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Author Manuscript

Proteins. Author manuscript; available in PMC 2015 November 01.

Published in final edited form as:

Proteins. 2014 November ; 82(11): 3013–3022. doi:10.1002/prot.24662.

Influence of Oligomerization State on the Structural Properties of Invasion Plasmid Antigen B (IpaB) from *Shigella flexneri* in the Presence and Absence of Phospholipid Membranes[†]

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Abstract

Shigella flexneri causes bacillary dysentery, an important cause of mortality among children in the developing world. *Shigella* secretes effector proteins via its type III secretion system (T3SS) to promote bacterial uptake into human colonic epithelial cells. The T3SS basal body spans the bacterial cell envelope anchoring a surface-exposed needle. A pentamer of invasion plasmid antigen D (IpaD) lies at the nascent needle tip and IpaB is recruited into the needle tip complex upon exposure to bile salts. From here, IpaB forms a translocon pore in the host cell membrane. Although the mechanism by which IpaB inserts into the membrane is unknown, it was recently shown that recombinant IpaB can exist as either a monomer or tetramer. Both of these forms of IpaB associate with membranes, however, only the tetramer forms pores in liposomes. To reveal differences between these membrane-binding events, Cys mutations were introduced throughout IpaB, allowing site-specific fluorescence labeling. Fluorescence quenching was used to determine the influence of oligomerization and/or membrane association on the accessibility of different IpaB regions to small solutes. The data show that the hydrophobic region of tetrameric IpaB is more accessible to solvent relative to the monomer. The hydrophobic region appears to promote membrane interaction for both forms of IpaB, however, more of the hydrophobic region is protected from solvent for the tetramer after membrane association. Limited proteolysis demonstrated that changes in IpaB's oligomeric state may determine the manner by which it associates with phospholipid membranes and the subsequent outcome of this association.

Keywords

Fluorescence Quenching; Protein-Lipid; Membrane; Stern-Volmer; *Shigella*

[†]This work was supported by funding to WLP (NIH R01 AI067858) and WDP (Oklahoma Health Research Program Funding HR10-128S) and WDP (NIH R01 AI099489).

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Introduction

Type III secretion systems (T3SS) are used by numerous Gram-negative bacteria to introduce effector proteins into target eukaryotic cells.¹⁻³ The type III secretion apparatus (T3SA) consists of a cytoplasmic bulb, a basal body that spans the entire bacterial envelope, a surface-exposed needle,⁴⁻⁶ and a complex of proteins poised at the distal end of the needle that controls secretion.^{7,8} *Shigella flexneri* uses its T3SS to cause apoptosis in macrophages and invade cells of the human colon as a first step in causing shigellosis.³ The *S. flexneri* T3SA needle is a polymer of MxiH arranged around a helical axis and its nascent tip complex consists of a pentamer of invasion plasmid antigen D (IpaD).⁸ In the presence of bile salts, IpaD undergoes a structural transition that leads to the recruitment of the hydrophobic translocator protein IpaB to the T3SA needle tip.^{7,9-11} While the precise function of IpaB at the needle tip is not yet clear, it appears to be required for sensing contact with host cell membranes to facilitate the injection of additional T3SS proteins. Upon contact with phospholipid membranes, a second hydrophobic translocator protein, IpaC, is recruited to the needle tip and secretion becomes fully activated.¹² It has been proposed that IpaB and IpaC form the translocon pore through which effector proteins are injected to induce the cytoskeletal rearrangements that ultimately lead to bacterial entry into the host cell.¹³⁻¹⁵

IpaB and IpaC are maintained in a soluble state within the *Shigella* cytoplasm by individually associating with the chaperone invasion plasmid gene C (IpgC).^{16,17} Previous work suggests that the IpaB-IpgC complex is a heterodimer with the interaction mediated by the primary sequence within the N-terminal portion of IpaB.¹⁷⁻¹⁹ Transit of IpaB to the T3SA needle tip occurs after separation from IpgC, which remains in the bacterial cytoplasm to carry out other processes.¹⁶ Once at the tip, IpaB's hydrophobic nature allows it to recognize and penetrate mammalian cell membranes following recognition of specific lipid components.^{12,20} The structural state of IpaB as part of the maturing T3SA needle tip complex is unknown, however, evidence from our lab and others indicates that purified IpaB can exist as an oligomer and this may have implications with regard to IpaB's multiple functions.^{21,22} Unfortunately, detailed structure analysis of full-length IpaB is complicated by its hydrophobic nature. Purification has been enhanced by co-expression with IpgC followed by removal of the chaperone using mild detergents.¹⁷ We recently discovered that the physical state of IpaB is influenced by the detergents used in its preparation.²¹ IpaB prepared in the mild zwitterionic detergent lauryl-N,N-dimethyldodecylamine N-oxide (LDAO) exists primarily as a monomer in solution, while IpaB prepared with the non-ionic detergent octyl-oligo-oxyethylene (OPOE) exists in a primarily tetrameric state.²¹ Both forms of IpaB readily interact with liposomes, but only the tetrameric form promotes the release of small molecules from liposomes due to the formation of a pore.²¹ While the relationship of this structure to the translocon pore at the T3SA needle tip remains to be determined, others have found that alternate oligomeric forms of IpaB can form ion channels in macrophage membranes that lead to cell death.²²

In this study, spectroscopic analyses were used to analyze the nature of IpaB's interaction with phospholipid vesicles. Because IpaB exists in different oligomeric forms that may be relevant to its function at different stages of infection, it is important to explore the nature of

IpaB's interaction with phospholipid membranes and with itself. To identify the IpaB regions that promote membrane interaction, we introduced single-cysteine residue replacements along the length of the protein. The resulting mutant *ipaB* genes were co-expressed in *Escherichia coli* with *ipgC* and the resulting protein complex was purified as a translocator/chaperone pair. The complex was then dissociated with either LDAO or OPOE to give rise to monomeric or tetrameric IpaB, respectively. Each protein was then labeled with a sulfhydryl-reactive fluorophore to probe different microenvironments within IpaB, including the predicted hydrophobic region (approximately spanning residues 310–420)²³ under different conditions. From our findings, it appears that residues within the IpaB hydrophobic region are differentially accessible to quenching agents depending upon oligomeric state. Surprisingly, oligomerization appeared to make the hydrophobic region more accessible to a charged quenching agent. Furthermore, the oligomeric state directly influences the way in which the hydrophobic region interacts with phospholipid membranes and the susceptibility of IpaB to proteolysis. A possible model for the organization of membrane-interacting regions of IpaB is presented.

Materials and Methods

Cloning, expression, and purification of recombinant proteins

The *ipaB* coding sequence was cloned into pHS2.²⁴ Primers were designed to mutate residues G308 and C309 to Ala-Ser eliminating the native Cys (C309) in IpaB. This change had no effect on IpaB's role in invasiveness or contact-mediated hemolysis activity (data not shown) when expressed in the *ipaB* null mutant *S. flexneri* SF620 (provided by P. Sansonetti, Institut Pasteur, Paris, France). This construct was used as a template for inverse PCR in which paired primers were designed to substitute Cys for residues S58, S107, S149, S237, A254, A353, S486, and S519. After sequence confirmation, the mutant *ipaB* genes were sub-cloned into pET15b (Novagen, Madison, WI) and used to co-transform *E. coli* Tuner (DE3) (Novagen, Madison, WI) with *ipgC/pACYC-Duet 1*¹⁷ and grown in Terrific Broth containing ampicillin (100 µg / ml) and chloramphenicol (37 µg / ml) at 37°C with shaking at 200 rpm to an OD₆₀₀ of 1.0, moved to 17°C, and induced for 16 hours with isopropyl-β-d-thiogalactopyranoside (IPTG). The His-tagged IpaB/IpgC complex was purified from the clarified supernatant of the lysed bacteria by immobilized metal affinity chromatography (IMAC) followed by hydrophobic interaction chromatography as previously described.²⁵ Peak fractions of each mutant IpaB/IpgC complex were collected, split into two populations, and bound again to an IMAC column and incubated with either 0.1% LDAO or 1% OPOE to dissociate the translocator/chaperone complex. The chaperone was washed away in the flow-through. To maintain IpaB solubility, the His-tagged IpaB was eluted from the IMAC columns in the presence of either 0.05% LDAO or 0.5% OPOE. To isolate the monomer from any soluble aggregates, IpaB in 0.05% LDAO was further purified using size-exclusion chromatography on a HiLoad 16/60 Superdex-200 preparation grade column (GE Health Care, Piscataway, NJ). The proteins were then concentrated and dialyzed into 10 mM phosphate (pH 7.4) containing 150 mM NaCl (PBS) containing either 0.05% LDAO or 0.5% OPOE.

Shigella contact-mediated hemolysis and invasion of cultured cells

The ability for *Shigella* expressing mutant forms of IpaB to form translocon pores was determined by contact-mediated hemolysis as previously described.²⁶ This assay measures the release of hemoglobin from erythrocytes as determined by absorbance at 595 nm. SF620 strains expressing the IpaB mutants were tested for their contact-hemolytic activity relative to SF620 expressing wild-type *ipaB* (100% contact-mediated hemolysis). This assay provided an assessment of IpaB's ability to properly interact with phospholipid membranes *in vivo*. As a measure of IpaB invasion-related function, the effect of the IpaB mutations on the invasive capacity of *Shigella* was monitored using HeLa cells in a standard gentamycin protection assay using SF620 expressing wild-type *ipaB* as the positive control (100% relative invasion).²⁶

Preparation of phospholipid vesicles

All phospholipids and extrusion equipment were purchased from Avanti Polar Lipids (Alabaster, AL). All phospholipids were reconstituted in chloroform at 25 mg/ml. Lipid formulations were made to contain 66.5 mol % dioleoylphosphatidylcholine (DOPC), 23.5 mol % dioleoylphosphatidylglycerol (DOPG), and 10 mol % cholesterol and dried under a stream of nitrogen. Films were rehydrated in PBS to a lipid concentration of 8.7 mg/ml. Films were briefly sonicated using a probe sonicator followed by extrusion through a 100 nm pore-size membrane at 45°C. Phospholipid vesicles were stored at 4°C and used within two weeks.

Phospholipid vesicle dye release assay

To monitor the IpaB-dependent release of small molecules from phospholipid vesicles, vesicles were made as described above except that 100 mM sulfo-rhodamine-B (SRB, Molecular Probes, Eugene, OR) was included during extrusion. After extrusion, the free dye was removed by Sephadex G50 size exclusion chromatography. SRB auto-quenches at this concentration within the liposomes. The auto-quenching is relieved upon release from the liposomes, which is seen as a time-dependent increase in SRB fluorescence intensity. Fluorescence emission was monitored at 584 nm on a Jobin-Yvon FluoroMax-4 spectrofluorometer as a function of time. The excitation wavelength was 562 nm and the slits were set to 2.5 nm. Phospholipid vesicles containing 100 mM SRB were diluted in PBS and scanned for 20 sec to establish a baseline level of fluorescence. IpaB mutants in PBS containing 0.05% LDAO or 0.5% OPOE were added to a final protein concentration of 100 nM, mixed, and scanned for 240 sec. Triton X-100 was then added to a final concentration of 0.1% to release all fluorophore and the sample was scanned for an additional 20 sec. Percent SRB release was calculated by dividing the fluorescence intensity after 240 sec of protein-induced fluorophore release by the final fluorescence intensity after complete release by Triton X-100. Tetrameric, wild-type IpaB was used as a positive control and IpaD was used as a negative control. The concentrations of the detergents used here did not lead to any substantial release of SRB (see Suppl. Fig. S1).

Fluorescence labeling of proteins

IpaB cysteine point mutants were labeled with fluorescein maleimide (FM) as previously described.¹⁸ Proteins were dialyzed into labeling buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM tris(2-carboxyethyl)phosphine (TCEP) and either 0.05% LDAO or 0.5% OPOE). The TCEP concentration was then reduced to 1 mM by dialysis prior to labeling with a ten-fold molar excess FM dissolved in N',N'-dimethylformamide (DMF). Labeling reactions were stirred under a nitrogen stream for 2 h at 25°C. Unreacted dye and the DMF were removed from the labeled proteins by binding His-tagged FM-IpaB to an IMAC column and washing with binding buffer containing either 0.05% LDAO or 0.5% OPOE. The protein was then eluted and dialyzed as described above. It was noted that in all cases the monomeric IpaB labeled with better efficiency than did the tetramer (data not shown).

Quenching of FM-IpaB with potassium iodide

Fluorescence emission spectra were collected on a Jobin-Yvon FluoroMax-4 spectrofluorometer. The excitation wavelength was 493 nm with 5 nm slits. Emission spectra were collected from 500–560 nm with 2.5 nm slits with an integration time of 0.1 sec. Samples were made to contain 100 nM FM-IpaB in PBS, which was incubated for 1 min in the presence or absence of 0.150 mg/ml liposomes. For each sample, an initial scan was taken in the absence of potassium iodide (KI), and then scans were taken following the addition of iodide (as KI) to the sample. F_o/F values were determined from the fluorescence intensities observed at 518 nm and plotted as a function of iodide concentration. The data were fit to the Stern-Volmer equation (Equation 1), where F_o and F represent the fluorescence intensity of fluorescein in the absence and presence of KI, respectively, and K_{SV} represents the Stern-Volmer quenching constant and is given by the slope of the resulting plot.²⁷

$$F_o/F = 1 + K_{SV} [KI] \quad \text{Equation 1}$$

To visually present the differences in K_{SV} among the IpaB mutants, a protection index (PI) resulting from the addition of phospholipid vesicles was calculated. PI is the ratio of the K_{SV} in the absence of phospholipid vesicles to the K_{SV} in the presence of phospholipid vesicles, according to the work of Nalefski and Falke (Equation 2).²⁸

$$K_{SV}^{-\text{Liposomes}} / K_{SV}^{+\text{Liposomes}} = \text{Protection Index (PI)} \quad \text{Equation 2}$$

We also calculated a protection index resulting from the association of IpaB to form an oligomeric state in the absence of liposomes. This is the ratio of the K_{SV}^{Monomer} to the K_{SV}^{Tetramer} in the presence of LDAO and OPOE, respectively (Equation 3).

$$K_{SV}^{\text{Monomer}} / K_{SV}^{\text{Tetramer}} = \text{Protection Index (PI)} \quad \text{Equation 3}$$

In either case, a PI value >1 indicates that a particular residue was more protected from quenching, whereas a PI value <1 indicates increased exposure of that residue to the

quenching agent.²⁸ A PI value near 1 indicates that there was no significant change in the solvent accessibility for the fluorophore at that residue.²⁸

Proteolysis and identification of proteolytic products

Subtilisin-A (Sigma-Aldrich, St. Louis, MO) was used to detect temporal differences in the proteolytic degradation of IpaB monomers and tetramers. Subtilisin A (stock concentration = 7–15 units/mg) was used at a final dilution of 1:80,000. Monomeric or tetrameric IpaB (6 μ M) was incubated with 4.4 μ g/ μ l liposomes or an equivalent volume of PBS at room temperature for 10 min. Protease was then added and the solution mixed. Samples were taken at specified time points and added to SDS-PAGE sample buffer pre-heated to 99°C to rapidly stop the proteolytic digestion. Samples were then separated on 12% SDS-polyacrylamide gels, stained with Coomassie brilliant blue R250 and imaged on an Odyssey infrared imager (LI-COR, Lincoln, NE). Selected bands were subjected to LC-MS/MS as previously described to determine their sequences.²⁹

Statistical analysis of experimental data

All multi-group analyses were performed using GraphPad Prism Version 6.02 (GraphPad Software, Inc. La Jolla, CA). Where indicated, the data were subjected to a one-way ANOVA with multiple pairwise comparisons using Tukey's test. Data were normally distributed according to the D'Agostino and Pearson test, and had equal variance according to the Brown-Forsythe test.

Results

Cysteine substitutions do not abrogate IpaB's ability to form membrane pores

IpaB contains a native Cys at position 309 that was mutagenized to Ser to provide a Cys-free background for generating the following IpaB single-cysteine variants: S58C, S107C, S149C, S237C, A254C, A353C, S486C, and S519C (refer below to Fig. 4A for a schematic of residue placement). To ensure the mutations did not negatively affect the ability for IpaB to form translocon pores when delivered by the bacterium, an *ipaB* null strain of *S. flexneri* (SF620) was transformed with a plasmid encoding each *ipaB* variant. Each new strain was then evaluated for its ability to insert a functional pore into erythrocyte membranes (contact-mediated hemolysis, Table I) and for its ability to restore invasiveness to SF620 using HeLa cells, as previously described²⁴ (Suppl. Table S1). Hemolysis was not dramatically altered for any of the IpaB variants, indicating little or no effect on the membrane recognition or insertion properties of the protein *in vivo* (*i.e.* translocon pore formation). There was a moderate decrease in invasiveness for *Shigella* expressing the IpaB mutants S107C, S149C, A254C and A353C, but this was not deemed an obstacle in characterizing the membrane-interacting potential of the protein.

To demonstrate that the purified IpaB mutants retain the previously described membrane-active properties when prepared in LDAO and OPOE,^{17,21} they were used in a liposome disruption assay to provide an *in vitro* measure of pore formation. We previously demonstrated that wild-type, tetrameric IpaB prepared in OPOE causes efficient release of SRB from liposomes while monomeric IpaB prepared in LDAO does not.²¹ As expected, all

the IpaB mutants in the tetrameric state were able to disrupt the liposomes (Suppl. Fig. S1). The IpaB mutants labeled with fluorescein maleimide (FM-IpaB) behaved similarly to the unlabeled proteins, indicating that attachment of the FM probe does not alter the membrane active properties of IpaB (Suppl. Fig. S2). It is worth noting that IpaB S58C was reduced in its ability to disrupt liposomes *in vitro* (before or after labeling with FM), however, it retained approximately wild-type levels of contact-hemolysis and invasiveness *in vivo* when expressed in *S. flexneri* SF620 (Suppl. Fig. S1 and S2; Table I and Suppl. Table I). It is not clear why IpaB S58C shows this slightly modified behavior *in vitro* but not *in vivo*, however, it could be due to having a role in stable IpaB oligomerization that is overcome *in vivo* by the presence of other T3SS components (*e.g.* IpaC or IpaD) within the context of the complete at the T3SA needle tip. Nevertheless, this reduced lipolytic activity should be kept in mind while considering other data pertaining to IpaB S58C because, while not likely, its position within the IpaB tetramer structure *in vitro* may be different than what occurs for wild-type IpaB. Meanwhile, the monomeric forms of IpaB allowed very little fluorophore release from the liposomes (Suppl. Fig. S1). Chemical crosslinking confirmed that each IpaB variant was monomeric or tetrameric in LDAO or OPOE, respectively (data not shown). Thus, introduction of these Cys residues did not abrogate IpaB's previously described properties *in vivo* or *in vitro*.

Specific sites within IpaB's hydrophobic region are more accessible when in a tetrameric state

FM-IpaB prepared in either LDAO or OPOE was subjected to fluorescence quenching using the collisional quencher iodide. The data were plotted according to the Stern- Volmer equation (see Suppl. Fig. S3) and the quenching constant (K_{SV}) was calculated for each (Table II). When the iodide quenching efficiency of the site-specific FM probes was compared, some sites on IpaB were more accessible to quenching agent than others and this accessibility was influenced by the IpaB oligomeric state. Because no change in solute quenching was observed for the probe at position 107 for the two protein states, this residue was used as a reference point for determining the significance of changes at other positions. Where appropriate, the pairwise comparison of PI values is discussed. Based upon the protective index (PI) calculated for the probe at each position, residue 58 near the N terminus experienced the greatest protection from quenching upon oligomerization (Fig. 1), however, this mutant also showed a reduced level of lipolytic activity *in vitro* (see above). Small but statistically significant increases in the PI occurred for the probes at positions 149 and 237 relative to position 107, whereas the PI for the probe at position 254 was small and not significantly different from position 107. The PI values for residues 149, 237, and 254 were not statistically different from each other. When the PI values were compared pairwise, residues 237 and 519 were not significantly different from each other. These data suggest that outside of position 58, the solvent accessibility of the N-terminal half of IpaB is minimally impacted by the protein's oligomeric state.

In contrast to what was observed for residues within the N-terminal portion of IpaB, sites located near or within the hydrophobic region of the protein became more accessible to iodide quenching (having low PI values) within the tetrameric IpaB (Fig. 1). Residues 309, 353, and 486 displayed increased quenching when IpaB was tetrameric, with the probe at

residue 353 being particularly susceptible to quenching (Fig. 1). These three residues were significantly different from the reference residue 107, however, a pairwise comparison revealed that the PI values of residues 309 and 486 were not statistically different from each other, but they were both quite different from residue 353 which was the most exposed residue upon tetramer formation. It thus appears that the IpaB hydrophobic region becomes more accessible to polar solutes upon tetramer formation. This was an unexpected result that may reveal important information regarding IpaB's structural organization and its function within the context of a tetrameric complex.

The hydrophobic region of tetrameric IpaB is protected from quenching after association with liposomes

IpaB is able to associate with phospholipid membranes regardless of oligomeric state.²¹ The efficiency of IpaB-dependent release of small molecules from liposomes is inversely related to the size of those molecules,²¹ suggesting that tetrameric IpaB is a pore-forming protein. Thus, we compared the influence of membrane association on the quenching of site-specific probes on tetrameric and monomeric IpaB. Iodide quenching was performed in the presence and absence of liposomes and the protection index (PI) was calculated from these data (Fig. 2, Table II). Carboxyfluorescein (CF) was included as a control for the influence of liposome addition on the emission properties of fluorescein. The K_{SV} of CF was not altered by liposome addition, indicating that the fluorescein moiety did not nonspecifically interact with the liposomes (Fig. 2).

In these quenching analyses, the probe at residue 107 was more protected from solute interaction after association with liposomes for both monomeric and tetrameric IpaB. Likewise, sites within the hydrophobic region of IpaB were protected from iodide quenching with the probe at residue 353 becoming least accessible to iodide for both forms of IpaB following liposome addition (Table II). Interestingly, the PI and K_{SV} data suggested that all three sites within the hydrophobic region (residues 309, 353, and 486) of tetrameric IpaB were more protected from quenching agent following association with liposomes than they were for monomeric IpaB (Table II, Fig. 2). This may indicate that the hydrophobic domain is poised specifically for membrane recognition after IpaB is oligomerized. When the oligomeric versus monomeric state was compared in the presence of liposomes, however, the majority of the residues had a PI that was much closer to 1.0 (Suppl. Fig. S4). With the exception of residues 58 and 353 (Suppl. Fig. S4), these data suggest that after membrane association, IpaB monomers and tetramers are largely in the same environment, however, only the latter lead to the release of small molecules trapped in membrane vesicles.

The significant increase in the PI value at residue 353 in the IpaB tetramer after membrane association (Fig. 2) is partly attributable to the high K_{SV} of this probe for the tetramer in the absence of liposomes (Table II). The raw K_{SV} value for the probe at this position on the IpaB tetramer is much greater than for the probe at the same position on monomeric IpaB prior to liposome addition (Table II). The K_{SV} is reduced at residue 353 on both species after liposome addition, however, it is reduced substantially enough upon liposome addition for the tetramer that it gives rise to the largest PI value observed in this study (Table II, Fig. 2). Perhaps more importantly, there is a greater degree of protection for the two positions

flanking residue 353 within the IpaB tetramer following membrane association than for the monomer. For wild-type, tetrameric IpaB (Cys309), the K_{SV} is higher prior to liposome addition relative to the monomer, but this is reversed once liposomes are added. The same phenomenon occurred for the probe at position 486, though to a slightly smaller degree. This could be a result of differences in the location of the entire hydrophobic region of the tetramer relative to that of the monomer with respect to the membrane.

Time-dependent proteolysis suggests differences in how monomeric and tetrameric IpaB interact with membranes

Monomeric and tetrameric wild-type IpaB were subjected to proteolysis with subtilisin-A in the absence and presence of liposomes to determine how membrane association influences the resulting hydrolysis patterns (Fig. 3). We reasoned that oligomerization and/or liposome association could cause some regions to be shielded from proteolysis by steric constraint. Surprisingly, tetrameric IpaB was more rapidly degraded by subtilisin than monomeric IpaB (Fig. 3). We anticipated that the oligomeric form would be more protease resistant due to the multiple protein-protein contacts needed for oligomerization. The finding that the monomer was more stable in the presence of subtilisin could indicate that LDAO has intrinsic stabilizing properties or inhibits subtilisin activity. Therefore, we used IpaD, a *Shigella* needle tip protein that does not interact with liposomes, as a control protein. Subtilisin-A degraded IpaD equally well in the presence of LDAO or OPOE (Suppl. Fig. S5). Similar results were found for the more hydrophobic protein, bovine serum albumin (Suppl. Fig. S6). Therefore, differences in the patterns seen for IpaB after treatment with subtilisin were not due to the impact of these detergents on the protease. Thus, it appears that monomeric IpaB is more resistant to protease degradation than is the tetramer. These findings suggest there are structural differences between the monomer and oligomer, despite the fact that they retain quite similar secondary structures.²¹

The addition of liposomes to monomeric IpaB resulted in a more rapid loss of high molecular weight species (Fig. 3) and the prolonged presence of intermediate (approximately 45 kDa) polypeptides. These peptides may contain sites that become inaccessible to protease following membrane association, while the more rapid loss of full-length protein could mean that structural changes that expose certain sites within the protein occur following peripheral association with the membrane. In contrast, liposomes caused little change in the proteolysis pattern of the IpaB tetramer. This suggests that oligomerization exposes regions of the protein to proteolysis in the absence of membranes and that these same regions remain available for digestion following association with liposomes.

Discussion

An essential feature of the *Shigella* T3SS is its ability to form a translocon pore in target cell membranes to facilitate the delivery of host-altering effector proteins. The first hydrophobic translocator delivered into the host membrane appears to be IpaB. This is based on the fact that *ipaC* null mutants (lacking the second hydrophobic translocator protein) still retain a measureable amount of contact-mediated hemolysis activity, whereas *ipaB* null mutants

possess no such activity.³⁰ Furthermore, purified recombinant IpaB associates with phospholipid membranes and is a cholesterol binding protein.^{20,21} In addition to associating with phospholipid membranes, the ability to form higher order complexes capable of inducing macrophage death²², smaller oligomers (*e.g.* tetramers) that can form pores in liposomes²¹, and heterodimers with the chaperone IpgC^{17,18} show that IpaB can adopt multiple states, perhaps all having physiologically relevant functions. We recently demonstrated that IpaB's quaternary state can be influenced by the detergent used for its purification.²¹ When prepared in LDAO or OPOE, IpaB is a monomer or tetramer, respectively.²¹ Both IpaB oligomeric states retain nearly identical secondary structure.²¹ Although both forms of IpaB associate with phospholipid membranes, only the tetramer forms pores that release small molecules from liposomes.²¹ Here, we used a fluorescence quenching strategy to identify regions that are affected during IpaB oligomerization and interaction with phospholipid membranes.

Residues within the hydrophobic region of IpaB became more accessible to quenching by iodide upon oligomerization. While surprising, this observation does suggest that tetramer formation is not a result of nonspecific hydrophobic interactions that should result in the hydrophobic region becoming less accessible to solvent. This is supported by the formation of a distinct tetramer in OPOE rather than a series of heterogeneous species. Furthermore, the relatively small size of this complex (tetramer) and the ability to form pores may indicate that this form of IpaB is related to the one observed at the T3SA needle tip immediately prior to IpaB delivery into target cell membranes. This is speculative and requires detailed ultrastructure analysis of the T3SA needle tip for comparison with the purified IpaB tetramers and these studies are under way. Nevertheless, one could speculate that the observation of increased accessibility of the IpaB hydrophobic region to quenching agent in the tetramer would provide a hydrophobic "patch" for the T3SA needle tip to recognize or sense contact with a host cell membrane. Loss of this accessibility to quenching agents within the IpaB hydrophobic region following membrane interaction could indicate that the collective presence of an exposed hydrophobic surface allows the insertion of IpaB tetramers into the membranes as a first step in translocon pore formation.

Because the fluorescence data presented here suggest structural differences between monomeric and tetrameric IpaB, limited proteolysis experiments were performed. The resulting proteolytic patterns suggested that there are structural differences between the IpaB monomer and tetramer, however, the results were not entirely expected. It appears that IpaB is more sensitive to digestion by subtilisin when the protein is tetrameric rather than monomeric. These data may indicate that monomeric IpaB possesses a more compact structure that protects its hydrophobic region and renders it less sensitive to proteolysis. Interestingly, the IpaB monomer also had the more pronounced change in proteolytic pattern after incubation with liposomes, suggesting membrane association alters either accessibility to protease cleavage sites or overall protein structure. In contrast, tetrameric IpaB demonstrated more subtle changes in proteolytic patterns following membrane association. This suggests that sites that are accessible to subtilisin in the absence of membranes largely remain accessible following association with a membrane. This occurs along with protection of the IpaB hydrophobic region from interaction with the quenching agent iodide. Perhaps

tetrameric IpaB, which is capable of forming pores in liposomes, exposes portions of the protein outside of the hydrophobic region that are sensitive to subtilisin cleavage and remain accessible to solvent after membrane association.

We previously published the crystal structure of an N-terminal region of IpaB and its *Salmonella* homolog SipB and found significant structural homology to the coiled-coil domains of the membrane-disrupting colicin pore-forming toxins.³¹ Further analysis indicated that residues 310–370 of IpaB and 320–380 of SipB share sequence homology to an α -helical hairpin within the pore-forming domain of colicin Ia, indicating that such a hairpin may exist within the hydrophobic regions of IpaB and its *Salmonella* homologue SipB.³¹ The α -helical hairpin in colicin Ia is responsible for anchoring the colicin pore within the targeted phospholipid membrane.³² Thus, the predicted α -helical hairpin in IpaB may serve a similar purpose. IpaB's α -helical hairpin could initiate interaction with a host membrane and promote further intimate contact between the IpaB hydrophobic region and the membrane. This possibility is supported by the high PIs observed for residues in and around this region, most notably residue 353. Recent work suggested that colicin Ia forms an oligomeric structure when forming channels in phospholipid membranes,³² which is consistent with IpaB's oligomeric form being more efficient at releasing molecules from liposomes. Comparisons between IpaB and colicin Ia are possible since both proteins contain an extended coiled-coil,³¹ an α -helical hairpin that may serve as a membrane anchor, and require oligomer formation in order to form a pore.

Based on these and prior findings, we propose an updated organizational model for IpaB interactions with itself, IpgC and with model phospholipid membranes (Fig. 4). IpaB contains two protein-protein interaction sites in the N-terminal domain (spanning residues 16–35 and 51–70) which are recognized by the chaperone IpgC.¹⁸ Upon release of the chaperone, the IpaB N terminus may be involved in intermolecular protein-protein contacts within the tetramer, which is supported by the data presented in Figure 1 where residue 58 near the N terminus was significantly protected by oligomer formation. Other groups have proposed that the IpaB C terminus may also be involved in making intermolecular contacts that are important for IpaB binding to the T3SS needle tip³³, but we do not see obvious protection of the C terminus upon IpaB oligomerization. The resulting quaternary arrangement exposes the hydrophobic domain to the aqueous environment (Fig. 1). The putative α -helical hairpin within the hydrophobic domain may then anchor IpaB in the membrane in both physical states (Fig. 2, residue 353), and residues within the tetramer regions surrounding the initial contact site may be recruited to allow intimate contact and formation of a pre-translocon pore.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank Michael Barta for helpful discussions, Joshua P. Encinas for technical assistance, and Kelly S. Harrison for critical reading and assistance with the statistical analyses. The research reported here was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award

Number R01 AI099489 to WDP. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

References

1. Mueller CA, Broz P, Cornelis GR. The type III secretion system tip complex and translocon. *Mol Microbiol.* 2008; 68(5):1085–95. [PubMed: 18430138]
2. Cornelis GR. The type III secretion injectisome, a complex nanomachine for intracellular 'toxin' delivery. *Biol Chem.* 2010; 391(7):745–51. [PubMed: 20482311]
3. Schroeder GN, Hilbi H. Molecular pathogenesis of *Shigella* spp.: controlling host cell signaling, invasion, and death by type III secretion. *Clin Microbiol Rev.* 2008; 21(1):134–56. [PubMed: 18202440]
4. Espina M, Olive AJ, Kenjale R, Moore DS, Ausar SF, Kaminski RW, Oaks EV, Middaugh CR, Picking WD, Picking WL. IpaD Localizes to the Tip of the Type III Secretion System Needle of *Shigella flexneri*. *Infect Immun.* 2006; 74(8):4391–400. [PubMed: 16861624]
5. Blocker A, Jouihri N, Larquet E, Gounon P, Ebel F, Parsot C, Sansonetti P, Allaoui A. Structure and composition of the *Shigella flexneri* "needle complex", a part of its type III secretin. *Mol Microbiol.* 2001; 39(3):652–63. [PubMed: 11169106]
6. Darboe N, Kenjale R, Picking WL, Picking WD, Middaugh CR. Physical characterization of MxiH and PrgI, the needle component of the type III secretion apparatus from *Shigella* and *Salmonella*. *Protein Sci.* 2006; 15(3):543–52. [PubMed: 16501225]
7. Stensrud KF, Adam PR, La Mar CD, Olive AJ, Lushington GH, Sudharsan R, Shelton NL, Givens RS, Picking WL, Picking WD. Deoxycholate interacts with IpaD of *Shigella flexneri* in inducing the recruitment of IpaB to the type III secretion apparatus needle tip. *J Biol Chem.* 2008; 283(27):18646–54. [PubMed: 18450744]
8. Epler CR, Dickenson NE, Bullitt E, Picking WL. Ultrastructural analysis of IpaD at the tip of the nascent MxiH type III secretion apparatus of *Shigella flexneri*. *J Mol Biol.* 2012; 420(1–2):29–39. [PubMed: 22480614]
9. Dickenson NE, Zhang L, Epler CR, Adam PR, Picking WL, Picking WD. Conformational Changes in IpaD from *Shigella flexneri* upon Binding Bile Salts Provide Insight into the Second Step of Type III Secretion. *Biochemistry.* 2010
10. Olive AJ, Kenjale R, Espina M, Moore DS, Picking WL, Picking WD. Bile salts stimulate recruitment of IpaB to the *Shigella flexneri* surface, where it colocalizes with IpaD at the tip of the type III secretion needle. *Infect Immun.* 2007; 75(5):2626–9. [PubMed: 17296762]
11. Barta ML, Guragain M, Adam P, Dickenson NE, Patil M, Geisbrecht BV, Picking WL, Picking WD. Identification of the bile salt binding site on IpaD from *Shigella flexneri* and the influence of ligand binding on IpaD structure. *Proteins.* 2012; 80(3):935–45. [PubMed: 22423359]
12. Epler CR, Dickenson NE, Olive AJ, Picking WL, Picking WD. Liposomes recruit IpaC to the *Shigella flexneri* type III secretion apparatus needle as a final step in secretion induction. *Infect Immun.* 2009; 77(7):2754–61. [PubMed: 19433542]
13. Blocker A, Gounon P, Larquet E, Niebuhr K, Cabiaux V, Parsot C, Sansonetti P. The tripartite type III secretin of *Shigella flexneri* inserts IpaB and IpaC into host membranes. *J Cell Biol.* 1999; 147(3):683–93. [PubMed: 10545510]
14. Veenendaal AKJ, Hodgkinson JL, Schwarzer L, Stabat D, Zenk SF, Blocker AJ. The type III secretion system needle tip complex mediates host cell sensing and translocon insertion. *Molecular Microbiology.* 2007; 63(6):1719–1730. [PubMed: 17367391]
15. Mounier J, Popoff MR, Enninga J, Frame MC, Sansonetti PJ, Van Nhieu GT. The IpaC Carboxyterminal Effector Domain Mediates Src-Dependent Actin Polymerization during *Shigella* Invasion of Epithelial Cells. *PLoS Pathog.* 2009; 5(1):e1000271. [PubMed: 19165331]
16. Parsot C, Hamiaux C, Page AL. The various and varying roles of specific chaperones in type III secretion systems. *Curr Opin Microbiol.* 2003; 6(1):7–14. [PubMed: 12615213]
17. Birket SE, Harrington AT, Espina M, Smith ND, Terry CM, Darboe N, Markham AP, Middaugh CR, Picking WL, Picking WD. Preparation and characterization of translocator/chaperone

- complexes and their component proteins from *Shigella flexneri*. *Biochemistry*. 2007; 46(27):8128–37. [PubMed: 17571858]
18. Adam PR, Patil MK, Dickenson NE, Choudhari S, Barta M, Geisbrecht BV, Picking WL, Picking WD. Binding Affects the Tertiary and Quaternary Structures of the *Shigella* Translocator Protein IpaB and Its Chaperone IpgC. *Biochemistry*. 2012; 51(19):4062–4071. [PubMed: 22497344]
 19. Lokareddy RK, Lunelli M, Eilers B, Wolter V, Kolbe M. Combination of two separate binding domains defines stoichiometry between type III secretion system chaperone IpgC and translocator protein IpaB. *J Biol Chem*. 2010; 285(51):39965–75. [PubMed: 20937829]
 20. Hayward RD, Cain RJ, McGhie EJ, Phillips N, Garner MJ, Koronakis V. Cholesterol binding by the bacterial type III translocon is essential for virulence effector delivery into mammalian cells. *Mol Microbiol*. 2005; 56(3):590–603. [PubMed: 15819617]
 21. Dickenson NE, Choudhari SP, Adam PR, Kramer RM, Joshi SB, Middaugh CR, Picking WL, Picking WD. Oligomeric states of the *Shigella* translocator protein IpaB provide structural insights into formation of the type III secretion translocon. *Protein Sci*. 2013; 22(5):614–27. [PubMed: 23456854]
 22. Senerovic L, Tsunoda SP, Goosmann C, Brinkmann V, Zychlinsky A, Meissner F, Kolbe M. Spontaneous formation of IpaB ion channels in host cell membranes reveals how *Shigella* induces pyroptosis in macrophages. *Cell Death Dis*. 2012; 3:e384. [PubMed: 22951981]
 23. Hume PJ, McGhie EJ, Hayward RD, Koronakis V. The purified *Shigella* IpaB and *Salmonella* SipB translocators share biochemical properties and membrane topology. *Mol Microbiol*. 2003; 49(2):425–39. [PubMed: 12828640]
 24. Picking WL, Coye L, Osiecki JC, Barnoski Serfis A, Schaper E, Picking WD. Identification of functional regions within invasion plasmid antigen C (IpaC) of *Shigella flexneri*. *Mol Microbiol*. 2001; 39(1):100–11. [PubMed: 11123692]
 25. Martinez-Becerra FJ, Kissmann JM, Diaz-McNair J, Choudhari SP, Quick AM, Mellado-Sanchez G, Clements JD, Pasetti MF, Picking WL. Broadly protective *Shigella* vaccine based on type III secretion apparatus proteins. *Infect Immun*. 2012; 80(3):1222–31. [PubMed: 22202122]
 26. Harrington AT, Hearn PD, Picking WL, Barker JR, Wessel A, Picking WD. Structural characterization of the N terminus of IpaC from *Shigella flexneri*. *Infect Immun*. 2003; 71(3):1255–64. [PubMed: 12595440]
 27. Stern O, Volmer M. On the quenching-time of fluorescence *Physik. Zeitschr*. 1919; 20:183–188.
 28. Nalefski EA, Falke JJ. Location of the Membrane-Docking Face on the Ca²⁺-Activated C2 Domain of Cytosolic Phospholipase A₂[†]. *Biochemistry*. 1998; 37(51):17642–17650. [PubMed: 9922129]
 29. Kinter, M.; Sherman, NE. *Protein Sequencing and Identification Using Tandem Mass Spectrometry*. John Wiley & Sons, Inc; 2005. *Mass Spectrometric Analysis of Tryptic Digests*; p. 166-206.
 30. Terry CM, Picking WL, Birket SE, Flentie K, Hoffman BM, Barker JR, Picking WD. The C-terminus of IpaC is required for effector activities related to *Shigella* invasion of host cells. *Microb Pathog*. 2008; 45(4):282–9. [PubMed: 18656530]
 31. Barta ML, Dickenson NE, Patil M, Keightley A, Wyckoff GJ, Picking WD, Picking WL, Geisbrecht BV. The structures of coiled-coil domains from type III secretion system translocators reveal homology to pore-forming toxins. *J Mol Biol*. 2012; 417(5):395–405. [PubMed: 22321794]
 32. Greig SL, Radjainia M, Mitra AK. Oligomeric structure of colicin ia channel in lipid bilayer membranes. *J Biol Chem*. 2009; 284(24):16126–34. [PubMed: 19357078]
 33. Roehrich AD, Martinez-Argudo I, Johnson S, Blocker AJ, Veenendaal AK. The extreme C-terminus of *Shigella* IpaB is required for regulation of T3SS secretion, needle-tip composition and binding. *Infect Immun*. 2010

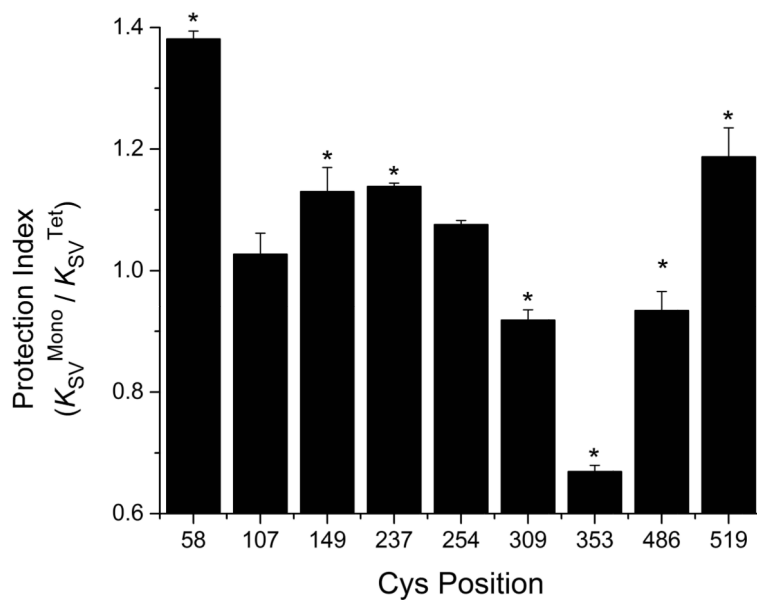


Figure 1. Oligomeric state affects quencher accessibility to the hydrophobic region of IpaB
 FM-IpaB (100 nM) prepared in LDAO (monomeric) and OPOE (oligomeric) was quenched by titration of KI in the absence of liposomes. The protection index (PI) for oligomer formation was calculated using Equation 3 as described in Materials and Methods. Mono = monomeric IpaB; Tet = tetrameric IpaB. Error bars represent standard deviation of the PI's derived from at least three independent experiments performed in triplicate. The mean PI values were found to be significantly different for those residues designated with an * relative to the PI value for the probe at position 107 ($P < 0.05$, one-way ANOVA).

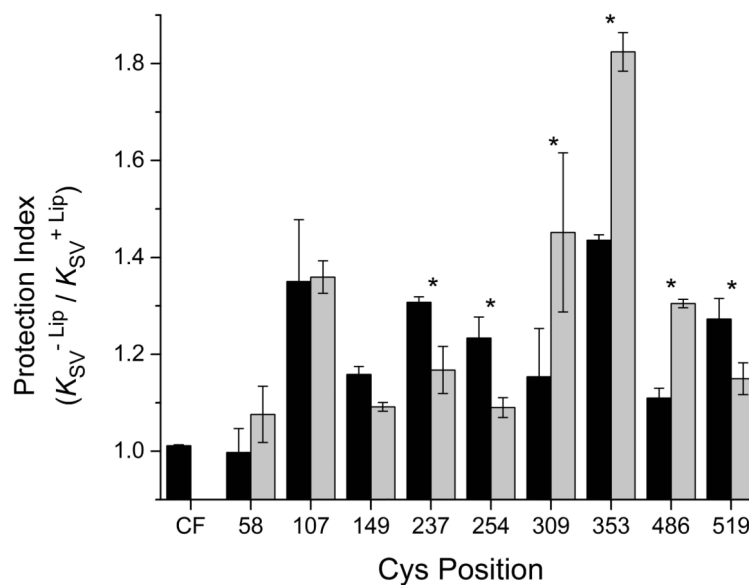


Figure 2. Protection of FM-IpaB mutants from KI quenching by liposomes

Monomeric and oligomeric FM-IpaB (100 nM) were subjected to quenching in the absence or presence of liposomes composed of DOPC, DOPG and cholesterol. The protection index (PI) for liposome association was calculated using Equation 2 for each FM-labeled IpaB point mutant in both the monomeric (black bars) and tetrameric (shaded bars) states. -Lip = no liposomes; +Lip = plus 0.150 mg/ml liposomes. Carboxyfluorescein (CF) was used as a control to demonstrate that the addition of liposomes does not arbitrarily affect the quenching of fully exposed fluorescein. Error bars represent standard deviation of the PI's derived from three independent experiments performed in triplicate. Statistically significant differences between the monomer and tetramer for each IpaB variant in response to membrane association are indicated by *, $P < 0.05$ using Tukey's multiple comparisons test.

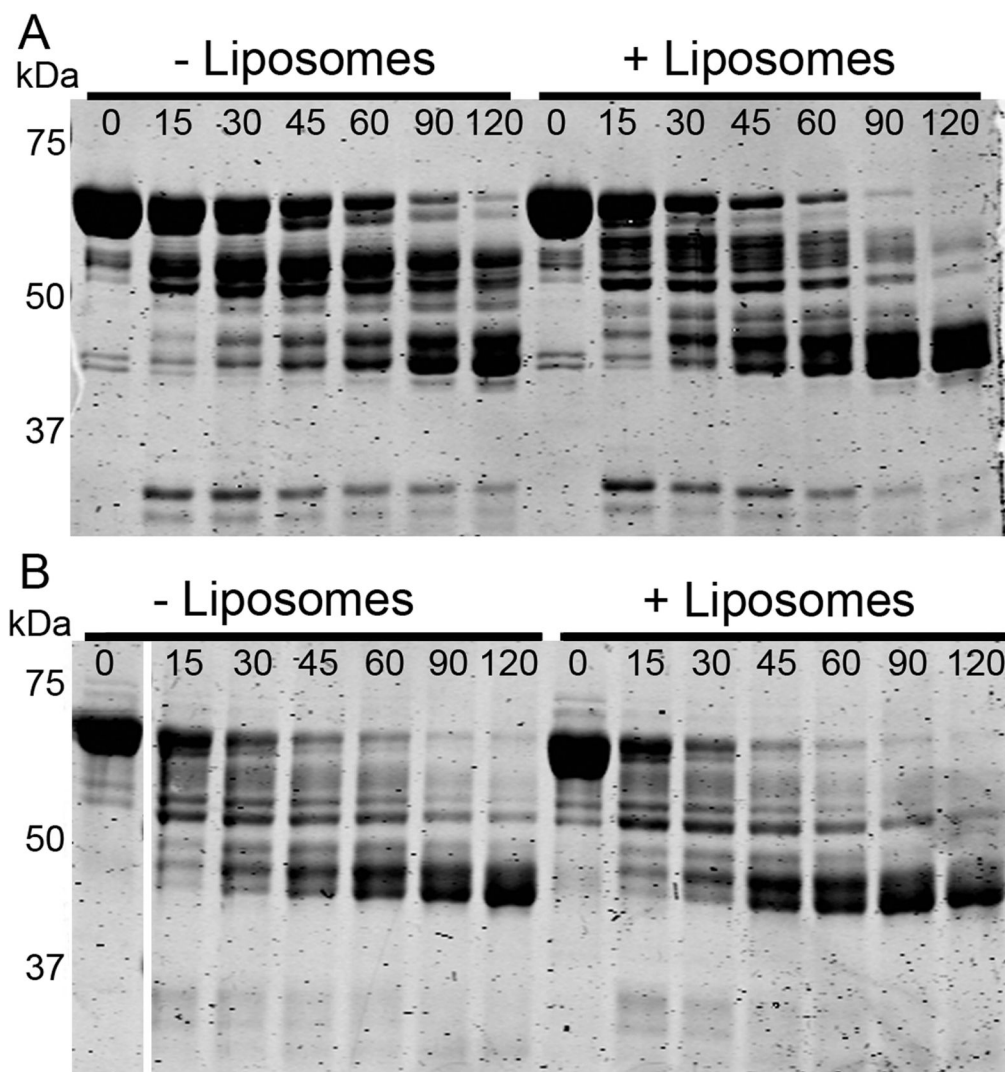


Figure 3. Proteolytic stability of IpaB is influenced by oligomeric state and liposome interaction Monomeric (A) or tetrameric (B) IpaB was subjected to proteolysis in the absence or presence of liposomes, as indicated above the gel images. Subtilisin A was used at the concentrations given in Materials and Methods. Time points (in seconds) are indicated above each lane in the gels. Gels were stained with Coomassie blue and imaged on an Odyssey infrared imager (LI-COR, Lincoln, NE) to detect low-abundance products. Gels shown are representative of at least three independent experiments.

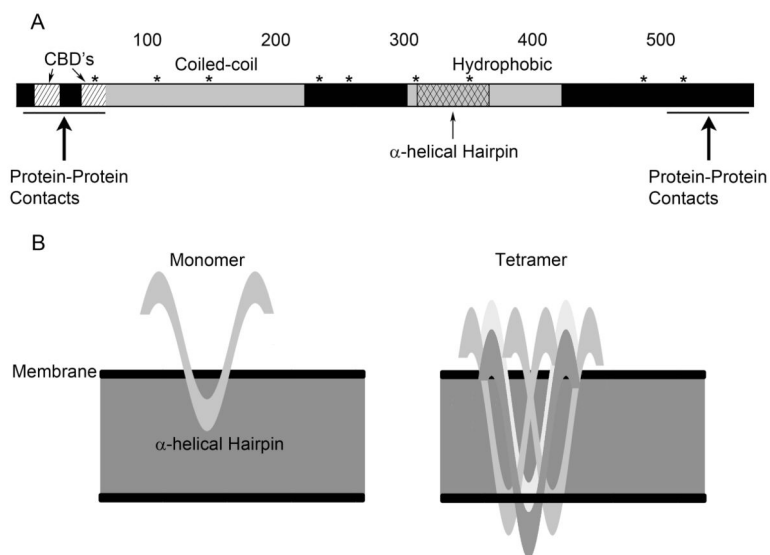


Figure 4. Functional organization of IpaB with regard to oligomerization, chaperone binding and membrane association

(A) IpaB is shown as a single bar with regions believed to be involved in specific macromolecular interactions. Residue numbers (100, 200, etc.) are indicated above the bar. Chaperone-binding domains (CBD's) based on experiments performed using a soluble IpaB fragment¹⁹ are indicated near the N-terminus. This region and sequences near the C-terminus may also be involved in intermolecular protein-protein interactions (large arrows). A coiled-coil domain for which a crystal structure has been solved is also located in the N-terminal half of IpaB. Less is known about the structure of the C-terminal half of IpaB, which contains a prominent hydrophobic region. It is in this region that IpaB may possess a hydrophobic α -helical hairpin (indicated) similar to what has been observed in colicins. (B) A closer look at the possible position of monomeric (left) and tetrameric (right) IpaB within the phospholipid membrane. Initial contact may occur via the immediate region surrounding residue 353 with the oligomeric arrangement of the protein then allowing a more intimate interaction with the hydrophobic core of the membrane.

Table IContact-Mediated Hemolysis by *Shigella* Expressing Single-Cys IpaB mutants.

<i>Shigella</i> strain	Hemolysis (% of positive control)
SF620	3.2 ± 0.2
SF620 + WT IpaB	100 ± 11.8
SF620 + S58C	114.5 ± 4.0
SF620 + S107C	94.4 ± 1.9
SF620 + S149C	85.2 ± 3.4
SF620 + S237C	112.0 ± 4.0
SF620 + S254C	93.1 ± 8.3
SF620 + A353C	94.4 ± 5.2
SF620 + S486C	97.8 ± 1.8
SF620 + S519C	83.2 ± 3.4

Contact-mediated hemolysis using sheep red blood cells was performed as previously described to determine the impact of individual Cys mutations on IpaB's ability to be delivered into target cell membranes by *Shigella*.²⁶

The values shown are an average (n = 3) from one of three representative experiments ± standard deviation. WT = wild-type.

Table II K_{SV} Values for the Quenching of IpaB by Iodide.

Mutant	K_{SV}			
	- Liposomes		+ Liposomes	
	Monomer	Tetramer	Monomer	Tetramer
S58C	6.68 ± 0.11	4.81 ± 0.02	6.62 ± 0.11	4.41 ± 0.02
S107C	5.26 ± 0.19	5.21 ± 0.01	3.98 ± 0.04	3.94 ± 0.02
S149C	7.26 ± 0.18	6.39 ± 0.09	6.22 ± 0.13	5.99 ± 0.07
S237C	5.29 ± 0.06	4.63 ± 0.02	4.01 ± 0.05	4.16 ± 0.02
A254C	7.07 ± 0.24	6.53 ± 0.16	5.66 ± 0.18	5.94 ± 0.13
WT (309C)	4.91 ± 0.05	5.24 ± 0.11	3.96 ± 0.06	3.41 ± 0.04
A353C	4.79 ± 0.02	7.08 ± 0.08	3.29 ± 0.02	3.97 ± 0.02
S486C	5.23 ± 0.07	5.57 ± 0.03	4.62 ± 0.06	4.24 ± 0.02
S519C	5.84 ± 0.04	4.82 ± 0.06	4.71 ± 0.04	4.28 ± 0.03
Carboxyfluorescein (CF)	10.83 ± 0.42		10.69 ± 0.38	

100 nM FM-labeled IpaB was subjected to fluorescence quenching by KI as described in Experimental procedures. Shown here are representative K_{SV} values with standard errors of linear regression given (n = 3). WT = wild-type.