during entry into epithelial cells. *Salmonella* appears to induce overall protein dephosphorylation (Yixin and Galán, 1998; our unpublished observations), whereas *Shigella* clearly induces the phosphorylation of several proteins including cortactin (this report; Dehio et al., 1995). When analyzing *Shigella*-induced pattern of tyrosine phosphorylation during cell entry, a consistent reduction in the phosphorylation level of two proteins was observed in IFN- $\alpha$ -treated cells. These proteins consisted of an unidentified p260 and cortactin (Fig. 4). Since cortactin was described as a Src substrate, we sought to investigate the potential role of a Src-dependent signal triggered by *Shigella* and inhibited by IFN- $\alpha$ .

#### A Role for pp60c-src Kinase Activity during *Shigella* Entry

To study the potential role of Src during *Shigella* entry, we generated stable transfectants overexpressing a kinase defective dominant interfering mutant (srcK-). Expression of the dominant interfering form of pp60c-src decreased *Shigella* uptake, *Shigella*-induced actin rearrangements as

well as cortactin phosphorylation during entry. Surprisingly, overexpression of wild-type Src or of a constitutively active form of Src also led to reduced *Shigella* uptake and to altered formation of entry foci. These latter appeared to form and to disappear faster than in control cells (Fig. 7 g). Altogether, these results argue in favor of a dual role for Src during *Shigella* entry. Src appears to be involved in the induction of the entry foci as well as in their downregulation. This dual role of Src is reminiscent of its involvement in focal adhesion formation, a well-studied process that presents several analogies with *Shigella* entry. Data obtained from fibroblasts derived from knock-out mice indicate that Src may participate, in concert with Fyn, in the formation of focal adhesions (Thomas et al., 1995). Focal adhesion formation, however, was shown to be independent of Src kinase activity, since a truncated form of Src lacking the kinase domain could restore spreading onto fibronectin-coated surfaces and focal adhesion to wild-type levels (Kaplan et al., 1995). On the other hand, it has been proposed that Src kinase activity could control the turnover of focal adhesions since expression of a kinase inactive mutant of v-src induced the formation of unusually large focal adhesions (Fincham and Frame, 1998). It is possible that during *Shigella* entry, Src activity is regulated by the sequential recruitment of substrates at the entry foci, and it could up- or downregulate entry foci by phosphorylating different sets of substrates.

#### Protein Phosphorylation and *Shigella* Entry

We found that *Shigella* induces the phosphorylation of at least three proteins in two kinetically distinct waves of phosphorylation. The individual role of these proteins, relative to foci formation, remains to be defined. In immunofluorescence studies, cortactin was shown to be recruited early in entry foci, whereas pp60c-src localized most abundantly at the periphery of the phagosome (Dehio et al., 1995). Also, tyrosine phosphorylation of cortactin was found to be weak in nascent foci and to increase as entry foci develop. These results suggest that cortactin phosphorylation takes place after the actual initiation of foci formation and thus could be involved in downmodulation of entry foci. This hypothesis would be consistent with findings showing that cortactin, once phosphorylated by pp60c-src, loses its ability to cross-link actin filaments (Huang et al., 1997).

Another likely substrate of pp60c-src is p260, because the kinetics of phosphorylation of p260 parallel that of cortactin during *Shigella* entry. In addition, *Shigella*-induced p260 phosphorylation is increased in cells overexpressing pp60c-src (data not shown). Interestingly, plating of HeLa cells on fibronectin did not lead to p260 phosphorylation while phosphorylation of FAK was observed (Duménil, G., unpublished data). Although, Src is involved in both *Shigella* invasion and focal adhesion formation, the differences in the phosphorylation patterns are probably indicative of another level of regulation of Src activity. The subcellular localization of Src or its interaction with specific partners may determine differences between *Shigella* entry and focal adhesion formation. The identification of p260 will be important to further define the role of pp60c-src in *Shigella* entry.

The phosphorylation of p40, identified as ERK2, is probably part of a different signaling pathway than that of p260 and cortactin, since ERK2 phosphorylation is induced later during *Shigella* entry. Also, overexpression of the dominant-interfering form of Src, or IFN- $\alpha$  treatment, have little effect on p40 phosphorylation. Altogether the results suggest that p40 phosphorylation may not participate in foci formation. Phosphorylation of MAP kinases has been described during *Salmonella* and *Listeria* infections (Tang et al., 1994, 1998; Hobbie et al., 1997) and may reflect cellular responses that are not specifically linked to actin rearrangements during *Shigella* entry.

### IFN- $\alpha$ and pp60c-src

We found that *Shigella*-induced foci formation is dependent upon a signaling pathway involving Src tyrosine kinase activity. Moreover, IFN- $\alpha$  exerts its inhibitory role on *Shigella* entry by interfering with this Src-dependent pathway, as expression of a constitutively activated Src prevented IFN- $\alpha$ -mediated inhibition (Fig. 8). Known cellular effects of IFNs are mediated by the activation of the JAK/STAT pathway which leads to the modulation of gene transcription (Pellegrini and Dusanter-Fourt, 1997). This pathway, monitored by accumulation of an IFN-induced protein (PKR), does not seem to be perturbed by the expression of activated Src. To inhibit *Shigella* entry, a minimum of 6 h IFN- $\alpha$  treatment is required, suggesting that the inhibitory effect requires modulation of gene transcription. Since no change in the levels of Src occurs upon IFN- $\alpha$  treatment, the effects of IFN- $\alpha$  on *Shigella* entry cannot be attributed to a decreased expression of Src. It therefore appears that IFN- $\alpha$  affects the regulation of Src activity during *Shigella* entry.

Src intrinsic activity is dependent upon its conformation, which is regulated by its phosphorylation state and by its interaction with other proteins (Brown and Cooper, 1996). Src activity is also regulated by its subcellular location relative to its substrates. Thus, IFN- $\alpha$  could inhibit recruitment of Src or of Src substrates, to the site of *Shigella* entry. IFN- $\alpha$  could also affect an early step in the pathway induced by *Shigella* invasive determinants and leading to the catalytic activation of Src. For example, both focal adhesion formation and *Shigella* entry are dependent on the function of Src and Rho, although the sequence of events leading to their activation remains unclear. During focal adhesion formation, a tyrosine-sensitive tyrosine kinase is required for Rho activation (Nobes et al., 1995). Src family members are good candidates to play such a role. The activity of Rho may also be downregulated by Src via the phosphorylation of p190RhoGAP as seen during EGF-mediated cytoskeletal rearrangements (Chang et al., 1995). Alternatively, Src may play a role downstream of Rho proteins, since translocation of Src to the cell membrane requires a cytoskeletal network regulated by these small G proteins (Fincham et al., 1996). Unraveling the mechanism of inhibition of Src function should reveal unexpected IFN- $\alpha$  targets and help clarifying the early cellular events that trigger *Shigella* uptake.

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