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Soluble Invasion Plasmid Antigen C (IpaC) from *Shigella flexneri* Elicits Epithelial Cell Responses Related to Pathogen Invasion

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Shigella flexneri **invades colonic epithelial cells by pathogen-induced phagocytosis. The three proposed effectors of *S. flexneri* internalization are invasion plasmid antigens B (IpaB), IpaC, and IpaD, which are encoded on the pathogen's 230-kb virulence plasmid and translocated to the extracellular milieu via the Mxi-Spa translocon. To date, there are no definitive functional data for any purified Ipa protein. Here, we describe the first characterization of highly purified recombinant IpaC, which elicits numerous epithelial cell responses related to events that take place during pathogen invasion. ^{125}I-labeled IpaC binds cultured Henle 407 intestinal cells with an apparent dissociation constant in the low micromolar range. Moreover, incubation of epithelial cells with IpaC results in general changes in cellular phosphoprotein content, demonstrating this protein's ability to influence cellular protein kinase activities. These results contrast dramatically with those seen for recombinant IpaD, which does not bind to or induce detectable changes in the normal activities of cultured epithelial cells. In addition to influencing host cell activities, preincubation of epithelial cells with purified IpaC enhances uptake of *S. flexneri* by host cells. A similar result is seen when the cells are preincubated with a highly concentrated water extract of virulent *S. flexneri* 2a (strain 2457T). Interestingly, soluble IpaC also appears to promote uptake of the noninvasive *S. flexneri* 2a strain BS103. Purified IpaD failed to enhance the uptake of virulent *S. flexneri* and did not facilitate uptake of BS103. Taken together, the data suggest that IpaC is a potential effector of the host cell biological activities and may be responsible for entry of *S. flexneri* into target cells.**

Shigella flexneri is a gram-negative bacillus responsible for bacillary dysentery, an important cause of infant mortality in underdeveloped regions of the world. Establishment of infection by *S. flexneri* is characterized by (i) bacterial invasion of the colonic epithelium (14), (ii) intracellular movement and multiplication of the pathogen (3, 31), and (iii) direct infection of neighboring epithelial cells (also called intercellular spread) (3, 16, 30). Localized trauma results as *S. flexneri* invasion of the colonic epithelium elicits localized cell death and inflammation (8). These events ultimately lead to the overt manifestations of dysentery, which include fever, abdominal cramping, and scanty, loose stools containing mucus and blood (8).

Initial invasion by *S. flexneri* resembles phagocytosis and has thus been called "pathogen-induced phagocytosis" (4, 5). Uptake of *S. flexneri* occurs when the pathogen stimulates localized accumulation of filamentous actin and myosin at the inner face of the host cytoplasmic membrane at the site of bacterial contact (4). Both the pathogen and the host cell actively contribute to this process (9, 10), and subsequent lysis of the resulting phagocytic vacuole allows the bacterium to gain access to the target cell cytoplasm (11, 31).

All the determinants necessary for mediating *S. flexneri* invasion are located on a 31-kb fragment of this pathogen's large virulence plasmid (19, 33). An important region within this fragment contains an operon encoding four polypeptides that are major targets of the host humoral immune response during *S. flexneri* infection (26). These proteins are the invasion plasmid antigens (Ipa proteins) and include IpaA (70 kDa), IpaB (62 kDa), IpaC (42 kDa), and IpaD (38 kDa) (26, 32). Synthesis of IpaB, IpaC, and IpaD is required for the *S. flexneri* invasive phenotype (21), as is the transport of these invasins to the bacterial surface and/or the external medium via products of the *mxi* and *spa* operons (1, 2, 22, 28, 37, 39).

Well-characterized nonpolar mutants possessing inactivated forms of either IpaB, IpaC, or IpaD have been instrumental in providing initial evidence for the importance of these proteins in the *S. flexneri* entry mechanism (21). Complementation analyses have further implicated IpaB as a participant in bacterial internalization and lysis of the resulting phagosomal membrane (11). Additional evidence linking IpaB and IpaC with the invasion process was provided by studies in which monoclonal antibodies known to bind specific epitopes on these proteins were shown to influence the ability of *S. flexneri* to invade epithelial cell monolayers (24, 34). With further information that IpaC possesses immunogenic domains that may have important roles in determining disease outcome following exposure to *Shigella* spp. (35), it is becoming clear that the Ipa proteins, IpaB and IpaC in particular, contribute directly to the onset of shigellosis. Unfortunately, no biochemical data are yet available to define the roles of the individual Ipa proteins in pathogen uptake, and few studies have been initiated to investigate directly the individual in vitro activities of these invasins (18).

It was recently suggested that the Ipa proteins are rapidly secreted from the *S. flexneri* outer membrane and from intracellular stores upon incubation of the bacterium with epithelial cells (22, 28, 39, 40). In these studies, IpaB and IpaC were found to exist as part of a secreted protein complex (23, 28). IpaD was not found to associate significantly with the extracellular IpaB-IpaC complex and was proposed to function as a transient effector of IpaB secretion within the context of the

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Mxi-Spa translocon (28). The latter suggestion was based upon observations that IpaD appeared to form a complex with IpaB but only within the *S. flexneri* membrane (22). The putative extracellular IpaB-IpaC complex has been proposed to mediate pathogen entry (20, 22, 23, 39, 40), possibly through binding of $\alpha_5\beta_1$ integrin receptors (38). After binding, the Ipa complex may induce signaling cascades that trigger changes in target cell protein kinase activities (6, 38) and promote lysis of the resulting phagosomal membrane (11). Unfortunately, no invasion-related biochemical activity has yet been attributed to a purified form of any of the Ipa proteins, thus compromising definitive assignment of specific roles for any of these invasins.

Here, we provide the first report of possible invasion-related activities for a highly purified recombinant Ipa protein. The *ipaC* and *ipaD* genes (18, 29) were cloned from the invasion plasmid of *S. flexneri* and inserted into a plasmid expression vector. Following expression, each was purified and its effects on cultured epithelial cells were investigated. IpaD had no detectable activity in any of these experiments; however, IpaC was found to specifically bind epithelial cells and induce a number of cellular responses. These responses are believed to be related to IpaC-mediated events occurring in the *S. flexneri* internalization process.

MATERIALS AND METHODS

Materials. $Na^{125}I$ and [^{32}P]orthophosphoric acid in water were obtained from New England Nuclear (Boston, Mass.); 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril was from Sigma Chemical Co. (St. Louis, Mo.). Dulbecco's modified Eagle's medium was from Gibco-BRL (Grand Island, N.Y.), and Eagle's modified minimal essential medium (MEME) was from Fisher Scientific (St. Louis, Mo.). All other chemicals were of reagent grade.

Growth and maintenance of cultures. *S. flexneri* 2a (strain 2457T) was provided by A. T. Maurelli (Uniformed Services University of the Health Sciences, Bethesda, Md.) and routinely grown at 37°C on Trypticase soy agar containing 0.025% Congo red. Where stated, *S. flexneri* 2a which had been cured of its invasion plasmid (strain BS103) was used and similarly maintained. The latter strain appeared white on Congo red plates, while strain 2457T appeared red on this medium because of the *crb* locus (8). The plasmid expression vectors pWPD10 (*ipaD*/pET15b) and *ipaC*/pET15b (now called pWPC15) were maintained in *Escherichia coli* BL21(DE3)(pLysS) (Novagen, Madison, Wis.), which is routinely grown at 37°C in Luria-Bertani broth containing 50 μg of ampicillin per ml. Henle 407 epithelial cells (ATCC CCL6) were routinely grown in monolayers with 5% CO_2 in MEME supplemented with 10% newborn calf serum.

Plasmid construction. Construction of pWPD10 has been described previously (18), and *ipaC*-containing pET15b (pWPC15) was constructed as described elsewhere (29). Briefly, PCR primers based on published sequences (36) were made to copy the *ipa* gene from the virulence plasmid of *S. flexneri* 2a strain 2457T. In the case of pWPD10, the gene fragment was digested with *Nde*I and *Bam*HI and inserted into *Nde*I-*Bam*HI-digested pET15b from Novagen. In the case of pWPC15, the *ipa* PCR fragment was first trapped in the pCRII plasmid of Invitrogen (San Diego, Calif.) and then subcloned into pET15b. For expression of the recombinant protein, each plasmid was transformed into *E. coli* BL21(DE3)(pLysS).

Purification of recombinant Ipa proteins. Recombinant HisTag-IpaC was expressed by inducing early-log-phase cultures of BL21(DE3)(pLysS) containing pWPC15 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were grown at 30°C with mild shaking (150 rpm) to slow bacterial growth, thereby enhancing the yield of IpaC expressed in a soluble form (29). After 2 h, the bacteria were harvested by centrifugation, resuspended in column binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl [pH 7.9]), and sonicated, and the resulting lysate was clarified by centrifugation at 39,000 × *g* for 20 min. The resulting IpaC (or IpaD) fusion protein was purified by virtue of its short leader peptide which contains six tandem histidine residues for HisBind (Ni^{2+}-chelation resin) affinity column chromatography according to the instructions of the manufacturer (Novagen). Following elution with imidazole, purified IpaC was dialyzed against phosphate-buffered saline (PBS) (10 mM sodium phosphate [pH 7.2] containing 150 mM NaCl). Protein concentrations were determined by bicinchoninic acid microprotein determination (Sigma Chemical Co.).

Immunoblot analysis of the Ipa proteins. The identification of Ipa proteins in purified protein preparations, or in water extracts of virulent *S. flexneri* (26), could be carried out by immunoblot analysis as described previously (18). Briefly, proteins were first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (15) and electrophoretically transferred to a polyvinylidene difluoride membrane with a Transblot semi-dry blotting apparatus (Bio-Rad, Madison, Wis.). The membrane was incubated with 3% nonfat dried milk in 20 mM Tris-HCl containing 150 mM NaCl (TBS) to block nonspecific protein binding sites. The blots were then incubated with antibody diluted in TBS containing 0.05% Nonidet P-40. Monkey prechallenge and convalescent-phase sera, monoclonal antibody to IpaC (2G2), and polyclonal rabbit serum to IpaD were provided by E. V. Oaks (Walter Reed Army Institute for Research, Washington, D.C.). After being washed in TBS containing Nonidet P-40, the membranes were incubated with ^{125}I-labeled protein G. The membranes were washed in TBS containing 1 M NaCl and 4% sodium *N*-lauroylsarcosine and exposed to X-ray film for autoradiography.

Changes in the phosphorylation of host cell proteins in the presence of IpaC. To monitor Ipa protein effects on host cell protein kinase activities (6), Henle 407 cells in 25-cm^2 flasks (approximately 4×10^6 cells) were incubated for 30 min in 2 ml of high-glucose Dulbecco's modified Eagle's medium lacking phosphate which was supplemented with 50 μCi of [^{32}P]orthophosphoric acid. Next, PBS alone or PBS containing IpaC or IpaD (1 ml) was added to the monolayers, and the flasks were incubated for another 30 min at 37°C. The flasks were placed on ice, and the cells were washed three times with 2 ml of ice-cold PBS. The cells were then scraped into 1 ml of PBS, collected by centrifugation, and resuspended in 200 μl of water to which 200 μl of 2× SDS-PAGE sample buffer was added. The samples were boiled for 5 min, and 25 μg of protein was separated on an SDS–10% polyacrylamide gel. The gel was stained with Coomassie blue R250, destained, dried, and exposed to X-ray film. Qualitative changes in the phosphoprotein profile of the cells could be observed by autoradiography.

Protein binding to cultured epithelial cells. To examine the ability of IpaC or IpaD to associate with cultured Henle 407 cells, each protein was labeled with ^{125}I by a standard protocol for iodination using 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (7). Radiolabeled IpaD or IpaC was incubated for 30 min with monolayers of Henle 407 cells seeded on 24-well culture plates (approximately 2×10^5 cells per well). The amount of radiolabeled protein bound to the cells (on a per-cell basis) was monitored by washing the cells extensively with PBS and determining the radioactivity present in a uniformly distributed cell suspension incubated with 0.25% trypsin. Similar results were obtained when the cells were solubilized with 1% Triton X-100 in water (data not shown).

Assay of *S. flexneri* uptake by cultured epithelial cells. Uptake assays were performed essentially by the method of Niesel et al. (25). Henle 407 cells were grown to near confluence in 24-well tissue culture plates. *S. flexneri* 2a strain 2457T, grown to an A_{600} of about 0.4, was diluted with MEME containing 0.67 μg of $FeCl_3$ per ml and 0.45% glucose (MEME-Fe) so that a final multiplicity of infection (MOI) of about 3 was reached. To each well containing Henle 407 cells, MEME-Fe was added alone or with increasing concentrations of protein extracted from the surface of *S. flexneri* cells with water (water-extracted *Shigella* proteins) (26), IpaD or IpaC. The cells were incubated for 30 min at 37°C, after which the liquid was removed by aspiration. *S. flexneri* organisms (in 250 μl of MEME-Fe) were added and incubated with the cells for 30 min at 37°C. Following incubation, the bacterial suspension was removed by aspiration, and the cells were washed six times with MEME containing 5% newborn calf serum and 40 μg of gentamicin per ml (with a 1-min incubation during each wash). The cells were then incubated with the last gentamicin-containing wash for 2 h prior to its removal. This treatment killed the bacteria remaining exposed to the medium but not those that had been internalized. The cells were then rinsed with MEME-Fe lacking serum and gentamicin. Each monolayer was overlaid with 250 μl of 0.5% agarose. After solidification, the agarose was overlaid with 0.5% agar containing 2× Luria-Bertani medium. The plate was then incubated at room temperature for 30 min and inverted for incubation at 37°C overnight. The *S. flexneri* cells protected from gentamicin because of uptake by the Henle 407 cells were seen as subsurface colonies and counted with a dissecting microscope. This uptake assay was also used with the noninvasive *S. flexneri* 2a strain BS103 to investigate the ability of IpaC to promote entry of a noninvasive bacterium. The same assay could be modified to monitor *S. flexneri* binding to Henle 407 cells by omitting gentamicin from the wash steps. It should be pointed out, however, that this modification of the assay does not distinguish between bacteria adhering to the surface of the Henle 407 cells and organisms adhering to open spaces on the surface of the culture dish.

RESULTS

Preparation of recombinant IpaC and IpaD. After transformation of *E. coli* BL21(DE3)(pLysS) with pWPC15 (or pWPD10), high-level expression of IpaC (or IpaD) could be induced with IPTG and seen by SDS-PAGE with Coomassie staining (Fig. 1A). Because each protein possesses a short leader peptide containing six consecutive histidine residues, it was possible to affinity purify each overexpressed protein product on a Ni^{2+} chelation resin (Fig. 1A, lanes 3 and 4). In samples of purified IpaC, the full-size IpaC product was visible as the major protein product on Coomassie-stained gels, with a molecular mass of about 45 kDa. Also visible are some smaller protein products which are stable IpaC degradation

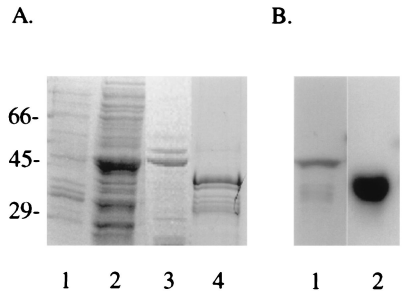

FIG. 1. Expression and purification of IpaC and IpaD. *E. coli* BL21(DE3)(pLysS) was transformed with plasmid pET15b containing *ipaC* (pWPC15) or *ipaD* (pWPD10). (A) Coomassie-stained SDS–10% polyacrylamide gel. Lane 1, whole cells harboring pWPC15 prior to induction of recombinant protein synthesis; lane 2, extract of cells containing pWPC15 after 2 h of induction with IPTG; lane 3, recombinant IpaC following purification on the HisTag-resin; and lane 4, IpaD following its purification by the same method. Molecular masses (in kilodaltons) are shown on the left. (B) The purified IpaC (lane 1) and purified IpaD (lane 2) were analyzed by immunoblotting with an anti-IpaC monoclonal antibody (2G2) or polyclonal antibodies prepared against synthetic peptides from IpaD.

products (see below). Identification of purified IpaC and IpaD was carried out by immunoblot analysis using anti-IpaC monoclonal antibody (2G2) or rabbit polyclonal antiserum raised against synthetic peptides from IpaD (Fig. 1B). The purified antigens were also seen to react with sera from convalescent-phase monkeys but not with sera from the same monkeys prior to challenge with *S. flexneri* (data not shown). Moreover, the 2G2 monoclonal antibody reacted with the small polypeptides present in the IpaC sample (Fig. 1B), which indicates that these are stable IpaC degradation products. Degradation products of a similar size were seen when the same monoclonal antibody preparation was used with concentrated *S. flexneri* 2a water-extracted proteins (data not shown). It is not yet known if the presence of the predominant IpaC degradation product (about 25 to 30 kDa) has any link to *S. flexneri* pathogenesis.

Binding of ^{125}I-labeled IpaC to Henle 407 cells. An effector of the host cell events that lead to pathogen-induced phagocytosis would be anticipated to interact in some way with the host cell, most likely through a specific receptor-ligand interaction. To determine whether Henle 407 cells can interact with IpaD or IpaC, each protein was radioiodinated and its binding to these cells was monitored. There did not appear to be a significant interaction between ^{125}I-IpaD and the cell monolayers (Fig. 2A), and the minor amount of IpaD binding that was observed did not appear to saturate within the IpaD concentration range examined (giving an apparent dissociation constant [K_d] near 0.5 mM). In contrast, ^{125}I-IpaC bound well to the cells (Fig. 2A) and had an apparent dissociation constant near 5 μM. To test the specificity of ^{125}I-IpaC association with Henle 407 cells, unlabeled IpaC and IpaD were used as competitive inhibitors of ^{125}I-IpaC binding. While excess unlabeled IpaC was found to effectively block the binding of ^{125}I-IpaC, IpaD had no effect on this binding (Fig. 2B). The specific interaction of IpaC with the surface of epithelial cells suggests that IpaC is involved in the early steps of host cell invasion.

Changes in cellular phosphoprotein profile. It has been suggested that an early event in the uptake of *S. flexneri* by epithelial cells is the stimulation of host cell tyrosine kinase activities (6). The observation that IpaC binds to epithelial cells indicates that this important *S. flexneri* virulence protein may be capable of triggering specific signal transduction pathways. Because protein phosphorylation events have been postulated to play a role in host cell signaling during *S. flexneri* invasion, IpaC was incubated with Henle 407 cells and changes in the cellular profile of phosphoproteins were examined. In these experiments, the cells were incubated in phosphate-free MEME to which $^{32}P_i$ was added. After a 30-min incubation (to allow equilibration of the cells with the radioactive phosphate), either PBS alone or PBS containing IpaC or IpaD was added to the medium and the cells were incubated for another 30 min. At the end of the second incubation, the profile of phosphorylated proteins was monitored by separating the cellular proteins by SDS-PAGE and using the dried gel for autoradiography. IpaD had only a minor effect on the phosphoprotein profile of the Henle 407 cells relative to that of control cells incubated with PBS (Fig. 3). In contrast, IpaC caused a major decrease in the overall extent of protein phosphorylation in the cells which was not accompanied by any apparent qualitative change in the phosphoprotein profile (Fig. 3). These results

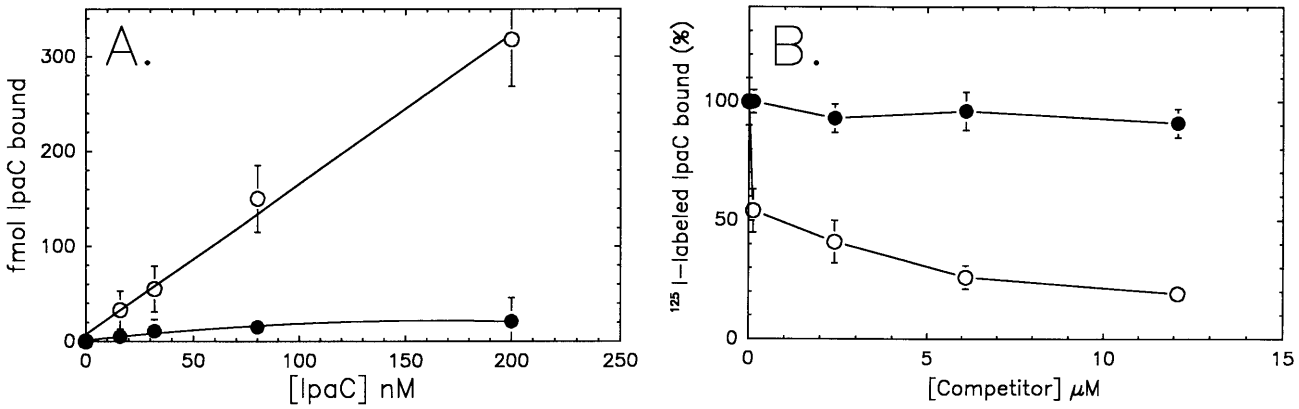

FIG. 2. IpaC and IpaD were labeled with ^{125}I so that their association with Henle 407 cells could be monitored. Radioiodinated protein (specific activity = 10^6 cpm/μg) was incubated for 30 min with monolayers of Henle 407 cells (2×10^5) seeded onto 24-well plates. Following the incubation, the monolayers were washed five times with PBS. The cells were then released from their monolayers with 0.25% trypsin, and aliquots of a uniform cell suspension were used to determine the counts per minute that had remained with the cells after extensive washing. The concentration of IpaC or IpaD that remained with the cells was determined on the basis of the known molecular weight of each protein and the known amount of protein (in micrograms) incubated in each well. (A) Binding of ^{125}I-IpaC to the cells (open symbols) and binding of ^{125}I-IpaD (closed symbols). The points given are an average of four samples (± standard error [SE]). (B) The binding of approximately 300 fmol of ^{125}I-IpaC was competitively blocked by adding excess unlabeled IpaC (open symbols). In contrast, ^{125}I-IpaC binding was essentially unaffected by adding excess unlabeled IpaD (closed symbols). The points shown are an average of four samples ± SE.

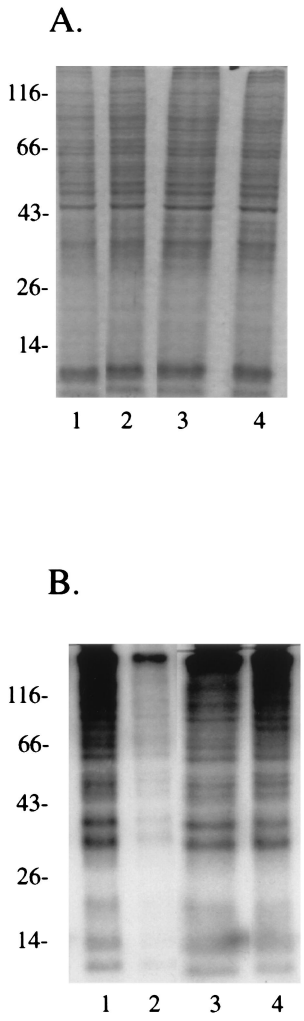

FIG. 3. Changes in the phosphoprotein content of Henle 407 cells caused by incubation with IpaC. Henle 407 cells were incubated for 30 min in 2 ml of PBS containing 50 μCi of $^{32}P_i$. The Henle 407 cells were then incubated for another 30 min following the addition of 1 ml of PBS (lane 1) or PBS containing IpaC (lane 2), IpaD (lane 3), or intact virulent *S. flexneri* 2a (lane 4). The cells were then washed, scraped loose from the monolayers, pelleted by mild centrifugation, and lysed by adding 200 μl of water, 200 μl of SDS sample buffer, and 10 μl of β-mercaptoethanol. (A) The proteins in the sample (50 μg of protein) were separated by SDS-PAGE. The gels were stained with Coomassie blue R250 so that the amounts of protein in the lanes could be compared. (B) The dried gels were exposed to X-ray film to monitor the phosphoprotein profile in each lane. Molecular masses (in kilodaltons) are shown on the left.

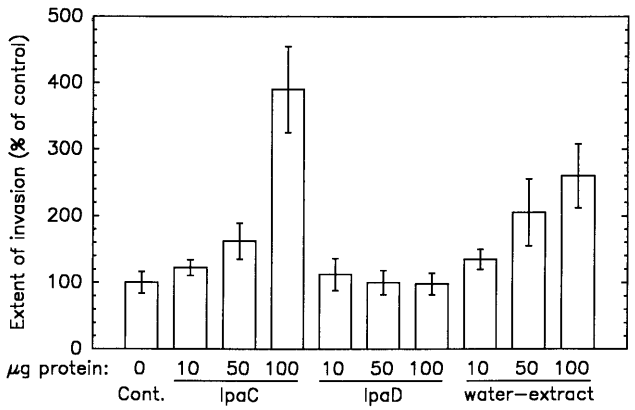

FIG. 4. *S. flexneri* 2a invasion of Henle 407 cell monolayers in 24-well culture plates monitored as a function of increasing concentrations of IpaC, IpaD, or proteins extracted from intact virulent *S. flexneri* 2a with water and dialyzed against PBS. The cells were preincubated for 30 min with PBS alone or PBS containing IpaC, IpaD, or water-extracted proteins. The cells were then rinsed, and 250 μl of MEME-Fe containing *S. flexneri* 2a was added to give an MOI of 3. The bacteria were incubated with the cells for 30 min, and the monolayers were then washed with MEME containing gentamicin. After being washed, the cells were overlaid with agarose and then agar containing 2× Luria-Bertani medium. The effect of preincubation on invasion was determined by comparison with controls in which either no *Shigella* organisms (negative control) were added or in which *Shigella* organisms were added but the preincubation mixture did not contain any test protein (positive control). The values given are the averages (±SE) of five experiments, with 100% invasion being 116 colonies (±31). Statistical significances of differences relative to the control (Cont.) sample (using the Student's *t* test) were $P < 0.1$ for 50 μg of IpaC and 50 μg of water extract protein, $P < 0.05$ for 100 μg of water extract protein, and $P < 0.01$ for 100 μg of IpaC.

provide evidence that IpaC exerts effects on Henle 407 cells that influence intracellular signaling processes. Interestingly, no such obvious effect on the cellular phosphoprotein content was seen when the Henle 407 cells were incubated with intact virulent *S. flexneri* at an MOI of 5 (Fig. 3). The latter observations could be due to the relative concentration of IpaC in the *S. flexneri* sample or due to the presence of factors associated with the intact pathogen that temper or modify the activity of IpaC. At a higher MOI, *S. flexneri* did cause a general decrease in Henle 407 cell phosphoprotein content (data not shown); however, this was accompanied by an increase in cell killing. IpaC was not found to be cytotoxic to the cells as determined by exclusion of trypan blue.

IpaC effects on the uptake of *S. flexneri* by Henle 407 cells. To further investigate the role of IpaC as a soluble invasin, Henle 407 cells were preincubated with PBS alone or with PBS containing IpaD, IpaC, or proteins extracted from *S. flexneri* 2a with water, so that the influence each preparation has on subsequent pathogen uptake could be determined. Preincubation of the cells with micromolar concentrations of IpaC enhanced the apparent uptake of *S. flexneri* (Fig. 4). Because the assay used measures the protection of bacteria from gentamicin, these results are interpreted to reflect an enhancement of pathogen uptake via macropinocytosis; however, it is also possible that protection from gentamicin occurs as bacteria become trapped within membrane folds that form on the host cell surface in response to IpaC binding. Either possibility suggests that IpaC evokes host cell changes that contribute to invasion. A similar event was observed when Henle 407 cells were preincubated with concentrated water extracts of *S. flexneri* 2a (Fig. 4), while no such effect was seen when any concentration of IpaD was preincubated with the epithelial cells (Fig. 4).

To further investigate this phenomenon, the effect of IpaC on *S. flexneri* adherence to Henle 407 cells was monitored and also found to be enhanced (from 620 to 1,364 adherent organisms per well at an MOI of 0.3; average of three wells). These data complicated matters in that increased adherence of virulent *S. flexneri* organisms (capable of facilitating their own invasion following IpaC-mediated host cell binding) could perhaps account entirely for the enhanced internalization of the pathogen. To determine whether pathogen binding alone could explain the enhancement of uptake of internalized organisms, noninvasive *S. flexneri* 2a strain BS103 was added to the Henle 407 cells after they had been incubated with IpaC to determine if uptake of these cells could be achieved. Indeed, preincubation of the epithelial cells with IpaC promoted uptake of BS103, albeit at a much lower rate than that of 2457T (Fig. 5). IpaD was not able to stimulate the uptake of BS103

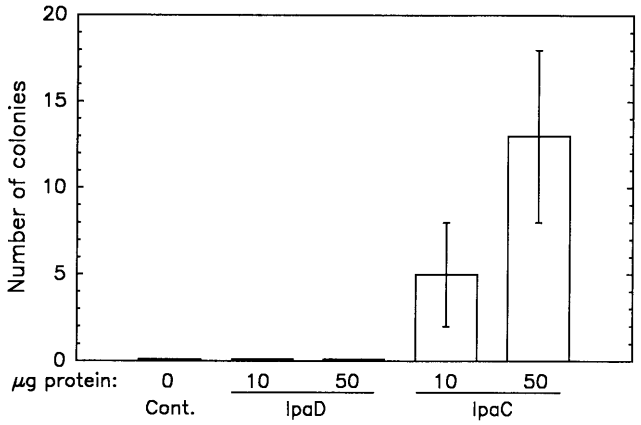

FIG. 5. Purified IpaC induces invasion of Henle 407 cells by *S. flexneri* 2a strain BS103. The same invasion assay as used for Fig. 4 was carried out except that noninvasive *S. flexneri* strain BS103 (cured of the virulence plasmid) was used. Controls (Cont.) containing either no protein in the preincubation mix or no *Shigella* organisms in the subsequent incubation had no growth. The graph shows the number of invasive bacteria (averages of six experiments ± SE) seen for each concentration of IpaC or IpaD.

and had no effect on the adherence of 2457T. These data suggest that purified recombinant IpaC is capable of inducing uptake of a noninvasive strain of *S. flexneri*, further indicating the importance of IpaC as a major component of the invasiveness apparatus of *S. flexneri.*

DISCUSSION

S. flexneri and related species are major causes of morbidity and mortality in humans, particularly children, around the world. The low dose of organisms required for the establishment of infection makes shigellosis a particularly vexing health problem for a major segment of the world's population. A detailed description of the biochemistry of this organism's pathogenesis would provide a major step toward developing measures for the treatment and prevention of shigellosis.

Genetic analyses have demonstrated that the invasive phenotype of *S. flexneri* requires secretion of IpaB, IpaC, and IpaD via a type III (Mxi-Spa) secretory pathway (22). Inactivation of *ipaB*, *ipaC*, or *ipaD* or interference with the secretion of their gene products is sufficient for elimination of the *S. flexneri* invasive phenotype (1, 2, 22, 28, 39). Following their secretion, IpaB and IpaC form a complex that is postulated to be of importance in invasion (20, 22, 28). Indeed, when this complex is immunoadsorbed from a crude mixture of proteins to the surface of latex beads, the uptake of these particles by epithelial cells appears to be stimulated (20). While these data were interpreted to demonstrate the importance of soluble IpaB-IpaC complexes in the pathogenesis of *S. flexneri*, they perhaps better represent what would occur if the Ipa proteins were immobilized at the pathogen surface. In this respect, the IpaB-IpaC complex appears to mimic invasin from *Yersinia pseudotuberculosis* (12) except that the latter promotes internalization by a zippering mechanism while the IpaB-IpaC complex promotes uptake via a less well defined mechanism (20). Watarai and coworkers found that a culture filtrate containing soluble Ipa proteins could stimulate the uptake of noninvasive *spa32* mutants of *S. flexneri* 2a but not plasmid-cured *S. flexneri* 2a (39). Interestingly, the *spa32* mutants are able to expose the Ipa proteins on the bacterial surface but are unable to release these proteins into the surrounding medium (39). In this respect, the *spa32* mutant resembles Ipa protein-coated latex beads except that the coated beads are able to enter HeLa cells while the *spa32* mutants are not able to enter MK2 cells except in the presence of Ipa protein-containing culture filtrates (20, 39). These apparently contradictory results further demonstrate the complexity of the *S. flexneri* invasion mechanism and underscore the need for functional analyses using purified forms of the Ipa proteins.

There are currently no functional data available that precisely define the role of an individual Ipa protein in *S. flexneri* invasion. To alleviate this oversight, we have cloned, expressed, and purified IpaB, IpaC, and IpaD from *S. flexneri* 2a to study the in vitro biochemical properties of these proteins (29). Here, we report the first direct evidence of a biological activity for IpaC which relates to the invasion of Henle 407 cells by *S. flexneri*. Purified recombinant IpaC binds epithelial cells to trigger changes in host phosphoprotein content and uptake of *S. flexneri* 2a (strain 2457T). Moreover, preincubation of the epithelial cells with recombinant IpaC causes the apparent uptake of the noninvasive BS103 strain of *S. flexneri* 2a. None of these properties were observed for an identically prepared recombinant form of IpaD. The fact that IpaC stimulates uptake of a noninvasive bacterium strongly suggests a prominent role for this antigen in the initial stages of pathogen internalization.

The action of the Ipa invasins is dependent upon their surface localization and subsequent release from surface and cytoplasmic stores via the Mxi-Spa translocon system (1, 22, 37). It is perhaps not surprising that a highly concentrated water extract of *S. flexneri* enhances invasion by this pathogen via secreted invasins. This is consistent with a model in which the action of the secreted Ipa invasins is highly concentration dependent. Release of IpaB and IpaC upon contact with epithelial cells would result in localized high concentrations of these invasins over a relatively small area of the host cell surface. These high concentrations would be required to elicit the host cell cytoskeletal changes that are ultimately responsible for pathogen internalization (4, 5). Once the pathogens are enclosed in a macropinocytotic vesicle, further Ipa protein secretion would result in the massive accumulation of Ipa protein within the confines of the vesicle, which could be a prerequisite for lysis of the phagosomal membrane. Such a model is consistent with the need for contact between *S. flexneri* and erythrocytes to elicit an otherwise inefficient hemolysis (11, 31; unpublished observations) and with the need for relatively high concentrations of IpaC (in the micromolar range) for eliciting the observed effects in cultured cells. Interestingly, this relatively large K_d is consistent with associations that involve integrin receptors which have recently been proposed as being the Ipa protein receptor (38).

Possibly the most curious finding presented here is that incubation of Henle 407 cells with IpaC results in an overall decrease in cellular protein phosphorylation. Previous studies have implicated increased tyrosine phosphorylation of $pp60^{src}$ targets in the early stages of *S. flexneri* internalization (6, 38). At first glance, the results presented here appear to be in conflict with these previous findings (although this study does implicate changes in cellular protein phosphorylation in the action of an Ipa invasin). It is possible, however, that the high concentrations of IpaC used here, although not toxic to the host cell, may overload intracellular signaling processes and result in an apparent diminution of protein phosphorylation in general. This would be consistent with recent observations that invasion of HeLa cells by *S. flexneri* causes a decrease in the intracellular pools of nucleotide triphosphates (17).

Taken together, the data presented here provide exciting

new information on the cell biology of *S. flexneri* invasion. It will now be interesting to examine the biochemical properties of highly purified recombinant IpaB for comparison with the findings presented here and with findings on the function of a reconstituted IpaB-IpaC complex. Furthermore, the door is now open to more sensitively investigate the complex immunobiology of shigellosis. Studies of the host immune response to IpaD have revealed that this protein antigen may provide an extremely useful tool for the immunosurveillance of *Shigella* infections (27). Now, with findings that host immune recognition of specific epitopes on IpaC may be correlated with disease onset, it may be possible to use IpaB, IpaC, and IpaD to create a battery of immunodiagnostic tools for testing the efficacy of the live oral vaccines that are being developed (13). Perhaps it will even be possible to augment available live oral vaccines by using purified preparations of these recombinant Ipa invasins.

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