Interaction of *Shigella flexneri* IpaC with Model Membranes Correlates with Effects on Cultured Cells

NGOC TRAN,1 ALEXA BARNOSKI SERFIS,1 JOHN C. OSIECKI,2 WENDY L. PICKING,2 LISETTE COYE,3 REBECCA DAVIS,3 AND WILLIAM D. PICKING2*

*Department of Chemistry1 and Department of Biology,3 Saint Louis University, St. Louis, Missouri,* and *Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas2*

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Invasion of enterocytes by *Shigella flexneri* requires the properly timed release of IpaB and IpaC at the host-pathogen interface; however, only IpaC has been found to possess quantifiable activities in vitro. We demonstrate here that when added to cultured cells, purified IpaC elicits cytoskeletal changes similar to those that occur during *Shigella* invasion. This IpaC effect may correlate with its ability to interact with model membranes at physiological pH and to promote entry by an *ipaC* mutant of *S. flexneri.*

*Shigella flexneri* is an important cause of dysentery with a high incidence of infant mortality in developing nations. An early step in *Shigella* infection is bacterial invasion of colonic epithelial cells (8). Invasion is characterized by host cytoskeletal rearrangements at the site of bacterial contact, which leads to the formation of filopodia that coalesce and trap the pathogen within a membrane-bound vacuole (1). The resulting phagosomal membrane is rapidly lysed following pathogen uptake (16). IpaB and IpaC have been identified as being the effectors of *Shigella* invasion (9–11) following their secretion at the host-pathogen interface via the *mix-spa* secretory system (4, 12, 21). Once released within this localized area, IpaB and IpaC form a complex (2, 14) that is reported to be responsible for pathogen entry (10, 12, 18, 20, 21).

It was originally proposed that IpaB alone induces bacterial uptake and promotes the subsequent escape of *S. flexneri* into the host cell cytoplasm (6). This role was revised when purified IpaB could not be shown to possess membranolytic activity in vitro (13) and when purified IpaC was found to possess both a potential effector role in invasion (9, 19) and the ability to cause the release of small molecules from phospholipid vesicles at low pH (3). In the work presented here, a novel model membrane system is used to show that purified IpaC penetrates phospholipid membranes at neutral pH and that this activity may be correlated with IpaC’s role as an effector molecule that elicits cytoskeletal changes in cultured cells when it is added to the extracellular environment. The ability for IpaC to penetrate phospholipid membranes and to promote cellular effects may provide a significant step forward in our understanding of the mechanism by which *S. flexneri* directs its own uptake by enterocytes. Moreover, the data presented here may have a broader impact in the area of bacterial pathogenesis since *Salmonella enterica* initiates its entry into epithelial cells by an extraordinarily similar mechanism that requires SipC, a putative IpaC homologue.

**IpaC interacts with membranes at neutral pH.** Protein penetration of cell membranes occurs as a part of numerous biological processes. The incorporation of proteins into phospholipid Langmuir monolayers (17) provides a potentially valuable model for exploring specific protein-lipid interactions that have an important role in the pathogenesis of *S. flexneri*. The Langmuir membrane system used here is composed of a dipalmitoylphosphatidylcholine (DPPC) monolayer generated on top of a buffered aqueous subphase. This system mimics the outer faces of the cytoplasmic membranes of most mammalian cell types, as they are encountered by proteins secreted from *S. flexneri*. Although such a monolayer is structurally different from the phospholipid bilayer that makes up a cell’s cytoplasmic membrane, it provides a suitable model membrane for sensitively detecting specific lipid-protein interactions such as those that occur at the surface of a cell.

When a protein is injected into an aqueous subphase and given time to interact with an overlying lipid monolayer, changes in surface pressure can be monitored to deduce the nature of the protein-lipid interaction. Penetration of a protein into the phospholipid monolayer forces the phospholipids to become more tightly packed due to the space taken up by the protein (thereby giving rise to an increase in surface pressure). While the phospholipid monolayer model used here does not reproduce the entire structure of the cellular phospholipid bilayer, it has been shown to provide an effective system for monitoring protein-membrane interactions (17). Moreover, the sensitivity for detecting protein penetration of membranes using Langmuir monolayers far surpasses that seen using conventional approaches that detect protein-membrane interactions only after they result in the disruption of phospholipid vesicles (3). Because IpaC-mediated effects on cultured cells have not been found to be cytotoxic, it is important to explore this protein’s interaction with model membranes under conditions that, during invasion, may induce only subtle changes in the structure of the cytoplasmic membrane.

For proteins that do not interact with phospholipid membranes, maximum accumulation of the protein at the air-water interface occurs in the absence of phospholipids or when the phospholipids are very loosely packed and provide gaps at the air-water interface for the proteins to occupy. The ability of such a protein to accumulate at the air-water interface is rapidly diminished when the initial monolayer density is increased until, at high initial lipid densities, the protein’s access to the air-water interface is blocked (17). In contrast, a protein that possesses the ability to penetrate a phospholipid membrane tends to interact poorly at the air-water interface but demonstrates an enhanced ability to migrate to this site as the initial phospholipid monolayer density is increased. This behavior is common for known membrane-penetrating proteins such as...
factor VII, a protein involved in the blood-clotting cascade (17). The ipaC coding sequence was cloned and expressed, and IpaC was purified as described previously (15). The presence of a short leader sequence containing a His6 tag allowed affinity purification of IpaC in the presence of 6 M urea (2). When prepared with urea, IpaC was dialyzed against 20 mM phosphate (pH 7.2)–150 mM NaCl (phosphate-buffered saline [PBS]) containing 2 M urea. Refolding of the protein was facilitated by rapid dilution as described previously (2), and protein concentrations were measured by a bicinchoninic acid protein assay (Sigma Chemical Co.). To monitor potential interactions between purified recombinant IpaC and a DPPC monolayer, a NIMA model 611D Langmuir-Blodgett trough equipped with a model PS4 pressure sensor was used to monitor changes in membrane surface pressure (17). An aqueous subphase containing 0.1 M Tris (pH 7.4), and 500 μl was injected into the subphase to give a final concentration of 3 μg/ml. IpaC and factor VII (500 μl each) were added to the subphase to give a final concentration of 0.02 μg/ml each. The proteins were allowed to interact with the monolayer for 75 min, and the degree of protein accumulation at the interfacial region was measured as a time-dependent increase in surface pressure.

The largest changes in surface pressure for the negative-control protein (fibrinogen) were observed at the lowest initial lipid pressures (Fig. 1A), with no increase in surface pressure seen when the monolayer was compressed to 10 mN/m or higher. In contrast, IpaC showed the highest changes in surface pressure at the highest initial lipid pressures (Fig. 1B), with no surface activity seen in the absence of phospholipids. Membrane-binding proteins typically display this type of increased surface activity in the presence of a compacted phospholipid monolayer (17). Even at initial surface pressures approaching 30 mN/m (the estimated average density of phospholipids in the cell membrane), IpaC efficiently penetrated the DPPC monolayer, although at a rate that was somewhat lower than when the initial surface pressure was 15 mN/m (data not shown), indicating that this activity has physiological relevance.

The magnitude of the increased surface pressure caused by IpaC was comparable to that seen with the same concentration of factor VII, which also displays an enhanced ability to penetrate phospholipid monolayers as the initial surface pressure is increased (Table 1). These data indicate that IpaC interactions with phospholipid membranes at neutral pH are similar to that of a known membrane-penetrating protein. Unlike factor VII, whose penetration of phospholipid membranes is calcium dependent (17), IpaC interacts with the DPPC monolayer in the absence or presence of added calcium (Table 2). Interestingly, while the absolute value of the maximum surface pressure change for the phospholipid monolayers caused by IpaC is greater in the presence of 10 mM calcium, the ratio of IpaC penetration at high initial surface pressures relative to that at low initial surface pressures is greater in the absence of calcium (Table 2). This indicates that IpaC penetration of phospholipid membranes, unlike that of factor VII, is not calcium dependent.

Interestingly, when IpaC is freshly diluted from solutions containing urea, the observed protein-induced surface pressure changes are higher (Table 2). In control experiments, urea...
alone (at concentrations up to 50 mM) did not cause changes in surface pressure when it was injected beneath compressed phospholipid monolayers at any initial lipid pressure (data not shown), indicating that urea in the IpaC samples did not contribute directly to the larger observed surface pressure changes. From Table 2, it is clear that IpaC interacts more extensively with phospholipid membranes when it starts out in a partially unfolded state. This is interesting since, like SipC from *Salmonella* (7), IpaC appears to be secreted via a supramolecular “needle complex” that spans the inner and outer membranes of the pathogen (5). In *Salmonella*, the needle complex is proposed to have a relatively narrow inner diameter (5), indicating that SipC is probably released in a partially unfolded state. It is anticipated, therefore, that IpaC and SipC rapidly generate their final tertiary and quaternary structures at the host-pathogen interface (concomitant with formation of IpaC [SipC]-containing protein complexes).

De Geyter and coworkers suggest that IpaC possesses membrane-lysing potential based on its ability to release calcine trapped in vesicles composed of phosphatidylserine (an acidic phospholipid typically found at the inner face of the cytoplasmic membrane) and phosphatidylcholine (a neutral phospholipid) (3). This lytic activity is largely pH dependent, with little activity seen at neutral pH and with most efficient membrane lysis occurring below pH 6.0 (3). This is distinct from the penetration of DPPC membranes at neutral pH as described here. Moreover, it was shown in previous work that IpaC enhances the invasive capacity of *S. flexneri* without lysing host cells (9) and IpaC does not appear to possess a pH-dependent hemolytic activity in vitro (data not shown).

Because of the sensitivity of the assay described here for monitoring protein-membrane interactions under a variety of conditions, it is now possible to monitor the effect that IpaC and IpaC-containing protein complexes have on membranes composed of different phospholipids. This will provide a convenient model for deducing the events occurring at the host-pathogen interface immediately prior to *S. flexneri* entry into host cells. It is difficult at this point to determine whether IpaC penetration of DPPC monolayers is comparable to that of pore-forming toxins as previously proposed by De Geyter and coworkers (3); however, it is important to note that IpaC does not lyse and is not cytotoxic for cultured Henle 407 cells (9; data not shown). Therefore, if IpaC does form a pore following interaction with a host cell membrane, this pore does not result in the rapid death of the host cell. Alternatively, it is possible that IpaC penetrates phospholipid membranes at neutral pH but forms a pore only as the pH is lowered to that found in early endosomal compartments. This would be consistent with the work reported by De Geyter et al. (3). It should be possible to determine whether IpaC possesses properties consistent with pore formation by monitoring protein-protein interactions (see reference 2) involving membrane-imbbed IpaC.

It has been shown that IpaB-IpaC complexes being immunoprecipitated onto the surfaces of latex beads is sufficient for promoting the uptake of these beads by cultured cells, suggesting that both IpaB and IpaC have important roles in cellular invasion by *S. flexneri* (10). It is therefore possible that IpaB influences the action of IpaC at the host-pathogen interface and that it has a profound effect on IpaC’s ability to interact with phospholipid membranes. It will be important to compare the interactions that IpaC has with phospholipid membranes before and after its recruitment into protein complexes containing IpaB. Because Ipa complexes presumably represent IpaC in its natural extracellular context, their formation can be expected to enhance the membrane interactions seen here. It is also possible that recruitment into Ipa complexes may diminish IpaC’s interaction with phospholipid membranes while increasing its ability to interact with cellular integrins, which has been described as being a potential receptor for the Ipa complex by Terajima et al. (18) and Watarai et al. (20). In addition to using Langmuir films to explore the action of IpaC, it will be important to explore the same parameters for *Salmonella* invasion protein C (SipC), which is a putative homologue of IpaC (7). A comparison of the membrane-penetrating potentials of these two proteins would be enlightening since *Salmonella* invades epithelial cells in much the same way as *S. flexneri*, but without lysing the resulting membrane-bound vacuole (7).

**Addition of purified IpaC to Henle 407 cells promotes cytoskeletal changes.** Because IpaC interacts with DPPC membranes and has been shown to promote the uptake of virulence plasmid-cured *S. flexneri* when added at high concentrations, its effect on the cytoskeleton of Henle 407 cells was explored. Henle 407 cells (ATCC CCL6) were grown in Eagle’s modified minimal essential medium (MEME; Fisher Scientific) containing 10% calf serum (Gibco-BRL) and incubated in 5% CO₂. When incubated in 2 μM IpaC in serum-free medium, Henle 407 cells become rounded after 1 h, with some of the cells detaching after even longer incubations (data not shown). At no point during exposure to IpaC do the attached cells display IpaC-related cytotoxicity as monitored by their ability to exclude trypan blue or their failure to release lactate dehydrogenase into the culture supernatant (data not shown). These morphological changes suggest that IpaC induces cytoskeletal rearrangements in Henle 407 cells. To confirm this, Henle 407

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**TABLE 1. Changes in the surface pressures of DPPC membranes caused by factor VII and IpaC**

<table>
<thead>
<tr>
<th>Initial lipid pressure (dynes/cm)</th>
<th>Change in lipid pressure upon addition of the protein (ΔΠ)c</th>
<th>Factor VII</th>
<th>IpaC</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.8</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.6</td>
<td>2.1</td>
<td>1.4</td>
<td>2.1</td>
</tr>
<tr>
<td>10</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The data shown are representative results from a series of at least three independent experiments with similar results (deviation, ±10%). Factor VII requires the presence of 10 mM CaCl₂ for significant membrane penetration, while the results given for IpaC were obtained in the absence of added calcium.

**TABLE 2. The effect of urea and calcium on surface pressure changes caused by IpaC**

| Urea presenta | Ca²⁺ addedb | Change in lipid pressure upon addition of IpaC (ΔΠ)c at an initial lipid pressure (dynes/cm) of: |
|---------------|------------|---------------------------------------------------------------------------------|---|---|---|---|---|
|               |            | 3   | 5   | 10  | 15  |
| −             | −          | 0.3 | 1.0 | 1.4 | 2.1 | 7.0 |
| −             | +          | 0.6 | 1.0 | 1.7 | 2.1 | 3.5 |
| +             | −          | 1.1 | 1.3 | 2.4 | 2.9 | 2.6 |
| +             | +          | 2.2 | 2.8 | 3.4 | 4.1 | 1.9 |

a The data shown are representative results from a series of at least three independent experiments that generated similar results (deviation, ±10%).

b Samples were prepared in 2 M urea as described in the text and rapidly diluted into 0.1 M Tris (pH 7.4) to promote protein refolding. The final concentration of urea was less than 50 mM following addition to the aqueous subphase in all cases.

c CaCl₂ was added to a final concentration of 10 mM.
cells were grown on coverslips and incubated with 2 μM IpaC in serum-free MEME. Actin staining was carried out by simultaneously fixing, permeabilizing, and staining the cells for 15 min in a solution containing 3.7% formaldehyde, 1% palmitoyl-lysophosphatidylethylamine, and rhodamine-phalloidin. The stained cells were washed with PBS and overlaid with PBS containing 50% glycerol and 1 mg of n-propylgalactarate per ml. Stained actin was viewed by fluorescence microscopy on an Olympus BX60 microscope equipped with a charge-coupled device camera (Optronics) and videocapture card (Truevision).

After a 7-min incubation with IpaC, f-actin appeared to accumulate at the cell edges with the appearance of numerous microspikes (Fig. 2). After 30 min, the cells started to show signs of rounding up while appearing to have less overall f-actin content relative to that seen at 7 min as judged by a decrease in overall fluorescence intensity (Fig. 2). These time points correlate reasonably well with the reported times for cytoskeletal changes observed during S. flexneri invasion (1). It was recently reported that IpaC induces actin polymerization in (i) 3T3 cells permeabilized with saponin, (ii) 3T3 cells microinjected with IpaC, and (iii) HeLa cells expressing ipaC (19). In this work, IpaC added to the extracellular environment elicits a similar effect except that, unlike with permeabilized cells (19), IpaC does not cause detectable cytotoxic effects (at low IpaC concentrations) at times exceeding 1 h (data not shown). This observation suggests that permeabilization introduces cellular changes that ultimately disrupt ongoing functions and that such disruptions do not occur when IpaC is added to the extracellular milieu.

FIG. 2. Extracellular IpaC promotes cytoskeletal changes in Henle 407 cells. The cells were incubated at 37°C in serum-free MEME and immediately fixed (A) or fixed after 7 or 30 min in the serum-free MEME (B and C, respectively). Alternatively, the cells were incubated in serum-free MEME containing 2 μM IpaC for 7 min (D) or 30 min (E) and stained for analysis of polymerized actin by fluorescence microscopy (at a magnification of ×400). 1/8, 1/8-s exposure.
The ability of IpaC to elicit changes in cultured cells suggests that it interacts with the host cell surface to trigger a cascade of events. An earlier study implicated αβ₁ integrins as potential receptors for Ipa protein complexes (20). In previous work from our laboratory, the need for high IpaC concentrations to promote overt cellular effects may indicate that there is a large number of possible IpaC binding sites (9), perhaps because the IpaC interaction with host cells is rather general. Such an interaction could be explained if IpaC was able to interact directly with the host cell membrane in addition to, or instead of, integrin receptors.

Exogenously added IpaC promotes uptake of a S. flexneri ipaC mutant. In a recent report, it was shown that expression of ipaC in HeLa cells leads to a fivefold enhancement in the uptake of S. flexneri (19). Earlier work showed that exogenously added IpaC enhances invasion over fourfold for IpaC prepared in 20 mM phosphate (pH 7.2) with 150 mM NaCl (PBS) (9) and eightfold for IpaC prepared in PBS containing 2 M urea (2). Moreover, high concentrations of IpaC promote the uptake of small numbers of plasmid-cured S. flexneri (9). To determine the potential importance of IpaC-induced cytoskeletal changes and its potential relationship with IpaC-membrane interactions, the protein was added to Henle 407 cells and the subsequent effect on the uptake of different strains of S. flexneri was monitored. S. flexneri 2a strain 2457T was provided by A. T. Maurelli (Uniformed Services University of the Health Sciences, Bethesda, Md.), and S. flexneri strain SF621 (carrying a nonpolar mutation in ipaC) (11) was provided by Philippe Sansonetti (Unité de Pathogénie Microbiennne Moléculaire, Institut Pasteur, Paris, France). S. flexneri entry into Henle 407 cells was quantified using a gentamicin protection assay as described previously (9) except that the bacteria were centrifuged onto the surfaces of the Henle 407 cells to promote efficient contact between the bacteria and the host cells. S. flexneri strain 2457T was used at a multiplicity of infection (MOI) of 1.0, while the noninvasive strains BS103 and SF621 were used at an MOI of 10 or greater. Bacteria were added to the monolayers in serum-free MEME and incubated for 30 min at 37°C. The monolayers were then washed with MEME containing 5% newborn calf serum and 50 μg of gentamicin per ml, rinsed with serum-free MEME, and overlaid with 0.5% agarose and 0.5% agar containing 2× Luria-Bertani medium. The plates were incubated overnight at 37°C, and the resulting colonies were counted.

Exogenously added IpaC does not induce uptake of the plasmid-cured S. flexneri strain BS103 at high nanomolar concentrations, even at a high MOI, while purified IpaC does promote uptake of strain SF621 (data not shown). While an ipaC null mutant of S. flexneri is noninvasive and avirulent (11), exogenously added IpaC (50 nM) restores a significant portion of its invasive capacity (up to 5% of wild-type activity in some experiments). An important observation here is that because IpaC is a relatively efficient extracellular effector for the uptake of SF621 but not for BS103, there is a factor in addition to IpaC, perhaps IpaB and/or IpaD, that is an important participant in the invasion process.

Taken together, the data indicate that purified IpaC penetrates model phospholipid membranes, induces cytoskeletal changes in cultured epithelial cells, and promotes uptake of an ipaC null mutant of S. flexneri by cultured cells. It is not yet clear whether penetration of phospholipid membranes by IpaC is directly related to the observed changes in actin polymerization in Henle 407 cells; however, invasion data may support this possibility. As shown in Table 3, the uptake of wild-type S. flexneri is enhanced over eightfold by exogenously added IpaC when the IpaC protein is present in its fully folded state prior to its addition to the reaction mixture of the modified invasion assay used here. In contrast, IpaC enhances invasion by wild-type S. flexneri invasion by nearly 15-fold when it is freshly refolded from a stock solution containing 2 M urea (Table 3). These data are consistent with earlier results (2), and they parallel results from experiments designed to explore IpaC’s ability to more efficiently penetrate phospholipid membranes when starting out in a partially folded state (Table 2). Therefore, it appears likely that there is a correlation between IpaC-mediated effects on epithelial cells and IpaC-dependent penetration of phospholipid membranes.

The data presented here provide the first evidence that purified IpaC elicits cytoskeletal changes in cultured cells when it is presented as part of the extracellular environment. The ability for IpaC to carry out this activity, and thus its role in Shigella pathogenesis, may be related to its ability to interact with and possibly integrate into the cytoplasmic membranes of host cells. This is a significant observation that should contribute greatly to our understanding of the events responsible for the early steps in pathogenesis. Clearly the potential relationship between IpaC-membrane interactions and IpaC-mediated changes in the cytoskeletons of cultured epithelial cells warrants continued investigation.

Interestingly, the invasive phenotype of S. flexneri requires the properly timed secretion of IpaB and IpaD along with IpaC. Elimination of the gene encoding any of these proteins does not prevent secretion of the others (12), but it does completely eliminate the invasive phenotype (11). The importance of IpaD has been suggested to be at the level of secretion (12); however, IpaD has also been described as being part of a complex that (i) associates with αβ₁ integrins (20) and (ii) is involved in the uptake of noninvasive Escherichia coli (18). Other evidence suggests that an IpaB-IpaC complex is the effector of S. flexneri invasion (10), while data from our laboratory and that of others indicate that IpaC alone has a central role in the entry of S. flexneri into cultured cells (9, 19). In its soluble form, IpaC exists as part of a complex that involves both IpaC-IpaC and IpaC-IpaB interactions (2). It is therefore important to consider the potential effect that IpaB may have on IpaC’s ability to penetrate the host cell membrane following the release of both proteins at the host-pathogen interface. As mentioned previously, IpaB may have a profound influence on IpaC’s ability to penetrate phospholipid membranes; however, continuing work will be needed to determine what form these effects will take. Determining the regions on IpaC that are

### Table 3. Exogenously added IpaC enhances invasion by wild-type S. flexneri

<table>
<thead>
<tr>
<th>S. flexneri strain</th>
<th>Form of IpaC added</th>
<th>Relative invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS103</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>BS103</td>
<td>PBS</td>
<td>0</td>
</tr>
<tr>
<td>BS103</td>
<td>PBS + 2 M urea</td>
<td>0</td>
</tr>
<tr>
<td>2457T</td>
<td>None</td>
<td>1.00</td>
</tr>
<tr>
<td>2457T</td>
<td>PBS</td>
<td>8.33</td>
</tr>
<tr>
<td>2457T</td>
<td>PBS + 2 M urea</td>
<td>14.81</td>
</tr>
</tbody>
</table>

* IpaC was either prepared in the absence of denaturing agents and was added from a PBS stock solution or prepared in the presence of urea and dialyzed against PBS containing 2 M urea prior to being added to the invasion assay. In all experiments, the final concentration of IpaC was 100 nM and the concentration of urea never exceeded 40 mM.

* Invasion by wild-type S. flexneri in the absence of added IpaC was 54 colonies per well and was given the relative value of 1.00. All values are the averages (+15%) of triplicate results, and although values varied slightly from one experiment to the next, these data are representative of at least three independent experiments.
important for membrane penetration and those important for the protein-protein interactions involving IpaC may provide a great deal of insight into the effects that IpaB may have on the results presented here. Moreover, the prominent hydrophobic domains identified on IpaB may indicate that this protein, in concert with IpaC, has an important role in phagosomal escape by \textit{S. flexneri}. This would be consistent with the fact that IpaB appears to be important for phagosomal escape (6) while purified IpaB is not hemolytic (14). Such a scenario suggests that the IpaB-IpaC complex retains the ability to penetrate phospholipid membranes. Indeed, a tremendous amount of work remains; however, the membrane penetration assay described here and the protein-protein interactions described in a previous report from this laboratory (2) should provide pertinent information on the detailed mechanism of \textit{S. flexneri} entry into epithelial cells.

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