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Unipolar Localization and ATPase Activity of IcsA, a *Shigella flexneri* Protein Involved in Intracellular Movement

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Shigella flexneri **uses elements of the host cell cytoskeleton to move within cells and from cell to cell. IcsA, an *S. flexneri* protein involved in this movement, was purified and studied in vitro. IcsA bound the radiolabelled ATP analog 3′(2′)-*O*-(4-benzoyl)benzoyl-ATP and hydrolyzed ATP. In addition, the surface localization of IcsA on both extracellular and intracellular shigellae was unipolar. Further, in HeLa cells infected with shigellae, IcsA antiserum labelled the actin tail throughout its length, thereby suggesting that IcsA interacts with elements within the tail. Localization of IcsA within the tail at a distance from the bacterium would require its secretion; we demonstrate here that in vitro IcsA is secreted into the culture supernatant in a cleaved form.**

Shigellae are gram-negative bacterial pathogens that cause colonic mucosal ulcerations and abscesses in humans by invading and killing colonic epithelial cells. Infection of epithelial cells is a multistep process, involving entry of bacteria by induced phagocytosis, escape from the phagocytic vacuole, multiplication and spread within the cytoplasm, and passage into adjacent cells (7, 29, 32, 33). The last two steps involve interaction of the intracellular bacteria with the cellular cytoskeleton. When *Shigella flexneri* infects semiconfluent HeLa cells, short filaments of actin organize around individual intracellular bacteria and are then mobilized and tightly linked into a long bundle at one end of the bacterial body. This creates a tail of actin several micrometers long behind the bacterium as it moves forward in rapid spurts and passes into an adjacent cell by way of a finger-like protrusion from the cell surface (7, 24, 36). Similar findings have been made with *Listeria monocytogenes* infection of macrophages (44, 46) and epithelial cells (31, 43).

The respective roles of bacterial and host cell proteins in the reorganization of actin filaments associated with bacterial movement have yet to be elucidated. The finger-like protrusions by which bacteria spread from one cell to another generally contain a dividing bacterium or two bacteria at their tips (36), suggesting a correlation between bacterial division and initiation of movement. We have recently demonstrated that at the stage of elongation and septation of the bacterial body immediately preceding division, the coat of actin around the bacterium is disrupted and rapidly relocated to the distal end of one of the daughter bacteria (36). The actin filaments near the bacteria become tightly bundled, and antiserum to plastin (an isoform of fimbrin) (10, 17), a cellular actin-binding protein, strongly recognizes the actin tail at its junction with the bacteria. Plastin is unique in that it has two actin-binding sites per monomer that are located close to one another, so that it forms very tight actin bundles. The active bundling of filaments that have been organized at one pole of the bacterium may participate in bacterial movement.

We have previously constructed shigella mutants that are unable to spread within and between cells (7, 20, 40). Each of these strains contains a mutation in the gene that encodes IcsA (VirG), a 120-kDa outer membrane protein encoded on the 220-kbp virulence plasmid pWR100 (7, 28, 29). In infection of semiconfluent HeLa cells, the *icsA* mutant (i) does not diffuse within the cell cytoplasm but rather forms microcolonies near the cell nucleus, (ii) does not elicit the accumulation of polymerized actin on the surface of the bacteria that is observed with the wild-type strain, and (iii) does not spread into adjacent cells (7, 20, 29, 48, 49). Upon infection of macaque monkeys, the *icsA* mutant causes markedly less mucosal destruction than the wild-type strain (40), demonstrating that IcsA is an essential virulence determinant in vivo.

The phenotype of the *icsA* mutant suggests that IcsA is involved in the use of host cell cytoskeletal elements to produce intracellular movement of the bacteria. Because the interchange between ATP and ADP is important in the polymerization and depolymerization of actin, in this study we examined whether IcsA is able to bind and hydrolyze ATP. We found that purified IcsA binds the radiolabelled ATP analog 3′(2′)-*O*-(4-benzoyl)benzoyl-ATP (Bz_2ATP) and hydrolyzes ATP. In addition, we found that the surface localization of IcsA on both extracellular and intracellular shigellae is unipolar. Further, in HeLa cells infected with shigellae, IcsA antiserum recognized the actin tail throughout its length, suggesting that IcsA interacts with elements within the tail. Localization of IcsA within the tail at a distance from the bacterium would require its secretion; in support of this, we demonstrated in vitro that IcsA is secreted into the culture supernatant in a cleaved form.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. All *S. flexneri* strains were derived from wild-type serotype 5 strain M90T (41). M90T carries virulence plasmid pWR100, which encodes *icsA*, as well as other genes involved in virulence. SC560 is M90T with pWR100Δ*icsA*::Ω, where the omega interposon has been inserted after the first 300 bp of the *icsA* coding sequence (20). SF401 is M90T with pWR100 *mxiD1*, which contains a nonpolar insertion of *aphA-3* into *mxiD*, a gene that is necessary for secretion of invasins IpaB and IpaC (2). *Escherichia coli* JM109 carries *recA1 supE44*

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endA1 hsdR17 gyrA96 relA1 thi Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) (52). *E. coli* MC1061 carries *hsdR mcrB araD139* Δ(*araABC-leu*)*7679* Δ*lacX74 galU galK rpsL thi* (30).

Bacteria were grown in Trypticase soy broth (Diagnostics Pasteur, Marnes la Coquette, France) or L broth. Ampicillin and spectinomycin were each added to a final concentration of 100 μg/ml where appropriate. IcsA expression vector pMBG158 consists of the 3.4-kbp *Xmn*I-*Sma*I restriction fragment that contains 40 bp of DNA upstream of the *icsA* ATG start codon, the entire coding sequence for *icsA*, and 0.6 kbp of DNA downstream of *icsA*, under control of the inducible *tac* promoter in vector pKK223-3 (Pharmacia LKB Biotechnology, Uppsala, Sweden). Plasmid pHS3199 consists of a fragment that contains 1.5 kbp of DNA upstream of *icsA*, the entire coding sequence for *icsA*, and 0.6 kbp of DNA downstream of *icsA* inserted into vector pBR322 (20).

Genetic procedures and DNA analysis. Genetic procedures and DNA analysis, including DNA preparation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and bacterial transformation, were performed in accordance with standard procedures (38).

Chemicals. Bz_2ATP was purchased from Sigma (La Verpillière, France), and carrier-free P_i was from Radiochemical Centre (Amersham, United Kingdom). [γ-^{32}P]Bz_2ATP was synthesized from Bz_2ATP and $^{32}P_i$ (22). Ultrogel AcA22 was purchased from IBF Biotechnics (Villeneuve-la-Garenne, France).

Protein preparation and analysis. Whole-cell protein extracts were prepared as previously described (23). Supernatant protein extracts, membrane protein extracts, and cytoplasmic protein extracts were prepared as previously described (1). The distribution of IcsA into different extracts (i.e., whole cell versus supernatant) was estimated on the basis of the relative intensities of the signals produced on Western blots (immunoblots). Western blotting was performed as previously described (12), with either serum of a monkey convalescent from *S. flexneri* infection which recognizes IcsA (7) or a rabbit IcsA antiserum, with subsequent visualization by either ^{35}S- or ^{125}I-labelled protein A. Antiserum against IcsA was prepared by immunizing rabbits with purified IcsA (35). The antiserum was subsequently affinity purified on the 125-kDa IcsA polypeptide (11). Amino-terminal sequencing of the 95-kDa supernatant species of IcsA was performed by automated Edman degradation.

Nucleotide binding and hydrolysis by purified IcsA. ATPase activity of IcsA was measured by incubating purified protein (0.08 to 0.17 μM final concentration) in a reaction mixture containing 1 mM [γ-^{32}P]ATP, 30 mM Tris-HCl (pH 7.4), 100 mM KCl, and 1 mM $MgCl_2$ at 25°C. Prior to the assay, IcsA in 6 M urea–50 mM Tris-HCl (pH 7.4)–0.1% β-mercaptoethanol was dialyzed against 30 mM Tris-HCl (pH 7.4)–100 mM KCl–0.1% β-mercaptoethanol at 4°C. At various time points over 60 min, 45-μl aliquots were withdrawn from the reaction mixture. $^{32}P_i$ released from [γ-^{32}P]ATP was extracted as a phosphomolybdate complex (47).

Photolabelling experiments were performed by exposing 4 μM purified IcsA in a reaction mixture containing 0.3 mM [γ-^{32}P]Bz_2ATP, 10 mM Tris-HCl (pH 7.4), and 0.01% Triton X-100 to the longwave UV emission from a UVSL58 Mineralight at a distance of 5 cm from the sample for 10 min on ice. Soybean trypsin inhibitor (0.125 mg/ml) (type I-S; Sigma) and a mixture of 0.004 U each of phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase per μl in the presence of 0.3 mM [γ-^{32}P]Bz_2ATP were used as negative and positive controls, respectively. Following photolabelling, aliquots were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiographed. Purified IcsA was separated from urea by chromatography on Sephadex G-25 in 10 mM Tris-HCl (pH 7.4)–0.01% Triton X-100.

Labelling of bacteria grown in vitro and bacteria infecting HeLa cells with IcsA antiserum. For labelling of bacteria in vitro, freshly grown bacteria were centrifuged onto coverslips (700 × *g* for 10 min) and fixed with 3.7% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature. For labelling of bacteria in HeLa cells, cells were grown to semiconfluence, infected, and permeabilized as previously described (13, 31). After fixation with 3.7% paraformaldehyde in phosphate-buffered saline, labelling with IcsA antiserum was performed as previously described (31), with either rabbit IcsA antiserum (5.4 mg/ml) or affinity-purified IcsA antiserum (150 μg/ml) and, as a second antibody, a 1/80 dilution of goat anti-rabbit rhodamine-conjugated immunoglobulin G. As a control for nonspecific signals due to the second antibody, HeLa cells infected with M90T were labelled only with goat anti-rabbit rhodamine-conjugated immunoglobulin G; these cells did not show the fluorescent labelling observed in cells infected with M90T and labelled with IcsA antiserum. Labelling of polymerized actin was performed as previously described (31), with 7-nitrobenz-2-oxa-1,3-diazole-phallacidin (Molecular Probes, Inc., Junction City, Oreg.).

Standard fluorescent microscopy was performed with a BHS microscope (Olympus Optical Co., Ltd.). Confocal microscopy was performed with a Leica confocal microscope with a 100× lens and a blue (488-nm) or yellow-green (567-nm) filter and recovery of the signal across an RG610 filter (Schott). Observations were made from the adherence zone of the cell to the top. The distance between adjacent sections was 0.3 μM. Photographic records were taken from a flat-screen monitor with high linearity.

RESULTS

Purification of IcsA. IcsA is produced in relatively small amounts by shigellae; the 125-kDa band representing IcsA was barely visible in Coomassie-stained whole-cell protein extracts from wild-type *S. flexneri* M90T (data not shown). Therefore, to purify IcsA, we used a system of overexpression in *E. coli*. We constructed the recombinant plasmid pMBG158 that expresses *icsA* under control of the *tac* promoter; induction of the promoter leads to production of abundant IcsA (Fig. 1, lane 2). Strain JM109(pMBG158), grown at 37°C in L broth containing ampicillin, was supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside when the optical density at 600 nm reached 0.4. Bacteria grown for an additional 4.5 h were harvested by centrifugation, suspended in 10 mM Tris-HCl (pH 8.0)–5 mM $MgCl_2$–0.1% β-mercaptoethanol at 4°C, and sonicated. More than 90% of the IcsA was recovered in the pellet fraction after centrifugation at 1,700 × *g* at 4°C for 10 min. Insoluble proteins were treated with 8 M urea in the same Tris-$MgCl_2$ buffer and passed over a Ultrogel AcA22 column which had been pre-equilibrated with 6 M urea in 50 mM Tris-HCl (pH 7.4)–0.1% β-mercaptoethanol. Fractions containing IcsA were identified by SDS-PAGE and pooled (Fig. 1, lane 3). Verification that the band observed in lane 3 of Fig. 1 was IcsA was obtained by Western blotting with serum from a monkey convalescent from shigellosis (data not shown); the serum had been previously shown to recognize IcsA in whole-cell proteins prepared from wild-type strain M90T but

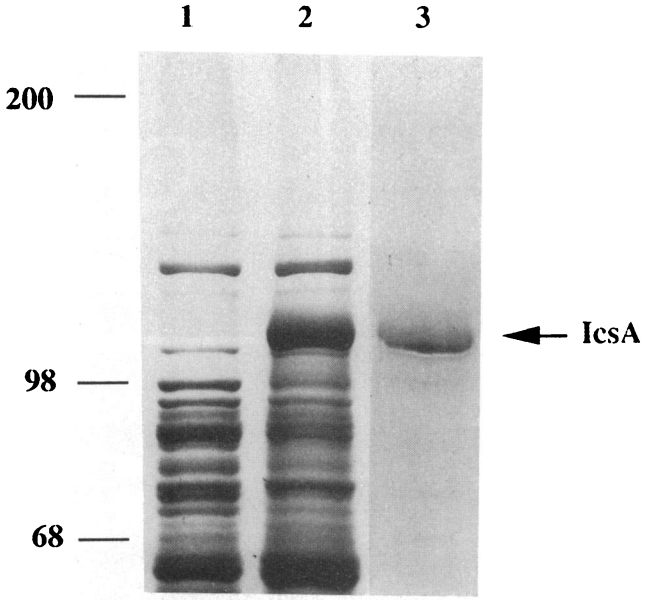

FIG. 1. Purification of IcsA. SDS-PAGE of whole-cell protein extracts (lanes 1 and 2) and purified IcsA (lane 3). Lanes: 1, *E. coli* JM109(pKK223.3); 2, *E. coli* JM109(pMBG158) for overexpression of IcsA; 3, purified IcsA. Apparent molecular masses are indicated in kilodaltons.

not in whole-cell proteins prepared from strain SC560 (*icsA* mutant) (data not shown).

Characterization of the ATP-binding and ATPase activities of IcsA. Because of the importance of the interchange of ATP with ADP in the polymerization and depolymerization of actin, we examined whether purified IcsA binds and hydrolyzes ATP. First, we investigated the binding of the ATP analog Bz_2ATP to IcsA. [γ-^{32}P]Bz_2ATP bound covalently to purified IcsA after 10 min of exposure of the reaction mixture to UV light (Fig. 2a). The signal produced by the radioactively labelled IcsA-Bz_2ATP complex increased in intensity with increasing concentrations of unlabelled ATP up to 10 mM in the reaction mixture (Fig. 2a, lanes 2 to 6). This result suggests cooperativity in the binding of Bz_2ATP with IcsA by ATP. It is unclear whether the signal subsequently decreased in the presence of much higher concentrations of unlabelled ATP. As expected, the mixture of phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase, which served as a positive control, was also covalently labelled and soybean trypsin inhibitor, the negative control, was not (Fig. 2a, lanes 8 and 7, respectively).

In the presence of 1 mM ATP, the rate of hydrolysis of ATP by purified IcsA was 22 ± 9 nmol min^{-1} mg^{-1} (mean ± standard deviation) over 15 min and decreased to 12 ± 6 nmol min^{-1} mg^{-1} over 45 min (Fig. 2b). The binding of Bz_2ATP to IcsA (see above) strongly suggests that the

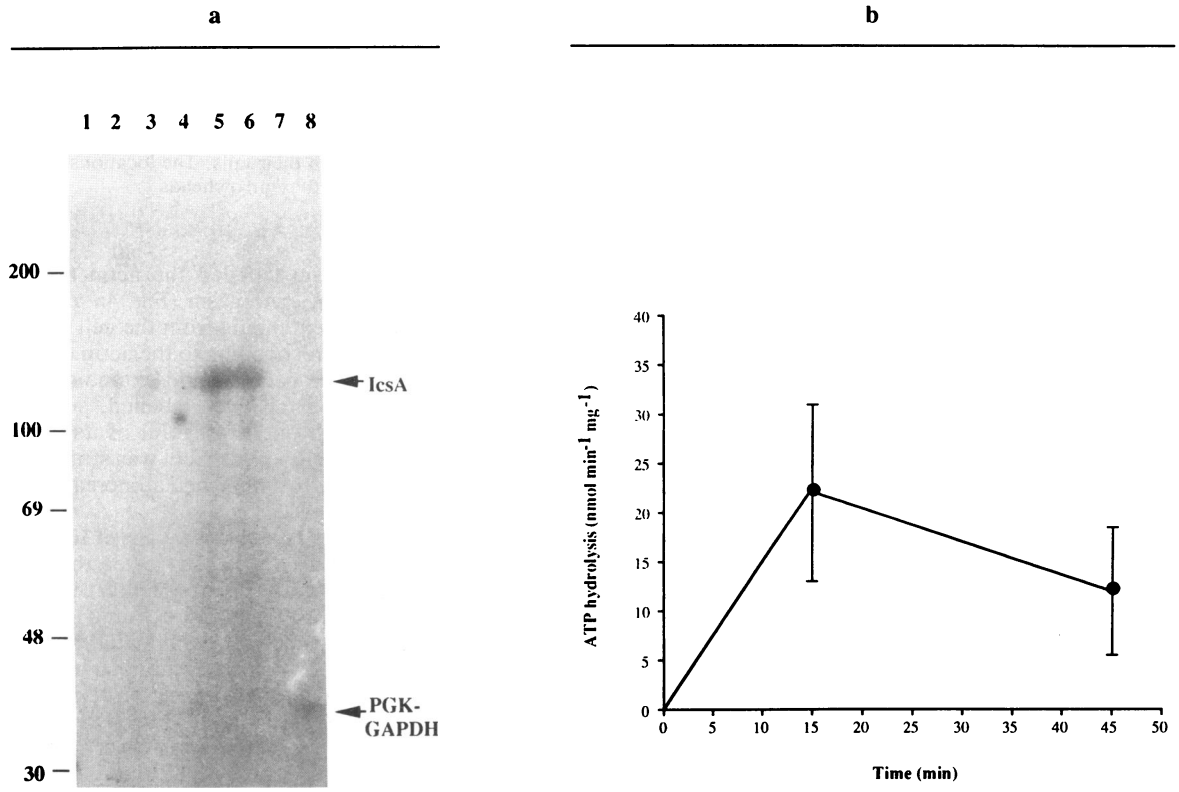

FIG. 2. ATP binding and hydrolysis by IcsA. (a) Binding of ATP with IcsA. Autoradiograph of ATP-binding blot. Lanes contained the following samples: 1, purified IcsA and [^{32}P]Bz_2ATP without unlabelled ATP; 2 to 6, purified IcsA and [^{32}P]Bz_2ATP with unlabelled ATP at final concentrations of 10 μM, 100 μM, 1 mM, 10 mM, and 100 mM, respectively; 7, soybean trypsin inhibitor (negative control) and [^{32}P]Bz_2ATP; 8, mixture of phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase (positive control) and [^{32}P]Bz_2ATP. Apparent molecular masses are indicated in kilodaltons. PGK-GAPDH, mixture of phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase. (b) Hydrolysis of ATP by IcsA. Each point represents the mean of 11 independent measurements. Vertical bars correspond to 1 standard deviation from the mean.

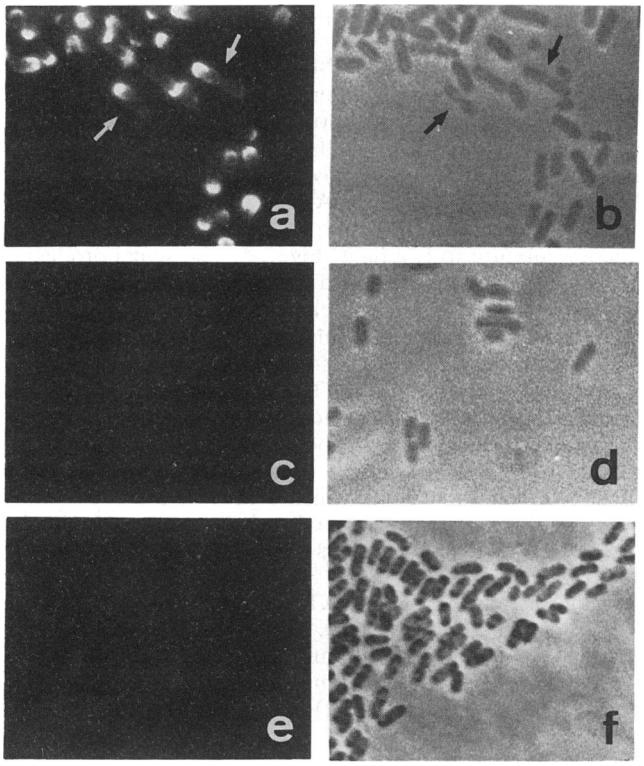

FIG. 3. Labelling of bacteria grown in vitro with affinity-purified IcsA antibody. Fluorescence microscopy of labelled bacteria (panels a, c, and e) and phase-contrast microscopy of the corresponding microscopic field (panels b, d, and f, respectively) are shown. Panels: a and b, wild-type *S. flexneri* M90T; c and d, *S. flexneri icsA* mutant SC560; e and f, *E. coli* MC1061 complemented with pHS3199, which contains *icsA*. Arrows indicate bacteria in the process of division.

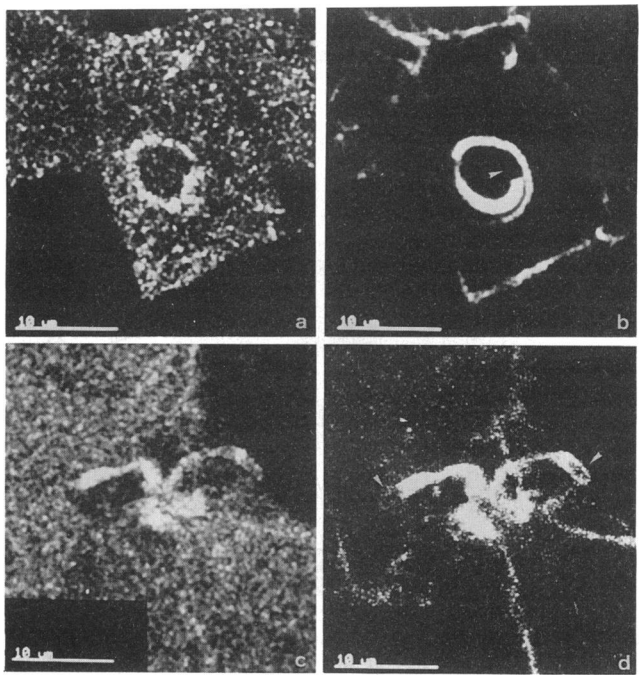

FIG. 4. Labelling of IcsA and polymerized actin in semiconfluent HeLa cell monolayers infected with wild-type strain M90T, observed with confocal fluorescence microscopy. Labelling with affinity-purified IcsA antiserum is shown in panels a and c. Labelling of the corresponding microscopic fields with 7-nitrobenz-2-oxa-1,3-diazole-phallacidin (which labels polymerized actin) is shown in panels b and d, respectively. Panels: a and b, a moving bacterium within the cytoplasm, trailed by its actin tail; c and d, bacteria at the tips of two protrusions from the cell surface, each trailed by its actin tail. The images represent the reconstitution of all focal planes containing the bacteria and their tails. The locations of bacteria are indicated in panels b and d by arrowheads.

hydrolysis of ATP by the IcsA preparation was due to IcsA rather than to a contaminant. Taken together, these data suggest that IcsA binds and hydrolyzes ATP.

Localization of IcsA on extracellular bacteria. By using affinity-purified IcsA antiserum, we examined the localization of IcsA on extracellular bacteria prior to infection of HeLa cells. Wild-type strain M90T was strongly labelled at one extremity of the bacillus and was minimally labelled over the remainder of its surface (Fig. 3a and b). Moreover, bacteria that were in the process of division were strongly labelled at the distal extremity of one of the daughter bacteria and to various degrees at the distal extremity of the second (Fig. 3a, arrows). As expected, *icsA* mutant strain SC560 was not labelled (Fig. 3c and d).

To determine whether the factors necessary for unipolar localization of IcsA are present in *E. coli*, we examined the distribution of IcsA on *E. coli* MC1061 carrying pHS3199, which encodes IcsA. This strain was not labelled by IcsA antiserum (Fig. 3e and f). Production of IcsA by this strain was confirmed by Western blotting of protein preparations (see below); therefore, the absence of surface labelling indicates that IcsA, although produced in *E. coli*, is not expressed on its surface.

Distribution of IcsA in infected HeLa cells. As we observed in extracellular bacteria, after infection of semiconfluent HeLa cells with wild-type strain M90T, intracellular bacteria appeared to be labelled only at their posterior poles. In addition, IcsA antiserum labelled the actin tail that trails bacteria within both the cytoplasm (Fig. 4a and b) and the finger-like protrusions extending from the cell surfaces (Fig. 4c and d). The area corresponding to the actin tail, beginning at the junction with the bacteria and extending throughout the entire length of the tail, was labelled, suggesting that IcsA is associated with one or several of its components. Furthermore, the intensity of the label was strongest close to the bacteria and gradually diminished at increasing distances from them.

Secretion and carboxy-terminal cleavage of IcsA. The association of IcsA with the actin tail suggested that IcsA might be secreted by the bacteria. Consequently, we looked for IcsA in the supernatant of bacterial cultures in vitro. When bacteria were grown to the late exponential phase at 37°C, Western blot analysis of whole-cell, supernatant, membrane, periplasm-enriched, and cytoplasmic-protein extracts of wild-type strain M90T, separated by SDS-PAGE under reducing conditions, demonstrated that approximately equivalent amounts of IcsA were found as a 125-kDa polypeptide in whole-cell and membrane extracts prepared from the same bacterial culture volume (Fig. 5, lanes 1 and 4, respectively). In addition, we observed no IcsA in the cytoplasmic protein extract and an extremely faint band at 125 kDa in the periplasmic protein extract (data not shown). These data indicate that virtually all IcsA within or bound to the bacteria is located in the membrane fraction. In addition,

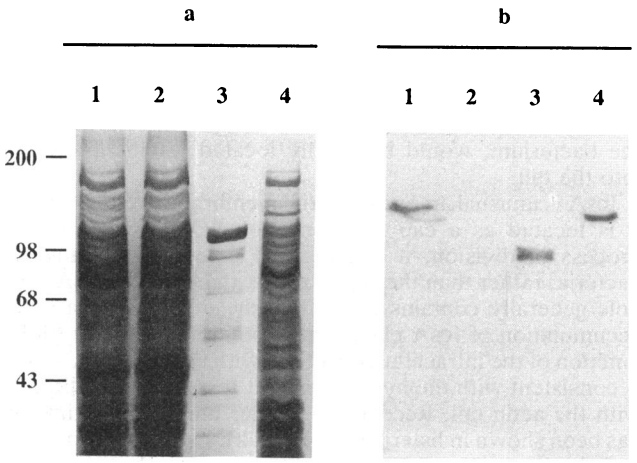

FIG. 5. Secretion of IcsA. SDS-PAGE and Western blot analysis of protein extracts of wild-type strain M90T and *icsA* mutant strain SC560. (a) SDS-PAGE. Lanes: 1, M90T whole-cell proteins; 2, SC560 whole-cell proteins; 3, M90T supernatant proteins; 4, M90T membrane proteins. (b) Western blot analysis performed with affinity-purified IcsA antibody. The lanes are identical to those in panel a. The sample in lane 3 of panel a was prepared from five times the culture volume as those in each of the other lanes, and that in lane 3 of panel b was prepared from 2.5 times the volume. Apparent molecular masses are indicated in kilodaltons.

an approximately equivalent amount of IcsA was observed as a polypeptide of 95 kDa in the supernatant extract (Fig. 5, lane 3). The 95-kDa polypeptide was not detectable in either the whole-cell or the membrane extract (Fig. 5, lanes 1 and 4, respectively).

To determine whether the 95-kDa polypeptide detected in the supernatant resulted from amino- or carboxy-terminal processing of the 125-kDa species, we performed amino-terminal sequence analysis on the supernatant polypeptide. This analysis yielded the sequence X-Pro-Leu-Ser (where X is indeterminate), which corresponds uniquely to amino acids 53 through 56 of the protein sequence deduced from the *icsA* gene (28). The amino terminus of the mature protein thus results from cleavage after a hydrophobic stretch of amino acids (positions 32 through 52) that terminates in the sequence Ala-Phe-Ala and likely corresponds to an atypical signal sequence (Fig. 6a). Cleavage of this 52-amino-acid amino-terminal peptide releases a 6-kDa fragment, which represents only a very small percentage of the decrease in molecular mass of the supernatant species compared with the membrane species (125 versus 95 kDa). This implies either that cleavage also occurs at approximately 220 amino acid residues from the carboxy terminus of the 125-kDa protein (Fig. 6b) or, less likely, that internal deletion occurs.

Maturation of IcsA is independent of the Mxi secretion apparatus. We tested whether secretion of IcsA was dependent upon the specialized transport system of shigellae that is responsible for surface expression of invasins IpaB and IpaC (2, 4, 51). Normal surface expression of these proteins relies upon several gene products of the *mxi* and *spa* loci. Included among the gene products known to be essential to this transport system is MxiD. We analyzed by Western blotting the presence or absence of IcsA in whole-cell and supernatant protein extracts of strain SF401, which contains a mutation in *mxiD*. IcsA was still secreted and cleaved in the *mxiD* mutant (data not shown), indicating that its secretion and cleavage are independent of the specialized Ipa transport system.

Secretion and carboxy-terminal cleavage of IcsA in *E. coli*. To determine whether the pathway and signals necessary for secretion and cleavage of IcsA are also present in *E. coli*, we examined the localization of IcsA in protein extracts from MC1061 carrying plasmid pHS3199, which encodes IcsA. In supernatant protein extracts, IcsA was present in MC1061 (pHS3199) as a band at 95 kDa (Fig. 7, lane 3), at the same molecular mass and in approximately the same amount as in M90T (Fig. 7, lane 1). A second band, at 80 kDa, probably represents IcsA breakdown products. In membrane fractions, IcsA was present in almost undetectable amounts in MC1061(pHS3199) (Fig. 7, lane 4), compared with the prominant band found in M90T (Fig. 7, lane 2). Thus, the pathways and signals necessary for secretion and cleavage of IcsA in shigellae are also present in *E. coli*, but IcsA either

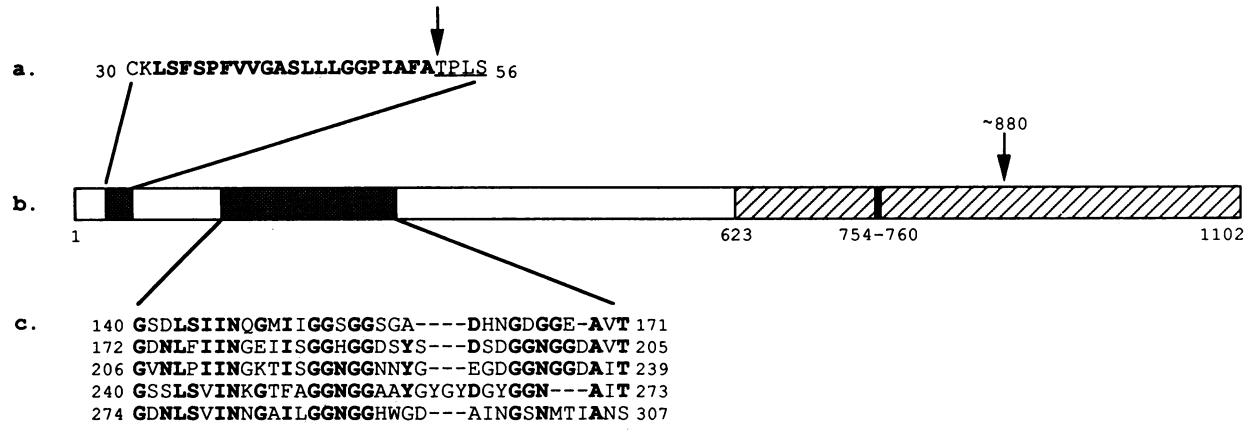

FIG. 6. Schema of IcsA. (a) Site of amino-terminal cleavage (arrow). The stretch of 21 hydrophobic amino acid residues just preceding the cleavage site is indicated in bold lettering. The amino-terminal amino acid sequence of the 95-kDa polypeptide is underlined. (b) Overall organization of the full-length protein. The approximate location of carboxy-terminal cleavage is indicated (arrow). The site of phosphorylation (20) is indicated by a solid band at residues 754 to 760. The region of carboxy-terminal homology to *E. coli* 2787 protein AIDA-I is indicated by cross-hatching. (c) Amino-terminal repetitive sequences. Residues that are identical in three or more of the repetitive sequences are indicated in bold lettering. Dashes indicate spacing introduced into the sequences to improve alignment.

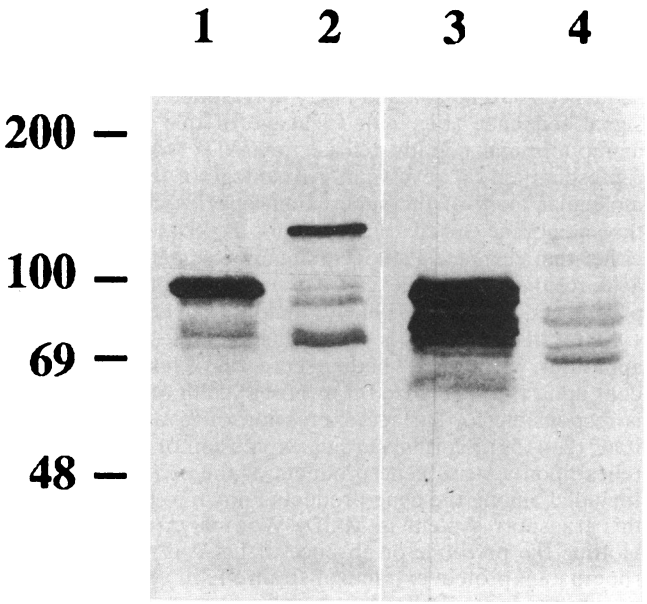

FIG. 7. Secretion of IcsA in *E. coli* compared with *S. flexneri*. Western blot analysis of protein extracts of wild-type *S. flexneri* M90T and *E. coli* MC1061 carrying pHS3199, which encodes *icsA*, performed with IcsA antiserum. Lanes: 1, M90T supernatant proteins; 2, M90T membrane proteins; 3, MC1061(pHS3199) supernatant proteins; 4, MC1061(pHS3199) membrane proteins. Approximately equivalent amounts of comparable protein preparations were loaded in lanes 1 and 3, as well as in lanes 2 and 4. Apparent molecular masses are indicated in kilodaltons.

is not anchored in the membrane of *E. coli*, as it normally is in shigellae, or is more efficiently processed at the surface of *E. coli*.

DISCUSSION

S. flexneri uses the host cell cytoskeleton to move within cells and from cell to cell. Shigella outer membrane protein IcsA was previously demonstrated to be required for intra- and intercellular movement (7, 28, 29, 48, 49). The ATPase activity, unipolar localization, and processing and secretion of IcsA, which are described in this work, suggest that it serves a unique role in bacterial movement.

We found that IcsA binds and hydrolyzes ATP. The levels of ATPase activity we observed (12 to 22 nmol min^{-1} mg^{-1}) are in the lower ranges of those observed for many classes of ATPases, including eukaryotic myosin ATPases, such as the 110-kDa protein–calmodulin complex of intestinal microvilli (14 to 500 nmol min^{-1} mg^{-1}) (14, 15, 27). These relatively low levels of activity may be due in part to the denaturing conditions required in the purification process.

The association of IcsA with the actin tail in infected HeLa cells suggests that ATP hydrolysis provides energy for actin cross-linking or another step in the organization and stabilization of the actin tail. Alternatively, as suggested recently by Theriot et al. (43), the exchange of ATP for ADP may play an important role in the induction of spontaneous polymerization of cytoplasmic monomeric actin. It has been demonstrated in listeriae that actin filaments within the tail remain stationary and that actin monomers are incorporated into filaments that are added to the tail at the junction of the tail with the bacterium, in conjunction with forward movement (16, 39, 43, 45). Further, it has been suggested that coordinated addition of actin filaments at this site is sufficient to generate the propulsive force for bacterial movement (39, 43). Similarly for shigellae, actin filaments formed in the vicinity of surface-bound IcsA, i.e., adjacent to the pole of the bacterium, would be ideally located for incorporation into the tail.

IcsA is unusual among bacterial membrane proteins in that it is located as a cap over one pole. On bacteria in the process of division, it is the distal poles of the daughter bacteria, rather than the septal plane, that contain IcsA; one pole generally contains more than the other. Such a polar accumulation of IcsA places the protein in proximity to the junction of the intracellular bacteria with the actin tail, which is consistent with the hypothesis that IcsA interacts directly with the actin tail. Recently, a similar unipolar localization has been shown in listeriae for the 67-kDa surface-associated protein ActA (25). Further, an *actA* mutant phenotypically resembles the *icsA* mutant in that it does not associate polymerized actin with its surface, is unable to spread intra- or intercellularly, and does not form protrusions from the cell surface (26). However, no significant amino acid sequence similarity between ActA and IcsA was detected. The striking similarity in localization of IcsA and ActA, as well as the similar phenotypes of the corresponding mutants, further implicates the putative roles of these proteins in the interaction of the bacteria with the actin tail.

Other than certain complex structures such as pili and flagella, very few bacterial membrane-bound proteins are known to be unipolarly localized. One of these is *Caulobacter crescentus* integral membrane protein McpA, a chemotaxis signal transducer (3). When the homologous *E. coli* chemoreceptor protein Tsr is expressed in *C. crescentus*, it is also segregated to the flagellum-bearing progeny cell, but it is not clear whether it is actually localized to the pole in *C. crescentus* or where it is localized in *E. coli*. The carboxy-terminal regions of these two proteins are very similar to one another and to those of several other bacterial chemoreceptors, including Tap, Trg, and Tar in *E. coli* and Tse and Tas in *Enterobacter aerogenes*. When this region is deleted from McpA, the protein continues to be inserted into the membrane but is no longer localized to the pole; rather, it is distributed evenly around the cell (3). There is no sequence similarity between IcsA and these chemoreceptor proteins, and the factors that govern the polar localization of IcsA are not known. Other bacterial structures that are known to be asymmetrically localized include *E. coli* protein FtsZ, which forms a ring at the septum during cell division (8), and *E. coli* peptidoglycan, which has a distinct muropeptide composition at the bacterial pole, in comparison with the lateral bacterial wall (19).

IcsA is secreted into the culture supernatant as a 95-kDa polypeptide, which has been cleaved both near the amino terminus and in the carboxy-terminal region. These data are in agreement with those of Venkatesan et al. (51), who have recently noted the presence of IcsA as a 95-kDa species in water extracts of whole organisms. By Edman degradation, we determined that amino-terminal processing occurs following the alanine at position 52 (Fig. 6). The cleaved fragment is characteristic of a signal peptide in that it contains a positively charged amino-terminal stretch followed by a hydrophobic stretch (21 amino acids long) that terminates in the sequence Ala-Phe-Ala (34, 50). While 52 amino acids is longer than a typical signal peptide, unusually long signal peptides are known to occur occasionally within members of the family *Enterobacteriaceae*. These include

the F-pilin subunit of *E. coli* (TraA) (21), the ferric-pseudobactin receptor of *Pseudomonas putida* (PupA) (9), and the phospholipase C of *P. aeruginosa* (37), which have signal peptides 51, 47, and 38 amino acids long, respectively.

To account for the decrease in size between the membrane-bound and secreted proteins, carboxy-terminal cleavage of IcsA should also occur at approximately 220 amino acids from the carboxy terminus (Fig. 6). IcsA found in the membrane fraction is unprocessed in this region, suggesting that the 125-kDa polypeptide is anchored in the membrane by its carboxy terminus and that carboxy-terminal cleavage releases the 95-kDa polypeptide into the supernatant. If IcsA is present within the actin tail, as suggested by the binding of IcsA antiserum to the tail, release of the 95-kDa polypeptide would enable it to remain incorporated within the tail at a distance from the bacterium. The 25-kDa polypeptide predicted to result from carboxy-terminal cleavage was not detected in either membrane or whole-cell extracts (data not shown), suggesting that either the fragment is degraded or the antiserum does not recognize epitopes in this region of the protein.

Benz and Schmidt (5) have recently shown significant sequence similarity between the carboxy-terminal regions of IcsA and the 132-kDa adhesin of diffusely adherent *E. coli* 2787, AIDA-I. In addition, towards the amino terminus of AIDA-I is a region of 21 repeated sequences of various lengths. Similarly, towards the amino terminus of IcsA, we identified a region that contains five repeats of a conserved 34-amino-acid sequence (Fig. 6c) which, however, have little similarity to those of AIDA-I. Benz and Schmidt have further demonstrated that AIDA-I is evenly distributed over the bacterial surface (6) and that the 132-kDa protein is processed near the carboxy terminus to produce a 100-kDa outer membrane protein (5). A notable difference is that the processed form of AIDA-I is found in the outer membrane, whereas the processed form of IcsA is found only in the culture supernatant. It is not known whether the processed form of AIDA-I is also secreted. Thus, the carboxy-terminal sequence similarity between the two proteins appears to correlate with a similarity in processing rather than with similarities in function or localization, suggesting that a common system of processing of surface proteins involving carboxy-terminal cleavage exists in shigellae and *E. coli*. Further supporting this hypothesis is our finding that IcsA expressed in *E. coli* is also secreted as a 95-kDa polypeptide. Thus, the system for normal secretion and carboxy-terminal cleavage of IcsA must be functional in *E. coli*. In contrast, IcsA is not found in membrane fractions prepared from *E. coli* strains that express IcsA, implying that the system for anchoring IcsA in the membrane is absent in *E. coli* and that anchorage in the membrane is not required for carboxy-terminal cleavage.

A mutation in *mxiD*, a gene essential to the alternative secretion pathway of shigellae (2), did not impair secretion or cleavage of IcsA. This alternative pathway in shigellae is responsible for the secretion of several plasmid-encoded virulence factors that lack signal peptides, including IpaB and IpaC (2, 4, 51). The normal secretion of IcsA in both the *mxiD* mutant and *E. coli*, as well as the presence of a cleavable signal peptide, suggests that it uses a Sec-like secretory pathway, since signal peptide-based secretion is dependent on several *sec* gene products in *E. coli* (42). The existence of a Sec-like system in shigellae is suggested by the finding that polyclonal antisera to *E. coli* SecA and SecB recognize proteins of similar molecular masses in whole-cell protein extracts of *S. flexneri* and *S. dysenteriae* (18).

In conclusion, we have demonstrated that IcsA is an ATPase that is located at one pole on the surface of the bacterium, thereby placing it in immediate proximity to the actin tail that trails moving intracellular bacteria. In infected HeLa cells, IcsA antiserum labels the actin tail throughout its length, thereby suggesting that IcsA interacts with elements within the tail. The localization of IcsA within the tail at a distance from the bacterium is consistent with our finding that IcsA is secreted into the culture supernatant and that, in conjunction with its secretion, it is cleaved near the carboxy terminus.

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