The Tripartite Type III Secreton of *Shigella flexneri* Inserts IpaB and IpaC into Host Membranes

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Abstract. Bacterial type III secretion systems serve to translocate proteins into eukaryotic cells, requiring a secreton and a translocator for proteins to pass the bacterial and host membranes. We used the contact hemolytic activity of *Shigella flexneri* to investigate its putative translocator. Hemolysis was caused by formation of a 25-Å pore within the red blood cell (RBC) membrane. Of the five proteins secreted by *Shigella* upon activation of its type III secretion system, only the hydrophobic IpaB and IpaC were tightly associated with RBC membranes isolated after hemolysis. Ipa protein secretion and hemolysis were kinetically coupled processes. However, Ipa protein secretion in the immediate vicinity of RBCs was not sufficient to cause hemolysis in the absence of centrifugation. Centrifugation reduced the distance between bacterial and RBC membranes beyond a critical threshold. Electron microscopy analysis indicated that secretons were constitutively assembled at 37°C before any host contact. They were composed of three parts: (a) an external needle, (b) a neck domain, and (c) a large proximal bulb. Secreton morphology did not change upon activation of secretion. In mutants of some genes encoding the secretion machinery the organelle was absent, whereas *ipaB* and *ipaC* mutants displayed normal secretons.

Key words: microbial pathogenesis • type III secretion • contact hemolysis • pore formation • membrane protein insertion

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Shigella flexneri is the etiological agent of the endemic form of bacillary dysentery. This gram-negative bacterium causes disease by invading the colonic mucosa (Hale, 1998). All bacterial genes necessary for entry into host cells have been identified. They are clustered within a 30-kb region of a large virulence plasmid (Parsot, 1994). This region carries two types of genes: the *ipa* and *ipg* genes encoding the entry-mediating proteins and their individual intrabacterial chaperones, and the *mxi* and *spa* genes which code for proteins forming a type III secretion apparatus (secreton) required for secretion of the invasins (IpaA-D and IpgD)

Type III secretons are found in many other pathogenic gram-negative bacterial species that have developed diverse survival strategies within their hosts. Several components of type III secretons share sequence similarities with components of flagellar basal bodies (Hueck, 1998). Kubori et al. (1998) recently identified and biochemically isolated the macromolecular structure formed by the *Salmonella* type III secreton. They described this organelle as a cylindrically symmetrical object composed of two parts: (a) a 7–8-nm-wide and 60-nm-long needle emanating from the bacterial surface and (b) a shorter cylinder, formed by a succession of plates (20–40 nm in diameter) presumed to traverse the inner and outer bacterial membranes and the peptidoglycan. A major function of the type III secretons is to transport, upon contact with host cells, proteins from the bacterial cytoplasm into the host cell cytoplasm or its limiting membrane (Cornelis and Wolf-Watz, 1997; Kenny et al., 1997; Galan and Collmer, 1999).

In *S. flexneri*, the *mxi* and *spa* operons and the *ipa* and *ipg* operons are expressed at 37°C, and Ipa proteins remain in the bacterial cytoplasm until the secretion machinery is activated by host contact or external signals such as serum or the small amphipatic dye molecule Congo red (Ménard et al., 1994a; Bahrani et al., 1997). Physical contact of the bacterium with the host cell induces formation of a localized macropinocytic-like ruffle on the host cell surface that internalizes the bacterium (Clerc and San-

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sonetti, 1987; Adam et al., 1995). This initiates a cycle of intra- and intercellular spreading (Niebuhr and Sansonetti, 1999).

Only three Ipa proteins are essential for entry into HeLa cells and contact hemolysis (Clerc et al., 1986; Sansonetti et al., 1986): IpaB, IpaC, and IpaD (Sasakawa et al., 1989; Ménard et al., 1993). IpaB and IpaC have hydrophobic regions that could form transmembrane helices. Both proteins form separate complexes with the same chaperone, IpgC, within the bacterial cytoplasm (Ménard et al., 1994b). A small amount of IpaB is present in bacterial outer membrane fractions, from where it can be coprecipitated with IpaD (Ménard et al., 1994a). IpaD is a hydrophilic 35-kD protein that is also stored intracellularly and secreted upon host contact. *ipaB* and *ipaD* mutants constitutively secrete the remaining Ipa proteins suggesting that IpaB and IpaD play a role in secretion regulation (Ménard et al., 1994a; Parsot et al., 1995). IpaB and IpaC form a complex upon secretion from the bacterium (Ménard et al., 1994b). This complex is sufficient to induce membrane ruffling and entry into mammalian cells (High et al., 1992; Ménard et al., 1996; Tran Van Nhieu et al., 1999). As both proteins are hydrophobic, they could interact with the host plasma membrane to form a structure through which the remaining Ipa proteins become translocated. Indeed, IpaB and IpaC share structural similarities with YopB and YopD, respectively, from *Yersinia* species. These proteins are secreted by a type III secretion system and are required for translocation of other Yops into host cells (Sory and Cornelis, 1994).

Two other proteins, IpaA and IpgD, are secreted in response to Congo red and are likely to be secreted upon contact of bacteria with eukaryotic cells. Inactivation of *ipaA* and *ipgD* alters but does not abolish entry into HeLa cells (Allaoui et al., 1993b; Ménard et al., 1993). IpaA interacts with vinculin, an intracytoplasmic actin-associated host protein, during HeLa cell invasion (Tran Van Nhieu et al., 1997). IpgD shows homology to SopB, a protein found in *Salmonella* species. The latter is a phosphatidylinositol phosphatase which is translocated into eukaryotic cells and involved in tissue inflammation (Galyov et al., 1997; Norris et al., 1998). These data and the predicted hydrophilic nature of IpaA and IpgD suggest that they are translocated into the host cell cytoplasm where they modulate the invasiveness of *S. flexneri*.

How do type III secretons mediate insertion and translocation of proteins into the host cell? In flagellar biogenesis, there is evidence for a canal within the structure (Morgan et al., 1995) through which flagellin may traffic until it reaches the tip of the structure and inserts into the growing flagellum (Emerson et al., 1970). By analogy, the type III secreton may function by allowing protein traffic through a canal within its length, with insertion at the flagellum tip being replaced by insertion into the host membrane. The molecular mechanisms underlying these hypothetical functions and the subsequent translocation of substrate proteins remain to be established.

Contact hemolysis is the ability of *Shigella* to lyse red blood cells with which they have been brought into contact by centrifugation. It occurs also with *Yersinia* species, requiring the *yopB* gene product for formation of 1.2–3.2-nm-pore within RBCs (Håkansson et al., 1996). To gain a mechanistic understanding of how the IpaB, IpaC, and IpaD proteins mediate the initial interaction of the bacterium with the host cell, we used contact hemolysis as a model of bacterial protein interaction with host membranes. In addition, we performed morphological analysis of the RBC/bacteria interaction and of type III secretons. By correlating biochemical and morphological data we propose a model of the earliest molecular events leading to *S. flexneri* entry into host cells.

Materials and Methods

Contact Hemolysis

Assay Protocol. Sheep blood was obtained from BioMérieux (Marcy L'Etoile, France). Human blood was obtained from the Etablissement Français de Transfusion Sanguine (Paris, France). Bacteria were grown from overnight precultures diluted 1:100 in aerated flasks of Trypticase Soy Broth (TSB; Becton Dickinson) for 4 h at 37°C to $OD_{600} = 4$. They were collected by centrifugation for 5,000 *g* for 7 min at 4°C, washed in saline (150 mM NaCl) and resuspended at 10^{10} bacteria/ml at 4°C. RBCs were washed 3× in saline by centrifugation at 2,000 *g* for 5 min at 4°C and resuspended at 5.10^8/ml. The final resuspension buffer of both bacteria and RBCs contained 30 mM Tris at pH 7.5 (Tris-saline). 100 μl of bacteria were mixed with 100 μl of RBCs in round bottom 96-well plates, centrifuged at 1,500 *g* for 10 min at 10°C, and incubated at 37°C for 1 h. The cells were resuspended and the samples recentrifuged. 100 μl of supernatant was transferred to a fresh plate where its optical density at 595 nm was measured. The baseline (B) of the assay was set with RBCs incubated with Tris-saline instead of bacteria and total hemolysis (T) was the value obtained when RBCs were incubated with saline containing 0.1% sodium dodecyl sulfate. The percentage of total hemolysis (P) was calculated using the equation $P = [(X - B)/(T - B)] \times 100$, where X is the optical density value of the sample analyzed. All data presented result from at least three independent experiments. Errors given are standard deviations.

When AfaE-expressing *Shigella* (Clerc and Sansonetti, 1987) were used to lyse human RBCs, bacteria were resuspended at only 10^9 bacteria/ml. Avirulent *S. flexneri* expressing *E. coli* hemolysin A are described by Zychlinski et al. (1994). To obtain the hemolysin containing culture supernatants, bacteria were diluted 1:100 from overnight precultures into 8 ml of TSB and grown for 3 h at 37°C. The bacteria were eliminated by centrifugation and the supernatant used immediately. The *ipaA ipgD* mutant strain was constructed by integration of pLAC-A carrying an ipaA::LacZ fusion (Ménard et al., 1993) into the large plasmid of the *ipgD* mutant strain.

Osmoprotection Experiments. RBCs were resuspended in a 60-mM solution of sucrose, raffinose (Sigma), or PEG1000 through 3000 (Fluka) made in Tris-saline. After incubation at 37°C, the samples were cooled to stop the reaction, resuspended and immediately recentrifuged.

Neuraminidase Treatment of RBC. 5×10^7 washed RBCs in 100 μl were treated with 0.5 U of neuraminidase, type V from *Clostridium perfringens* (Sigma) for 60 min on ice. The cells were washed 3× in Tris-saline and resuspended at the same concentration for use in the hemolytic assay. Complete removal of sialic acid was verified by loss of the ability of HA virus, the cell receptor of which is sialic acid, to hemagglutinate the treated RBCs.

Red Blood Cell Membrane Isolation

Bacteria and sheep RBCs were prepared as described above, except that they were resuspended in Tris-saline at 2.10^{11} and $1.5.10^{10}$ cells/ml, respectively. Hemolytic reactions were prepared in 50-ml conical tubes with 600 μl RBCs, 120 μl bacteria, 860 μl Tris-saline, and a protease inhibitor cocktail (PIC[1]; Complete™, Boehringer Mannheim). Samples were centrifuged at 2,000 *g* at 4°C for 10 min, incubated at 37 or 4°C for 1 h, resuspended by vortexing, and recentrifuged. Hemolysis was assessed spectrometrically as above. 800 μl of distilled water was added to each sample to lyse all RBCs nonspecifically (so that lysed membranes could be isolated even from those samples where no hemolysis had occurred), and these were vor-

1. *Abbreviations used in this paper:* LPS, lipopolysaccharide; PIC, protease inhibitor cocktail.

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texed and centrifuged again to remove bacteria. 2 ml of supernatant was collected, adjusted to 2.4 ml with Tris-saline and brought to 46% sucrose with 7 ml of 62% sucrose in Tris-saline containing the PIC. The mixtures were deposited at the bottom of SW41 centrifuge tubes (Beckman) and layered with 2 ml of 44% and then 25% sucrose in Tris-saline containing the PIC. Gradients were centrifuged at 15,000 *g* for 16 h at 4°C. The material at the 44/25% sucrose interface was collected, diluted in Tris-saline and concentrated by centrifugation at 450,000 *g* for 20 min 4°C in a TLA 100.3 rotor (Beckman). The pellets were resuspended in a minimal volume of buffer. Such samples were checked by transmission electron microscopy but no contaminating bacteria or bacterial ghosts were seen. When ^{35}S-labeled bacteria were used less than 0.01% of the initial radioactive input was recovered in RBC membranes. The protein content of the samples was estimated using Bradford's assay (Bio Rad) and an equivalent protein amount of each was separated by SDS-PAGE and Western blotted.

To assess the strength of association of Ipa proteins with RBC membranes, 100 μl of purified *Shigella*-lysed RBC membranes were incubated at 4°C for 1 h in Tris-saline or Tris-saline with 5 M NaCl, 8 M urea, or 0.2 M carbonate, pH 11. 300 μl of 62% sucrose in Tris-saline was then mixed with the membranes that were deposited at the bottom of 0.8-ml SW55 tubes (Beckman) and overlaid with 150 and 100 μl of 44 and 25% sucrose in Tris-saline. The gradients were spun overnight at 4°C at 15,000 *g*. The top 150 μl was collected from the gradients, diluted in Tris-saline, and concentrated by pelleting in a TLA 100.2 rotor for 20 min at 450,000 *g* at 4°C. The pellets were resuspended in a minimal volume of buffer.

Measurement of Ipa Protein Secretion

With Congo Red. 1-ml aliquots of *Shigella* cultures, grown as above were washed in Tris-saline and resuspended in 500 μl of the same buffer. 200 μM of Congo red was added to the bacteria and the cells were incubated for 10 min at 37°C. The bacteria were pelleted at 14,000 *g* at 4°C for 15 min. 10 μl of supernatant and of the pellet resuspended in 500 μl of Tris-saline were separated by SDS-PAGE and Western blotted.

In Hemolysis. After the final centrifugation 20 μl of hemolytic assay supernatant and 20 μl of a resuspended reaction containing an equivalent number of bacteria incubated in the absence of any inducer of secretion were separated by SDS-PAGE and Western blotted.

Antibodies

mAbs to IpaB and IpaC were a gift from Armelle Phalipon. mAbs against IpaA and IpgD were generated by immunizing female BALB/c mice with Ipa protein containing supernatants obtained after induction of secretion in *Shigella flexneri* with Congo red. In brief, 10–20 mg of protein was injected subcutaneously into both hind legs (5 injections at 3-d intervals) and the popliteal lymph nodes were fused with X63Ag8 lymphoma cells according to standard protocols. Hybridoma supernatants were screened by ELISA and immunoblotting and clones positive for IpaA and IpgD were subcloned by limiting dilution.

Immunoblotting

Conventional SDS-PAGE was performed and immunoblotting was performed using a semi-dry apparatus onto nitrocellulose or PVDF membranes and developed using the ECL or ECL Plus kits (Amersham) and X-OMAT film (Kodak).

Electron Microscopy

Whole Mount Negative Stain. Bacteria were grown for 2 h at 37°C from 1:50 dilutions of overnight precultures in 8 ml of TSB. 2 ml of bacteria were collected, washed in filter sterilized PBS, and resuspended in 20 μl of the same buffer. The bacteria were osmotically and mechanically shocked by a 1:4 dilution into a 60-μl drop of distilled water deposited on parafilm. Often lysis of dividing bacteria was favored by incubation of the samples on ice for a few hours before dilution in water. After a few minutes, 200 mesh Formvar-coated copper microscopy grids were placed on top of the drops. After a brief incubation at room temperature, the grids were rinsed with a flow of drops of 2% phosphotungstic acid at pH 7. After drying, the samples were observed in a Philips CM12 electron microscope working in standard conditions.

Image Processing. Negative micrographs were digitized on a rotary drum scanner using a 10-μm/pixel square scanning aperture. Windowing and processing of the particle images was performed on digitized micrographs, using SPIDER software and WEB interactive selection program (Franck et al., 1996) on a Unix Digital Workstation. After contrast normalization, the whole set of selected image was subjected to a reference free alignment process (Penczek et al., 1992) to generate an average projection image of the secreton. The resolution limit estimation was performed using both differential phase-residual (Franck et al., 1981) and Fourier ring correlation criterion (Saxton and Baumeister, 1982).

Resin Embedding of RBC and Bacteria. AfaE-expressing bacteria and human red blood cells were mixed as in for the hemolytic assay but in 500-μl tubes. Hemagglutination occurred and samples were either centrifuged at various *g* forces for 10 min or left to sediment at 1 *g* at 10°C. Samples were fixed for 1 h at room temperature with 1.2% glutaraldehyde, and 0.05% ruthenium red (RR) in 0.1 M cacodylate buffer, pH 7.4, to better visualize the carbohydrate surrounding cell membranes (Handley, 1991). Samples were rinsed with 0.05% RR in cacodylate buffer and post-fixed with 1% OsO4, 0.05% RR in cacodylate buffer for 1 h at room temperature. Cells were rinsed with H_2O and embedded in 2% agarose (Type VII; Sigma). After gelling on ice samples were dehydrated with an increasing acetone series and embedded in Epon. Thin sections were conventionally stained.

Results

The Hemolytic Activity of S. flexneri Is Due to Formation of a 25-Å Pore within RBC Membranes

To study the interaction of *S. flexneri* with a simple cell membrane, we reinvestigated its contact hemolytic activity. Bacteria were mixed with RBCs, centrifuged at 1,500 *g* for 10 min at 10°C and incubated at 37°C for 1 h. Release of haemoglobin was monitored as described in Materials and Methods. As previously reported, contact hemolysis did not occur without centrifugation (Fig. 1 A), and strains carrying mutations in *mxiD*, *ipaB*, or *ipaD* were nonhemolytic, whereas strains carrying mutations in *ipaA*, *ipgD*, or in both *ipaA* and *ipgD* displayed normal hemolysis (Allaoui et al., 1993a,b; Ménard et al., 1993). The *ipaC* mutant had ~10% hemolytic activity. This low activity had not been detected before because previously the wild-type strain induced only 40% hemolysis (Barzû et al., 1997), whereas, in the current assay, it caused 75–100% hemolysis. These results confirmed that integrity of the secretion machinery and IpaB, IpaC, and IpaD, but neither IpaA nor IpgD, were required for hemolysis. The residual activity of the *ipaC* mutant suggested that, in the absence of IpaC, another factor could still destabilize the RBC membrane, albeit inefficiently.

To determine whether hemolysis was due to the formation of a pore within the RBC membrane, we performed osmoprotection experiments. Lysis of RBCs by bacterial toxins forming hydrophilic pores in membranes proceeds through osmotic shock (Menestrina et al., 1994). This can be prevented by addition of osmotic protectants at 30 mM to the medium. If the molecule is too large to pass through the pores, it counterbalances the increased intracellular pressure. Osmoprotectants of intermediate sizes induce a size-dependent increase of the half time of hemolysis that can be used to estimate pore size. Molecules larger than PEG1000 allowed significant protection against lysis generated by wild-type bacteria and protection increased with the size of the molecule (Fig. 1 B). A time course analysis of hemolysis in the presence of different osmoprotectants (for example, Fig. 1 C) was used to derive a Renkin plot (Renkin, 1954; Ginsburg and Stein, 1987) showing the relative permeability of the osmoprotectants versus their size

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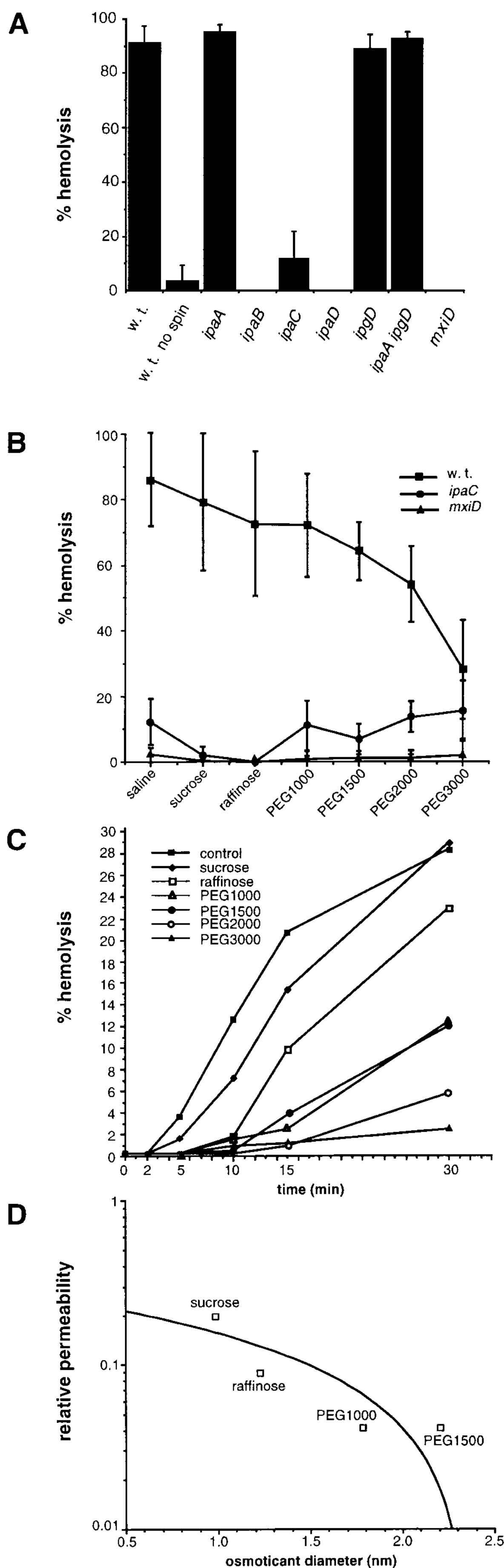

(Fig. 1 D). This allowed estimation of the functional inner radius of the pore at 26 Å (±0.4 Å, standard deviation, $n = 3$). No osmoprotection was observed in lysis mediated by the *ipaC* strain (Fig. 1 B). This suggested that hemolysis induced by the *ipaC* mutant resulted either from formation of a pore larger than 32 Å in diameter (the size of PEG 3000) or from destabilization of the membrane by another mechanism.

IpaB and IpaC Are Tightly Associated with Lysed RBC Membranes

To determine the components of the pore formed in the membrane during contact hemolysis, we isolated the lysed RBC membranes by floatation in a sucrose density gradient. The protein content of the membranes was separated by SDS-PAGE, blotted and probed with antibodies against IpaA-D and IpgD. IpaB, IpaC, IpgD, and IpaA were present in membranes recovered after incubation of RBCs with the wild-type at 37°C, but not in fractions recovered after incubation with the *mxiD* mutant at 37°C or with the wild-type at 4°C (Fig. 2 A). By semiquantitative immunoblotting, the amount of IpaB, IpaC, IpgD, and IpaA recovered in the membrane fraction corresponded to ~0.1% of the total amount of these proteins present initially in bacteria. IpaD was not detected in the membrane fraction, although the detection procedure was sensitive enough to reveal 0.1% of IpaD in bacterial extracts (not shown).

Membranes isolated after contact with the wild-type at 37°C were then incubated in the presence of agents known to release peripheral membrane proteins. The amount of Ipa and Ipg proteins associated with membranes was measured after a second floatation in a sucrose density gradient. After stripping with 5 M NaCl, 0.2 M carbonate, pH 11, or 8 M urea, the majority of IpaB and various amounts of IpaC remained associated with the membranes (Fig. 2 B). In contrast, IpgD and IpaA were lost even in the mock-treated sample (not shown). This indicated that IpaB, and to a lesser extent IpaC, were strongly associated with the

Figure 1. Contact hemolytic activity of *S. flexneri.* Osmoprotective analysis of the lytic activity. (A) The hemolytic assay was performed with bacteria and sheep RBCs incubated for 1 h at 37°C; w.t. stands for strain M90T, for w.t. no spin the centrifugation step before incubation at 37°C was omitted. (B) Wild-type and *ipaC* or *mxiD* strains were incubated with sheep RBCs for 1 h at 37°C in the presence of 30 mM of the different sugars indicated. (C) Wild-type bacteria were incubated with RBCs for various times in the presence of 30 mM of the different sugars. (D) The lysis kinetics were evaluated from the data shown in B by the time necessary to obtain 50% of maximal lysis, $t_{1/2}$. We used $t_{1/2}$–$t0_{1/2}$ ($t0_{1/2}$ being the time without osmoprotectants) as an estimate of the time necessary for the osmoticant to diffuse inside the cell through the bacterially induced lesions. Accordingly $1/(t_{1/2}\text{-}t0_{1/2})$ is an estimate of the permeability of the sugar through the pore and was used to build a Renkin plot showing the relative permeability of the molecule versus its size. In this graph, the line was the best fitting curve determined by the computer program Cricket Graph and its intersection point with the x-axis puts the pore diameter at 2.25 nm. Consistently, PEG 3000 (estimated molecular diameter 3.2 nm) offers nearly complete osmoprotection.

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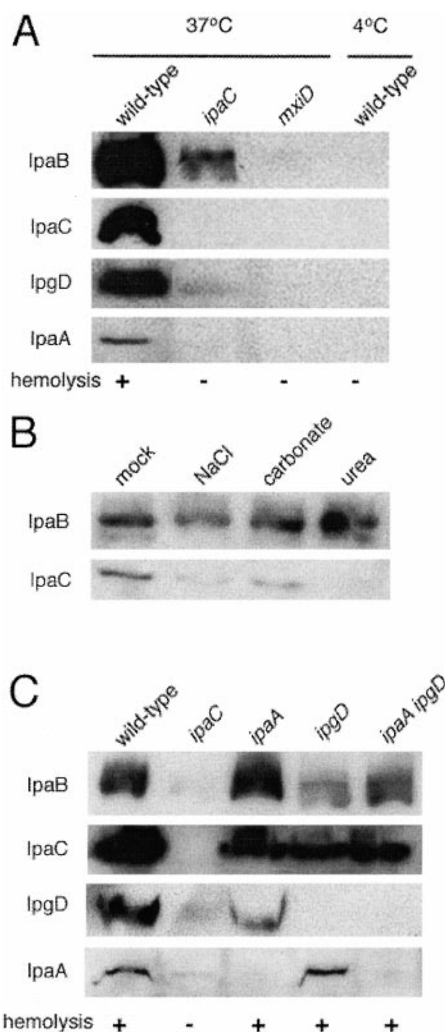

Figure 2. Analysis of the Ipa proteins associated with RBCs membranes after contact hemolysis. RBCs membranes exposed to contact with various strains under different condition were isolated and examined for their Ipa protein content using SDS-PAGE and immunoblotting. Blots were revealed with the H16 anti-IpaB mAb, a mixture of K24, N9, H10, and J22 anti-IpaC mAbs or the 20G9 anti-IpgD and 322F7 anti-IpaA mAbs. The occurrence of contact hemolysis is indicated under each panel. (A) Ipa/Ipg protein associated with RBCs membranes after contact hemolysis with wild-type, *ipaC*, or *mxiD* strains at 37°C or 4°C. The blot probed with the anti-IpaB antibody was overexposed to visualize the small amount of IpaB associated with RBC membranes lysed by the *ipaC* mutant. (B) Association of IpaB and IpaC with RBC membranes isolated after contact with wild-type bacteria at 37°C and stripped, or not (mock), with 5 M NaCl (NaCl), 0.2 M carbonate, pH 11 (carbonate), and 8 M urea (urea). (C) Analysis of the association of IpaB, IpaC, IpgD, and IpaA with RBCs membranes brought into contact with various mutants.

membranes, while IpgD and IpaA were located at the membrane periphery.

To investigate further the mode of interaction of Ipa and Ipg proteins with RBCs membranes, we examined their presence in this fraction after contact of the *ipaC*, *ipaA*, *ipgD*, and *ipaA ipgD* mutants with RBCs. Membranes exposed to the *ipaA*, *ipgD*, or *ipaA ipgD* mutants contained relatively high amounts of IpaB and IpaC, and of IpgD or IpaA, or neither of the latter two proteins, respectively. This indicated that IpaA and IpgD were not required for association of IpaB and IpaC with the membranes or for association of each other with membranes. In the *ipaC* mutant, reduced amounts of IpaB, IpaA and IpgD were associated with the membranes (see Fig. 2 A, in which the blots were overexposed to show more clearly the low levels of these proteins in these samples). Although this amount was vastly reduced as compared with that observed with the wild-type, it was significant as incubation of RBCs with the *mxiD* mutant at 37°C or with the wild-type at 4°C led to undetectable amounts of these proteins in the membranes. The small amount of IpaB associated with membranes after incubation of RBCs with the *ipaC* mutant could account for the residual hemolysis of this strain. The low amount of IpaA and IpgD in these membranes suggested that these proteins require the presence of IpaC and/or IpaB to associate with membranes.

Ipa Protein Secretion Is Necessary but Not Sufficient for Contact Hemolysis

To gain an understanding of how IpaB and IpaC were transferred into target membranes, we studied the relationship between hemolysis and Ipa protein secretion. We investigated the efficiency of hemolysis after incubation for 1 h between 4 and 42°C. No hemolysis was observed below 25°C and the reaction proceeded normally above 30°C (Fig. 3 A). The temperature dependence of lysis correlated with that of Ipa protein secretion after induction of the machinery with Congo red (Fig. 3 B).

The *spa47* gene encodes a protein related to the β subunit of F1 mitochondrial ATPases which is essential for secretion (Venkatesan et al., 1992; Sasakawa et al., 1993). This led us to investigate the effect of sodium azide, an inhibitor of oxidative phosphorylation (to which RBCs should be insensitive), on secretion and hemolysis. Addition of increasing amounts of azide progressively inhibited Ipa secretion induced by Congo red (Fig. 3 D), indicating that Ipa secretion required energy. However, the presence of 25 mM azide inhibited Congo red–induced secretion of IpaB ~20-fold but decreased hemolysis only 4-fold (Fig. 3, C and D). This suggested that not all of the Ipa proteins that could be secreted were released during hemolysis. Indeed, during a hemolytic assay, only a small amount of Ipa proteins were recovered in the medium, the rest of these proteins remaining within the bacterium (inaccessible to a protease added to the medium; Fig. 3 F and not shown). In addition, not all the released Ipa proteins may be required to observe full hemolysis, for which a single pore per RBC is in theory sufficient. In support of this, pretreatment of bacteria with Congo red, which released ~50% of Ipa proteins (Fig. 3 F), had no effect on the efficiency of a subsequent hemolytic assay, even when novel protein synthesis was blocked (not shown). This may explain why lysed RBC membranes contained <0.1% of the amount of Ipa proteins that were initially present in bacteria. In summary, Ipa secretion was an active process and secretion at the moment of contact was necessary for lysis.

To determine whether the proteins responsible for he-

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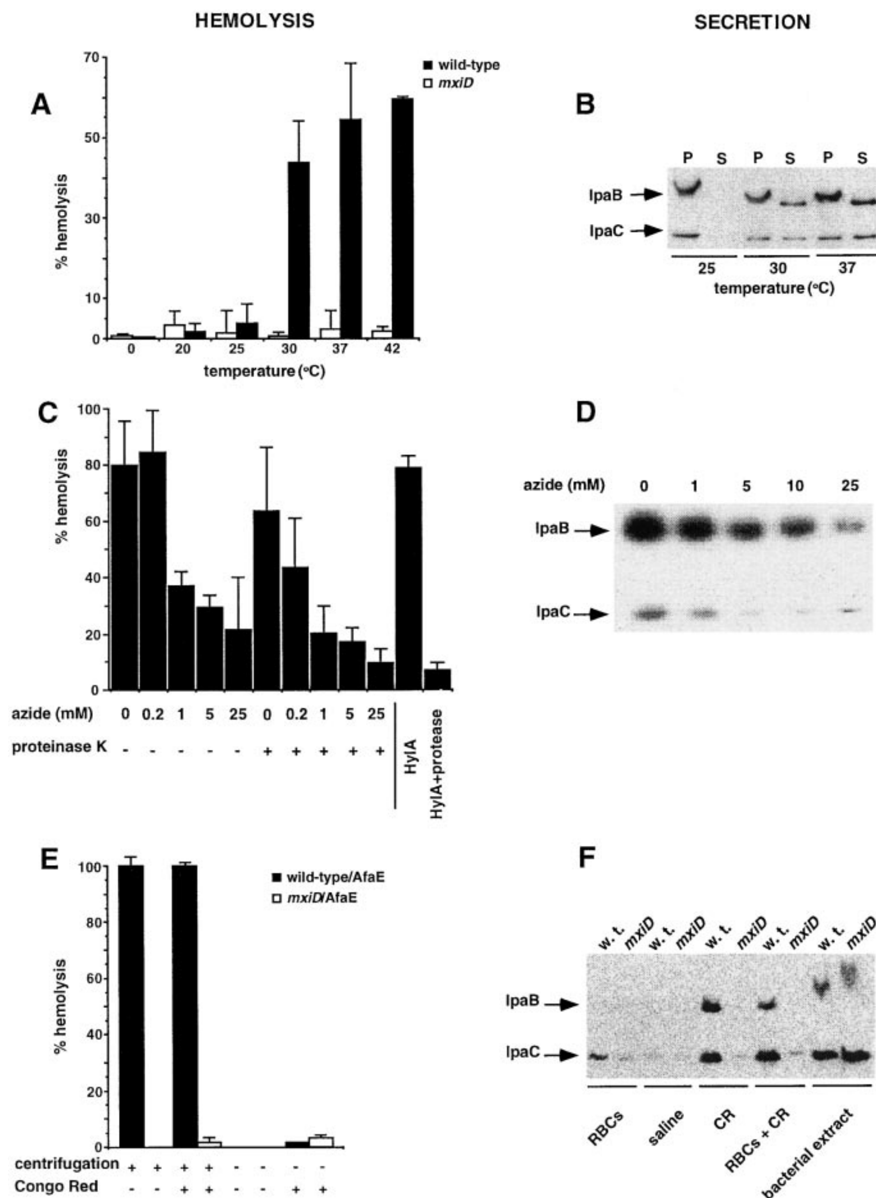

Figure 3. Parallel analysis of hemolysis and Ipa protein secretion. (A) The hemolytic assay was performed with bacteria and sheep RBCs incubated for 1 h at the indicated temperatures. (B) Congo red–induced secretion in the absence of any RBCs was measured at 25, 30, and 37°C. P stands for bacterial pellet and S for supernatant. (C) Wild-type bacteria were mixed with sheep RBCs in the presence or in the absence of the indicated concentrations of sodium azide and/or 1 mg/ml of proteinase K. As a control for the activity of the protease, RBCs were also exposed to a bacterial culture supernatant containing *E. coli* hemolysin A (HylA) or to the same supernatant to which proteinase K had been added (HylA+protease). (D) Congo red–induced secretion was measured in the presence of increasing concentrations of azide. IpaB and IpaC content of the supernatants is shown. (E) Wild-type or *mxiD* bacteria expressing the afimbrial adhesin AfaE were mixed with human RBCs in the presence or in the absence of 30 μM Congo red with or without centrifugation at 1,500 *g* for 10 min at 10°C, and incubated for 1 h at 37°C. (F) Wild-type (w.t.) or mxiD bacteria expressing AfaE were incubated with human RBCs, saline alone, saline with 30 μM Congo red (saline + CR), or RBCs with Congo red (RBCs + CR). They were treated as in the hemolytic assay, as was an identical volume of bacteria (bacterial extract) at the same concentration. Subsequently, 20 μl of the supernatants (for the bacterial extract, the bacterial pellet was resuspended) were separated on a 10% SDS-PAGE gel, blotted and probed with anti-IpaB and anti-IpaC mAbs, respectively.

molysis ever became exposed to the external medium, we added antibodies to IpaB, IpaC, and/or IpaD to the hemolytic reaction. These reagents had no effect on hemolysis at any concentration tested (up to 100 μg/ml), whether alone or in combination or even upon preincubation of bacteria (not shown). Next we added 1 mg/ml of proteinase K to the assay. This led to only a minor decrease in hemolytic efficiency (Fig. 3 C). This amount of protease instantly degraded *E. coli* hemolysin A, completely preventing hemolysis mediated by this protein (Fig. 3 C). Interestingly, the inhibitory effects of azide and proteinase K on hemolysis were additive (Fig. 3 C). This suggested that azide was acting by reducing secretion and proteinase K by degrading some of the secreted proteins as they were exiting the bacterium. Individual pretreatment of RBCs and bacteria with azide and proteinase K did not affect hemolysis (not shown), suggesting that any Ipa proteins at the bacterial surface before contact were not involved in hemolysis. These data confirmed that Ipa protein secretion and hemolysis were coupled in time and suggested that the process of Ipa protein transfer into the target membrane was poorly accessible from the surrounding medium.

To investigate the requirement for contact between bacteria and RBCs to obtain hemolysis, we used a derivative of *S. flexneri* carrying the AfaE afimbrial adhesin from enteropathogenic *E. coli.* This adhesin binds the decay accelerating factor, a ubiquitous surface glycoprotein, and confers upon bacteria the ability to hemagglutinate human RBCs (Pham et al., 1995). Despite their close binding to RBCs, these bacteria were unable to lyse RBCs in the absence of centrifugation (Fig. 3 E). However, upon centrifugation, they lysed RBCs efficiently. Since the adhesion mediated by AfaE was not sufficient to induce extensive Ipa protein secretion (Fig. 3 F), AfaE-expressing bacteria were incubated at 37°C in the presence of human RBCs

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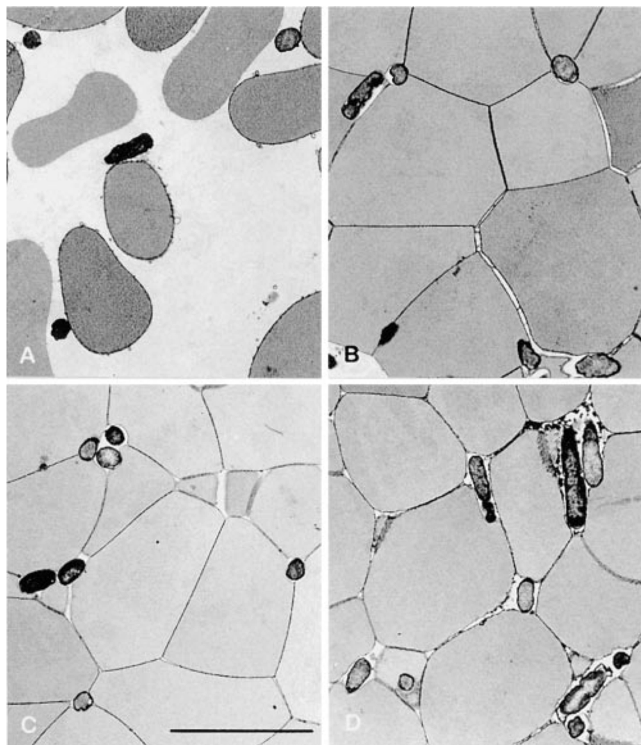

Figure 4. Electron microscopy analysis of the interaction between bacteria and RBCs. Derivatives of wild-type bacteria expressing the afimbrial adhesin AfaE were mixed with human RBCs and either left to sediment at (A) 1 *g*, or centrifuged at (B) 1,400 *g* for 10 min at 10°C. In C and D RBCs were mock treated or treated with sialidase, respectively, before mixing with the bacteria, and the samples were centrifuged at 160 *g*. The samples were fixed and prepared for transmission electron microscopy. Bar, 5 μm.

Table I. Effect of Centrifugation Force on Hemolytic Efficiency

RCF	RBCs		
	Untreated	Sialidase treated	Mock treated
g			
1	0 ± 0	0 ± 0	0 ± 0
160	1.1 ± 1.6	23.3 ± 11.1	3.9 ± 1.9
350	33.2 ± 5.4	35 ± 10.1	45.4 ± 2.6
630	55.7 ± 13.5	36.5 ± 9.4	45.0 ± 10.4
1400	69.7 ± 9.4	ND	ND

The values presented in this table are percentages of total hemolysis. All reactions were performed with wild-type bacteria expressing the AfaE adhesin and human RBCs untreated, sialidase treated, or mock treated.

and Congo red. Induction of Ipa protein secretion in the immediate vicinity of RBCs did not lead to lysis but the same procedure in the presence of centrifugation did (Fig. 3 E). This indicated that Congo red did not inhibit hemolysis and suggested that Ipa proteins could not insert into the RBC membrane after release from the bacterium. These data indicated that Ipa secretion was necessary but not sufficient for hemolysis.

Hemolysis Requires Extensive Surface Apposition and Reduced Distance between Bacteria and RBCs

Given that bacteria adhering to cells via the AfaE adhesin were unable to cause hemolysis without centrifugation at 1,500 *g*, we examined the consequence of this physical treatment on the cells. Mixtures of RBCs and AfaE-expressing bacteria were exposed to different centrifugation forces at 10°C and then either incubated at 37°C to assay hemolysis or prepared for examination by transmission electron microscopy. Hemolysis did not occur at 160 *g* but was complete at 630 *g* (Table I). In samples centrifuged at 160–630 *g*, the RBC membrane was deformed to line that of bacteria (Fig. 4 B). In the sample which had not been centrifuged, bacteria associated with RBCs adhered at a tangent rather than over their entire surface (Fig. 4 A). This indicated that centrifugation increased the surface of bacteria in contact with RBCs.

Although samples treated at 160 and 630 *g* looked similar, lysis occurred in the latter but not in the former (Table I), suggesting that the distance between bacterial and RBC membranes might also be critical for hemolysis. To test this possibility, we shortened this distance artificially, by removing either bacterial lipopolysaccharide (LPS) or sialic acid, a major constituent of the RBC surface (Shotton, 1998). We compared the lysis efficiency of wild-type *Shigella* and that of an *rfe* mutant that expressed a complete LPS core but lacked the O-antigen (Sandlin et al., 1995; Rathman, M., P.J. Sansonetti, C. Parsot, and G. Tran Van Nhieu, manuscript submitted for publication). The *rfe* mutant was as efficient as the wild-type at lysing RBCs at any g force applied (not shown). Then, we treated RBCs with neuraminidase to remove sialic acid. This treatment did not prevent AfaE carrying *Shigella* from hemagglutinating RBCs (not shown), as the site on the DAF molecule which is sialylated is distant from the SCR-3 domain to which AfaE binds (Pham et al., 1995). This treatment allowed lysis to occur efficiently at 160 *g* (Fig. 4 D) whereas mock-treated RBCs remained intact after exposure to bacteria at 160 *g* (Fig. 4 C and Table I). Surface charge is known to affect the ability of proteins to insert into artificial membranes (van der Goot et al., 1991) and the absence of LPS or the removal of sialic acid affect the surface changes of bacteria and RBCs. Thus, both the distance and the charge of the target membrane may be critical for induction of hemolysis.

The Type III Secreton Is a Multicopy Tripartite Structure Constitutively Assembled at 37°C

To understand why contact was critical for hemolysis, we searched by electron microscopy for the structure encoded by the *mxi* and *spa* genes in osmotically shocked and negatively stained bacteria. Most bacteria were unaffected by osmotic shocking, while some, mostly those undergoing binary fission, were partially lysed and a few appeared as complete membrane ghosts. No structures were visible on intact bacteria. Some structures, resembling the type III secretion apparatus of *Salmonella*, were visible at the periphery of partially lysed bacteria (Fig. 5 A, inset). In the ghosts, many structures were visible dispersed over the whole bacterial surface (50–100/cell) and they were not identical to that seen in partially lysed bacteria (Fig. 5 A).

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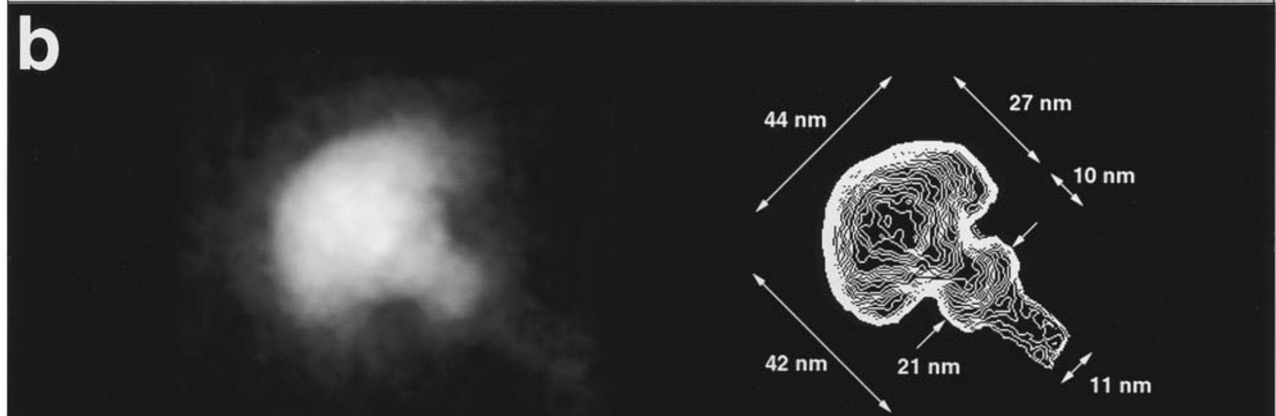

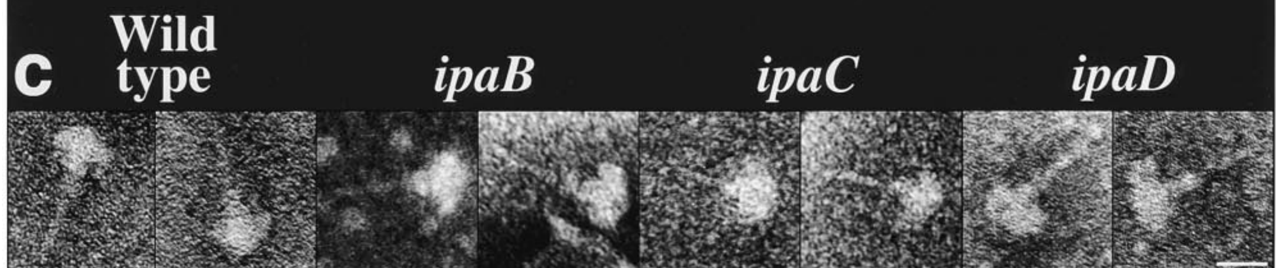

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These structures appeared as composed of three parts: (a) a needle 11 nm in diameter, (b) a neck 10 nm high and 21 nm wide, and (c) a bulb 44 nm wide and 27 nm high (Fig. 5 B). This bulb did not appear to have cylindrical symmetry since we saw several different views of it (Fig. 5 A). Upon alignment and averaging of a gallery of the 22 best preserved structures on the micrograph displayed in Fig. 5 A, we obtained a two-dimensional density map of the bulb at 2.8 nm resolution (Fig. 5 B). This tripartite structure is not identical to that seen by Kubori et al. (1998). They presented partially lysed bacteria in which secretons were visualized at the cell periphery and lacked the bulb. When examined by this method, intact bacteria and, to a lesser extent, partially lysed ones have a thickness which could explain why secreton structures seemed absent or partial, respectively, at their surface.

No structures were found in *mxiD*, *mxiJ*, and *mxiG* mutants (not shown). As these three strains carry mutations within genes coding for components of the type III secretion system, we concluded that the structures observed in the wild-type corresponded to the type III secretons. No obvious differences were detected in the morphology of secretons in *ipaB*, *ipaC*, and *ipaD* mutants relative to the wild-type (Fig. 5 C), nor in their distribution or number (not shown). Likewise, we saw no differences in secreton morphology or distribution of the wild-type which had been induced to secrete with Congo red (Fig. 5, A and C). This indicated that activation of secretion, as seen in *ipaB* and *ipaD* mutants or upon incubation of the wild-type with Congo red, did not result in or from gross alterations of secreton structure or number. We also concluded that the nonhemolytic phenotypes of the *ipaB*, *ipaC*, and *ipaD* mutants did not result from major anomalies in the structure of their secretons.

Discussion

Nature and Composition of the Translocator

Osmoprotection experiments showed that *S. flexneri* inserts a hydrophilic pore into RBCs. Amongst the five proteins secreted upon contact with eukaryotic cells, only IpaB and IpaC were tightly associated with RBC membranes isolated after contact-hemolysis. IpaA and IpgD were peripherally associated with RBC membranes while IpaD was not found in the membrane fraction. Since *ipaA* and *ipgD* mutants are not impaired in hemolysis, association of IpaA and IpgD with membranes is unrelated to pore formation. As IpaB and IpaC are the only secreted proteins with predicted α-helical hydrophobic regions capable of spanning membrane bilayers, it is likely that they are the sole bacterial components inserted into the host membrane during cell entry. Whether the IpaB/IpaC pore structure contains host components also can not be assessed with our experimental setup. However, studies of the association of Ipa proteins secreted in the presence of Congo red with artificial membranes have yielded only the same four proteins associated with liposomes and these proteins form a nonselective, gated channel of 91 pS in conductance within lipid bilayers (De Geyter, C., F. Homblé, R. Wattiez, P.J. Sansonetti, P. Falmagne, J.M. Ruysschaert, C. Parsot, and V. Cabiaux, manuscript submitted for publication). We also showed that IpaB and IpaC are both required to form the pore. Indeed IpaB alone is unable to form the pore structure since lysis mediated by the *ipaC* strain was insensitive to osmoprotection yet led to a small amount of IpaB protein becoming associated with lysed RBC membranes (Figs. 2 B and 3 A). When secretion occurs in the absence of any membrane, IpaB and IpaC are found solely as a complex in the medium (Blocker, A., unpublished observation), suggesting that they associate as they exit the secreton. Hence, to be inserted efficiently into the target membrane IpaB and IpaC must form a complex which may remain associated within lipid bilayers.

Although the *ipaD* mutant was nonhemolytic, we did not recover IpaD in lysed RBC membranes. Given the constitutive secretion phenotype of the *ipaD* mutant, the requirement of IpaD for hemolysis probably reflects the role of this protein in controlling secretion. IpaA and IpgD were associated peripherally with lysed RBC membranes, yet they were dispensable for hemolysis. As these proteins were found associated with RBC membranes lysed by the wild-type but not the *ipaC* mutant, they probably associate with the membranes via IpaB and/or IpaC. Evidence is mounting that both IpaA and IpgD proteins are translocated within the host cell at the time of entry when they modulate the actin rearrangements induced by IpaC (Tran Van Nhieu et al., 1997, 1999; Bourdet-Sicard, 1999; Niebuhr, K., N. Jourhi, A. Allaoui, P. Sansonetti, and C. Parsot, manuscript in preparation). This would suggest that IpaA and IpgD associate with the cytoplasmic face of the host membrane. Further work is required to establish the domains in each protein involved in these interactions, their topology relative to the membrane as well as the role of these interactions in cell entry.

Structure of the Type III Secreton

Electron microscopy studies showed that the type III secretons are assembled before any contact with target cells. In the *mxiD*, *mxiG*, and *mxiJ* mutants, no secretons were detected, which suggests that components of the secreton are degraded when the machinery can not be assembled. This is consistent with evidence that MxiG is unstable in the *mxiJ* and *mxiD* mutants (Bahrani, F., and C.

Figure 5. Morphological identification and analysis of the type III secretons of *S. flexneri.* (A) Osmotically shocked and negatively stained wild-type *Shigella* which had been induced to secrete with Congo red were visualized by electron microscopy. Arrows show the position of the secreton at the margin of bacteria. The neck and bulb are inside the body of the bacterium while the needle is protruding outside the outer membrane. Inset shows the periphery of partially lysed bacteria. Only the protruding needle is clearly visible. The neck is poorly distinguished and the bulb is masked inside the body of the bacterium. With the method used here one can not assume that the two lines at the margin of the bacterium are its inner and outer membranes. (B) Image of the secreton bulb resulting from alignment and averaging of the 22 best preserved secretons found in A and deduced projection density map of the averaged image at 2.8 nm resolution. (C) Typical secretons found in wild-type, *ipaB*, *ipaC* and *ipaD* strains. Bars: (A) 200 nm; (B) 50 nm.

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Parsot, unpublished observation). The secreton structure is composed of three different parts: (a) an external needle, (b) a neck, and (c) a large proximal bulb. In our images, it is difficult to discern the two bacterial membranes and thus to judge the localization of the bulb. Nevertheless the length of the neck domain is sufficient to traverse both membranes, implying that the bulb lies within bacterial cytosol. This putative cytoplasmic part was not recovered by Kubori et al. (1998) in secreton preparations, which were purified using a protocol derived from that for isolation of flagellar basal bodies (Aizawa et al., 1985). A cytoplasmic structure (other than the C-ring) is known to be associated with the bacterial flagellum and thought to contain the cytoplasmic F1-type ATPase FliI as well as several other components. This structure is not recovered in purified basal body preparations and has never been visualized (MacNab, 1996; Minamino and MacNab, 1999).

Inactivation of *spa47*, the homologue of the flagellar biosynthesis gene *fliI*, abolishes secretion. However, so far, it was unclear whether this ATPase was required for assembly and/or activity of the secretion machinery (Dreyfus et al., 1993). We showed that type III secretons were assembled by *S. flexneri* before host contact and that their structure did not grossly change when secretion was activated (in the presence of Congo red or in the *ipaD* or *ipaB* mutants). We also showed that secretion required energy and temperatures above 30°C (Fig. 3, B and D). Low temperatures did not disassemble secretons (not shown). Thus ATP hydrolysis and conformational change(s) are likely to be required for Ipa secretion, as for flagellin export (Vogler et al., 1991). We propose that Spa47 is the azide-sensitive element of the secretion machine.

Mechanism for Assembly of the Translocator

Intimate contact between bacteria and RBCs is essential for hemolysis. Indeed, secretion is necessary but not sufficient for hemolysis since inducing protein secretion from AfaE-expressing bacteria bound to RBCs did not provoke hemolysis in the absence of centrifugation. The increased hemolysis which is seen after centrifugation could be due to the greater surface contact which either activates more secretons to secrete and/or allows Ipas to reach the membrane still in active form after release. Both notions are supported by the sharp threshold of *g* forces required to obtain lysis and by the sialic acid removal experiment (Table I). Wild-type *Shigella* do not need centrifugation or the AfaE adhesin to enter HeLa cells, these agents simply make the process more efficient (Clerc and Sansonetti, 1987). The shortened glycocalyx, especially on the basal surface of transformed epithelial cells, might explain why AfaE can increase the efficiency of HeLa cell entry but not of hemolysis. Electron microscopy images indicate that the minimal distance required for lysis is $\sim$100 nm; the secreton needle is 60 nm long. Yet, contact with the needle alone is unable to cause lysis as (a) the diameter of the pore (25 Å) is smaller than that of the needle (80–100 Å), and (b) *ipaB*, *ipaC* and *ipaD* mutants display normal secretons yet no hemolysis.

Two hypotheses have been put forward for how Ipas associate with host cells (reviewed in Hueck, 1998). First, Ipa proteins could be presented at the bacterial surface and inserted from there upon contact. This corresponds to a two-step model. Second, contact may induce secretion and the newly secreted Ipas may insert into the target membrane at this stage. This is a one-step model. We detected only minute amounts of Ipas at the bacterial surface by immunoelectron microscopy and these Ipa proteins never colocalized with secretons revealed by negative staining (Gounon, P., and A. Blocker, unpublished observations). In addition, hemolysis was insensitive to protease pretreatment of bacteria, suggesting that the biologically active Ipa proteins are not at the bacterial surface before contact. This is not in favor of a two-step mechanism for Ipa protein transfer into the target membrane. On the other hand, the temperature and energetic correlation between secretion and lysis and the additive inhibition of hemolysis by azide and proteinase K (Fig. 3) support a process where secretion or surface presentation upon contact is kinetically coupled to insertion.

How might secretion and membrane association of the pore forming Ipas be coupled? The approximate diameter of the canal known to exist in the bacterial flagellum is 20–30 Å (Morgan et al., 1995). As the secreton structure is evolutionarily derived from flagella there could exist a canal of similar diameter through the needle. The semi-folded state from which IpaB and IpaC would necessarily exit the secreton needle (given the small diameter of the putative canal) might be required for their membrane association. As the role of the Spa47 ATPase is probably to push the secreted proteins into the secreton canal, ATP hydrolysis by this protein could also indirectly serve to allow membrane insertion. Accordingly, the needle may be required both as a tactile sensor for activation of the secreton and to present the Ipas directly at the host membrane surface in a sufficiently unfolded state to allow their insertion. What we can not yet assess is whether the semifolded IpaB/IpaC complex enters the membrane immediately upon exiting the secreton or whether there is a short-lived intermediate in a different conformation which travels $<$100 Å through the medium to reach the membrane. Nevertheless, as 25 Å is the functional inner diameter of the IpaB/IpaC pore we find in RBCs, we propose that the IpaB/IpaC complex fits around the end of the putative canal within the needle. This linkage is likely to be dynamic in time and/or fragile because otherwise osmoprotection would be difficult to rationalize and bacteria would copurify with floated RBC membranes after hemolysis. The 25 Å IpaB/IpaC pore would then let the other Ipa proteins destined to be translocated through in the semi-folded state generated by Spa47-powered extrusion from a similarly seized secreton canal.

This work is dedicated to the memory of Thomas E. Kreis. We are grateful to Jacomine Krinjse-Locker for instructions on the use of neuraminidase and to Michel Chignard and Jean-Claude Manuguerra for human blood and HA assistance. A. Blocker wishes to thank in particular Philippe Clerc for his recollections of 15-yr-old unpublished data on the contact hemolytic activity of *S. flexneri.* She acknowledges Chantal LeBougenec, Tony Pugsley, and Elie Dassa for local advice and reagents and Lina Bernardini, Guy Cornelis, Mike Donnenberg, Bob MacNab, Ira Mellman, and Dennis Thomas for inspiring discussions, communication of results before publication, and/or critical reading of the manuscript. Finally, she is indebted to Gisou van der Goot, Gareth Griffiths, David Holden, and Paul Lazarow for their supportive friendship.

A. Blocker and K. Niebuhr were supported by National Institutes of

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Health and European Molecular Biology Organization postdoctoral fellowships, respectively. V. Cabiaux is a research associate from the Fonds National de la Recherche Scientifique, Belgium.

Submitted: 1 July 1999
Revised: 21 September 1999
Accepted: 27 September 1999

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