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Liposomes Recruit IpaC to the *Shigella flexneri* Type III Secretion Apparatus Needle as a Final Step in Secretion Induction▽

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Shigella flexneri **contact with enterocytes induces a burst of protein secretion via its type III secretion apparatus (TTSA) as an initial step in cellular invasion. We have previously reported that IpaD is positioned at the TTSA needle tip (M. Espina et al., Infect. Immuno. 74:4391–4400, 2006). From this position, IpaD senses small molecules in the environment to control the presentation of IpaB to the needle tip. This step occurs without type III secretion induction or IpaC recruitment to the *S. flexneri* surface. IpaC is then transported to the *S. flexneri* surface when target cell lipids are added, and this event presumably mimics host cell contact. Unlike IpaB mobilization, IpaC surface presentation is closely linked to secretion induction. This study demonstrates that sphingomyelin and cholesterol are key players in type III secretion induction and that they appear to interact with IpaB to elicit IpaC presentation at the TTSA needle tip. Furthermore, IpaB localization at the needle tip prior to membrane contact provides the optimal set of conditions for host cell invasion. Thus, the *S. flexneri* type III secretion system can be induced in a stepwise manner, with the first step being the stable association of IpaD with the needle tip, the second step being the sensing of small molecules by IpaD to mobilize IpaB to the tip, and the third step being the interaction of lipids with IpaB to induce IpaC localization at the needle tip concomitant with translocon insertion into the host membrane and type III secretion induction.**

Shigella flexneri, the causative agent of shigellosis, is responsible for more than 1 million deaths each year, especially among children in developing regions (www.who.int/vaccines-documents/DocsPDF99/www9947.pdf). Once ingested, *S. flexneri* crosses M cells and passes into the underlying gut-associated lymphoid tissues of the colon (20), where it kills macrophages (29) and then invades epithelial cells by macropinocytosis (17). The *S. flexneri* invasive phenotype localizes genetically to a 31-kb region of its large virulence plasmid and is absolutely tied to its type III secretion system (TTSS) (6, 23).

TTSSs are used by numerous gram-negative bacteria to introduce bacterially derived effector proteins into the membrane and cytoplasm of a target cell, resulting in the subversion of normal cell functions (8). Linking the bacterium and host cell in this process is the type III secretion apparatus (TTSA), which structurally resembles a molecular needle and syringe. The system is controlled by a basal body (the syringe) that spans both bacterial membranes and an external needle that provides a conduit from the basal body to the sensory needle tip complex (8, 28). The needle in *S. flexneri* is comprised of a polymer of MxiH and is approximately 50 nm long and 7 nm in diameter, with a central channel that is about 2.5 nm in diameter (5). At the top of the MxiH needle resides the tip protein IpaD, most likely as a pentamer, which serves as an environmental sensor for the MxiH-IpaD tip complex (4, 7). When the presence of bile salts such as deoxycholate (DOC) is sensed by IpaD, the first translocator protein, IpaB, is mobilized to the TTSA needle tip to form an MxiH-IpaD-IpaB ternary complex. At this stage, the TTSA structure is primed for subsequent host cell contact (19, 24). In previous studies IpaC had not been found to localize to the *S. flexneri* surface of the log-phase bacterium (7, 19).

As a next step in describing the process of type III secretion, we show here that liposomes trigger mobilization of IpaC to the needle tip complex, where it is immediately inserted into the host cell membrane, along with IpaB, to complete the TTSA conduit into the host cell just prior to initiating host cytoskeleton rearrangements. IpaC is most efficiently recruited to the *S. flexneri* surface with a defined liposome composition that includes phospholipids, sphingomyelin (SM), and cholesterol (Chol). Furthermore, IpaC recruitment occurs concomitantly with induction of type III secretion of IpaB, IpaC, and IpaD into the *S. flexneri* culture supernatant. It thus appears that IpaB mobilization to the *S. flexneri* TTSA needle tip represents a second discrete step in TTSA assembly, with the final third step being IpaC recruitment to the needle tip, which occurs after IpaB contacts and inserts into the host cell membrane.

MATERIALS AND METHODS

Materials. The *S. flexneri ipaC* null mutant (strain SF621) and *ipaA-ipgD* null mutant were from P. J. Sansonetti (Institut Pasteur, Paris, France) (18). The *S. flexneri mxiH* null strain was generated by A. Allaoui (Brussels, Belgium) (3). Antibodies were from E. V. Oaks, Walter Reed Army Institute for Research (Silver Springs, MD). Sheep and goat red blood cells were from the Colorado Serum Company (Denver, CO). Alexa Fluor-labeled secondary antibodies were from Molecular Probes (Eugene, OR). DOPC (1,2-dioleoyl-sn-glycero-3-phos-

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phocholine), DOPG {1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]}, and SM were from Avanti Polar-Lipids, Inc. (Alabaster, AL). Hank's balanced salt solution (HBSS) and Chol were from the Sigma Chemical Co. (St. Louis, MO). Minimum essential medium with Earle's salts (MEME) was from Gibco-BRL. All other chemicals were reagent grade.

Preparation of liposomes from sheep erythrocytes. Liposomes were prepared from sheep erythrocytes as described previously (2, 12). Briefly, 15 ml of erythrocyte suspension was collected by centrifugation, washed three times with 10 mM sodium phosphate (pH 7.0)–150 mM NaCl (phosphate-buffered saline [PBS]), and subjected to hypotonic lysis with deionized water. The resulting erythrocyte ghosts were collected by centrifugation and washed four times with PBS. The ghosts were used directly to assay for IpaC mobilization to the *S. flexneri* surface, or the lipids were extracted with chloroform and methanol (2). The extracted lipid fraction was then dried under vacuum conditions and resuspended in 2 ml of PBS. To ensure that the procedure resulted in liposome preparations of uniform size, the suspension was pulse sonicated, centrifuged, and extruded 10 times at 45°C through a 100-nm-pore-size membrane (1).

Preparation of liposomes having a defined composition. Commercial lipids were resuspended in chloroform and mixed in the following ratios to achieve 1 mg/ml: (i) DOPC; (ii) DOPG:DOPC (1:3); (iii) SM:DOPC (1:3); (iv) Chol:DOPC (1:3); and (v) SM:Chol:DOPC (1:1:2). Lipid mixtures were dried under vacuum conditions. The resulting lipid films were resuspended in 1 ml of PBS, sonicated to form liposomes, and extruded through a 100-nm-pore-size membrane as described above (1).

Incubation of *S. flexneri* with liposomes. To determine the ability of liposomes to promote the mobilization of IpaC to the *S. flexneri* TTSA needle tip, a modification of the standard contact-mediated hemolysis assay was used (21, 22). Briefly, *S. flexneri* were grown to the log phase at 37°C in tryptic soy broth (TSB) containing 0.1% DOC and 50 μg/ml kanamycin (100 μg/ml ampicillin was included when appropriate). The bacteria were collected, resuspended in 200 μl of PBS, and combined with either 200 μl of erythrocyte-derived liposomes or defined liposomes. After 90 min at 37°C, the bacteria and associated proteins were fixed with formaldehyde and the reaction was quenched with glycine. The presence of IpaC on the *S. flexneri* surface was determined by immunofluorescence microscopy. To reduce exposure risks, an *ipaA-ipgD* null strain was initially used for many of these experiments. This strain has a wild-type phenotype for secretion control, invasion, and hemolysis (18). To determine the region of IpaC required for its recruitment to and maintenance at the *S. flexneri* TTSA needle tip, previously characterized *ipaC* mutants of *S. flexneri ipaC* null strain SF621 were used (21, 26).

Fluorescence microscopy. Bacteria were dried on a coverslip, blocked with 1% bovine serum albumin in PBS, and immunolabeled with rabbit polyclonal anti-MxiH, anti-IpaB, and/or mouse monoclonal anti-IpaC antibodies. Alexa Fluor 488 goat anti-rabbit and/or Alexa Fluor 488 or 568 anti-mouse immunoglobulin G (IgG) was utilized for fluorescence detection. Imaging was performed using an Olympus IX-81 microscope equipped with 488- and 568-nm-excitation laser lines, a 60× objective (numerical aperture, 1.45), and a Hamamatsu electronic microscopy charge-coupled device. Phase-contrast images were also obtained. Student's *t* tests were performed using the resulting data, as needed.

Electron microscopy. When *mxiH* is subcloned behind the inducible *lac* promoter of pRK2 and protein expression is induced in *mxiH* null mutant SH116 (giving *mxiH* pRK2/SH116), long needles (some more than 300 nm in length) are generated, making them more readily visible on the bacterial surface (11, 25). This strain was grown at 37°C in TSB containing 100 μg/ml ampicillin, 50 μg/ml kanamycin, 0.1% DOC, and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were harvested by centrifugation, resuspended in the appropriate liposome preparation, and incubated for 90 min at 37°C. Glutaraldehyde was added to achieve a final concentration of 4%, and the samples were placed on either carbon-Formvar or holey-carbon electronic microscopy grids. The samples were treated with rabbit polyclonal anti-IpaB antibodies followed by 5-nm-diameter gold-labeled goat anti-rabbit IgG $F(ab')_2$ and with mouse monoclonal anti-IpaC antibodies followed by 10-nm-diameter gold-labeled anti-mouse IgG $F(ab')_2$. Negative staining with 2% uranyl acetate permitted visualization of the samples. Samples were imaged using an FEI Tecnai G^2 field emission transmission electron microscope at an electron acceleration voltage of 200 KeV.

Induced secretion of TTSS proteins. Induction of type III secretion in *S. flexneri* can be examined by measuring protein secretion following the addition of Congo red (CR). CR stimulates a rapid burst of protein secretion by the *S. flexneri* TTSS, thus mimicking the response seen upon host cell contact (11). Bacteria were grown in a single broth culture, and then the culture was divided equally for incubation with liposomes or other treatments to ensure that identical numbers of bacteria were represented in the Western blot analysis. After a 20-min incubation with various liposome preparations, bacteria were removed by centrifugation. The resulting supernatant fraction from identical numbers of cells was assessed for IpaB, IpaC, and IpaD secretion by Western blot analysis using rabbit anti-IpaC antibodies and mouse monoclonal anti-IpaB and -IpaD antibodies and the corresponding Alexa Fluor 680 goat anti-mouse IgG and Alexa Fluor 800 goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Images were obtained and relative protein ratios quantified using an Odyssey Infrared Imager (LI-COR, Lincoln, NE).

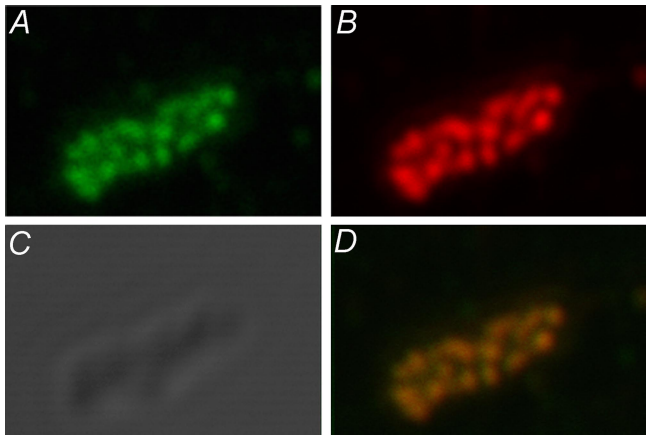

FIG. 1. Immunofluorescence microscopy was used to demonstrate the presence of IpaC on the *S. flexneri* surface following incubation with sheep erythrocyte ghosts. Panel A shows an immunofluorescence micrograph of IpaB localization. IpaC localization is seen in panel B, and a merged image composed of the images presented in panels A and B is seen in panel D. Panel C shows a differential interference contrast micrograph.

Invasion of cultured HeLa cells. The ability of *S. flexneri* to invade cultured cells was measured by a modified gentamicin protection method (11). To assess the impact of IpaB localization on invasion, these assays were performed using HBSS to avoid the effects that MEME has on tip complex composition (data not shown). Bacteria for these assays were grown under several sets of conditions, including those that promote localization of IpaB or of IpaB plus IpaC on the bacterial surface. Student's *t* tests were performed using the resulting data as needed.

RESULTS

IpaC can be induced to localize to the *S. flexneri* surface as part of the TTSA needle tip complex. We have previously shown that IpaD alone localizes to the TTSA needle tip of *S. flexneri* in the log phase in TSB. IpaB colocalizes with IpaD only when *S. flexneri* bacteria are incubated in the presence of DOC during the log phase; however, IpaC has not previously been observed on the *S. flexneri* surface (7, 19, 24). Because IpaC is required for translocon pore formation and contact-mediated hemolysis, it is likely that it must become a part of the tip complex upon contact with erythrocytes. Therefore, the contact-mediated hemolysis assay was modified to visualize IpaC on the bacterial surface. Bacteria were grown in the presence of DOC to the log phase and then incubated with the erythrocytes. Indeed, mouse anti-IpaC antibodies and Alexa Fluor 488 goat anti-mouse IgG detected IpaC on the *S. flexneri* surface; however, the released hemoglobin interfered with microscopy (data not shown). Confirmation of this event was achieved when erythrocyte ghosts were used. Under these conditions, IpaC could be clearly visualized on the surface of 25% of the observed *S. flexneri* bacteria (Fig. 1B). Furthermore, IpaC was visible only in areas where IpaB was also detected (Fig. 1D), indicating colocalization.

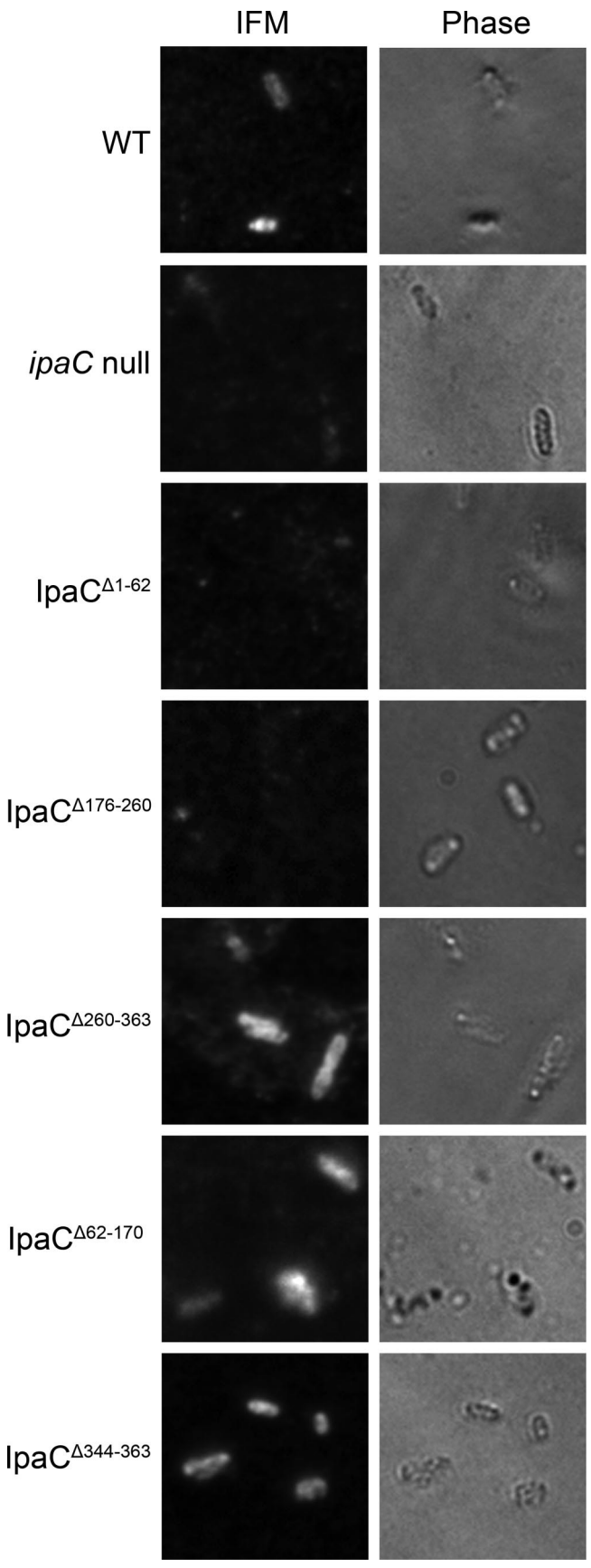

FIG. 2. Immunofluorescence microscopy (IFM) was used to determine whether different IpaC deletion mutants could be retained at the *S. flexneri* surface following incubation with sheep erythrocyte-derived liposomes. No IpaC was observed on the surface of *S. flexneri ipaC* null strain SF621 bacteria or of bacteria that synthesized IpaC with either an IpaC$^{\Delta 1-62}$ or IpaC$^{\Delta 176-260}$ deletion mutant. IpaC$^{\Delta 260-363}$, IpaC$^{\Delta 344-363}$, and IpaC$^{\Delta 62-170}$ deletion mutants all stained positively for IpaC on the *S. flexneri* surface after incubation with liposomes. WT, wild type.

The central hydrophilic region of IpaC may be important for its maintenance at the *S. flexneri* surface. We have previously shown that IpaC possesses distinct functional domains (9, 21, 26). Therefore, we sought to use these constructs to determine the regions within IpaC that are required for its stable maintenance at the *S. flexneri* surface. Erythrocyte liposomes were used in these experiments and induced IpaC surface localization in 20% of the observed bacteria. Because the TTSS signal is located at the IpaC N terminus, an IpaC$^{\Delta 1-62}$ mutant lacking residues 1 to 62 (IpaC$^{\Delta I}$; 21) is not secreted. Thus, as expected, IpaC$^{\Delta 1-62}$ failed to be detected on the *S. flexneri* surface after addition of the liposomes (Fig. 2). Although an IpaC$^{\Delta 176-260}$ mutant lacking central hydrophilic residues 176 to 260 (IpaC$^{\Delta II}$; 21) exhibits residual invasiveness (10%) in *S. flexneri* SF621 (21), the mutant protein did not associate with the *S. flexneri* surface (Fig. 2). In contrast, truncations of the C-terminal portion of IpaC, including IpaC$^{\Delta 260-363}$ (IpaC$^{\Delta III}$; 21) and IpaC$^{\Delta 344-363}$ (an effector region deletion referred to as 1035; 26), did not prevent maintenance of IpaC on the *S. flexneri* surface after the addition of erythrocyte-derived liposomes. Similarly, an IpaC$^{\Delta 62-170}$ mutant with its hydrophobic region removed (IpaC$^{\Delta H}$; 21) still associated with the *S. flexneri* surface (Fig. 2). This mutant is noninvasive and has greatly reduced stability and secondary structure content as a purified recombinant protein (13). However, if the secondary structure of residues 176 to 260 remains intact, stability may be increased in vivo by its inclusion as a part of the tip complex. Based on these findings, it is possible that the central hydrophilic portion of IpaC is involved in forming a stable interaction with IpaB on the surface of *S. flexneri* following contact with host cells.

The importance of liposome composition in IpaC surface localization. The sheep erythrocyte-derived liposomes that mobilized IpaC to the *S. flexneri* surface are reported to have a high content of SM and Chol (12). With this in mind and to better define the requirements for IpaC recruitment to the *S. flexneri* TTSA surface, defined liposomes composed of DOPC, SM, and Chol were generated. Indeed, the SM/DOPC/Chol (1:2:1) liposomes were efficient at promoting the stable recruitment of IpaC to the *S. flexneri* surface (Fig. 3). This is consistent with work of van der Goot et al. that showed that liposomes rich in SM and Chol induced TTSS-dependent secretion of IpaB (27).

To further investigate the impact that defined liposomes have on IpaC surface localization in *S. flexneri*, a variety of liposomes prepared using DOPC, DOPG, Chol, or SM or combinations of these were examined. A summary of those results is given in Table 1. Although all of the lipids tested were found to increase IpaC recruitment to the *S. flexneri* surface, the results with respect to efficiency of recruitment differed

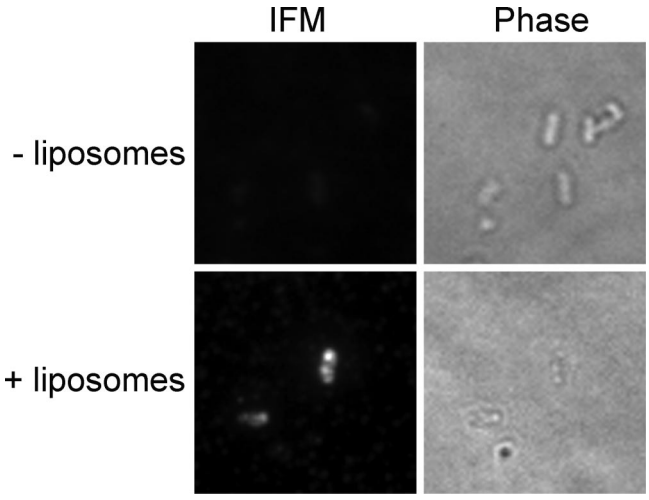

FIG. 3. Immunofluorescence microscopy (IFM) was used to demonstrate the recruitment of IpaC to the *S. flexneri* surface in the presence or absence of liposomes composed of DOPC, SM, and Chol. The negative control was formed by incubation of bacteria with PBS plus DOC.

depending upon the liposome composition (Table 1). Whereas 10% of the bacteria exposed to DOPC or Chol alone exhibited surface-localized IpaC, nearly 30% of the bacteria incubated with either SM or mixed liposomes composed of SM/DOPC/Chol (1:2:1) exhibited IpaC surface localization. These data suggest an important role for SM in promoting the recruitment of IpaC to the *S. flexneri* surface. The particulate nature of the liposomes is most likely responsible for the relatively small percentage of bacteria with IpaC detected on their surfaces.

IpaC recruitment to the *S. flexneri* surface occurs concomitantly with induction of type III secretion. Because SM and SM/DOPC/Chol liposomes increase the surface localization of IpaC and because liposomes containing SM and Chol were previously implicated in type III secretion induction (27), we examined the effects of the presence of erythrocyte and defined

TABLE 1. The influence of defined liposomes on IpaC recruitment on the *S. flexneri* surface[a]

Defined liposome content[b]	% Bacteria with IpaC on the surface (±SD)[c]
No lipid added (Cntrl)	0 ± 0
DOPC	10.2 ± 5.3
Chol	10.5 ± 2.7
SM	29.1 ± 8.1
SM/DOPC/Chol	29.7 ± 9.3

[a] When statistically evaluated utilizing Student's *t* tests, IpaC surface localization was increased following incubation with either DOPC or Chol containing liposomes compared to control results ($P < 0.01$). Furthermore, liposomes containing either SM alone or a mixed lipid content of SM, DOPC, and Chol resulted in a further increase in the percentages of bacteria expressing IpaC surface localization ($P < 0.01$).

[b] All bacteria were incubated with DOC prior to the addition of liposomes. The negative control sample (Cntrl) was incubated with DOC without subsequent incubation with liposomes.

[c] Bacteria having IpaC on their surface were detected by immunofluorescence microscopy. At least five different random fields from a series of three experiments were counted for total bacteria (approximately 100 bacteria per experiment) and then for bacteria within those fields that stained positively for the presence of IpaC on their surfaces.

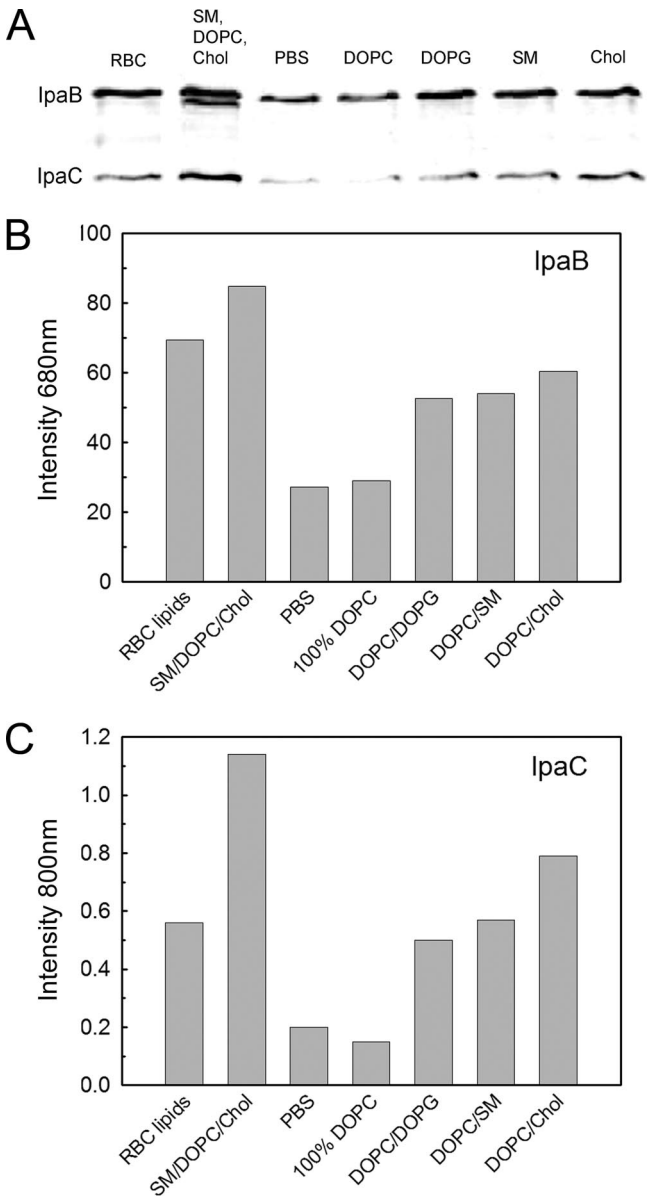

FIG. 4. Immunoblot analysis of IpaB and IpaC secreted by *S. flexneri* in the presence of different liposome types. (A) Results for PBS plus DOC (the control for uninduced secretion), liposomes derived from erythrocytes (red blood cells [RBC]), or defined liposomes composed of DOPC, DOPG, SM, and/or Chol. (B and C) Densitometry scans of IpaB (B) and IpaC (C) present in the immunoblot represented in panel A provide a quantitative measure of Ipa protein secretion by *S. flexneri* in the presence of different lipid preparations. The intensity of the signal at 680 nm was used to quantify the Alexa Fluor 680-labeled goat anti-rabbit IgG used to detect rabbit anti-IpaB antibodies. The intensity at 800 nm was used to quantify the Alexa Fluor 800-labeled goat anti-mouse IgG used to detect mouse anti-IpaC antibodies.

liposomes on the induced secretion of IpaB and IpaC by identical numbers of *S. flexneri* bacteria. As shown in Fig. 4, erythrocyte liposomes induced *S. flexneri* Ipa protein secretion, whereas a synthetic liposome mixture of SM/DOPC/Chol induced even greater secretion of Ipa proteins (Fig. 4). Neutral liposomes consisting only of DOPC had little or no effect on

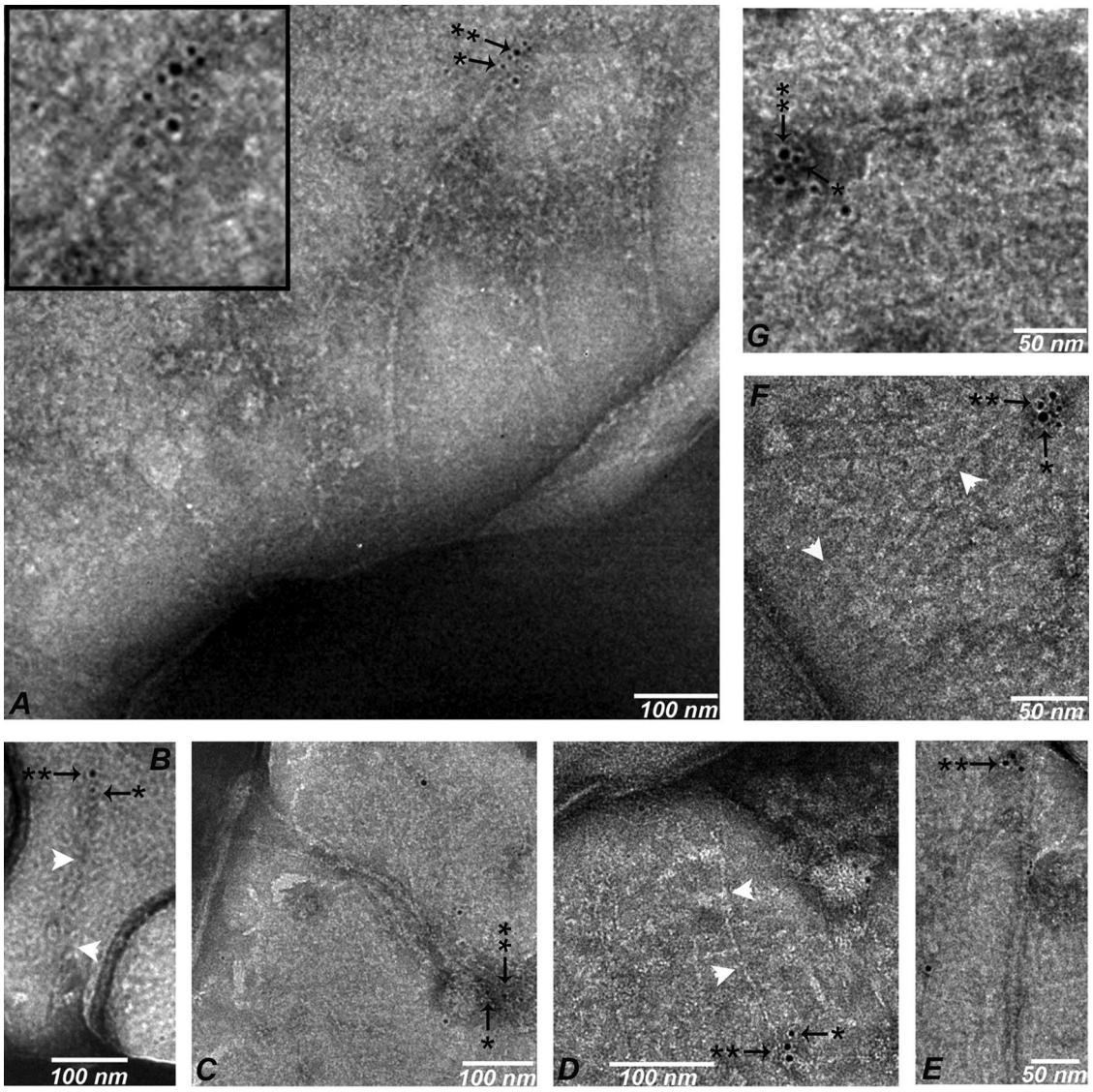

FIG. 5. Transmission electron microscopy of TTSA needles protruding from the *S. flexneri* surface (see white arrowheads) after incubation with liposomes composed of SM, DOPC, and Chol (1:2:1) was used with immuno-gold particle labeling. The presence of 5-nm-diameter gold particles indicates labeling for IpaB (*) and that of 10-nm-diameter gold particles indicates IpaC labeling (**). No IpaB or IpaC labeling was observed for an *mxiH* null mutant (not shown). IpaB labeling but no IpaC labeling was seen for the *ipaC* null SF621 prior to the addition of liposomes (not shown).

IpaB or IpaC secretion, but the addition of the acidic lipid DOPG also increased type III secretion (Fig. 4). When the data from this representative immunoblot were quantified (Fig. 4B and C), IpaB and IpaC levels reflected the qualitative trend represented in Fig. 4A, with the results for IpaC being the more striking (Fig. 4C). It thus appears that once IpaC is recruited to the *S. flexneri* surface, type III secretion is induced for all the Ipa proteins and, presumably, for the subsequently secreted TTSS effectors.

IpaC is located at the TTSA needle tip after liposome addition. We previously used immuno-gold labeling and electron microscopy to demonstrate that both IpaD and IpaB are associated with the TTSA needle tip (7, 19). To confirm that IpaC localizes to the TTSA needle tip complex upon lipid-induced type III secretion, this methodology was employed again (Fig. 5). In order to reduce the nonspecific background observed during the uranyl acetate staining of bacteria after erythrocyte ghosts or lipids were added, defined liposomes were used to promote IpaC mobilization. The liposome preparation used was DOPC/SM/Chol (1:2:1) based on the data presented in Table 1. Following incubation of bacteria with liposomes, they were doubly immuno-gold labeled using rabbit anti-IpaB antibodies with 5-nm-diameter gold-labeled goat anti-rabbit IgG $F(ab')_2$ for IpaB and mouse monoclonal anti-IpaC antibodies with 10-nm-diameter gold-labeled goat anti-mouse IgG $F(ab')_2$ for IpaC (Fig. 5). The resulting micrographs show that IpaB and IpaC are present at the tip of the needles, which confirms the colocalization shown by immunofluorescence (Fig. 1). Immuno-gold labeling appears only at the distal end of the needle structures, suggesting a specific association of these

TABLE 2. The influence of DOC and liposomes on the invasion of HeLa cells[a]

Bacterial strain	Supplement[b]	Relative invasion (% ± SD)[c]	
		Control A	Control B
SF621 (*ipaC* null)	None	0 ± 0	0 ± 0
SF621 + *ipaC*	None	100 ± 13	NA[e]
SF621 + *ipaC*	DOC	319 ± 3	100 ± 1
SF621 + *ipaC*	DOC + lipids[d]	246 ± 8	77 ± 3
SF621 + *ipaC*	0.07% Congo red	132 ± 24	41 ± 7
SF621 + *ipaC*	10% serum	71 ± 17	22 ± 5

[a] Student's *t* tests were performed in order to statistically evaluate the results. Although the addition of serum or Congo red did not result in an increase in the relative invasiveness of the S. *flexneri* bacteria compared to the control, the addition of either DOC alone or DOC plus lipids increased invasiveness compared to the results seen with any of the other populations ($P < 0.01$).

[b] Bacteria were grown in TSB to an A_{600} of 1.0 and then placed into TSB containing the supplements shown for 30 min. The bacteria were then used directly for invasion of HeLa cells in HBSS for 30 min. The data shown represent the results obtained with an average of three samples from one of three representative experiments.

[c] Invasion values are presented relative to those determined for *S. flexneri* SF621 complemented for *ipaC* expression and grown as described above and then incubated in TSB with no additional supplement (Control A). The values for relative invasion with respect to Control B represent bacteria incubated for 30 min in TSB containing DOC.

[d] The liposomes used here were a mixture of SM, DOPC, and Chol (see Fig. 3 and 5) to give maximal surface localization of IpaC and secretion of IpaB and IpaC.

[e] NA, not applicable.

proteins following secretion induction. It thus appears that IpaC, together with MxiH, IpaD, and IpaB, contributes to the formation of a quaternary complex at the needle tip of the *S. flexneri* TTSA following incubation of the bacteria with DOC and liposomes.

The status of the *S. flexneri* TTSA needle tip influences the efficiency of bacterial invasion of HeLa cells. We have now shown that the maturation process of the TTSA tip complex occurs in three steps. For step 1, IpaD alone is present at the needle tip of *S. flexneri* bacteria grown to the log phase in TSB. Addition of DOC induces step 2 by mobilizing IpaB to create the MxiH-IpaD-IpaB ternary tip complex. Here we show that step 3 is the mobilization of IpaC to the needle tip complex after incubation of bacteria with the ternary complex on their surfaces with specific liposomes. To understand the importance of these needle tip complexes with respect to *S. flexneri* invasion of cultured cells, bacteria were grown to the mid-log phase in TSB, exposed to conditions known to promote either IpaB or IpaB-IpaC recruitment to the *S. flexneri* surface, and then tested for their relative levels of invasion of HeLa cell monolayers. Since the presence of MEME, like that of DOC, results in the recruitment of IpaB to the bacterial surface without inducing type III secretion (unpublished observations), the gentamicin-protection assay was performed with HBSS to avoid possible bias in determining the importance of DOC-induced IpaB surface presentation for *S. flexneri* invasiveness. When the bacteria were incubated for 30 min in the absence or presence of DOC after growth in TSB, clear differences in invasion efficiency were observed (Table 2). The addition of DOC enhanced *S. flexneri* invasion more than threefold, indicating that the DOC-mediated recruitment of IpaB to the TTSA needle tip resulted in the formation of a productive complex at the tip of the TTSA needle and that the ternary complex is important in host cell invasion (Table 2). This finding supports the proposal that the ternary complex present on the *S. flexneri* surface is physiologically relevant. Thus, the DOC-mediated events represent the second step in TTSA complex maturation and may be a prerequisite step for type III secretion induction.

The MxiH-IpaD-IpaB complex appears to represent the mature tip complex that is required for optimally productive host cell contact. Thus, the subsequent membrane-IpaB interaction would logically represent the type III secretion trigger that promotes IpaC recruitment to the TTSA needle tip and the simultaneous injection of IpaB and IpaC into the host cell membrane and cytoplasm. If this is the case, when IpaC localizes prematurely to the tip, interaction with the host cell would no longer be optimal and there would be a reduction in invasion. In line with this, when the bacteria were incubated in DOC combined with the DOPC/SM/Chol liposomes, invasion levels still increased relative to those seen with *S. flexneri* incubated in the absence of any supplement; however, the levels were reduced relative to those seen with bacteria incubated with DOC alone (Table 2). When known artificial inducers of *S. flexneri* type III secretion, namely, CR and serum, were incubated with the bacteria (with no DOC present), invasion levels also decreased relative to the levels seen with the DOC control (Table 2). In fact, incubation with serum decreased invasion levels to below those seen without DOC. Based on these findings, it is possible to suggest that the optimal TTSA tip complex for *S. flexneri* invasion of cultured cells is the ternary complex that includes MxiH, IpaD, and IpaB. The subsequent induction and secretion of IpaC comprise the final step in triggering the induction of full type III secretion.

DISCUSSION

IpaD represents the first protein found to localize to the tip of the *S. flexneri* TTSA needle, where it is involved in controlling secretion and further tip assembly (7). IpaB subsequently associates with the needle tip complex (7), but only after incubation of the bacteria with certain small molecules such as those of bile salts (19) and those found in MEME (unpublished observations). It is noteworthy that the bile salt concentration in the gut lumen greatly exceeds that needed to trigger IpaB mobilization. The fact that tissue invasion occurs in the colon rather than where bile salt concentrations are highest (in the small intestine) suggests that additional factors contribute to the ultimate selection of the colon as the infection site. It should be noted, however, that conditions that promote the completion of the IpaD-IpaB tip complex do not result in type III secretion induction and do not give rise to detectable levels of IpaC on the bacterial surface (19). In contrast, these conditions do lead to optimal invasion efficiency. Thus, the MxiH-IpaD-IpaB ternary complex appears to represent the physiologically relevant complex that senses contact with the host cell and triggers subsequent invasion.

Another argument for the proposal that IpaC is not needed for initial TTSA interaction with host cells stems from past observations that the *ipaC* null mutant SF621 strain is capable of eliciting detectable levels of contact hemolysis whereas *ipaD* and *ipaB* null mutants do not lead to any detectable contact hemolysis, despite the fact that they are constitutive for type III

secretion (15, 16). This finding is explained by the fact that IpaD is needed for IpaB maintenance at the TTSA needle tip and IpaB is needed for initial interaction with the host cell membrane, perhaps via the Chol-binding activity of IpaB (10, 19), its interaction with raft-associated proteins such as CD44 (14), or interactions involving SM. Meanwhile, the *ipaC* null mutant fails to allow the formation of the full translocon pore, thus preventing the incorporation of the first major *S. flexneri* effectors needed for initial invasion-related events (26). This is consistent with IpaB sensing host cell membrane contact by inserting itself into the host membrane and simultaneously promoting the insertion of IpaC into the membrane to complete translocon pore formation as type III secretion is induced. This, in turn, is consistent with the observation that in the majority of obtained micrographs, it appears that IpaC is distal to IpaB in relation to the bacterial surface. This suggests a specific order to the needle tip complex (see Fig. 5B, D, and G); however, this proposal requires confirmation. The data presented in Fig. 2 may also show that the hydrophilic region of IpaC is required for its interaction with IpaB at the TTSA needle tip, but this interpretation is speculative at present.

We show here that incubation of *S. flexneri* with SM/Chol-containing liposomes induces the recruitment of IpaC to the TTSA needle tip. This appears to occur as the final step in the induced secretion of IpaB and IpaC into the extracellular environment, since detection of IpaC on the bacterial surface occurs concomitantly with full induction of type III secretion. The fact that liposome preparations that roughly mimic the composition found in lipid rafts (27) are able to stimulate full secretion suggests that this event normally occurs upon contact with a host cell. While treatment of *S. flexneri* with DOC and the SM/DOPC/Chol liposomes resulted in elevated invasion relative to untreated bacteria, invasion was reduced relative to that seen when the cells were preincubated with only DOC. This suggests that IpaB is at the TTSA needle tip prior to host cell contact whereas IpaC is inserted directly into the host cell membrane immediately following host cell contact. Premature release of IpaC, as caused by adding liposomes, therefore decreases overall invasion efficiency. It is possible that the reduced invasiveness is due to the fact that the MxiH-IpaD-IpaB-IpaC quaternary complex is the physiologically relevant needle tip "invasion complex" that is present only following host cell contact. Premature formation of this complex (prior to actual contact with a host cell) could give rise to a dead-end complex that cannot efficiently promote invasion. The possibility cannot be ruled out, however, that incubation of *S. flexneri* with lipids leads to depolarized type III secretion which could impair the proper targeting of effectors and cause a reduction in the observed levels of invasion.

From the findings presented here and in previous work, we propose that the complete *S. flexneri* TTSA is built in a stepwise manner. Upon completion of needle formation, IpaD localizes to the needle tip, where it serves as an environmental sensor for the mobilization of IpaB and formation of the MxiH-IpaD-IpaB ternary complex. It is this complex that then senses host cell contact to promote the simultaneous insertion of IpaB and IpaC into the target cell membrane and induction of type III secretion of effectors. Because of the large amounts of IpaB and IpaC that are secreted upon incubation with liposomes and because of the highly apolar nature of these proteins, it will be difficult to determine a precise structure for the MxiH-IpaD-IpaB-IpaC quaternary structure. It may be possible, however, to more closely examine the structure of the preinduction states of the TTSA needle tip to determine the structural and functional features of these complexes and to dissect the molecular basis for the stepwise induction of type III secretion in *S. flexneri*.

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