IpaC induces actin polymerization and filopodia formation during *Shigella* entry into epithelial cells

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***Shigella* proteins that are targeted to host cells by a type III secretion apparatus are essential for reorganization of the cytoskeleton during cell invasion. We have developed a semi-permeabilized cell assay that tests the effects of bacterial proteins on the actin cytoskeleton. The *Shigella* IpaC protein was found to induce the formation of filopodial and lamellipodial extensions in these semi-permeabilized cells. Micro-injection of IpaC into cells, or cellular expression of IpaC also led to the formation of filopodial structures. Monoclonal antibodies (mAbs) directed against the C-terminus of IpaC inhibited the IpaC-induced extensions, whereas an anti-N-terminal IpaC mAb stimulated extensive lamellae formation. *Shigella* induced foci of actin polymerization in the permeabilized cells and these were inhibited by anti-C-terminal IpaC mAbs. Consistent with a role for IpaC in *Shigella*-induced cytoskeletal rearrangements during entry, stable transfectants expressing IpaC challenged with *Shigella* showed increased bacterial internalization. IpaC-induced extensions were inhibited by a dominant-interfering form of Cdc42 or the Cdc42-binding domain of WASP, whereas a dominant-interfering form of Rac resulted in inhibition of lamellae formation. We conclude that IpaC leads to activation of Cdc42 which in turn, causes activation of Rac, both GTPases being required for *Shigella* entry.**

Keywords: actin/IpaC/Rho proteins/*Shigella* invasion

Introduction

Shigella flexneri is a Gram-negative bacterium responsible for dysentery in humans. After ingestion by the host, *Shigella* infection leads to severe destruction of the colonic mucosa, mainly due to the intense inflammatory reaction elicited by the bacterium during colonic invasion (Sansonetti, 1998). The ability of this pathogen to colonize the colonic mucosa is dependent on its ability to invade epithelial cells and to spread from cell to cell (Parsot and Sansonetti, 1996). These properties have been studied using cultured epithelial cells that can be invaded readily by *Shigella*. *In vitro*, *Shigella* enters cells by a process

resembling macropinocytosis. Shortly after internalization, *Shigella* lyses the phagocytic vacuole and multiplies freely in the cell cytosol. During this multiplication step, *Shigella* can move intracellularly by polymerizing actin at one pole of the bacterial body. This actin-based motility leads to the formation of cellular protrusions that are driven by the bacterium and that propel it into adjacent cells. After lysis of the protrusion-derived membrane and that of the recipient cell, *Shigella* reinitiates the infectious cycle and can spread in a cell monolayer without extracellular steps.

In contrast to other invasive pathogens for which entry into host cells is associated with binding in tight apposition with cellular pseudopods during internalization, *Shigella* shows little cell binding ability. Instead, within a bacterial population, only a few bacteria bind transiently to the cell surface (Mounier *et al.*, 1997). These bound bacteria induce the formation of cellular extensions that rise several microns above the cell surface and that engulf the bacterium in a large vacuole (Adam *et al.*, 1995). The activity of Mxi-Spa, a bacterial type III secretion apparatus, is critical for the induction of these cell extensions and for bacterial entry (Parsot and Sansonetti, 1996). As for the type III secretion apparatus described in other bacterial pathogens, the *Shigella* Mxi-Spa apparatus allows secretion of bacterial proteins upon cell contact (Ménard *et al.*, 1994a). Among these, the IpaA–D proteins play a central role in the entry process, because *Shigella* mutants for which individual *ipa* genes have been inactivated are either totally deficient for cell invasion in the case of the *ipaB–D* genes (Ménard *et al.*, 1993), or significantly impaired in the case of the *ipaA* gene (Tran Van Nhieu *et al.*, 1997). More evidence suggests that the IpaB and IpaC proteins can act as direct effectors of *Shigella* entry: IpaB and IpaC associate in the extracellular medium to form a complex (Ménard *et al.*, 1994b), and this complex was shown to bind to cell surface receptors such as $\beta 1$ integrins (Watarai *et al.*, 1996) and CD44 (A.Skoudy, J.Mounier, A.Aruffo, P.Gounon, P.J.Sansonetti and G.Tran Van Nhieu, submitted). As inert particles coated with this complex are internalized readily by epithelial cells (Ménard *et al.*, 1996), this suggested that the IpaB and IpaC proteins could trigger the entry process by binding directly to cell surface receptors. Beads coated with the IpaB,C complex, however, do not induce the massive cytoskeletal changes seen during *Shigella* entry (Ménard *et al.*, 1996). Also, during entry, the vast majority of the IpaB,C pool is not found associated with the bacterial surface, but is found in the extracellular medium or associated with host cells (Ménard *et al.*, 1994a). These results argue that mere binding of the IpaB and IpaC proteins to receptors on the cell surface is not sufficient to trigger cytoskeletal rearrangements induced by *Shigella* during entry. Alternatively, proteins that translocate from extracellular bacteria directly into the cell cytosol/

membranes via type III secretion apparatus (Hueck, 1998) may participate in *Shigella*-induced cytoskeletal reorganization during entry. For example, among proteins secreted by *Shigella* via the Mxi-Spa apparatus, IpaB and IpaC are found associated with lipid membranes *in vitro*, and electrophysiological measurements indicate that they participate in the formation of a channel across lipid bilayers (De Geyter *et al.*, 1997; V.Cabiaux, personal communication). Thus, cytoskeletal rearrangements could be induced either directly by the IpaB,C proteins, or indirectly, after IpaB,C-dependent translocation of other *Shigella* effectors into the cell cytosol.

Surface extensions induced by *Shigella* during entry into epithelial cells, however, are unique in the sense that they organize into a highly dynamic structure involving the recruitment of specific cytoskeletal proteins. Shortly after *Shigella* interaction, microspike-like or filopodial structures emerge in the vicinity of the bound bacterium (Adam *et al.*, 1995). These filopodia appear to fill in rapidly to form leaflets that organize around and merge over the bacterial body (Adam *et al.*, 1995). Immunolabelling indicates a recruitment of cytoskeletal proteins which are different in the close vicinity of the bacterium contacting the cell membrane from those in the periphery of the extensions. A dense meshwork of F-actin and vinculin is found in close contact with the bacterium initiating the entry process, whereas actin-bundling proteins such as α -actinin or cortactin co-localize with the bulk of actin polymerized in the cell extensions (Dehio *et al.*, 1995; Tran Van Nhieu *et al.*, 1997). Remarkably, ezrin, a protein presumed to link the cytoskeleton to the cell membrane, is highly enriched in the tips of the protrusions where little F-actin is found (Skoudy *et al.*, 1999). Because of the complexity of the structures induced by *Shigella* during entry, it was suggested that Rho GTPases are required for *Shigella* invasion by governing distinct steps during the entry process, and that a fine coordination of the three GTPases is required for *Shigella* entry. Treatment of cells with a recombinant form of the exoenzyme C3, that specifically inactivates Rho by ADP-ribosylation, inhibited *Shigella* entry (Adam *et al.*, 1996; Watarai *et al.*, 1997). Dominant-interfering forms of Cdc42 and Rac also lead to significant inhibition of *Shigella* entry (Mounier *et al.*, 1999). A role for Rho in *Shigella* entry was unexpected because Rho is generally considered to determine the formation of stress fibres and focal adhesions, whereas Cdc42 and Rac determine actin polymerization leading to filopodia or ruffles, respectively, on the cell surface (Nobes and Hall, 1995).

Using a semi-permeabilized cell assay, we show here that among *Shigella* proteins secreted by the Mxi-Spa type III secretion apparatus, IpaC triggers actin polymerization and the formation of filopodial and lamellipodial extensions that are dependent on Cdc42 and Rac GTPases. We also present data that are consistent with IpaC being the major effector responsible for initial steps of actin polymerization during *Shigella* entry.

Results

Cytoskeletal rearrangements induced by *Shigella*-secreted proteins

To test if bacterial proteins that have access to the cell cytosol could induce cytoskeletal reorganization, a

permeabilized cell assay was developed. Swiss 3T3 cells were chosen because of their defined actin structures and, to limit the effects of serum on signalling induced by *Shigella* effectors, cells were starved of serum for 36 h prior to the assay. To minimize the loss of viability linked to permeabilization procedures, low concentrations of saponin were used to permeabilize cells in the presence of 2 mg/ml bovine serum albumin (BSA). Under the conditions used, the permeabilization had little effect on the cell morphology, and the cytoskeletal organization did not undergo significant changes after 20 min incubation (compare Figure 1A and B). Furthermore, when the detergent was washed away and the cells were allowed to reseal after the permeabilization procedure, a significant percentage of cells survived the treatment, as assessed by trypan blue exclusion or by the ability to grow as adherent cells after transfer to cell medium. No significant loss of cell viability was observed after incubation for up to 20 min in the permeabilization buffer, whereas cell viability decreased steadily with a longer incubation period, with 50% viability after 30 min incubation and 25% viability after 60 min. These results indicate that the permeabilization procedures did not lead to major alterations of cell-vital processes in the time frame used for the experiments.

Shigella strains that do not express the IpaB or IpaD proteins present a constitutively active secretion apparatus (Parsot *et al.*, 1995). When such strains are grown in standard bacterial culture medium, protein species that correspond to those that are normally secreted upon cell contact are released into the culture supernatant (Figure 2). To test if such proteins could have an effect on the cell cytoskeleton, supernatants of exponential cultures of *Shigella* strains were normalized relative to their amount of total proteins, and added to semi-confluent Swiss 3T3 cells that had been serum starved for 48 h. Cells were then incubated for 10 min at 37°C, fixed and processed for F-actin staining.

As shown in Figure 1B, cells that were subjected to serum starvation were well spread and typically presented a well-defined belt of cortical actin at their periphery, as well as actin cables spanning the cell body under the conditions used. When proteins from a supernatant of an *ipaB* or an *ipaD* mutant strain were added to cells in buffer without detergent permeabilization, no significant changes were observed (not shown). When cells were incubated with the *ipaB* mutant supernatant in the presence of traces of saponin during the incubation procedure, however, cells showed a drastic reorganization of the actin cytoskeleton. Actin polymerization and ruffle-like structures were detectable at the cell periphery (Figure 1C). In most instances, these cell extensions appeared to initiate at the level of edges that anchor the cells to the substrate, although such extensions could encompass important areas of the cell periphery. Similar results were obtained when cells were incubated with *Shigella* proteins from a supernatant of an *ipaD* mutant strain (Figure 1D), but not with samples that were incubated with supernatants from a plasmid-cured strain (not shown) or from a strain that was deleted for the *ipa* locus (Δipa) (Figure 1E). Incubation became cytotoxic after 30 min as many cells rounded up and showed retraction fibres (not shown). This cytotoxicity, however, did not appear to be specific for

Fig. 1. Effects of *Shigella* strain supernatants on permeabilized Swiss 3T3 cells. Semi-confluent Swiss 3T3 cells were grown in serum-free medium for 36 h before the assay. Cells were incubated in permeabilization buffer in the presence of *Shigella* supernatant (Materials and methods) for 10 min at 37°C. Samples were fixed, processed for F-actin fluorescence labelling and analysed by direct fluorescence microscopy. (A) Non-permeabilized cells. Cells that were permeabilized in the presence of (B) buffer alone; (C) supernatant of the *Shigella ipaB* mutant strain; (D) supernatant of the *Shigella ipaD* mutant strain; (E) supernatant of the *Shigella Δipa* mutant strain; and (F) supernatant of the *ipaB* strain incubated with the anti-IpaC mAb K24. Supernatants of *ipaB* and *ipaD* *Shigella* strains induce the formation of ruffle-like structures at the cell periphery, that are not induced by the supernatant of the Δipa mutant strain. Scale bar = 10 μ m.

products encoded by the virulence plasmid, as rounded cells were also observed after prolonged incubation with the supernatant from the plasmid-cured *Shigella* strain (not shown).

These results indicate that *Shigella* secreted proteins can reorganize the cell cytoskeleton specifically when cells are permeabilized, and that cytoskeletal reorganization was dependent on the *ipa* locus, but could occur in the absence of IpaB and IpaD.

***IpaC* induces actin polymerization and filopodia formation**

The *ipa* operon encodes four major secreted products, IpaA–D, which are involved in *Shigella* entry into epithelial cells. Previous work has indicated that IpaA was not required for the initial polymerization induced by

Shigella, but rather was involved in converting the initial cell extensions into a response productive for bacterial internalization (Tran Van Nhieu *et al.*, 1997). As the results obtained in the previous section indicated that neither IpaB nor IpaD was responsible for actin polymerization, we undertook to purify IpaC to test if its addition to cells was by itself sufficient to promote actin polymerization.

IpaC was purified from the supernatant of a *Shigella ipaB* mutant as described below (Materials and methods). Briefly, the supernatant of an exponentially growing culture of a *Shigella ipaB* mutant was concentrated and proteins were fractionated by anion-exchange, followed by cation-exchange chromatography in the presence of 0.1% NP-40. Detergent was then removed by incubation with Bio-Beads SM-2, and samples were dialysed in perme-

Fig. 2. (A) Organization of the *ipa* operon and *Shigella* mutant strains used in this study. The *ipaA–D* genes are organized in a single transcriptional unit which also contains *ipgC*, a gene encoding a chaperone for the *ipaB* and *ipaC* products (Ménard *et al.*, 1994b). *ipaB* (b), *ipaD* (d) and *ipaC* (c) mutants were generated by in-frame insertion of a non-polar cassette that confers resistance to kanamycin (Km) (Ménard *et al.*, 1993). The Δ *ipa* mutant strain (Δ) containing a deletion in the *ipa* locus was described previously (Parsot *et al.*, 1995). (B) SDS-PAGE analysis of *Shigella* strain supernatants and of purified IpaC. Culture supernatants of *Shigella* strains that were growing in mid-exponential phase were concentrated (Materials and methods) and resuspended directly in Laemmli loading buffer. IpaC was purified from the supernatant of an *ipaB* *Shigella* mutant after ammonium sulfate precipitation and anion-exchange followed by cation-exchange chromatography (Materials and methods). Samples were analysed by SDS-PAGE on a gel containing 15% polyacrylamide and Coomassie staining. Supernatant from: lane b, *ipaB* *Shigella* mutant strain; lane d, *ipaD* *Shigella* mutant strain; lane Δ , *Shigella* mutant strain containing a deletion in the *ipa* operon and that does not express IpaA–D; lane bs, non-invasive *Shigella* strain cured from the large virulence plasmid; and lane c, purified IpaC. The molecular weight markers are shown in the left lane. Proteins that are secreted by the Mxi–Spa apparatus are visible in lanes b, d and Δ . IpaC is indicated by the arrowhead.

abilization buffer. As shown in Figure 3, purified IpaC induced significant cytoskeletal reorganization at concentrations as low as 0.1 μ g/ml (0.5 nM). Many filopodia could be detected at the cell periphery (Figure 3B, arrow). Lamellipodia-like structures could also be observed at the cell periphery and, again, in most instances, these structures appeared at the levels of the cell edges contacting the substrate. Videomicroscopy indicated that these small lamellae forming at the edges appeared to derive from numerous filopodia that appeared within the first few minutes of incubation and filled in with lamellae (not shown). When the IpaC concentration was increased to 1 μ g/ml, cells showed similar lamellae, although these looked wider and often appeared at the leading edges of the cell (Figure 3C). Figure 3D shows a higher magnification of such a structure obtained by reconstruction of a confocal microscopy analysis. Numerous small filopodia connected by lamellipodial leaflets and, in some instances, membrane leaflets rising from the apical surface could be

observed (Figure 4D). Higher concentrations of IpaC resulted in cell rounding up and cytotoxicity (not shown).

To analyse if IpaC could induce actin rearrangements in a different cell assay, IpaC was microinjected in quiescent sub-confluent Swiss 3T3 cells that were serum starved for 24 h. Cells were rendered quiescent by prolonged growth at 37°C, after reaching confluence. Cells were then starved of serum for 20 h and plated onto a fibronectin-coated surface prior to microinjection (Nobes and Hall, 1995). As shown in Figure 4, cells treated under these conditions showed few actin structures (Figure 4). Microinjection of IpaC into these cells, however, resulted in the rapid formation of numerous small filopodial structures at the cell periphery, but also microspikes over the apical surface (Figure 4, arrow). The formation of these cellular extensions was also accompanied by an overall increase in actin polymerization, as judged by the fluorescence intensity levels of F-actin staining. In contrast to the permeabilized cell assays, however, few lamellipodial structures were observed, even after prolonged incubation.

These results indicate that IpaC is sufficient to promote actin polymerization and the formation of filopodial and lamellipodial extensions when introduced into the cell cytosol.

Anti-IpaC mAbs against the C-terminal moiety of IpaC inhibit IpaC-induced actin polymerization

To confirm further the role of IpaC in the cytoskeletal rearrangements, monoclonal antibodies (mAbs) recognizing various domains of IpaC were incubated with IpaC prior to addition to permeabilized cells. To test specifically the effects of mAbs on IpaC, purified mAbs were used, at concentrations ranging from 20 to 50 μ g/ml, that recognized epitopes located at the N-terminal region between residues 25 and 33 (J22), 90 and 97 (H8) or the C-terminal region between residues 297 and 349 (K24 and N9) (Phalipon *et al.*, 1992; Figure 5E). All antibodies were shown to associate with soluble IpaC by immunoprecipitation (not shown).

When the H8 anti-IpaC mAb was added to IpaC, IpaC still induced cellular extensions that were similar to those observed in the absence of mAb, or with an irrelevant mAb (not shown). Interestingly, addition of the J22 mAb resulted in a strikingly accentuated phenotype (Figure 5B and D). In this case, filopodial structures were longer and appeared more frequently at the cell periphery (Figure 5D, arrows). In addition, lamellipodial structures induced by the IpaC–J22 complex appeared much larger than those induced by IpaC alone (Figure 5C). In contrast, the anti-IpaC mAb K24 that recognized the C-terminal domain of IpaC inhibited IpaC-induced cytoskeletal rearrangements, as no significant differences were observed in cells that were treated with K24–IpaC (Figure 5A) or N9–IpaC complexes (not shown), when compared with buffer-treated samples (Figure 3A). To quantitate the effects of antibodies on extensions induced by IpaC, cells were scored for the presence of filopodial and lamellipodial structures (Materials and methods). As shown in Table I, when incubated with IpaC, the vast majority of cells showed the formation of extensions (Table I, IpaC), with 92% of the cells showing spikes and filopodial structures, and 85% of the cells showing lamellipodial structures. Similar percentages were obtained when cells were treated

Fig. 3. Effects of purified IpaC on permeabilized cells. Swiss 3T3 cells were starved for 36 h and permeabilized in the presence of IpaC purified from the supernatant of the *Shigella ipaB* mutant strain (Materials and methods). (A) Non-permeabilized cells. Cells permeabilized with IpaC at a final concentration of (B) 0.1 µg/ml, (C) and (D) 1 µg/ml. Samples were fixed and processed for fluorescence staining of F-actin. (D) Confocal analysis of a structure at the cell edge induced by incubation with IpaC. Scale bar = 5 µm in (A–C), and 1 µm in (D). IpaC induces the formation of filopodial (arrow) and lamellipodial structures.

with IpaC after pre-incubation with an irrelevant antibody (Table I, mAb) or with the anti-IpaC mAb J22 (Table I, J22), although in this latter case the lamellae were clearly larger than those induced by IpaC alone (compare Figure 5C with 3C). These extensions were specific for IpaC because cell treatment with the buffer alone did not induce significant filopodia or lamellipodia formation (Table I, buffer). When IpaC was pre-incubated with the K24 anti-IpaC mAb, however, a drastic inhibition of the effects of IpaC was observed, with 83% of the cells not showing detectable extensions and only 15% of the cells showing filopodial extensions (Table I, K24; Figure 2F).

These results indicate that the C-terminal domain of IpaC is required for cytoskeletal rearrangements, whereas the N-terminal domain regulates its activity.

Anti-IpaC mAbs inhibit *Shigella*-induced cytoskeletal rearrangements

Beads coated with IpaB and IpaC are internalized efficiently by cultured epithelial cells, pointing to IpaB and IpaC as being primary determinants of *Shigella* entry (Ménard *et al.*, 1996). Attempts to inhibit *Shigella* entry using a variety of antibodies against *Shigella* proteins including IpaB or IpaC, however, have been unsuccessful. This lack of inhibition could be explained if *Shigella* Ipa proteins are present on the bacterial surface in a configuration that is not recognized by the various antibodies used or, alternatively, because Ipa proteins are absent from the bacterial surface and translocate directly from the bacterial cytosol into the cell cytoplasm/

membrane. With this latter hypothesis, Ipa proteins would be hindered from blocking antibodies during the entry process. To determine whether *Shigella*-induced cytoskeletal rearrangements could be affected by antibodies that have free access to the cytosol, bacterial challenge was performed using HeLa cells that were permeabilized using the same procedure as that used for the Swiss 3T3 cells.

We first analysed the capacity of *Shigella* to induce cytoskeletal rearrangements on semi-permeabilized cells. Bacteria that were recovered in mid-exponential phase were washed in permeabilization buffer and added to cells for 15 min at 37°C. Samples were then processed for immunofluorescence staining of F-actin and bacterial lipopolysaccharide (LPS). As shown in Figure 6, *Shigella* challenge showed some cytotoxic effects under these conditions as many round cells could be observed (Figure 6A). Foci of actin polymerization similar to *Shigella* entry foci, however, could be detected readily in permeabilized cells (Figure 6A, arrowhead). In many instances, however, these foci did not show associated bacteria, suggesting that the permeabilization procedure weakened bacterial association with host cell membranes, or that soluble IpaC that was released upon cell contact could trigger foci formation. When quantitated, these foci occurred on an average of $29 \pm 5\%$ of the cells scored. Foci of actin polymerization could also be observed when the H8 (Figure 6B) or the J22 mAb was added to the bacteria during challenge (Figure 6C), with a frequency of $34 \pm 4\%$ and $22 \pm 8\%$, respectively. When cells were challenged with *Shigella* in the presence of the J22 mAb, however,

Fig. 4. Effects of microinjection of IpaC in serum-starved Swiss 3T3 cells. Quiescent Swiss 3T3 cells were starved for 20 h before replating onto fibronectin-coated coverslips. Cells were microinjected with IpaC at a final concentration of 1 µg/ml and FITC-dextran as a microinjection tracer. Samples were incubated for 20 min at 37°C, fixed and processed for fluorescence labelling of F-actin by incubation with rhodamine-coupled phalloidin. Samples were analysed by direct fluorescence microscopy. The arrows indicate IpaC-microinjected cells. Scale bar = 10 µm. Microinjection of IpaC induces the formation of numerous microspikes and filopodial structures.

foci of actin polymerization often appeared larger (Figure 6C, arrow) than those induced by *Shigella* alone (Figure 6A) or when incubated in the presence of the H8 mAb (Figure 6B). In contrast, when the K24 anti-IpaC mAb was added during bacterial challenge, very few foci of actin polymerization could be detected, with a frequency of ~5% of foci/cell. Also, many fewer round cells were observed upon challenge with *Shigella* in the presence of the K24 mAb (Figure 6D), suggesting that the K24 mAb protected cells from IpaC-induced cytotoxicity. Results similar to those obtained with the K24 mAb were obtained with the anti-IpaC mAb N9 (data not shown).

These results indicate that IpaC is required for *Shigella*-induced cytoskeletal reorganization upon interaction with host cells.

IpaC expression in HeLa cells results in increased bacterial internalization

To show further the effects of IpaC in *Shigella* entry, a 1.2 kb fragment containing the *ipaC* gene was amplified by PCR and cloned under the control of the cytomegalovirus (CMV) promoter in pCDNA3, a eukaryotic expression vector, to generate plasmid pCDNA.C (Materials and methods). HeLa cells were transfected with the pCDNA.C plasmid by electroporation, and stable transfectants were cloned from G418-resistant colonies (Materials and methods). To check for IpaC expression, transfectants were expanded to prepare lysates, and lysates were then tested by anti-IpaC Western blotting. Two transfectants

were selected for this study. As shown in Figure 7D, a 43 kDa band cross-reacting with the anti-IpaC mAb J22 could be detected in transfectants (Figure 7D, lanes a6 and d4). This band was absent in lysates prepared from cells transfected by the pCDNA.3 plasmid vector alone (Figure 7D, lane H). Out of 25 transfectants analysed, however, the IpaC-related product appeared as a band of moderate intensity (not shown), suggesting that IpaC was expressed at low levels in the various transfectants. Consistent with this, little IpaC could be detected in the stable transfectants by immunofluorescence using anti-IpaC mAbs (not shown). Interestingly, IpaC transfectants showed major morphological changes, with numerous filopodial extensions at the leading edges of the cells (Figure 7B, arrow) that were absent from parental cells (Figure 7A). These features were particularly striking in cells that were freshly replated. IpaC transfectants also showed fewer actin cables than parental cells (compare Figure 7A and B). Instead, the IpaC-expressing transfectants showed numerous thin actin filaments that ramified and formed an extended network throughout the cell body (Figure 7C, arrow).

IpaC-expressing transfectants were challenged with wild-type *Shigella* for 30 min at 37°C to allow for bacterial invasion, and the number of internalized bacteria was determined by the gentamicin protection assay as previously described (Isberg and Falkow, 1985). As shown in Figure 7E, the levels of *Shigella* entry in the two transfectants were increased by up to 5-fold compared

Fig. 5. Effects of anti-IpaC mAbs on IpaC-induced cytoskeletal reorganization. IpaC was incubated with anti-IpaC mAb for 30 min before adding to permeabilized cells. After incubation for 20 min at 37°C, samples were fixed and processed for fluorescence labelling of F-actin. Cells were challenged with IpaC pre-incubated with: (A) anti-IpaC mAb K24 and (B–D) anti-IpaC mAb J22. Scale bar = 10 µm in (A–C) and 2 µm in (D). The arrow points to filopodial structures induced by IpaC in the presence of the J22 mAb. (E) Epitopes of the anti-IpaC mAbs. The epitopes recognized by the anti-IpaC mAbs J22, H8 and K24 (Phalipon *et al.*, 1992) are indicated by hatched bars, and the residues at each extremity of the regions are indicated by numbers. The grey area represents a large hydrophobic segment comprised of residues 100–150 predicted by the Kyte and Doolittle program. The solid lines indicate helical regions predicted by the SOPM program (POL, Lyon, France). The anti-IpaC mAb K24 inhibits IpaC-induced cytoskeletal reorganization, whereas the J22 mAb stimulates the formation of lamellipodial structures induced by IpaC.

with parental cells (Figure 7E, compare a6 and d4, relative to HeLa). This increase in bacterial internalization, however, was specific for invasive *Shigella*, as the levels of internalization of the plasmid-cured derivative of *Shigella* or of a *Shigella* strain deficient for IpaC did not show a significant increase in the transfectants (not shown). To characterize further *Shigella* entry in the two IpaC transfectants, *Shigella*-induced foci of actin polymerization were analysed. *Shigella* expressing the AfaE adhesin were incubated with the cells at 22°C to allow bacterial attachment to the cell surface and to synchronize the entry process, and samples were transferred at 37°C for 13 min. Samples were then fixed and processed for double-immunofluorescence labelling of bacteria and F-actin

(Materials and methods). As shown in Figure 7F, the frequency of foci of actin polymerization per cell was ~3-fold higher in the a6 transfectant relative to parental cells after 15 min, with foci appearing as early as 5 min after bacterial challenge and, unlike the situation in parental cells, did not appear to be down-modulated after 30 min (Figure 8E, compare closed circles and open squares). The d4 transfectant did not show a significant increase in the frequency of *Shigella*-induced foci of actin polymerization after 15 min, but showed a trend similar to the a6 transfectant, with more foci after 5 and 30 min compared with parental cells (Figure 7F, compare closed and open squares).

Taken together, these results indicate that expression

Table I. Effects of anti-IpaC antibodies on IpaC-induced extensions

	Buffer	IpaC	+mAb	+J22	+K24
No effect	94 ± 31	NS	NS	1 ± 3	83 ± 8
Filopodia	10 ± 4	92 ± 10	95 ± 28	98 ± 26	15 ± 8
Lamellae	NS	85 ± 8	82 ± 20	77 ± 30 (large)	2 ± 4

Swiss 3T3 cells were permeabilized in the presence of: buffer, buffer alone; IpaC, IpaC; +mAb, IpaC incubated with an irrelevant mAb; +J22, IpaC incubated with the J22 mAb; and +K24, IpaC incubated with the K24 mAb. After incubation, samples were fixed and processed for fluorescence staining of F-actin (Materials and methods). Cells were scored for the presence of filopodial or lamellipodial extensions such as those shown in Figures 3 and 5. The results are expressed as the percentage of cells showing extensions in relation to the total number of cells scored. No effect, cell with no significant extensions; filopodia, cells showing at least 20 filopodial structures; lamellae, cells showing lamellipodial structures. NS, not significant.

of IpaC in HeLa cells promotes an increased uptake of *Shigella*.

Effects of dominant-interfering forms of Rho proteins on IpaC-induced cytoskeletal rearrangements

Previous work has shown that the small GTPase Rho was required for *Shigella* entry (Adam *et al.*, 1996; Watarai *et al.*, 1996). More recently, it was shown that Rho GTPases (Cdc42, Rac and Rho) were required for the entry process (Mounier *et al.*, 1999). As the data presented above suggested that IpaC was the primary effector of actin reorganization leading to *Shigella* entry, we set out to determine the role of these GTPases in IpaC-mediated actin reorganization. Dominant-interfering forms of Cdc42 (N17Cdc42) and Rac (N17Rac) were purified from GST fusions after expression in *Escherichia coli* as previously described (Self and Hall, 1995). A WASP-derived peptide,

Fig. 6. Effects of anti-IpaC mAbs on *Shigella*-induced cytoskeletal reorganization in permeabilized HeLa cells. HeLa cell monolayers were washed in serum-free medium 2 h before the assay and challenged with *Shigella* wild-type strain in the presence of anti-IpaC mAbs. After 10 min incubation at 37°C, samples were fixed and processed for double fluorescence labelling of bacterial LPS (green) and F-actin (red). Cells challenged with wild-type *Shigella* in: (A) permeabilization buffer alone; (B) permeabilization buffer in the presence of the H8 mAb; (C) permeabilization buffer in the presence of the J22 mAb; and (D) permeabilization buffer in the presence of the K24 mAb. The scale bar corresponds to 8 µm. The anti-IpaC mAb K24 inhibits foci of actin polymerization induced by *Shigella*.

Fig. 7. Analysis of IpaC-expressing transfectants of HeLa cells. HeLa cells were transfected with the pCDNA.C plasmid containing a copy of the *ipaC* gene (Materials and methods). Two stable transfectants were isolated and analysed by fluorescence labelling of F-actin, as well as for their ability to internalize *Shigella*. (A–C) Analysis of the actin cytoskeleton of HeLa cell transfectants. Cells were allowed to spread onto glass coverslips for 36 h, fixed and processed for fluorescence labelling of F-actin. (A) HeLa cells transfected with the pCDNA.3 vector alone. (B and C) HeLa cells transfected with pCDNA.C. Scale bar = 10 μ m. IpaC transfectants show filopodial structures at the cell periphery. Some large cells show a reorganization of actin cables into a cross-bundled network. (D) HeLa cell stable transfectants were expanded and whole lysates were analysed by anti-IpaC Western blotting. Lane H: cells transfected with pCDNA.3 alone; lanes a6 and d4, cells transfected with pCDNA.C. A band that cross-reacted with the anti-IpaC mAb is detected in the a6 and d4 transfectants. (E) Analysis of *Shigella* internalization by IpaC-expressing transfectants. Cells were plated 36 h prior to the assay in 24-well plates at a density of 2×10^4 cells/well in antibiotic-free medium. Cells were washed twice with serum-free medium and challenged with wild-type *Shigella* at an m.o.i. of 100:1 (bacteria/cell). After 30 min incubation at 37°C, samples were resuspended in medium containing gentamicin and incubated further for 30 min to kill extracellular bacteria. Cells were washed, lysed in sodium deoxycholate-containing PBS, and lysates were plated onto agar plates for determination of bacterial viable counts. The data are representative of three independent experiments, and each value corresponds to the average of determinations performed in triplicate. The bars indicate the standard deviation. IpaC-expressing transfectants internalized wild-type *Shigella* 3–5 times more efficiently than HeLa cells transfected with the control plasmid. (F) Analysis of *Shigella*-induced foci of actin polymerization. Semi-confluent cells were challenged with wild-type *Shigella* expressing the AfaE adhesin (Labigne-Roussel *et al.*, 1984). At various time points, samples were fixed and processed for fluorescence staining of bacterial LPS and F-actin. Foci of actin polymerization were scored visually and are expressed as the average number of foci per cell on the y-axis. The time in minutes is indicated on the x-axis. Foci scored in: HeLa cells transfected with the control plasmid pCDNA.3 (□); d4 transfectant (○); and a6 transfectant (■). The values are representative of two independent experiments performed in triplicate. At least 200 foci were scored per sample. The bars indicate the standard deviation. Foci of actin polymerization appear earlier and do not down-regulate as rapidly in IpaC transfectants compared with HeLa cells.

consisting of residues 201–321 of WASP, that blocks the effector domain of Cdc42 was also used (Aspenstrom *et al.*, 1996; Rudolph *et al.*, 1998). Purified proteins were then added to IpaC before addition to permeabilized cells, and samples were processed for F-actin staining.

As shown in Figure 8A, addition of N17Cdc42 resulted

in inhibition of filopodia and lamellipodia formation induced by IpaC (Figure 8A). Only 28% of the cells showed extensions, and no cells showed lamellipodia resembling those induced by IpaC (Table II, N17Cdc42). In some instances, a few patches of actin could be detected at the cell periphery (not shown), but these did not appear

Fig. 8. Effects of dominant-interfering forms of Cdc42 (N17Cdc42) and Rac (N17Rac), and the C3 exoenzyme on IpaC-induced cytoskeletal reorganization. Swiss 3T3 cells were permeabilized in the presence of: (A and C) IpaC and purified recombinant N17Cdc42; (B and D) IpaC and purified recombinant N17Rac; (E) IpaC and the C3 exoenzyme; and (F) the C3 exoenzyme alone. (A), (B), (E) and (F) are at the same magnification, and the scale bar in (F) corresponds to 5 μ m. (C) and (D) are at the same magnification, and the scale bar in (D) corresponds to 1 μ m. Dominant-interfering Cdc42 inhibits IpaC-induced cell extensions and associated membrane ruffling, whereas dominant-interfering Rac inhibits IpaC-induced lamellipodial structures. IpaC prevents disappearance of actin cables and cell retraction induced by C3 treatment.

Table II. Effects of dominant-interfering forms of Cdc42 and Rac on IpaC-induced extensions

	Buffer	IpaC	+N17Cdc42	+WASP-I	+N17Rac
No effect	94 $\pm$ 31	NS	71 $\pm$ 10	90 $\pm$ 7	27 $\pm$ 5
Filopodia	10 $\pm$ 4	92 $\pm$ 10	28 $\pm$ 7	9 $\pm$ 3	68 $\pm$ 11
Lamellipodia	NS	85 $\pm$ 8	NS	NS	3 $\pm$ 2

Swiss 3T3 cells were permeabilized in the presence of: buffer, buffer alone; IpaC, IpaC; +N17Cdc42, IpaC incubated with N17Cdc42; +WASP-I, IpaC incubated with the WASP-derived peptide containing the Cdc42-binding domain; and +N17Rac, IpaC incubated with N17Rac. After incubation, samples were fixed and processed for fluorescence staining of F-actin (Materials and methods). Cells were scored for the presence of filopodial or lamellipodial extensions such as those shown in Figure 3. The results are expressed as the percentage of cells showing extensions in relation to the total number of cells scored. No effect, cells with no significant extensions; filopodia, cells showing at least 20 filopodial structures; lamellipodia, cells showing lamellipodial structures. NS, not significant.

to extend into filopodial structures. The inhibitory effects were even more pronounced when the Cdc42-binding domain of WASP was added to IpaC during the incubation, as only 8% of the cells scored showed filopodial extensions with no lamellipodial extensions (Table II, WASP-I). Although both samples treated with IpaC in the presence

of the dominant-interfering form of Cdc42 or the WASP-derived peptide showed a marked inhibition of the IpaC-induced extensions, a reorganization of actin cables could be observed when compared with cells treated with buffer alone (Figure 8A). In cells treated with IpaC and dominant-interfering forms of Cdc42, long stress fibres that spanned the cell length disappeared; instead, shorter and thinner actin cables could be observed that often branched to form a network throughout the cell body (Figure 8A).

When the dominant-interfering form of Rac was added with IpaC, filopodial extensions were still visible that often appeared at the cell leading edges but did not fill in to form leaflets (Figure 8B and D). These extensions were longer, though less numerous than those observed when cells were treated with IpaC alone, as between 10 and 30 filopodial extensions per cell could be observed in cells treated with IpaC in the presence of N17 Rac, whereas cells treated with IpaC alone showed >100 filopodia on average. Very few lamellipodial structures such as those induced by IpaC could be observed, as only 3% of the cells scored showed such structures.

To test for the role of Rho in the formation of IpaC-induced extensions, cells were permeabilized in the presence of the C3 exoenzyme that inactivates Rho by ADP ribosylation at Asn61. When C3 was added to the cells at

a concentration of 70 µg/ml, the majority of cells retracted and showed a disappearance of F-actin structures (Figure 8F). This effect was not observed when cells were incubated in the presence of C3 without permeabilization, but could be detected as early as 10 min after incubation with C3 under permeabilization conditions. When IpaC was added in combination with C3, however, no lamellipodial structures such as those induced by IpaC alone could be observed, although extensions that contained little F-actin were visible at the cell periphery (Figure 8E, arrow). A striking feature of cells simultaneously treated by IpaC and C3 was the absence of retraction associated with C3 treatment (compare Figure 8E and F).

These results indicated that the formation of IpaC-induced lamellipodial extensions was dependent on Cdc42 and Rac. IpaC-induced filopodial extensions were also dependent on Cdc42, and appeared partly dependent on Rac.

Discussion

Many Gram-negative bacteria express a type III secretion apparatus that allows translocation of bacterial products from the bacterium to the host cell cytosol upon cell contact (Hueck, 1998). In the case of enteroinvasive *Shigella*, bacterial products that are secreted by the Mxi-Spa apparatus are responsible for actin polymerization and reorganization of actin filaments required to promote entry into the host cell. In this report, we have developed a permeabilized cell assay to analyse the effects on the host cell cytosol induced by *Shigella* products that are secreted by the type III secretion apparatus. Because, under the conditions used and in contrast to other previously described permeabilization techniques (MacKay *et al.*, 1997), cells appear to survive the permeabilization treatment, this type of assay could be used for the study of bacterial effectors that affect longer term cell responses and that are not perturbed by the transient incubation with low levels of saponin. The advantage of such an assay over standard microinjection techniques lies in the possibility of testing various samples rapidly, and of detecting the effects on most cells for a given sample. We found that the effects of IpaC on the cell cytoskeleton were best visualized when cells were incubated in serum-free medium for 36–48 h prior to permeabilization. When cells that were grown in serum-containing medium were permeabilized in the presence of IpaC, fewer filopodial and lamellipodial structures could be detected (not shown). It is possible that soluble factors present in the serum interfere with IpaC-induced responses. Such antagonistic effects of the serum may have altered the cell responses obtained when recombinant IpaC was added to HeLa cells (Marquart *et al.*, 1996). Using this assay, we identify IpaC as a direct effector of *Shigella*-induced cytoskeletal reorganization. Purified IpaC was shown to induce actin polymerization and the formation of filopodial and lamellipodial extensions that were dependent on the Cdc42 and Rac GTPases.

We show here that filopodial and lamellipodial extensions are induced when IpaC has access to the cell cytosol of Swiss 3T3 or HeLa cells. Furthermore, antibodies recognizing the C-terminal region of IpaC between residues 297 and 349 blocked IpaC-induced extensions. These data argue that the C-terminal domain of IpaC

needs to interact with components present in the cell cytosol in order to promote actin polymerization. IpaC was shown to have a propensity to interact with lipid bilayers (De Geyter *et al.*, 1997). Analysis of the IpaC secondary structure predicts an α -helical hydrophobic segment that could correspond to two transmembrane domains located approximately between residues 100 and 150. Such a structure would be consistent with the existence of two domains: one located at the N-terminal part that would contain the epitope for the J22 mAb, and another α -helical C-terminal domain containing the epitope for the K24 and N9 mAbs, this latter domain being responsible for the polymerization of actin. The fact that IpaC complexed with the J22 mAb that recognizes the region between residues 25 and 33 of IpaC induces the formation of lamellipodia that are much larger than those induced by IpaC alone suggests that this N-terminal domain of IpaC negatively regulates the actin polymerization activity associated with the C-terminal domain.

Expression of IpaC in HeLa cells leads to the formation of filopodia and lamellipodia, along with a significant reorganization of the cell cytoskeleton into a network of branched actin cables. Consistent with a role in *Shigella* entry, expression of IpaC in HeLa cells leads to a significant increase in *Shigella* internalization. This increase, however, was specific for the wild-type *Shigella* strain, as no increase in internalization was observed for the non-invasive strain of *Shigella* in which the *ipaC* gene was inactivated. These results are not surprising, because full complementation of the entry defect would require IpaC, when expressed by the cell, to achieve the proper localization and concentration at the site of bacterial entry. This is unlikely to be the case in the IpaC transfectants for which low levels of IpaC expression already lead to significant alterations of the cytoskeleton. Moreover, during *Shigella* entry, the formation of the entry structure induced by the bacterium requires a coordination between several bacterial effectors (Tran Van Nhieu and Sansonetti, 1999). Alteration of the balance between these different *Shigella* effectors could perturb the formation of the entry structure. Finally, alterations of signalling pathways induced by *Shigella* effectors can also interfere with the formation of the *Shigella* entry structure. Although Rho proteins and the Src kinase are required for *Shigella* entry, overexpression of constitutively active forms of Rho GTPases or of the Src kinase interfere with *Shigella*-induced foci of actin polymerization (Duménil *et al.*, 1998; G.Duménil, P.J.Sansonetti and G.Tran Van Nhieu, submitted). This suggests that the levels of activation of Rho proteins and of Src are tightly controlled during the *Shigella* entry process. Remarkably, cells that express IpaC induce foci of actin polymerization more rapidly and in a more sustained manner than parental cells, when challenged with *Shigella*. This argues for a role for IpaC in the nucleation of foci induced by *Shigella*. During *Shigella* entry, only a small fraction of the bacteria elicit cytoskeletal rearrangements that lead to internalization (Mounier *et al.*, 1997). It is possible that IpaC expression in host cells lowers the critical concentration of IpaC secreted by *Shigella* that is required for the induction of actin polymerization foci.

Addition of IpaC to permeabilized cells or microinjection of IpaC into intact cells induces filopodial and

lamellipodial extensions, which appear within minutes. These kinetics are consistent with activation of the endogenous Cdc42 and Rac GTPases that have been shown to govern the formation of such extensions upon stimulation with various agonists. In Swiss 3T3 cells, Cdc42 was shown to control the formation of filopodia, whereas Rac controls the formation of lamellipodia (Hall, 1998). Also, activation of Cdc42 leads to rapid activation of Rac, thus allowing the conversion of the filopodial structures into lamellipodia (Nobes and Hall, 1995). IpaC-induced extensions are inhibited by dominant-interfering forms of Cdc42 or Rac, indicating that IpaC is sufficient to up-regulate these GTPases. The fact that the formation of IpaC-induced filopodial and lamellipodial extensions is inhibited completely by dominant-interfering Cdc42, whereas the formation of lamellipodial extensions is inhibited by dominant-interfering Rac, suggests that the effects of IpaC on the cytoskeleton are most likely to be mediated after activation of Cdc42. Specific inhibition of Rho by the C3 exoenzyme, however, led to inhibition of massive actin polymerization at the site of *Shigella* entry but did not prevent the polymerization of actin in the close vicinity of the bacterium interacting with the cell membrane (Adam *et al.*, 1996). Thus, the role of Rho in *Shigella*-induced actin polymerization appears less direct than that of Cdc42 and Rac. It is possible that during *Shigella* entry other bacterial effectors such as IpaA regulate Rho-dependent responses (Tran Van Nhieu *et al.*, 1997).

Several lines of evidence are consistent with IpaC being the main *Shigella* effector responsible for filopodial extensions during invasion of epithelial cells. First, although *Shigella ipaC* mutants are not impaired for *in vitro* secretion via the Mxi-Spa apparatus (Parsot *et al.*, 1995), these mutants are unable to induce foci of actin polymerization and are completely defective for entry (Ménard *et al.*, 1993). Also, the inhibitory effects of the dominant-interfering forms of Cdc42 and Rac on cell extensions induced by purified IpaC correlate with the implication of a role for these Rho GTPases in the *Shigella* entry process. Expression of dominant-interfering forms of either Cdc42 or Rac in HeLa cells led to a significant inhibition of *Shigella*-induced foci of actin polymerization (Mounier *et al.*, 1999), indicating that both of these GTPases are implicated in the entry process. Furthermore, antibodies directed against the C-terminal domain of IpaC inhibited *Shigella*-induced foci of actin polymerization. How IpaC up-regulates Cdc42 and Rac remains to be determined. In the case of the enteroinvasive bacterium *Salmonella typhimurium*, the bacterial protein SopE was shown to translocate into the cell cytosol during entry and to act as an exchange factor for Cdc42 and Rac (Hardt, 1998). Activation of Cdc42 and Rac linked to loading of GTP on these GTPases by SopE induces actin polymerization and the formation of ruffles associated with entry of *Salmonella* into epithelial cells, although it is likely that other bacterial factors also participate in this process (Hardt, 1998). Although the entry processes of *Salmonella* and *Shigella* share some morphological similarities, as well as similarities at the level of their type III secretion apparatus, a homologue of SopE does not appear to exist in *Shigella*. We were not able to detect any *in vitro* exchange factor activity on Cdc42 or Rac associated with IpaC (unpublished), suggesting that the mechanism that

leads to activation of these GTPases by IpaC differs from that of SopE.

Materials and methods

Bacterial strains, cell lines, antibodies and reagents

M90T was used as *Shigella* wild-type strain serotype V (Sansone *et al.*, 1982). *Shigella ipaB* and *ipaD* mutant strains carry a non-polar insertion of a kanamycin resistance cassette in the *ipaB* and *ipaD* genes, respectively (Ménard *et al.*, 1993). The *Shigella Δipa* mutant strain bears a deletion of the *ipa* locus (Parsot *et al.*, 1995). All three mutants, *ipaB*, *ipaD* and *Δipa*, were shown constitutively to secrete proteins that are translocated by the Mxi-Spa apparatus during *in vitro* growth (Parsot *et al.*, 1995). Bacterial strains were grown routinely in trypticase soy broth at 37°C with constant stirring. HeLa cells, an epithelial cell line from human larynx carcinoma, was from ATCC. Swiss 3T3 cells, a fibroblastic cell line, were used between passages 8 and 11. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) in a 37°C incubator supplemented with 10% CO₂. Anti-IpaC monoclonal antibodies and the anti-*Shigella* LPS antiserum have been described previously (Phalipon *et al.*, 1992). Rhodamine-coupled phalloidin was from Sigma. Fluorescein isothiocyanate (FITC)- and rhodamine-coupled secondary antibodies were from Amersham.

Plasmids

Plasmids pGEX-N17Cdc42 and pGEX-N17Rac expressing N17Cdc42 and N17Rac fused to GST, respectively, were described previously (Self and Hall, 1995). To obtain plasmid pCDNA.C, the *ipaC* gene was amplified by PCR using the following primers: 5'-CCGGAAGCTTGC-AACAACTACTGCTTGA-3' for the 5' end and 5'-GCGCTCTAGAGGAAGAGCCATATAT-3' for the 3' end. The PCR product was cloned into plasmid pCDNA.3 after digestion with *Hind*III and *Xba*I. IpaC expression was detected by Western blot using anti-IpaC antibody after transformation of pCDNA.C in *E. coli* JM109(DE3) [*recA supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)*] F'[*traD36 proAB⁺ lac^I lacZΔM15*] [*λcIts857 ind1 Sam7 nin5 lacUV5*-gene 1 of T7] and induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Purification of fusion proteins

GST fusion proteins were purified from *E. coli* lysates on glutathione-Sepharose 4B beads (Pharmacia), as described previously (Self and Hall, 1995). The GST moiety was eliminated after cleavage by thrombin, and thrombin was eliminated by incubation with *p*-aminobenzylamide-coated beads (Self and Hall, 1995).

Preparation of supernatant of *Shigella* strains and IpaC purification

To prepare supernatant from *Shigella* strains, bacterial cultures were inoculated from an overnight culture using a 1:100 dilution, and grown in trypticase soy broth until mid-logarithmic phase (OD₆₀₀ = 0.6). Bacteria were pelleted by centrifugation for 15 min at 5000 g, and the supernatant was filtered through a 0.22 μm filter (Falcon). The supernatant was then concentrated 10 times using a Centricon C10 concentrator (Amicon), and dialysed against U buffer. Concentrated supernatant was stored as frozen aliquots at -80°C and thawed before use.

IpaC was purified from the supernatant of the *Shigella ipaB* mutant strain that constitutively secretes Ipa proteins. The supernatant was precipitated by addition of ammonium sulfate at 55% final concentration. The ammonium sulfate cut pellet was resuspended in 20 mM Tris pH 7.5, 20 mM NaCl, 1 mM EDTA, containing 1 mM AEBSF and 0.1% NP-40, and dialysed extensively against the same buffer to remove the ammonium sulfate. The dialysed sample was loaded onto MonoQ Sepharose. This step allowed absorption of most proteins present in the supernatant. The flow-through was found to consist of three main protein species: a 120 kDa species that corresponded to SepA, an as yet unidentified 45 kDa species and a 43 kDa species corresponding to IpaC. The flow-through obtained after anion-exchange chromatography was dialysed against 25 mM HEPES pH 7.5, 25 mM NaCl, containing 1 mM AEBSF and 0.1% NP-40, and loaded onto a MonoS cation-exchange column. Proteins were eluted using a linear gradient ranging from 25 to 450 mM NaCl. IpaC typically eluted early as a homogenous peak at ~100 mM NaCl.

IpaC-containing fractions were pooled and concentrated using a C10 Amicon concentrator and the buffer was exchanged for U buffer. To

remove excess NP-40, samples were incubated with Bio-Beads SM-2 (Bio-Rad) according to the manufacturer's instructions. Samples were stored as frozen aliquots at -80°C and thawed as required before use.

Cell permeabilization

Swiss 3T3 cells were used between passages 8 and 12 and routinely grown in DMEM containing 10% FCS in a 37°C incubator supplemented with 10% CO_2 . Cells were plated onto 13 mm diameter coverslips at a density of 10^4 cells/coverslip. The following day, cells were washed once in DMEM and incubated in serum-free medium for another 36–48 h. Variations in the duration of the periods of serum starvation ranging from 36 to 48 h did not markedly influence the behaviour of the cells. Cells were then washed twice with U buffer prior to permeabilization. To permeabilize the cells, coverslips were inverted on a piece of parafilm onto which was deposited 30 μl of permeabilization buffer consisting of U buffer supplemented with 1 mM ATP, 100 μM GTP and 100 μM UTP, and containing saponin at a final concentration of 0.002% and the appropriate protein. Samples were then incubated at 37°C in a humid chamber for 20 min. After incubation, samples were fixed in phosphate-buffered saline (PBS) containing 3.7% paraformaldehyde for 15 min at 22°C , and processed for immunofluorescence staining of F-actin.

To test for the effects of anti-IpaC mAbs or dominant-interfering forms of Cdc42 or Rac on IpaC-induced cell extensions, IpaC was incubated at a concentration of 0.1 $\mu\text{g}/\text{ml}$ in permeabilization buffer with the mAbs at a final concentration of 100 $\mu\text{g}/\text{ml}$, or with a final concentration of 200 $\mu\text{g}/\text{ml}$ of purified N17Cdc42, N17Rac or the WASP-1 peptide, for 15 min prior to adding to the cells. With the exception of IpaC, each of these proteins under these conditions did not show any significant effects on the cell cytoskeleton in the permeabilized cell assay. Purified C3 was a kind gift from Michel R. Popoff (Institut Pasteur, Paris, France) and was used at a concentration of 70 $\mu\text{g}/\text{ml}$. Lower concentrations of C3, under the kinetics of the permeabilization assay, only led to moderate effects on the cells, with no obvious changes on the cell morphology and partial disappearance of actin stress fibres (not shown).

Cell microinjection

Quiescent Swiss 3T3 cells were serum starved and microinjected as described previously (Nobes and Hall, 1995). Cells were microinjected using a Narishige 200 microinjection device (Narishige USA, Inc.) and an IMT-2 inverted microscope (Olympus Co., Japan). An average of 100 cells were microinjected per sample, and each experiment was repeated at least three times.

Immunofluorescence analysis

Samples were fixed in PBS containing 3.7% paraformaldehyde for 15 min at 22°C . Cells were washed twice in PBS and permeabilized in PBS containing 0.1% Triton X-100 for 4 min, and washed three times with PBS. Samples were stained for F-actin using rhodamine-coupled phalloidin (Sigma, St Louis, MO), and coverslips were mounted onto slides in 80% glycerol and 10 mg/ml DABCO (Sigma). Samples were analysed using a conventional fluorescence microscope (BH2 RFCA, Olympus Optical Co., Ltd), or with a confocal laser scanning microscope (Zeiss Axiophot).

HeLa cell transfection and IpaC expression analysis

HeLa cells from ATCC were used at passage 5 and transfected by electroporation using a Gene-pulser II apparatus (Bio-Rad Laboratories), using a voltage of 450 V and a capacitance of 960 μF . Cells were transfected with the pCDNA.C or pCDNA.3 plasmids. After 48 h, cells were transferred to DMEM containing 10% FCS and 0.5 mg/ml G418 (Sigma). After 10 days incubation, individual cell colonies were cloned by the cloning ring technique and expanded. A total of 5×10^5 cells were scraped from tissue culture dishes and resuspended directly in Laemmli sample buffer. Samples were subjected to Western blot analysis using anti-IpaC antibody. Filters were probed with the J22 anti-IpaC mAb at a final concentration of 10 $\mu\text{g}/\text{ml}$, followed by anti-mouse antibody coupled to horseradish peroxidase. Samples were processed for chemiluminescence detection using the ECL kit (Amersham Co). When stable transfectants expressing IpaC were tested by immunofluorescence analysis, a weak and diffuse staining was observed which did not appear to label specific cell structures (not shown).

Bacterial invasion assay and analysis of *Shigella*-induced foci of actin polymerization

Bacterial invasion of HeLa cell transfectants was tested using the gentamicin protection assay as described previously (Isberg and Falkow,

1985). Briefly, cells were plated 36 h before the assay in a 24-well plate at a density of 2×10^4 cells/well in antibiotic-free DMEM containing 10% FCS. Cells were washed in serum-free medium and incubated in serum-free medium for 2 h prior to bacterial challenge. Cells were challenged with *Shigella* strains at a multiplicity of infection (m.o.i.) of 100:1 for 30 min at 37°C . Samples were then resuspended in DMEM containing 10% FCS and gentamicin at a final concentration of 25 $\mu\text{g}/\text{ml}$ and incubated for another 30 min at 37°C to kill extracellular bacteria. Samples were then washed three times in PBS and cells were lysed in PBS containing 0.2% sodium deoxycholate. Lysates were plated onto agar plates and the number of internalized bacteria was determined by counting the colony-forming units after overnight incubation at 37°C . Bacterial internalization was expressed as the ratio of internalized bacteria to the initial input of bacteria (Isberg and Falkow, 1985). Each sample determination was performed in triplicate, and experiments were repeated at least three times.

To analyse *Shigella*-induced cytoskeletal rearrangements, cells were plated on 13 mm diameter coverslips 30 h before the assay at a density of 2×10^4 cells/coverslip in antibiotic-free medium. Cells were washed with serum-free medium 2 h before the assay, and samples were incubated at 22°C with wild-type *Shigella* strain M90T expressing the AfaE adhesin for 10 min to synchronize the invasion process (Labigne-Roussel *et al.*, 1984) at an m.o.i. of 50:1. Samples were shifted to 37°C and, at various time points, samples were fixed with paraformaldehyde and processed for fluorescence staining of F-actin and bacterial LPS. Samples were analysed by direct immunofluorescence microscopy. At least 200 foci of actin polymerization were scored visually on 10 fields from three independent experiments.

Analysis of *Shigella*-induced foci of actin of polymerization in permeabilized cells

HeLa cells were plated 48 h before the experiment onto 13 mm diameter coverslips. At 2 h prior to bacterial challenge, cells were washed and incubated in serum-free medium. Cells were then washed twice in UB buffer and incubated with the bacterial suspension in permeabilization buffer. To prepare bacteria, *Shigella* strains were grown to mid-exponential phase and washed in UB buffer. Bacteria were resuspended at a density of 10^8 bacteria/ml in permeabilization buffer containing mAb at a final concentration of 20–50 $\mu\text{g}/\text{ml}$, immediately prior to adding to HeLa cells. After 10 min, samples were fixed with paraformaldehyde and processed for immunofluorescence labelling. Bacteria were visualized by probing with anti-LPS antiserum (Tran Van Nhieu *et al.*, 1997) at a dilution of 1:500, followed by anti-rabbit antibody coupled to FITC. F-actin was visualized by rhodamine-coupled phalloidin.

Acknowledgements

We would like to thank Michel R. Popoff for helpful discussions and for the gift of the C3 exoenzyme. We thank Armelle Phalipon for kindly providing us with anti-IpaC mAbs, and Claude Parsot for the gift of *Shigella* mutant strains. We thank Kate Nobes for the gift of the WASP derivative. The confocal microscope was purchased with a donation from Marcel and Liliane Pollack. This work was supported by a grant from the 'DRET' (Direction des Recherches et Techniques) No. 94092. E.C. and A.H. were supported by a Cancer Research Campaign programme grant.

References

- Adam, T., Arpin, M., Prévost, M.C., Gounon, P. and Sansonetti, P.J. (1995) Cytoskeletal rearrangements and the functional role of T-plastin during entry of *Shigella flexneri* into HeLa cells. *J. Cell Biol.*, **129**, 367–381.
- Adam, T., Giry, M., Boquet, P. and Sansonetti, P. (1996) Rho-dependent membrane folding causes *Shigella* entry into epithelial cells. *EMBO J.*, **15**, 3315–3321.
- Aspenstrom, P., Lindberg, U. and Hall, A. (1996) Two GTPases, Cdc42 and Rac, bind directly to a protein implicated in the immunodeficiency disorder Wiskott–Aldrich syndrome. *Curr. Biol.*, **6**, 70–75.
- De Geyer, C. *et al.* (1997) Purification of IpaC, a protein involved in entry of *Shigella flexneri* into epithelial cells and characterization of its interaction with lipid membranes. *FEBS Lett.*, **400**, 149–154.
- Dehio, C., Prévost, M.C. and Sansonetti, P.J. (1995) Invasion of epithelial cells by *Shigella flexneri* induces tyrosine phosphorylation of cortactin by a pp60^{src} mediated signalling pathway. *EMBO J.*, **14**, 2471–2482.
- Duménil, G., Olivo, J.-C., Pellegrini, S., Fellous, M., Sansonetti, P.J. and Tran Van Nhieu, G. (1998) Interferon- α inhibits a Src-mediated

- pathway necessary for *Shigella*-induced cytoskeletal rearrangements during entry into epithelial cells. *J. Cell Biol.*, **143**, 1003–1012.
- Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science*, **279**, 509–514.
- Hardt, W.-D. (1998) *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell*, **93**, 815–826.
- Hueck, C.J. (1998) Type III secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.*, **62**, 379–433.
- Isberg, R.R. and Falkow, S. (1985) A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K-12. *Nature*, **317**, 262–264.
- Labigne-Roussel, A.F., Lark, L., Schoolnik, G. and Falkow, S. (1984) Cloning and expression of an afimbrial adhesin (AFA) responsible for P blood group-independent mannose-resistant hemagglutination from a pyelonephritic *E. coli* strain. *Infect. Immun.*, **46**, 251–259.
- MacKay, D.J., Esch, F., Furthmayr, H. and Hall, A. (1997) Rho- and rac-dependent assembly of focal adhesion complexes and actin filaments in permeabilized fibroblasts: an essential role for ezrin/radixin/moesin proteins. *J. Cell Biol.*, **138**, 927–938.
- Marquart, M.E., Picking, W.L. and Picking, W.D. (1996) Soluble invasion plasmid antigen C (IpaC) from *Shigella flexneri* elicits epithelial cell responses related to pathogen invasion. *Infect. Immun.*, **64**, 4182–4187.
- Ménard, R., Sansonetti, P.J. and Parsot, C. (1993) Non polar mutagenesis of the *ipa* gene defines IpaB, IpaC and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J. Bacteriol.*, **175**, 5899–5906.
- Ménard, R., Sansonetti, P.J. and Parsot, C. (1994a) The secretion of the *Shigella flexneri* Ipa invasins is induced by the epithelial cell and controlled by IpaB and IpaD. *EMBO J.*, **13**, 5293–5302.
- Ménard, R., Sansonetti, P.J., Parsot, C. and Vasselon, T. (1994b) Extracellular association and cytoplasmic partitioning of the IpaB and IpaC invasins of *Shigella flexneri*. *Cell*, **79**, 515–525.
- Ménard, R., Prévost, M.C., Gounon, P., Sansonetti, P.J. and Dehio, C. (1996) The secreted Ipa complex of *Shigella flexneri* promotes entry into mammalian cells. *Proc. Natl Acad. Sci. USA*, **93**, 1254–1258.
- Mounier, J., Bahrani, F. and Sansonetti, P.J. (1997) Secretion of *Shigella flexneri* Ipa invasins on contact with epithelial cells and subsequent entry of the bacterium into cells are growth stage dependent. *Infect. Immun.*, **65**, 774–782.
- Mounier, J., Laurent, V., Hall, A., Fort, P., Carlier, M.-F., Sansonetti, P.J. and Egile, C. (1999) Rho family GTPases control entry of *Shigella flexneri* into epithelial cells but not intracellular motility. *J. Cell Sci.*, in press.
- Nobes, C.D. and Hall, A. (1995) Rho, Rac and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia and filopodia. *Cell*, **81**, 53–62.
- Parsot, C. and Sansonetti, P.J. (1996) Invasion and the pathogenesis of *Shigella* infections. In Miller, V.L. (ed.), *Bacterial Invasiveness*. Springer Verlag, pp. 25–42.
- Parsot, C., Ménard, R., Gounon, P. and Sansonetti, P.J. (1995) Enhanced secretion through the *Shigella flexneri* Mxi-Spa translocon leads to assembly of extracellular proteins into macromolecular structures. *Mol. Microbiol.*, **16**, 291–300.
- Phalipon, A., Arondel, J., Nato, F., Rouyre, S., Mazie, J.C. and Sansonetti, P.J. (1992) Identification and characterization of B-cell epitopes of IpaC, an invasion-associated protein of *Shigella flexneri*. *Infect. Immun.*, **60**, 1919–1926.
- Rudolph, M.G., Bayer, P., Abo, A., Kuhlmann, J., Vetter, I.R. and Wittinghofer, A. (1998) The Cdc42/Rac interactive binding region motif of the Wiskott-Aldrich syndrome protein (WASP) is necessary but not sufficient for tight binding to Cdc42 and structure formation. *J. Biol. Chem.*, **273**, 18067–18076.
- Sansonetti, P. (1998) Molecular and cellular mechanisms of invasion of the intestinal barrier by enteric pathogens. The paradigm of *Shigella*. *Folia Microbiol.*, **43**, 239–246.
- Sansonetti, P.J., Kopecko, D.J. and Formal, S.B. (1982) Involvement of a plasmid in the invasive ability of *Shigella flexneri*. *Infect. Immun.*, **35**, 852–860.
- Self, A.J. and Hall, A. (1995) Purification of recombinant Rho/Rac/G25K from *Escherichia coli*. *Methods Enzymol.*, **256**, 3–10.
- Skoudy, A., Tran Van Nhieu, G., Mantis, N., Arpin, M., Gounon, P. and Sansonetti, P.J. (1999) A functional role for ezrin during *Shigella flexneri* entry into epithelial cells. *J. Cell Sci.*, in press.
- Tran Van Nhieu, G. and Sansonetti, P.J. (1999) Mechanism of *Shigella* entry into epithelial cells. *Curr. Opin. Microbiol.*, **2**, 51–55.
- Tran Van Nhieu, G., Ben Ze'ev, A. and Sansonetti, P.J. (1997) Modulation of bacterial entry into epithelial cells by interaction between vinculin and the *Shigella* IpaA invasin. *EMBO J.*, **16**, 2717–2729.
- Watarai, M., Funato, S. and Sasakawa, C. (1996) Interaction of Ipa proteins of *Shigella flexneri* with $\alpha_5\beta_1$ integrin promotes entry of the bacteria into mammalian cells. *J. Exp. Med.*, **183**, 991–999.
- Watarai, M., Kamata, Y., Kozaki, S. and Sasakawa, C. (1997) Rho, a small GTP-binding protein, is essential for *Shigella* invasion of epithelial cells. *J. Exp. Med.*, **185**, 281–292.

Received March 23, 1999; revised and accepted April 20, 1999