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Lack of Cleavage of IcsA in *Shigella flexneri* Causes Aberrant Movement and Allows Demonstration of a Cross-Reactive Eukaryotic Protein

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Once in the cytoplasm of mammalian cells, *Shigella flexneri* expresses a motile phenotype caused by polar directional assembly of actin. This process depends on accumulation of IcsA (VirG), a 120-kDa protein with ATPase activity, at the pole of the bacterium opposite to that at which ongoing septation occurs. IcsA is also secreted into the bacterial supernatant as a 95-kDa species, after cleavage at an SSRRASS sequence which, when mutagenized, blocks processing. MAbF15, an anti-IcsA monoclonal antibody, recognizes an epitope located within repeated Gly-rich boxes in the N-terminal half of the protein. We used this monoclonal antibody to visualize the location of a noncleavable 120-kDa IcsA mutant protein expressed in *S. flexneri*. We found that this noncleavable IcsA protein no longer localized exclusively to the pole of the bacterium but also could be detected circumferentially. Whereas the monoclonal antibody detected the wild-type cleavable form of IcsA in only 40% of the cells expressing this protein, the noncleavable form was easily detectable in all the cells carrying the *icsA* mutant allele. Similar aberrant localization of the IcsA mutant protein on bacteria growing within the cytoplasm of HeLa cells was observed. The strains expressing the noncleavable IcsA protein expressed abnormal intracellular movement and were often observed moving in a direction perpendicular to their longitudinal axis. The putative protease which processes IcsA may therefore play a role in achieving polar expression of this protein and providing maximum asymmetry essential to directional movement. In addition, MAbF15 allowed us to identify a 70-kDa eukaryotic protein cross-reacting with IcsA. This protein accumulated in the actin tails of motile bacteria and in membrane ruffles of the cells.

Shigella flexneri is a gram-negative bacillus belonging to the family *Enterobacteriaceae* which causes bacillary dysentery in humans by invading epithelial cells of the colonic mucosa (18). A major effort has been made to understand the molecular and cellular bases of epithelial cell invasion by this pathogen, as recently reviewed (12, 27). Most of the genes responsible for invasion of eukaryotic cells are located on a 220-kb plasmid in *S. flexneri* (31). Three major steps can be recognized. (i) Entry into cells occurs via a bacterium-directed macropinocytic process involving the formation of localized membrane ruffles due to massive rearrangements of the host cell cytoskeleton (1, 9). The *Shigella* entry genes have been located on a 30-kb locus of the virulence plasmid (5, 22, 34, 35). This locus includes the *ipa* operon encoding the secreted invasins (i.e., IpaB, IpaC, and IpaD) and a divergently transcribed sublocus encoding the Mxi-Spa specialized secretory system of invasins which becomes activated upon contact with epithelial cells (2–4, 24, 38). Recent evidence suggests that an IpaB-IpaC complex accounts for induction of entry (22, 25). (ii) Rapid intracellular growth, which seems to result essentially from lysis of the phagocytic vacuole occurring a few minutes after entry, follows (33). (iii) Intra- and intercellular spread (14) of the bacterium starts as soon as the cytoplasmic compartment has been reached. This Ics phenotype, which is required for intracellular colonization of a confluent cell monolayer, is encoded by *icsA* (*virG*), a gene located on the virulence plasmid of *S. flexneri* (6, 20, 21). Motility is caused by accumulation of short actin filaments at one extremity of the bacterium (6). These filaments are tightly bundled, possibly by plastin (28), and form a sharp comet that trails the moving microorganism.

IcsA is essential for intra- and intercellular spread (6, 13, 20). It is found as a 120-kDa outer membrane protein localizing, both in bacterial suspension and in the host cell cytoplasm, to one pole of the bacterial body. In the latter case, it accumulates at the junction of the bacterium with the actin tail. It is likely that IcsA, directly or indirectly, induces actin nucleation and polymerization at the bacterial surface (29a). There is no convincing evidence that IcsA binds actin directly (13a). IcsA binds and hydrolyzes ATP (13), but no specific function has yet been ascribed to this property.

IcsA is also found as a 95-kDa secreted protein (13). Weak labelling of the actin tail by a polyclonal serum directed against IcsA suggested that this protein was present in this structure in association with actin (13). This suggested, but did not demonstrate, that the secreted form may have a physiological function in motility. It has been shown that an SSRRASS sequence located at residues 754 to 760 is a target for cyclic nucleotide-dependent kinases (10). Site-directed mutagenesis of this sequence prevents phosphorylation but also enhances the cell-to-cell spreading ability of bacteria (10). It was hypothesized that phosphorylation may be a defense mechanism used by the cell to control intracellular spread of invasive bacteria. In confluent cells, intracellular spread of shigellae occurs in the area of intermediate junctions, components of this structure such as

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the cadherins being used to transport the bacterium from one cell to its neighbors (32).

The present work addresses some of the major issues raised in the previous paragraph. It confirms recent evidence (11) that IcsA is cleaved at the SSRRASS site, previously characterized as a phosphorylation site. It is not clear at this point whether phosphorylation of the protein occurs inside the host cell cytoplasm and affects the cleavage. Unlike the situation with ActA, the functionally equivalent protein of *Listeria monocytogenes* (7), we have not observed in vivo phosphorylation, either by looking at migration upshift of IcsA by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or by immunoprecipitation of IcsA from infected cells cultivated in the presence of ^{32}P (10a). This work also shows that the lack of cleavage leads to an accumulation of IcsA at the bacterial surface with loss of its polar repartition. This leads to aberrant localization of IcsA on the bacterial surface inside the host cell cytoplasm and to aberrant movements. A monoclonal antibody (MAb) recognizing Gly-rich repetitive boxes of IcsA strongly labels IcsA on the bacterial surface; the actin tails, including those formed by bacteria expressing a site-directed mutant of IcsA which is no longer cleaved; and a 70-kDa protein present in both infected and noninfected HeLa cells.

MATERIALS AND METHODS

Media, strains, and plasmids. Bacterial strains used in this study were as follows. M90T is an *S. flexneri* serotype 5 isolate harboring the 220-kb invasion plasmid pWR100 (31). SC560 is an M90T derivative in which a Δ*icsA*::ΩSp^r mutation has been engineered in pWR100 (10). JM101 is an *E. coli* strain with the following genotype: *supE thi* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}*Z*ΔM15) (39). Bacteria were routinely grown at 37°C in tryptic soy (TS) broth with aeration or on TS agar plates (Diagnostics Pasteur, Marnes la Coquette, France). Ampicillin and spectinomycin were used at a final concentration of 100 μg/ml.

The following series of plasmids was also used: pHS3199, a 5.4-kb *Eco*RI-*Sma*I fragment of pWR100 containing the entire *icsA* gene cloned in pBR322 (10); pHS3262, a pHS3199 derivative that has overcome a site-directed mutation in the SSRRASS sequence to obtain an SSRDASS sequence (10); and pHS3264, a pHS3262 derivative that has overcome another site-directed mutation to obtain an SSDDASS sequence (10).

Construction of the *lacZ* gene fusion plasmid pHS3285. The extremities of a 931-bp *Xba*I-*Hin*dIII fragment of the *icsA* gene comprising the Gly-rich repeated boxes were filled in with Klenow polymerase, and the fragment was cloned in frame with the *lacZ* gene into the filled-in *Sma*I-*Hin*dIII sites of the expression vector pSKS105 (8), which placed this hybrid gene under the control of the *lac* promoter. The resulting plasmid, pHS3285, was used for expression and purification of the hybrid protein (p150kDa) in *E. coli* JM101.

Preparation of an anti-p150kDa polyclonal antiserum. The p150kDa–β-galactosidase fusion protein expressed in *E. coli* JM101 after 1.5 h of induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was purified on a *para*-amino-phenyl-β-D-thiogalactoside (TEPG) affinity column (37). Fractions containing the most β-galactosidase activity were pooled, dialyzed in 50 mM (pH 7.4) phosphate buffer with glycerol (1:1), and used for production of an anti-p150kDa rabbit polyclonal serum.

This antiserum was successively adsorbed against whole bacterial lysates of *S. flexneri* SC560 and IPTG-induced *E. coli* JM101(pSKS105) and then against a lysate of HeLa cells. All lysates were fixed on nitrocellulose membrane filters. Briefly, bacterial lysates were obtained from exponentially growing cultures by sonication in Dulbecco's phosphate-buffered saline (PBS) (pH 7.5)–1 mM EDTA. For HeLa cell lysates, 10^7 cells of a 24-h culture were lysed in lysis buffer (PBS [pH 7.5], 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 356,000 × *g* for 30 min at 4°C in a TL100 tabletop centrifuge, the supernatant was used as the adsorption lysate.

Preparation of proteins. Whole-cell and supernatant bacterial protein extracts were prepared as previously described (23). A 10-ml volume of a bacterial culture was centrifuged. After filtration through a 0.22-μm-pore-size filter, supernatant proteins were precipitated with a 1/10 dilution (vol/vol) of trichloroacetic acid, solubilized in 100 μl of Laemmli buffer, and then boiled for 5 min. Whole-cell protein extracts were obtained from the pellet of 1 ml of culture. The pellet was washed once with 10 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$, solubilized in 100 μl of Laemmli buffer, and boiled for 5 min before SDS-PAGE.

From infected or noninfected HeLa cells, supernatant or pellet extracts were obtained as follows. Cells were washed three times with PBS–1 mM EDTA, lysed with lysis buffer, and scraped off the culture dish with a rubber policeman. The resulting material was centrifuged at 14,000 × *g* for 10 min at 4°C. The resulting supernatant was centrifuged at 356,000 × *g* for 30 min at 4°C in a TL100 tabletop centrifuge. The pellet resulting from the first centrifugation, after being washed once in PBS–1 mM EDTA, was lysed with lysis buffer containing 0.1% SDS and centrifuged at 356,000 × *g* for 30 min at 4°C. Both supernatants and the pellet were used for immunoadsorption experiments or directly for SDS-PAGE and immunoblotting.

SDS-PAGE, immunoblotting and antisera. Electrophoresis of the proteins by SDS–7.5% PAGE was performed as described by Laemmli (19). After electrophoresis, proteins were transferred onto a nitrocellulose membrane. Immunoblotting procedures were carried out with either of two polyclonal rabbit antisera, one raised against the purified 120-kDa native form of IcsA (13) and used at a concentration of 15 μg/ml (1/400 dilution) and the other raised against the β-galactosidase–p150kDa fusion protein described above and used at a concentration of 40 μg/ml (1/400 dilution) or with a mouse MAb (MAbF15) raised against p120kDa IcsA and used at a concentration of 90 μg/ml (1/150 dilution). Horseradish peroxidase-labelled goat anti-mouse or goat anti-rabbit antibodies were used as secondary antibodies and visualized by enhanced chemiluminescence (Amersham International, Little Chalfont, England). The rabbit polyclonal serum raised against the native 120-kDa form of IcsA was also preadsorbed against lysates of JM101(pKK223-3), SC560, and HeLa cells.

Preparation of protein A- or G-Sepharose beads covalently coupled to polyclonal antibodies or MAbs. Procedures used for binding and coupling the polyclonal anti-p150kDa serum to protein A-Sepharose beads CL4B (Pharmacia, Uppsala, Sweden) or MAbF15 to protein G-Sepharose (Pharmacia) were previously described (15). Briefly, 3 mg of MAbs or polyclonal antibodies was bound to a 500-μl suspension of protein A or G beads by overnight incubation in PBS at 4°C, covalently attached to these beads with dimethylpimelimidate, and stored at 4°C in PBS containing 0.02% sodium azide.

Immunoadsorption experiments. Immunoadsorption experiments with infected or uninfected HeLa cell supernatant or pellet extracts were carried out as follows. Beads (10 μl) were added to HeLa cell extracts, and the mixture was rocked gently overnight at 4°C. Immunoprecipitates were washed twice in PBS (pH 7.5)–1 mM EDTA–1% Triton X-100–1 mM phenylmethylsulfonyl fluoride and three times in PBS (pH 7.5)–1 mM EDTA and then resuspended in 20 μl of Laemmli sample buffer and boiled for 5 min before SDS-PAGE.

Fluorescence labelling. In vitro immunolabelling of bacteria was performed as previously described (16) with 100 μl of bacteria grown to an optical density at 600 nm of 0.5 and then diluted fivefold with either anti-p120kDa rabbit polyclonal serum used at a 1/100 dilution or MAbF15 used at a 1/50 dilution. Fluorescence labelling was performed with either donkey anti-rabbit antibody or sheep anti-mouse antibody conjugated to Texas red (Amersham).

HeLa cell infections were performed as described elsewhere (26) on glass coverslips in 35-mm-diameter dishes. After fixation for 20 min in PBS containing 3.7% paraformaldehyde (Aldrich-Chemie, Steinheim, Germany), as previously described (9), infected HeLa cells were permeabilized with methanol at −20°C for 1 min before being labelled with MAbF15 or serotype-specific rabbit polyclonal antiserum. MAbF15 binding was then visualized by using Texas red-conjugated sheep anti-mouse immunoglobulin G (Amersham) and rabbit polyclonal antibodies with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.). Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min before being immunolabelled with NBD-phallacidin to show polymerized actin (Sigma).

The labelled preparations were observed by using a conventional fluorescence microscope (BH2-RFCA; Olympus Optical Co., Ltd.) or a confocal laser scanning microscope (Wild Leitz Instruments GmbH, Heidelberg, Germany).

RESULTS

Production of MAbs against IcsA. A battery of murine MAbs was obtained by immunizing mice with purified IcsA (13). All MAbs obtained belonged to the immunoglobulin G1 isotype and expressed various degrees of affinity towards the protein (as determined by enzyme-linked immunosorbent assay and Western blot [immunoblot]), towards bacterial suspensions, and towards infected cells in immunolabelling experiments (data not shown). MAbF15 was selected for further study because of its strong recognition of the protein under these different conditions. Its affinity constant for IcsA was 6×10^{-8} M.

Figure 1 shows that MAbF15, as well as an anti-IcsA rabbit polyclonal serum (13), recognizes both the native 120-kDa IcsA protein and its secreted 95-kDa cleaved form. Total bacterial extract and supernatant were obtained from the Δ*icsA* mutant SC560, carrying pHS3199 (10). SC560 strains carrying site-directed mutants of pHS3199 altered in the SSRRASS cleavage-phosphorylation site were also tested with MAbF15

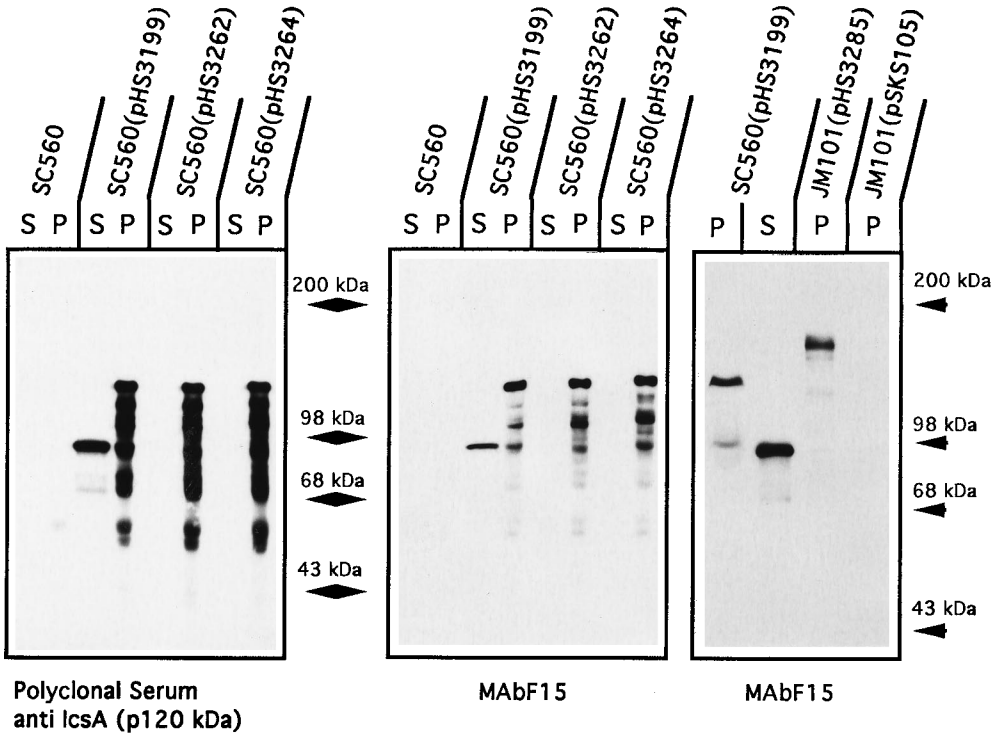

FIG. 1. (Left and center) Immunoblots of culture supernatant (S) and bacterial pellet extracts (P) with a rabbit polyclonal serum directed against purified native IcsA protein (p120kDa) (left) compared with immunoblots with MAbF15 (center). The pattern of multiple bands that is seen under the major 120-kDa form has already been observed. It is due both to proteolytic cleavage and to reinitiation of translation at several ATG start codons present in the *icsA* gene. (Right) Demonstration that MAbF15 recognizes the hybrid β-galactosidase–IcsA fusion protein (p150kDa) [JM101(pHS3285)] and not β-galactosidase alone [JM101(pSKS105)].

and the polyclonal serum, thus confirming the lack of cleaved 95-kDa molecular species in supernatants.

Further characterization showed that MAbF15 recognized an epitope contained in a 350-amino-acid (aa) sequence located between aa 62 and aa 412 in the N-terminal half of IcsA, which contains repeated Gly-rich boxes (20). This evidence was obtained by generating a *lacZ* fusion containing this sequence (i.e., plasmid pHS3285) and showing its strong recognition by MAbF15, as shown in the right part of Fig. 1. No further characterization could be obtained, because of the repetitive nature of the sequence.

Constant expression and aberrant localization of IcsA in the absence of cleavage. Bacteria grown in TS broth were labelled with MAbF15. As shown in Fig. 2, in which photographs of immunofluorescence labelling and the respective phase contrast views are combined, two major observations could be made. SC560 bacteria carrying plasmid pHS3199, expressing the cleavable form of the protein, were not constantly labelled since only approximately 40% of them showed significant fluorescence on their surfaces. In addition, strict localization of IcsA to the bacterial pole opposite the septation furrow was observed, as already described for wild-type strain M90T (13).

On the other hand, all SC560 bacteria carrying plasmid pHS3264 strongly expressed IcsA on their surfaces. The lack of cleavage caused major changes in repartition of this protein since IcsA was exposed along the entire contour of the bacilli, including the septation furrow, although it still exhibited polar reinforcement. The strain expressing plasmid pHS3264 was selected for having a double point mutation introduced by site-directed mutagenesis, thus warranting total destruction of the cleavage site. Results, however, were similar with SC560 harboring pHS3262 (data not shown). The same applies to the next series of experiments. Similar results have recently been reported for studies with *S. flexneri* 2a (11).

Lack of cleavage of mutagenized IcsA in intracellular bacteria. Before further experiments were carried out, we confirmed the lack of IcsA cleavage in intracellular SC560 harboring plasmid pHS3262 or pHS3264. Figure 3 compares levels of intracellular expression of the two IcsA forms by SC560 harboring either pHS3199 or pHS3264. The supernatant of the lysis product of HeLa cells infected by SC560 harboring plasmid pHS3199 indicated the presence of the cleaved 95-kDa species, whereas the pellet contained only the native 120-kDa protein. On the other hand, the supernatant of the lysis product of HeLa cells infected with SC560 harboring plasmid pHS3264 did not show detectable amounts of the 95-kDa species. These results, and similar results obtained with SC560 harboring pHS3262 (data not shown), confirm that in these mutants the cleavage of IcsA occurs neither in vitro nor in vivo.

Abnormal repartition of polymerized actin on the surfaces of intracellular bacteria and formation of aberrant actin tails. Figure 4 demonstrates aberrant localization of F-actin, revealed by NBD-phallacidin, on the surfaces of intracellular bacteria. Figure 4A shows a typical example of polar localization, with a narrow tail of F-actin caused by SC560 bacteria harboring pHS3199. Bacterial bodies, which are left unlabelled in this experiment in order to demonstrate actin exclusively, are indicated by small arrows in Fig. 4A. Figures 4B to D show examples of F-actin organization induced by SC560 harboring plasmid pHS3264 encoding noncleavable IcsA. Figure 4B shows nonpolar repartition of F-actin on the surface of a bacterial body and a bacterium (arrow) moving aberrantly, in a direction perpendicular to its longitudinal axis, leaving a large tail of F-actin whose width is equivalent to the length of the

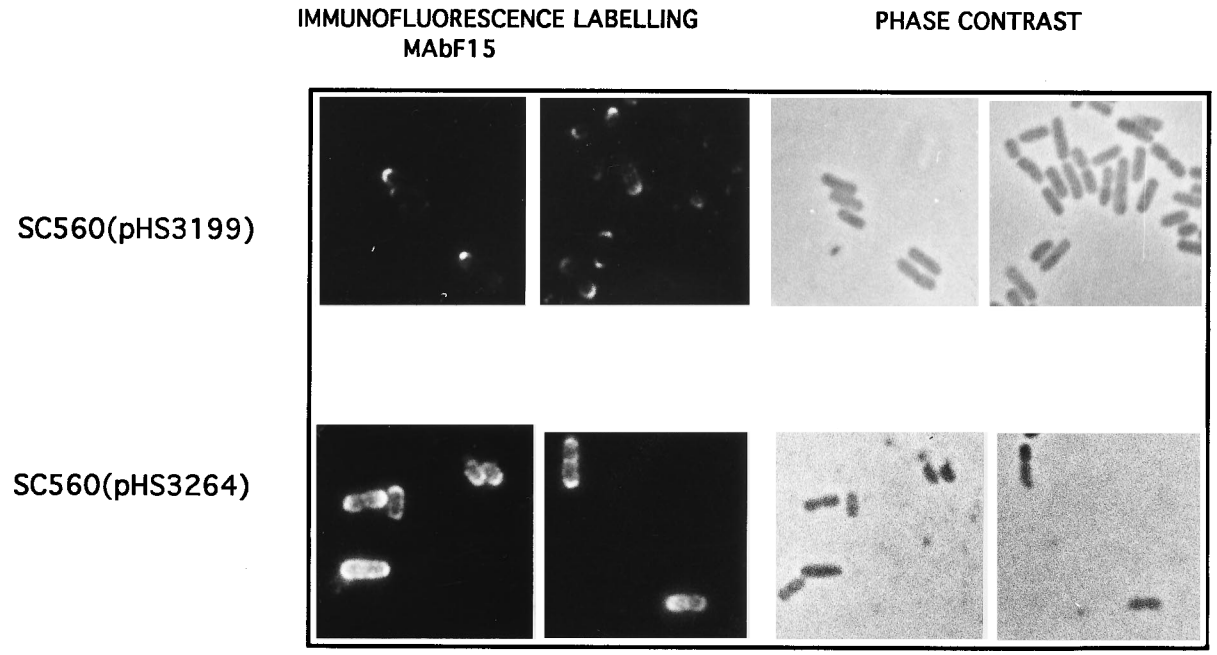

FIG. 2. Immunofluorescence labelling of *S. flexneri* by MAbF15 and corresponding phase-contrast images. The lack of IcsA processing leads to increased expression and circumferential localization of the protein and also to surface expression by all bacteria.

bacterium. Figure 4C shows subnormal movement, although the F-actin tail appears thicker, and one longitudinal side of the bacterial body is capped with actin (arrow). Figure 4D shows thicker actin tails, with another bacterium moving in a direction perpendicular to its longitudinal axis (arrow). In accordance with in vitro data showing constant expression of IcsA on the bacterial surface, all bacteria expressing noncleavable IcsA accumulated F-actin on their surfaces.

Immunolabelling of IcsA on intracellular bacteria confirms aberrant surface localization of the noncleavable protein and suggests the presence of a cross-reactive protein associated

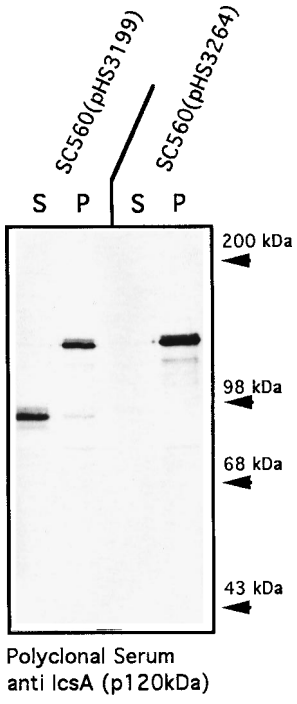

FIG. 3. Immunoblots of supernatant (S) and pellet extracts (P) of infected HeLa cells performed with a rabbit polyclonal serum directed against purified native IcsA protein (p120kDa). HeLa cells were infected either by *S. flexneri* SC560 carrying the gene encoding the cleavable form (pHS3199) or by SC560 carrying the gene encoding the noncleavable form (pHS3264) of IcsA. With the latter strain, this Western blot shows the lack of cleaved 95-kDa form in the soluble (S) fraction.

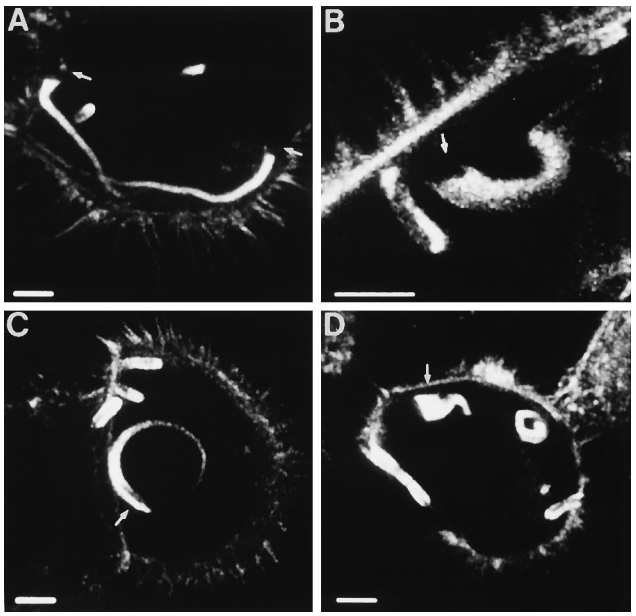

FIG. 4. HeLa cells infected with *S. flexneri* SC560(pHS3199) (A) and SC560(pHS3264) (B, C, and D). Labelling was done with NBD-phallacidin. Small arrows point to bacterial bodies which have not been specifically labelled in this experiment. They were observed in their respective positions by phase-contrast microscopy. Bars, 5 μm.

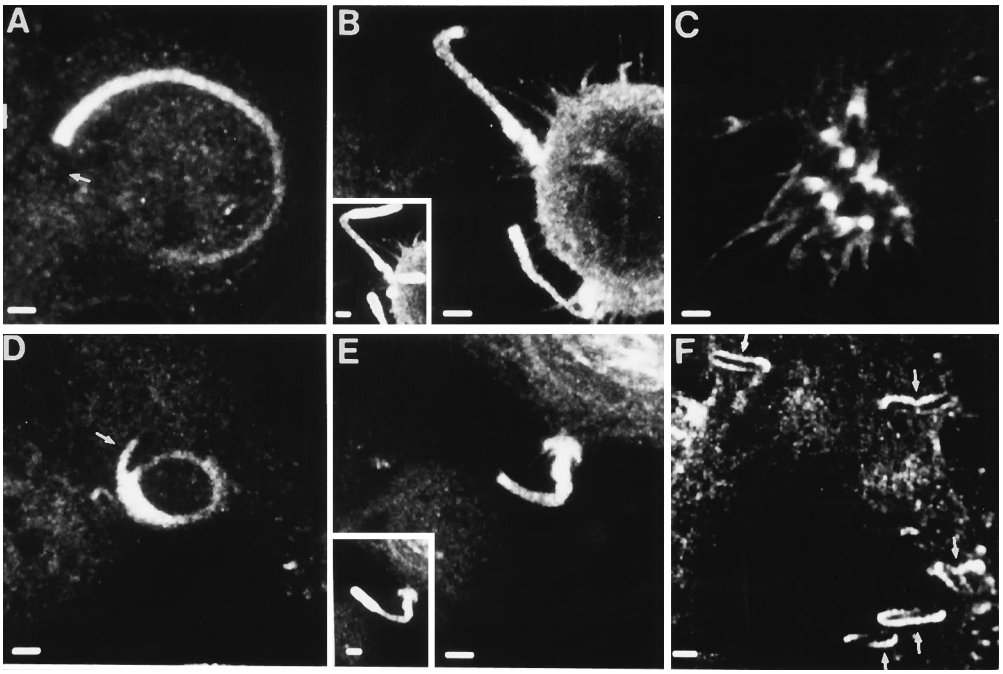

FIG. 5. HeLa cells infected with *S. flexneri* SC560(pHS3199) (A, B, and C) and SC560(pHS3264) (D, E, and F). Immunofluorescence labelling was done with MAbF15. (B and E, insets) Double immunofluorescence labelling confirms the protrusive nature of the structure shown, since a bacterium is present at the tip. Arrows point to bacteria when double labelling was not done. Bars, 5 μm.

with the F-actin tail. In Fig. 5, infected cells labelled with MAbF15 are shown.

Figures 5A to C show immunolabelling of cells infected by SC560 bacteria harboring pHS3199. Strong labelling of an F-actin tail is shown in Fig. 5A (bacterium indicated by an arrow). In Fig. 5B, strong labelling of the content of a protrusion is shown. A bacterium at the tip of this protrusion is labelled by antilipopolysaccharide polyclonal serum. Double immunofluorescence is shown in the insert in Fig. 5B. In Fig. 5C, strong IcsA labelling of bacteria is exclusively shown. Use of rabbit polyclonal anti-IcsA serum showed similar labelling of the bacterial surface but very weak labelling of both the actin tail and the protrusion (data not shown).

Figures 5D to F correspond to immunolabelling of cells infected by SC560 bacteria harboring pHS3264. Similarly strong labelling of the actin tail and the protrusions caused by the moving microorganisms is visible, in spite of the lack of cleavage of the protein, which should not appear in such a structure under these conditions. The bacterium in Fig. 5D shows a thicker tail than the one in Fig. 5A and, as shown in Fig. 4C for F-actin, lateral labelling of the bacterial body (arrow). Figure 5E again shows strong labelling of the content of a protrusion. Labelling of the cell ruffle where the protrusion emerges from the cell surface must also be noticed. Presence of the bacterium is shown in the insert in Fig. 5E, as in Fig. 5B. Figure 5F shows a totally different repartition of IcsA at the surfaces of intracellular bacteria (arrows). Bacterial bodies appear labelled either on their entire circumference or only on one of their sides. In all cases, labelling is much more extensive than the polarly restricted labelling shown in Fig. 5C. Again, similar results concerning bacterial surface labelling were seen with rabbit polyclonal anti-IcsA serum (data not shown).

MAbF15 identifies a eukaryotic protein that cross-reacts with IcsA. Strong immunolabelling of the F-actin tail and content of a protrusion by MAbF15, in spite of the lack of intracellular cleavage of IcsA, suggested that a eukaryotic cross-reactive protein existed in a soluble form in the cytoplasm of HeLa cells. In order to confirm the existence of such a protein, immunoprecipitation experiments were carried out with noninfected HeLa cells. Figure 6 illustrates three series of experiments. In the experiment shown in the left part of Fig. 6, Sepharose beads coated with the rabbit polyclonal serum directed against the 150-kDa β-galactosidase–IcsA hybrid protein were used to immunoprecipitate the putative cross-reactive species. This immunoprecipitation experiment recovered a protein of 70 kDa which reacted with the homologous serum

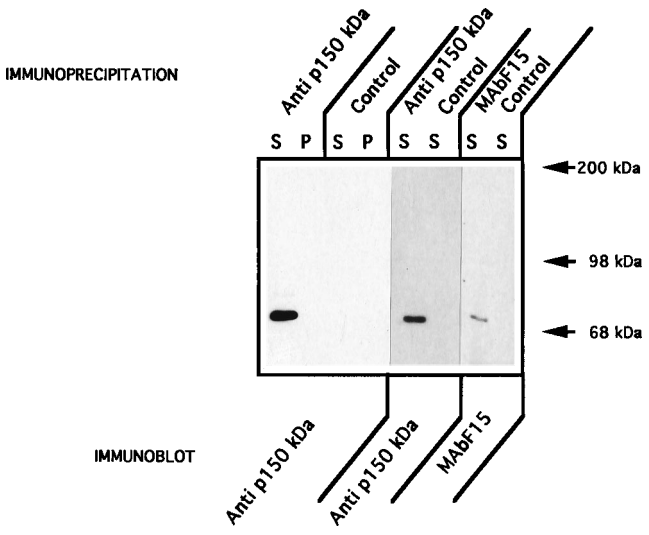

FIG. 6. Immunoblots performed either with rabbit polyclonal antiserum directed against the β-galactosidase–IcsA fusion protein (p150kDa) or with MAbF15 on immunoprecipitates obtained with either of these antisera from supernatant fractions (S) or pellet fractions (P) obtained by lysis of noninfected HeLa cells.

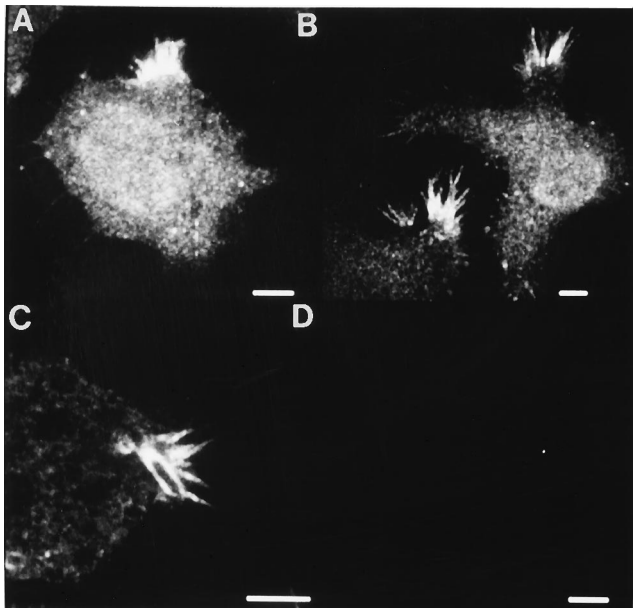

FIG. 7. (A, B, and C) Immunolabelling of noninfected HeLa cells with MAbF15; (D) negative control using only conjugated anti-mouse immunoglobulin G. Bars, 5 μm.

from the supernatant fraction. Nothing was recovered from the pellet fraction. Uncoated beads did not precipitate any HeLa cell protein from supernatant or pellet fractions, as shown in the control lanes in Fig. 6. The same material was blotted against MAbF15, and this allowed us to identify a protein of similar molecular weight. In addition, as shown in the right part of Fig. 6, beads coated with MAbF15 could also immunoprecipitate a protein of 70 kDa. This is strong evidence that a soluble eukaryotic protein which cross-reacts, at least with the Gly-rich boxes of IcsA, is present in HeLa cells. On the basis of this evidence, immunofluorescence labelling was performed with noninfected HeLa cells with MAbF15. Significant peripheral labelling was observed, in addition to a weak global background in the cytoplasm. However, as shown in Fig. 7, strong labelling could be observed, particularly in areas showing spontaneous ruffles.

DISCUSSION

The actin-mediated intracellular motility process caused by polar expression of IcsA is essential to the virulence of *S. flexneri*, since *icsA* null mutants, in spite of their retained capacity to penetrate into epithelial cells, are severely impaired both in vitro, in the plaque assay (6, 21), and in vivo, being unable to provoke keratoconjunctivitis in guinea pigs (6, 21) or to cause dysentery after intragastric infection of macaques (30).

A striking property of IcsA is its polar localization at the bacterial surface, opposite to the septation furrow (13). A progressive increase in expression of the native 120-kDa protein in this area has been shown, expression peaking during bacterial division (14). During this period, movement is often initiated and long protrusions are formed (28). It is likely that efficient motility is related to the amount of IcsA protein expressed at the bacterial pole. A similar process exists in *L. monocytogenes*, in which polar distribution of ActA, the functional equivalent of IcsA, has been demonstrated (16). Polar localization is likely to provide the system with an asymmetry that enhances directional polymerization of actin, thus orienting the movement. It has been shown that in *L. monocytogenes*, a mutation causing circumferential polymerization of actin severely impaired motility (17). No such mutation clearly affecting repartition of actin is available in *S. flexneri*. Nothing is known about the genetic or molecular mechanisms of polarization, in contrast to the situation for other recently reviewed systems such as that of *Caulobacter* spp. and the Mcp proteins of *Escherichia coli* (36). Only recently, a requirement for the O antigen for proper unipolar localization of IcsA in the bacterial outer membrane has been demonstrated (29). In the rough mutants used in this study, IcsA appeared to be expressed circumferentially on the bacterial surface, but the exact role of O side chains in influencing polarity of IcsA is not yet known.

IcsA is cleaved and secreted into the bacterial supernatant as a 95-kDa molecular species (14). Several consequences of this cleavage process may be anticipated. It could account in part for exclusive polar localization of the protein if the putative protease is located primarily at the level of the bacterial septum and beyond, along the side of the bacterial body. In this case, "shaving off" IcsA everywhere except at the bacterial pole opposite the septation furrow would be an efficient way of obtaining polarization. Cleavage of IcsA may also downregulate motility of the bacterium in the intracellular compartment, although the rationale for such a system is not clear. In a previous work, we had suggested that phosphorylation of IcsA on an SSRRASS cyclic nucleotide-dependent protein kinase consensus sequence could downregulate motility, thus representing a mechanism of defense for the cell (10). However, recently published evidence (11) and our own confirmatory data show that the SSRRASS sequence is also the target sequence for cleavage. Nothing is known about how phosphorylation could affect cleavage. We are currently addressing this question. Finally, on the basis of the weak labelling observed to occur in the actin tail of moving bacteria with an anti-IcsA polyclonal serum, it has been speculated that the incorporation of the cleaved form of IcsA into the actin tail may play a role in its formation and thus in the motility process (12).

The present work has addressed the above-mentioned three points. Destruction of the cleavage site of IcsA by site-directed mutagenesis has led to strains that constitutively accumulate the protein on their surfaces, both at the pole, where it remains predominant, and also circumferentially, including the area of the septation furrow. This result indicates that IcsA cleavage is not absolutely essential for polar localization but that it contributes significantly to it. In addition, in the absence of cleavage all bacteria expressed IcsA on their surfaces, in comparison with about 40% of those expressing a cleavable protein. Aberrant localization, higher frequency of expression on the bacterial surface, and increased expression indicate that proteolytic cleavage is important both quantitatively and qualitatively for the expression and role of IcsA in *Shigella* motility.

Aberrant localization of IcsA on the bacterial surface caused abnormal intracellular motility. Most obvious was a perpendicular movement relative to the bacterium's longitudinal axis, leaving a large tail of F-actin. It is likely that such movements are abortive, and we actually never observed such configurations leading to protrusions. It is therefore difficult to reconcile these observations with the previous demonstration that mutants with site-directed mutations in the SSRRASS sequence were more motile than wild-type strains (10). The fact that 100% of the strains express IcsA when its cleavage does not occur is likely to compensate for, and even outweigh, the expected negative effect on efficiency of the motility phenotype.

Finally, this work has demonstrated that the weak labelling of the actin tail observed with a polyclonal antiserum directed

against IcsA (13) was not due to accumulation of the cleaved 95-kDa species, at the same time eliminating a potential physiological role of this protein in stabilizing this structure. We have shown evidence that a soluble protein of 70 kDa is expressed in eukaryotic cells. This protein is recognized by MAbF15, especially in infected cells, where it may account for labelling of the actin tails of moving bacteria. It is also recruited, even in noninfected cells, into cytoskeletal structures that produce surface ruffles. Strong in vivo labelling may indicate that this antibody recognizes this epitope better when it acquires a certain conformation in vivo. These results indicate that there may be an IcsA equivalent in eukaryotic cells with similar biological functions of direct or indirect actin nucleation. We are currently trying to clone and characterize the HeLa cell cDNA encoding such an IcsA equivalent.

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