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The N-terminus of IpaB provides a potential anchor to the *Shigella* type III secretion system tip complex protein IpaD

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Abstract

The type III secretion system (T3SS) is an essential virulence factor for *Shigella flexneri*, providing a conduit through which host-altering effectors are injected directly into a host cell to promote uptake. The type III secretion apparatus (T3SA) is comprised of a basal body, external needle, and regulatory tip complex. The nascent needle is a polymer of MxiH capped by a pentamer of invasion plasmid antigen D (IpaD). Exposure to bile salts (e.g. deoxycholate) causes a conformational change in IpaD and promotes recruitment of IpaB to the needle tip. It has been proposed that IpaB senses contact with host cell membranes, recruiting IpaC and inducing full secretion of T3SS effectors. While the steps of T3SA maturation and their external triggers have been identified, details of specific protein interactions and mechanisms have remained difficult to study due to the hydrophobic nature of the IpaB and IpaC translocator proteins. Here we explored the ability for a series of soluble N-terminal IpaB peptides to interact with IpaD. We found that DOC is required for the interaction and that a region of IpaB between residues 11–27 is required for maximum binding, which was confirmed *in vivo*. Furthermore, intramolecular FRET measurements indicated that movement of the IpaD distal domain away from the protein core accompanied the binding of IpaB$^{11\text{-}226}$. Together these new findings provide important new insight into the interactions and potential mechanisms that define the maturation of the *Shigella* T3SA needle tip complex and provide a foundation for further studies probing T3SS activation.

Keywords

Shigella; type III secretion; deoxycholate; invasion plasmid antigen

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Supporting Information Available Supplemental figures include fluorescence polarization binding curves comparing the effect of DOC and DHC on IpaB$^{11\text{-}226}$ interaction with T3SS tip proteins, circular dichroism characterization of IpaB$^{11\text{-}226}$ and engineered cysteine point mutants, PSIPRED secondary structure predictions for N-terminal IpaB deletion mutants, FTIR analysis of fluorescein bound and unlabeled IpaB$^{11\text{-}226}$ S128C, and circular dichroism characterization of IpaB N-terminal deletion mutants. Supplemental tables include the effect of DOC and DHC on *S. flexneri* invasion phenotype, invasion phenotype of IpaB engineered cysteine point mutants, DichroWeb secondary structure prediction results for IpaB N-terminal deletion mutants, and quantification of uninduced secretion by *S. flexneri* expressing IpaB N-terminal deletion mutants. This material is available free of charge via the Internet at https:pubs.acs.org.

Shigella species cause a severe form of bacillary dysentery (shigellosis) that is readily spread via contaminated water. Shigellosis is of worldwide public health importance with an estimated 90 million cases and 100,000 deaths per year with especially high incidence in children in low income countries.[1,2] Those children that survive suffer impaired growth due to malnutrition which is exacerbated by repeated episodes.[3] Shigellosis outbreaks also occur on a regular basis in industrialized nations and the emergence of antibiotic resistant strains underscores the necessity for better understanding the virulence mechanism of *Shigella*.[4]

Shigella employs a type III secretion system (T3SS) as its primary virulence factor.[5] The type III secretion apparatus (T3SA) resembles a nano-syringe and needle and provides a physical link between the *Shigella* and the gastrointestinal epithelial cell.[5] It is comprised of three main components: the basal body which spans the inner and outer bacterial membranes serving as an anchor for the apparatus and providing a conduit through the bacterial envelope; an external needle, with an outer diameter of 7 nm with a 2.5 nm inner channel, consisting of multiple copies of a MxiH packed in a helical manner;[6] and an exposed tip complex consisting of a pentamer of invasion plasmid antigen D (IpaD).[7,8] IpaD serves as a sensor of environmental small molecules such as bile salts to control the context of the tip complex and secretion of effector proteins. Exposure to the bile salt dexoycholate (DOC) promotes recruitment of the first hydrophobic translocator, IpaB, to the maturing tip complex where an organized oligomer forms the translocon pre-pore.[9,10] From this exposed position, it has been proposed that IpaB can sense host membrane components, triggering recruitment of the second hydrophobic translocator, IpaC, to the *Shigella* exterior.[11] This completes formation of the translocon pore in the host cell membrane and induces secretion of T3SS host-altering effectors into the cell to promote pathogen entry.[11]

Although *Shigella* has provided a valuable system for identifying the steps encompassing T3SA tip complex maturation, the precise mechanisms and protein interactions that govern this process remain unknown. Recent studies have started to unravel some of these mechanisms by identifying an IpaD conformational change that occurs upon DOC binding. It is proposed that this conformational change allows IpaB access to the maturing needle tip where it associates with IpaD.[12,13] To date, verifying a direct association between IpaD and IpaB has been challenging due, in part, to the requirement of detergent to maintain soluble IpaB in aqueous conditions.

Previously, we generated stable IpaB N-terminal fragments that could be expressed in the absence of the cognate IpaB chaperone IpgC and are highly soluble in aqueous conditions.[14,15] In this study, we used these and additional IpaB fragments to explore conditions that may allow their stable interaction with purified IpaD. Fluorescence polarization (FP) and Förster resonance energy transfer (FRET) were employed to demonstrate that specific N-terminal fragments of IpaB are capable of binding with strong affinity to IpaD in a DOC dependent manner. Furthermore, we found that a short peptide sequence located near the N-terminus of IpaB is necessary for optimal binding. This region has recently been identified as one of two likely binding domains important for IpaB association with its chaperone IpgC,[15,16] suggesting there is a direct competition for binding at this site by IpaD and IpgC within the bacterial cytoplasm. Intramolecular FRET measurements revealed a relationship between DOC binding, flexibility within the IpaD distal domain, and the interaction between IpaD and IpaB. Overall, these data provide the first direct evidence of an IpaD-IpaB interaction *in vitro* and valuable mechanistic insight into this interaction. Generation of several N-terminal mutations in wild-type IpaB provided a correlation between the *in vitro* binding studies and virulence *Shigella* phenotypes *in vivo*. The resulting findings suggest that regions near the N-terminus of IpaB are necessary for its interaction with IpaD and the sequential maturation of the T3SA needle tip. As the first step in T3SA tip complex maturation and a prerequisite for the ability of *Shigella* to invade host

cells, determining the biochemical basis for the interaction between IpaD and IpaB provides a valuable contribution toward understanding the molecular basis for the onset of type three secretion induction in *Shigella* with ramifications that extend to related T3SSs. This could, in turn, help in the development of anti-infective measures against not only *Shigella*, but also other important pathogens that rely upon type III secretion as an essential component of their virulence arsenals.

Experimental Procedures

Materials

The *Shigella flexneri ipaB* null strain SF620, was provided by P.J. Sansonetti (Pasteur Institute, FR).[17] *E. coli* strains, protein expression plasmids, and Clonables 2X Ligation Premix were from Novagen (Madison, WI). Restriction enzymes were from New England Biolabs (Ipswich, MA). Alexa Fluor probes, fluorescein maleimide (FM), 7-Diethylamino-3-(4′-Maleimidylphenyl)-4-Methylcoumarin (CPM), and FlAsH-EDT_2 were from Invitrogen (Carlsbad, CA). Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). Iminodiacetic acid-Sepharose was from Sigma Chemical Company (St. Louis, MO). All other solutions and chemicals were reagent grade.

Plasmid constructs and expression of *ipaB* and *ipaD*

Wild-type *ipaD* and *ipaD* made to encode a tetracysteine binding pocket (the amino acid sequence CCPGCC) within the distal domain were produced as described previously.[13] The N-terminal IpaB peptides were generated in the plasmid pT7HMT or a modified version of the plasmid that introduced an N-terminal cysteine and expressed as previously described.[14,18] Mutations encoding the IpaB internal cysteine point mutants used for FRET were introduced into both full length *ipaB* and $ipaB^{11\text{-}226}$ using 5′ phosphorylated primers and inverse PCR. Residues 107, 120, 128, and 149 were chosen because they provide a mild mutation from serine to cysteine and all reside within the backbone of the solved $IpaB^{74\text{-}224}$ structure. For phenotypic characterization, the *ipaB* cysteine point mutants and deletion mutants were generated in the plasmid pWPsf4 and electroporated into the *Shigella flexneri ipaB* null strain, SF620. For protein expression, the cysteine mutants generated in $IpaB^{11\text{-}226}$ were cloned into pT7HMT and the IpaB N-terminal deletion mutants were subcloned into pET15b. The N-terminal IpaB fragments containing an N-terminal cysteine were generated using a modified version of the pT7HMT expression plasmid.[18] The resulting plasmids were introduced into *E. coli* Tuner(DE3). All of the *E. coli* Tuner(DE3) strains were grown at 37 °C in Terrific Broth to an OD_{600} of 1.0, protein expression induced with 1mM IPTG for 18 hours at 17 °C and the recombinant protein purified using standard nickel chelation chromatography as previously described.[14]

Characterization of novel IpaB peptides and mutants

The secondary structures and thermal stabilities of $IpaB^{1\text{-}226}$ and $IpaB^{28\text{-}226}$ were previously published.[15] The intermediate peptide, $IpaB^{11\text{-}226}$, the IpaB N-terminal deletion mutants, and the four cysteine point mutants generated for this study were also analyzed by far-UV CD using a Jasco Model J-815 spectropolarimeter equipped with a Peltier temperature controller (Jasco Inc., Easton, MD). Secondary structure content was assessed by collecting CD spectra from 190 nm to 260 nm at 10 °C using a 0.1 cm quartz cuvette. A scanning rate of 50 nm/min was used with a spectral resolution of 0.2 nm and a 2-sec data integration time. All spectra are an average of three measurements. The proteins' secondary structure thermal stability was determined by monitoring the CD signal at 222 nm over a temperature range from 10 °C to 90 °C. The CD signal was acquired every 2.5 °C as the temperature was increased at a rate of 15 °C/h and maintained at each interval for one minute to allow the solution temperature to equilibrate. The protein concentration was 0.3–0.5 mg/mL in 10 mM

phosphate (pH 7.4) with 150 mM NaCl (PBS) for all proteins and measurements were normalized by converting the output signal to mean residue molar ellipticity.

Phenotypic characterization of IpaB mutants

Three independent assays were used to characterize the effect of IpaB mutations.[8] First, 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% invasion). Second, the ability of *Shigella* to form a translocon pore through proper recruitment of the translocator proteins IpaB and IpaC can be determined by contact-mediated hemolysis. SF620 expressing the IpaB mutants were tested for their contact hemolytic activity and compared to SF620 expressing wild-type *ipaB* (100% contact mediated hemolysis). Third, uninduced secretion profiles measure the T3SA control of secretion during regular growth. The uninduced secretion of IpaD, IpaB, and IpaC into the culture media during overnight growth was assessed by Western blotting. Blots were probed with a primary antibody mixture containing mouse anti-IpaD, rabbit anti-IpaB, and rabbit anti-IpaC polyclonal antibodies and the corresponding Alexa Fluor 680 goat anti-rabbit IgG and Alexa Fluor 800 goat anti-mouse IgG (Molecular Probes, Eugene, OR). Images were obtained using an Odyssey Infrared Imager (LI-COR, Lincoln, NE).

Labeling of recombinant protein with fluorescent probes

The N-terminal cysteine introduced into IpaB peptides or the corresponding internal cysteine in the $IpaB^{11\text{-}226}$ point mutants were fluorescently labeled with FM. The purified proteins were dialyzed into 50 mM HEPES (pH 7.2), 140 mM NaCl, and 2.5 mM TCEP and degassed under N_2. FM prepared in N′,N′-dimethylformamide (DMF) was added to approximately a 10 molar excess to an aliquot of the protein and allowed to react for 2 h at room temperature under N_2. The unbound dye was removed by nickel chelation chromatography, the labeled protein eluted and dialyzed into PBS. The native cysteine of IpaD (residue 322) was labeled with the thiol-reactive 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) as described previously.[19] Protein secondary structure and thermal stability were verified by far-UV CD spectroscopy. Because of interference with CD signals after fluorescence labeling, the retention of structural features for labeled IpaB fragments was determined by Fourier-transform infrared (FTIR) spectroscopy. The infrared spectrum of each sample was collected at room temperature using a Vertex 70 FTIR spectrometer (Bruker Corp., Billerica, MA) using a BioATR cell (Harrick Scientific, Pleasantville, NY). The data were collected from 256 composite scans over a range of 4000 cm^{-1} to 900 cm^{-1} with a resolution of 4 cm^{-1}.

For the intramolecular FRET analysis, the CCPGCC tetracysteine motif introduced into the distal domain of purified IpaD was labeled with FlAsH-EDT_2 (a biarsenical fluorescein reagent that coordinates with the above tetracysteine motif) as described previously.[13] Both the donor only FlAsH-labeled IpaD and the donor and acceptor doubly-labeled IpaD were dialyzed into PBS and used within one week or stored at −80 °C.

Fluorescence polarization

The FM-labeled N-terminal cysteine mutants of $IpaB^{1\text{-}226}$, $IpaB^{11\text{-}226}$, and $IpaB^{28\text{-}226}$ were diluted to 80 nM in PBS and IpaD was titrated in at concentrations from 0 to 10 μM. The mixtures were incubated 30 min at room temperature and the fluorescence polarization of the IpaB-bound fluorescein was measured using a Molecular Devices SpectraMax M5 fluorescence plate reader equipped with excitation and emission polarizers. The polarization was measured in 96 well opaque plates using an excitation wavelength of 490 nm and emission wavelength of 527 nm. The measurements were repeated following the addition of either 1 mM DOC or the synthetic bile salt dehydrocholate (DHC). These measurements

were also used to test the binding of the IpaB$^{11-226}$ peptide to the IpaD homologs SipD (from *Salmonella enterica*)[20] and LcrV (from *Yersinia pestis*).[21] SigmaPlot 11.2 was used to plot the change in polarization as a function of IpaD (or homolog) concentration and the data were fit to a single-site saturation ligand binding equation to estimate the dissociation constant (K_d) for each interaction.

Förster resonance energy transfer (FRET) measurements

Fluorescence emission spectra of the FRET systems were collected at 20 °C with a FluoroMax-4 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ) (see Ref[22] for a detailed description of FRET). For the detection of intermolecular FRET between IpaD and the IpaB$^{11-226}$ cysteine point mutants, the excitation wavelength was 385 nm (specific for the coumarin donor) and emission was collected from 400–600 nm with an integration time of 0.05 sec. Donor-labeled IpaD (125 nM) was incubated with 1 μM of each IpaB$^{11-226}$ cysteine mutant either labeled with a fluorescein FRET acceptor (DA) or not (D only). Fluorescence emission spectra were collected for both conditions prior to and following 30-min incubation with 1 mM DOC at 20 °C to observe the effects of DOC on the IpaD/IpaB interaction. Intramolecular FRET measurements between the acceptor-labeled native cysteine in IpaD and the donor-labeled tetracysteine binding pocket engineered into the distal domain were acquired similarly using an excitation wavelength of 485 nm (specific for the FlAsH donor) and emission collected from 500–650 nm with an integration time of 0.05 seconds. Spectra were collected for 125 nM solutions of both IpaD labeled only with the FlAsH donor (D only) and IpaD dually-labeled with the FlAsH donor and the Alexa 568 acceptor (DA). The IpaD was split and incubated 30 min with either a PBS control or 1 mM DOC in PBS at 20 °C prior to titrating in IpaB$^{11-226}$ to 5 μM. The resulting data points were fit to a Hill1 binding model used to estimate the K_d of the interaction.

Calculating energy transfer efficiencies between FRET pairs

FRET efficiencies were measured spectrophotometrically, as described above, and calculated by quantifying the reduction in donor fluorescence intensity when in the presence of an acceptor.[13,15] For this method, it is important that the donor concentration and labeling efficiency remain identical in the conditions of donor only and donor in the presence of acceptor to ensure that the change in observed donor fluorescence intensity is solely due to FRET. This was achieved for the intermolecular FRET measurements because either acceptor-labeled or non-labeled ligand is added to the donor-labeled protein, ensuring that the donor conditions remain unchanged. For intramolecular FRET measurements, this is accounted for because the donor only sample was split just prior to labeling of the protein with the FRET acceptor. Thus, for both techniques the labeling efficiencies of the donor only and donor-acceptor conditions are identical and the energy transfer efficiencies can be calculated by:[23]

$$E = 1 - (F_{DA}/F_D) \quad \text{Eq.1}$$

where E is the energy transfer efficiency, F_D is the peak donor fluorescence intensity in the absence of the FRET acceptor, and F_{DA} is the peak donor fluorescence intensity in the presence of the FRET acceptor. Because the energy transfer efficiency is sensitive to the distance separating the FRET pair, comparing these efficiencies provides insight into changes in the spatial relationship between the donor and acceptor.

In vivo Shigella surface localization of IpaB

The ability of IpaB mutants to stably localize to the tip of the T3SA was determined by flow cytometry. *Shigella* were grown to an OD_{600} = 0.4 prior to the addition of 1 mg/mL DOC to the cultures. The bacteria were allowed to grow an additional 30 min, collected by

centrifugation, washed with PBS, and fixed by resuspension in freshly prepared 4% paraformaldehyde in PBS. The fixed cells were rinsed and diluted to ~5×10^8 bacteria/mL, blocked with a 1:1 mixture of Odyssey blocking buffer:PBS prior to addition of appropriate primary antibodies. The samples were incubated at room temperature for 2 h, rinsed, and incubated with Alexa Fluor 488 conjugated goat anti-rabbit secondary IgG for 1 h, rinsed again and diluted in PBS. The samples were then analyzed using a FACSCalibur flow cytometer set to collect 500,000 events for each mutant (BD Biosciences, San Jose, CA). The results were plotted and analyzed using FlowJo V10.0.6 (Tree Star, Ashland, OR).

Results

Deoxycholate promotes an interaction between IpaD and N-terminal IpaB peptides *in vitro*

Previous protease digestion studies identified several stable core fragments of the highly hydrophobic *Shigella* translocator IpaB.[14] Most of the stable regions clustered near the IpaB N-terminus with $IpaB^{28-226}$ being used to solve a 2.1 Å structure for $IpaB^{74-224}$.[14] Insights from this structure and the generation of soluble IpaB peptides capable of being expressed in the absence of IpgC have played an important role in dissecting interactions between IpaB and IpgC.[15] In this study, the cysteine residues introduced at the N-terminal extremity of the stable IpaB fragments $IpaB^{1-226}$, $IpaB^{11-226}$, and $IpaB^{28-226}$ were covalently labeled with a fluorescent probe to allow the use of fluorescence polarization (FP) to identify conditions that would permit their interaction with IpaD *in vitro*. Incubating each IpaB fragment with up to a 125-fold excess of IpaD (10 μM) in an aqueous buffer system resulted in essentially no change in polarization, suggesting that no interaction was occurring (Fig. 1A, open symbols). Following a 30-min incubation in the presence of 1 mM DOC, however, each labeled IpaB species demonstrated an IpaD-dependent increase in FP (Fig. 1A, filled symbols). This change in FP was seen as an initial sharp rise in polarization, indicating that an interaction was occurring. Interestingly, while the apparent K_d values for the interaction between IpaD and $IpaB^{1-226}$ and $IpaB^{11-226}$ in the presence of DOC were below 1 μM, the binding between $IpaB^{28-226}$ and IpaD was significantly weaker with an apparent K_d of 3.60 μM (Table 1), suggesting that a region within IpaB residues 11–27, together with DOC, play an important role in the interaction between IpaD and IpaB.

The specificity of DOC in promoting the interaction was tested by substituting DHC for DOC. Though structurally similar to DOC, DHC has been shown to neither interact with IpaD nor promote a conformational change within the protein.[13,19] Here, the addition of DHC resulted in no detectable binding of any of the IpaB fragments to IpaD (Supplementary Fig. S1A), supporting a specific DOC-dependent interaction rather than a non-specific detergent or small molecule effect. These results are consistent with the inability of DHC exposure to increase *S. flexneri* invasiveness of cultured cells. While exposure to 1 mM DOC substantially increased invasiveness, the addition of DHC had little effect, actually resulting in a slight decrease in invasion (Supplementary Table S1).

It is worth noting that although all three IpaB peptides could be expressed, purified, and fluorescently labeled at high levels, we found that $IpaB^{11-226}$ was stable longer (by SDS-PAGE) than the $IpaB^{1-226}$ peptide (data not shown). Although comparison of CD spectra of the IpaB peptides do not suggest significant differences in secondary structure (Supplementary Fig. S2 and reference[15]), $IpaB^{11-226}$ was selected over $IpaB^{1-226}$ for use in the remainder of the *in vitro* studies for this observed stability.

Deoxycholate promotes binding of IpaB$^{11-226}$ to the IpaD homolog *Salmonella* SipD but not *Yersinia* LcrV

To test the specificity for the observed IpaD-IpaB interaction, FP was used to determine the ability of IpaB$^{11-226}$ to bind the homologous T3SS tip proteins SipD from *Salmonella* and LcrV from *Yersinia*. As with IpaD, no change in the polarization of labeled IpaB$^{11-226}$ was observed following a 30-min incubation with either of these recombinant proteins (Fig. 1B). The addition of 1 mM DOC failed to promote an interaction between IpaB$^{11-226}$ and LcrV under any observed conditions and is likely attributed to its divergence from IpaD in both primary[24] and tertiary structure.[24,25] Titration of SipD in the presence of 1 mM DOC, however, resulted in binding (Fig. 1B) with an estimated dissociation constant of 1.71 μM, approximately four-fold weaker than the K_d for the interaction between IpaD and IpaB$^{11-226}$. Association with SipD might be expected due to similarities in their primary sequences[24], however, the IpaB and SipB N-terminal regions differ rather substantially with regard to sequence despite having notable structural similarity.[14] Although weaker than the IpaD/IpaB$^{11-226}$ interaction, the SipD/IpaB$^{11-226}$ interaction exhibits a similar specificity for DOC since DHC fails to promote binding between SipD and IpaB$^{11-226}$ (Supplementary Fig. S1B). While DOC binding to SipD with a concomitant conformational change has been reported by others,[26,27] there has not been a direct physiological link between DOC and changes in the *Salmonella* T3SA needle tip composition. In fact, growth in DOC decreases the invasiveness of *Salmonella* due, in part, to a down-regulation of gene expression.[28] Interestingly, however, short term (1 min) exposure of a mid-log culture to this bile salt also results in a decrease in invasion phenotype, which cannot be entirely contributed to down-regulation of gene expression (data not shown).

Intermolecular FRET provides additional insight into the association of IpaD with the IpaB N terminus

Förster resonance energy transfer (FRET) was measured between the CPM-labeled native cysteine (residue 322) of IpaD and the fluorescein-labeled cysteine residue introduced individually into each of four positions within IpaB$^{11-226}$ (S107C, S120C, S128C, and S149C) (Fig. 2A and 2B, respectively). The introduction of each of these cysteine mutations had little impact on secondary structure/stability of the IpaB$^{11-226}$ (Supplementary Fig. S2) and each mutation gave rise to a readily detectable invasive phenotype when introduced into full length IpaB and expressed in *S. flexneri* SF620 (Supplementary Table S2). FTIR analysis was used to compare the relative structure structural composition of unlabeled and fluorescein-labeled IpaB$^{11-226}$ S128C (Supplementary Fig. S3).[24,29] No difference in overall protein structure was detected, suggesting that the addition of the fluorescein probe does not have an adverse effect on the protein structure. FRET efficiency between the donor-labeled IpaD and each of the acceptor-labeled IpaB$^{11-226}$ mutants was determined by quantifying the decrease in donor fluorescence in the presence of a FRET acceptor (see Fig. 2C). The measured energy transfer efficiencies between the donor-labeled IpaD and all of the acceptor-labeled IpaB$^{11-226}$ cysteine mutants were minimal in the absence of DOC, which is consistent with the absence of an interaction (Table 2). After adding DOC, however, the measured FRET efficiencies increased, indicating the induction of an interaction between IpaD and the IpaB mutants (Table 2). When the *Yersinia* tip protein, LcrV, was substituted for IpaD in these experiments as a negative control, FRET efficiencies of ≤ 6.5% were calculated for all conditions and the addition of 1 mM DOC resulted in a change in FRET efficiencies of no more than 3.3% (data not shown), validating the technique and confirming the lack of interaction between LcrV and IpaB$^{11-226}$ seen in the fluorescence polarization measurements. The FRET efficiencies between the donor-labeled Cys322 of IpaD and acceptor-labeled Cys107 and Cys120 in IpaB$^{11-226}$ were both approximately 33% and the values decreased to 21.4% and 14.6% as the acceptor moved to Cys128 and Cys149, respectively, (Fig. 2A and B, Table 2) suggesting that the donor fluorophore on IpaD was

nearest to and approximately equidistant from the acceptor at positions 107 and 120 on IpaB$^{11-226}$. The acceptor probes at positions 128 and 149 then appeared to be located progressively further from the donor probe on IpaD, providing initial insight into the interaction between the proteins.

Intramolecular FRET implicates DOC in regulating IpaB interaction with IpaD via a conformation change within the IpaD distal domain

Recent EM reconstructions have demonstrated that the IpaD distal domain assumes an upward position within the context of the pentamer formed at the *Shigella* T3SA needle tip.[7] Therefore, we designed an intramolecular FRET system that is sensitive to movement of the IpaD distal domain relative to the native cysteine at the bottom of the central coiled coil. This would allow us to determine whether association with IpaB$^{11-226}$ is consistent with an upward movement of the IpaD distal domain (Fig. 3A). The FRET efficiency between the donor fluorophore at Cys322 (CPM) and the acceptor in the distal domain (FlAsH probe at position TC231) was determined based on the extent of donor quenching as for the intermolecular FRET described above. The measured energy transfer efficiencies followed a dose-dependent decrease with the titration of IpaB$^{11-226}$, indicating that the average position of the IpaD distal domain is directly affected by IpaB$^{11-226}$ binding (Fig. 3B). In the absence of DOC, however, titration of IpaB$^{11-226}$ has little effect on the measured intramolecular FRET efficiencies, supporting the idea that DOC promotes a stable IpaD/IpaB$^{11-226}$ interaction. Addition of 5 μM LcrV in place of IpaB$^{11-226}$ failed to produce a change in intramolecular FRET efficiency in either the presence or absence of DOC, indicating a level of specificity for IpaB (data not shown). Moreover, fitting the FRET efficiencies as a function of IpaB$^{11-226}$ concentration (1 mM DOC) to a Hill1 binding model with an adjusted R^2 value of 0.990, allowed us to calculate an apparent K_d value of 365 nM. The striking similarity of the K_d determined here and the K_d determined by FP (378 nM) suggests that the location of the IpaD distal domain is not only linked to the binding of IpaB, but that the correlation can be used as a direct measure of interaction. It is worth noting that while this method for measuring FRET efficiencies provides a reliable tool sensitive to Angstrom-level structural changes in the protein conformation, absolute FRET efficiencies and distances are difficult to quantify. This is evident as the FRET efficiencies reported here are approximately 20% lower than those reported previously,[13] however, the addition of DOC once again resulted in a measured decrease in FRET efficiency which was exaggerated upon the binding of IpaB$^{11-226}$.

Role of the N-terminus of IpaB in formation of the IpaD/IpaB complex

While the fluorescence data, as a whole, provide evidence for an interaction between IpaD and the N-terminal fragments of IpaB, the FP data identify a key region in IpaB that may be responsible for a maximal IpaD/IpaB interaction (IpaB$^{11-27}$). We therefore used the secondary structure prediction program PSIPRED[30] to guide in the design of deletion mutants within this region of full length IpaB for *in vivo* analysis (Supplementary Fig. S4). Three mutants, IpaB$^{\Delta 12-19}$, IpaB$^{\Delta 12-25}$, and IpaB$^{\Delta 12-27}$, were designed to probe the roles of specific elements of this region by taking into account the predicted secondary structure components (Supplementary Fig. S4) and maintaining the proposed secretion signal at the extreme N-terminus.[31] IpaB$^{\Delta 12-27}$ mimics the *in vitro* studies which identified these residues as instrumental in IpaD binding. The IpaB$^{\Delta 12-19}$ and IpaB$^{\Delta 12-25}$ mutants removed the first predicted α-helix and the region containing this helix plus the adjacent coil, respectively. The modified sequences were again analyzed by PSIPRED and while the IpaB$^{\Delta 12-19}$ and IpaB$^{\Delta 12-25}$ mutants resulted in predicted secondary structures consistent with simply removing the defined regions, the IpaB$^{\Delta 12-27}$ mutant was predicted to extend the second helix an additional five residues toward the N-terminus. The recombinant proteins containing the indicated deletions were purified, analyzed by CD, and secondary structure

contents estimated using the Dichroweb software package[32,33] including the CDSSTR algorithm.[34] Each of the mutants were found to maintain secondary structure composition and thermal stability characteristics similar to wild-type IpaB (Supplementary Fig. S5 and Supplementary Table S3.

The *ipaB* null *Shigella* strain SF620 strain was complemented with the above deletion mutants and the resulting virulence phenotypes characterized. The uninduced overnight secretion profiles of the T3SA Ipa tip proteins showed that all of the mutants were expressed and secreted at levels similar to SF620 complemented with full length *ipaB*, however IpaD and IpaC were hypersecreted with levels similar to those of the *ipaB* null strain (Fig. 4A and Supplementary Table S4), suggesting impaired secretion control by the IpaB deletion mutants. Analysis of the profiles of the whole cell extracts shows a typical pattern expected when a protein's secretion is uncontrolled, resulting in decreased levels remaining in the cytoplasm (Fig. 4B). The ability of the mutant complement strains to lyse erythrocytes through translocon insertion and to invade cultured epithelial cells were tested by hemolysis and gentamicin protection assays, respectively. Each of the mutants exhibited dramatically reduced phenotypes when compared to the full length *ipaB* complemented strain (Table 3), suggesting that while the mutants were expressed and secreted Fig. 4), they do not form a functioning T3SA tip complex, likely due to the impaired ability for IpaB to interact properly with IpaD.

The ability of IpaB and the IpaB deletion mutants to localize to the bacterial surface as a part of the T3SA was tested by using flow cytometry to detect levels of selectively immunolabeled IpaB. All of the strains were grown in the presence of 1 mM DOC to ensure appropriate signaling for IpaB translocation. Five hundred thousand events were recorded for each mutant and displayed as a histogram of fluorescence intensity (Fig. 5). The wild-type complemented strain exhibited the highest overall fluorescence intensity while the intensity of the IpaB deletion mutants was much lower and are nearly equivalent to those of the IpaB null strain. Thus, these data further suggest that the deleted regions are involved in maintaining a strong IpaD/IpaB interaction following DOC stimulation.

Discussion

Incubation of *Shigella* with the bile salt deoxycholate promotes recruitment of IpaB to the maturing T3SA needle tip which correlates with an enhanced ability of the bacteria to invade cultured epithelial cells.[9] This and other work clearly define IpaD as an important regulator of type III secretion from its position at the *Shigella* T3SA needle tip.[35,36] Nevertheless, until now there has been no demonstration of a direct interaction between the *Shigella* tip protein IpaD and the first hydrophobic translocator IpaB. In this study we used fragments from the N-terminus of IpaB to demonstrate this interaction, its dependence on DOC, and the involvement of a structural change in the IpaD distal domain using complementary fluorescence spectroscopy methods. Initially, FP measurements confirmed that DOC is required for the tested IpaB fragments to bind with IpaD, supporting the proposal that a DOC-induced conformational change in IpaD has a role in promoting stable maintenance of IpaB at the T3SA needle tip.[9,12,13] Intermolecular FRET measurements between a donor probe on IpaD and an acceptor at each of four individual sites on $IpaB^{11-226}$ then suggested that the N-terminal region of the coiled-coil in the solved structure of IpaB resides near the "bottom" of the IpaD coiled-coil (see Fig. 2). The intramolecular FRET results identified $IpaB^{11-226}$-dependent changes in FRET efficiency consistent with the IpaD distal domain moving further away from the C-terminus of IpaD (flipping upward) upon binding $IpaB^{11-226}$. This movement appears to be needed to accommodate proper interaction with IpaB, possibly by a providing $IpaB^{11-226}$ access to previously inaccessible contact points (see Fig. 3A). While the precise role of DOC in this

interaction and resulting movement of the distal domain remain unclear, previous work[13] together with these findings suggests that the IpaD distal domain location relative to the C-terminal cysteine is sensitive to DOC exposure and this exacerbated by the subsequent interaction with IpaB.

We also found through the initial FP studies that IpaB residues 11-27 play an important role in optimal binding to IpaD. We were able to confirm importance of this region *in vivo* and further narrow down the region responsible by creating deletion mutants and characterizing the resulting phenotype in *S. flexneri*. Hemolysis and invasion assays showed that loss of this region is detrimental to *Shigella* virulence activities although the protein was expressed at wild-type levels, was secreted through the T3SA (Fig. 4), and co-purified with IpgC when co-expressed in *E. coli* (data not shown). Consistent with these results, fluorescence immunolabeling of *Shigella* suggested that the IpaB mutants localized to the *Shigella* surface (T3SA) much less efficiently than the wild-type protein (Fig. 5), likely a result of the described deficiency in IpaD/IpaB interaction. Furthermore, it is worth noting that while IpaB residues 11–27 appear essential for optimum IpaD interaction and *Shigella* virulence mechanisms, we recently reported that a site within the N-terminal 27 residues of IpaB is also necessary for optimal IpaB binding of its chaperone IpgC.[15] Together, these studies suggest that IpaD and IpgC share an overlapping binding region near the N-terminus of IpaB, which acts to prevent a premature IpaD/IpaB interaction in the bacterial cytoplasm. Unfortunately, we could not directly address whether IpgC and IpaD binding to the IpaB N-terminal domain is competitive because while DOC is required for binding to IpaD, its detergent properties favor dissociation of the IpaB/IpgC heterodimer (data not shown).

From the data presented here, we propose an *in vitro* model in which exposure of IpaD to DOC induces a conformational change that generates the flexibility needed for the distal domain to move from the position observed in the IpaD crystal structure to permit the IpaD and IpaB coiled-coil cores to align parallel with one another. This is proposed to mimic the *in vivo* interaction where DOC induces flexibility within the IpaD needle tip pentamer that allows the individual five distal domains (already in an up position) to open up to allow IpaB to move to a position where it is now surface exposed. While it seems unlikely that the sole driving force for interaction between these two large proteins is a short peptide sequence near the N-terminus of IpaB, both *in vitro* and *in vivo* studies presented here suggest that a region as small as that from residues 12–19 in IpaB plays a crucial role in optimizing this interaction. Further investigation into the nature of this interaction, the proposed events leading up to this interaction, and its role in T3SA maturation is needed to fully understand how needle tip maturation occurs and its contribution to *Shigella* virulence, however, results from this study now provide a platform for addressing these questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IpaD	invasion plasmid antigen D
T3SS	type III secretion system
T3SA	type III secretion apparatus
DOC	deoxycholate
PMSF	phenylmethylsulfonyl fluoride
DHC	dehydrocholate
CD	circular dichroism
FRET	Förster resonance energy transfer
FP	fluorescence polarization
FTIR	Fourier-transform infrared spectroscopy
CPM	7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin
FM	fluorescein maleimide
TCEP	tris(2-carboxyethyl)phosphine
BME	β-mercaptoethanol
DMF	N′,N′-dimethylformamide
FlAsH-EDT$_2$	fluorescein-containing biarsenical reagent that coordinates with the tetracysteine motif CCPGCC
LcrV	low calcium response T3SA needle tip protein from *Yersinia*
SipD	*Salmonella* invasion protein D

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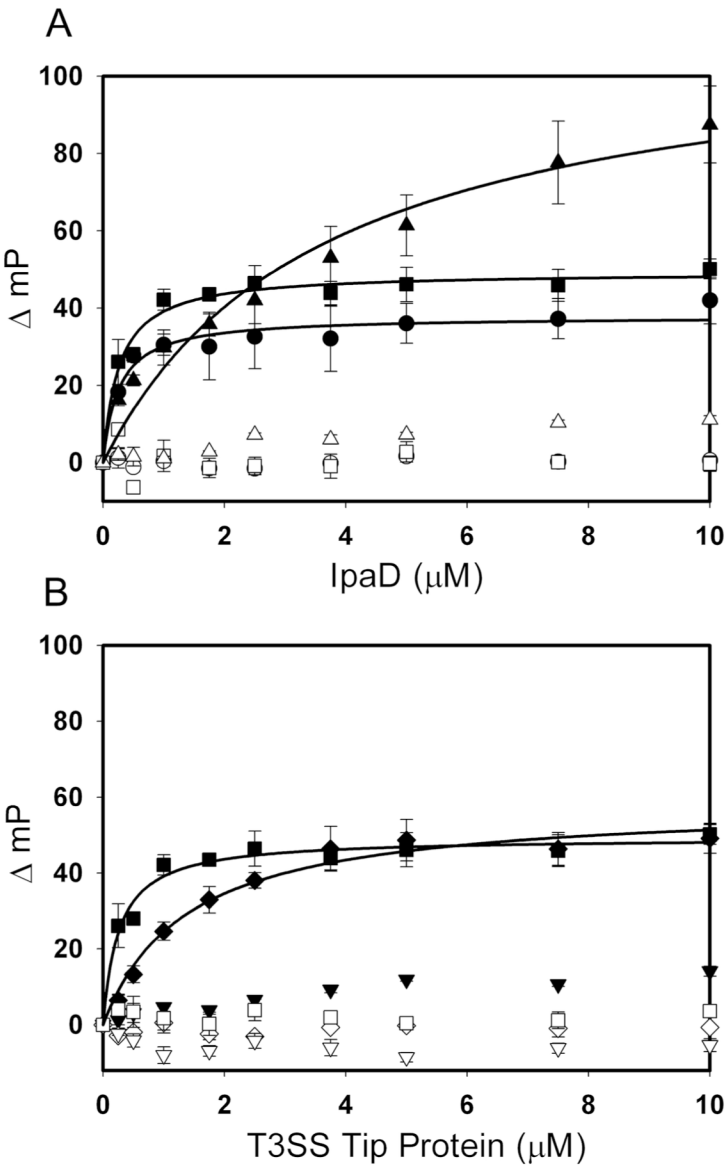

Figure 1. N-terminal IpaB peptides require deoxycholate for optimal interaction with IpaD

A) Fluorescence polarization of fluorescein-labeled IpaB peptides (80 nM) was monitored as the concentration of IpaD was increased from 0 to 10 μM. IpaD-dependent changes in polarization were monitored for $IpaB^{1-226}$ (●), $IpaB^{11-226}$ (■), and $IpaB^{28-226}$ (▲) following incubation with 1 mM DOC or in the absence of DOC (corresponding open symbols). **B)** The fluorescently-labeled *Shigella* $IpaB^{11-226}$ was incubated with the T3SS tip proteins from *Salmonella* (SipD, ◆) and *Yersinia* (LcrV, ▼) as well as IpaD (■) in the presence of 1 mM DOC or the absence of DOC (corresponding open symbols). Each graph is representative of three independent experiments with error bars indicating the standard deviation of triplicate measurements within a single experiment. The results were fit with a single site saturation model using SigmaPlot and the overlaid functions are included for those for which binding occurred ($R^2 > 0.90$).

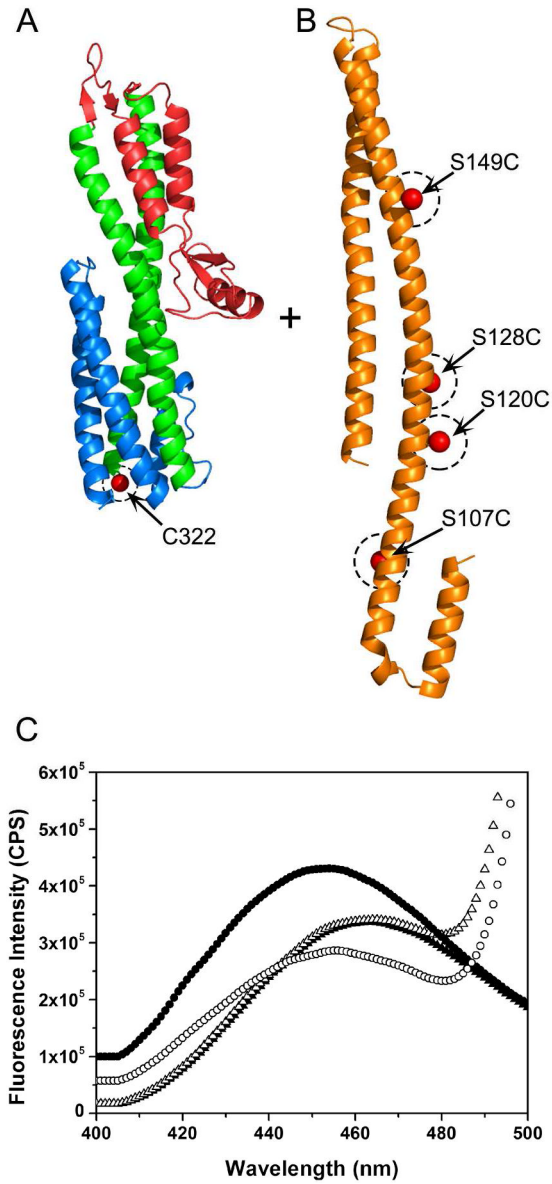

Figure 2. Using FRET to characterize the interaction between IpaD and IpaB

A) The FRET donor probe (CPM) was covalently bound to the thiol of the native IpaD cysteine at position 322. Individual cysteine point mutations were introduced into IpaB$^{11\text{-}226}$ at positions 107, 120, 128, and 149 to allow covalent attachment of a FRET acceptor probe (FM) **(B).** Dashed circles with diameters equal to those of the fluorophores are included at each labeling site. The FRET efficiency between the donor and acceptor was quantified for each acceptor position in the absence and presence of 1 mM DOC based on donor quenching. **Panel C** is a representative set of FRET donor emission spectra acquired for coumarin-labeled IpaD in the presence of acceptor-labeled IpaB$^{11\text{-}226}$ S107C (open triangles) and unlabeled IpaB$^{11\text{-}226}$ S107C (closed triangles). Spectra were re-acquired for donor only and donor plus acceptor conditions following the addition of 1 mM DOC (open circles and closed circles, respectively).

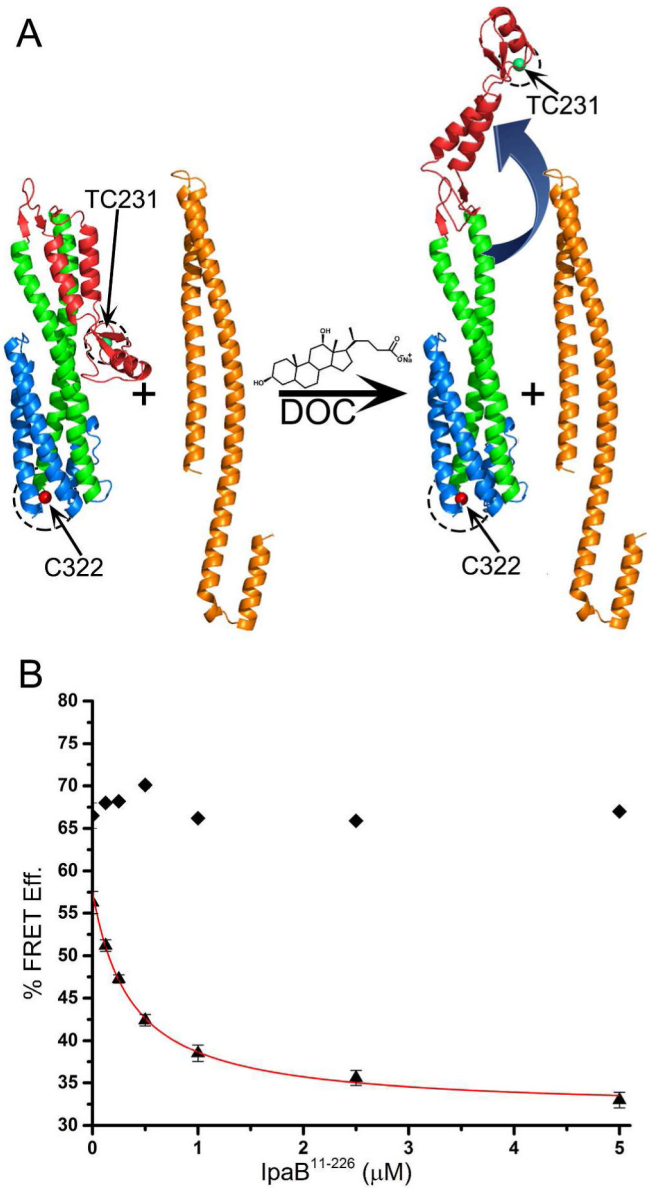

Figure 3. IpaD distal domain movement accompanies IpaB binding

The locations of FlAsH FRET donor and Alexa Fluor 568 FRET acceptor probes are identified at positions TC231 and Cys322, respectively. Dashed circles with diameters equal to those of the fluorophores are included at each labeling site **(A)**. Plotting IpaD intramolecular FRET efficiency in the absence of DOC (◆) and in the presence of 1 mM DOC (▲) as a function of $IpaB^{11-226}$ indicates a DOC initiated, dose-dependent decrease in FRET efficiency resulting from an increase in distance between the donor and acceptor fluorophores **(B)**, consistent with a movement such as the one illustrated in **panel A** (right side).

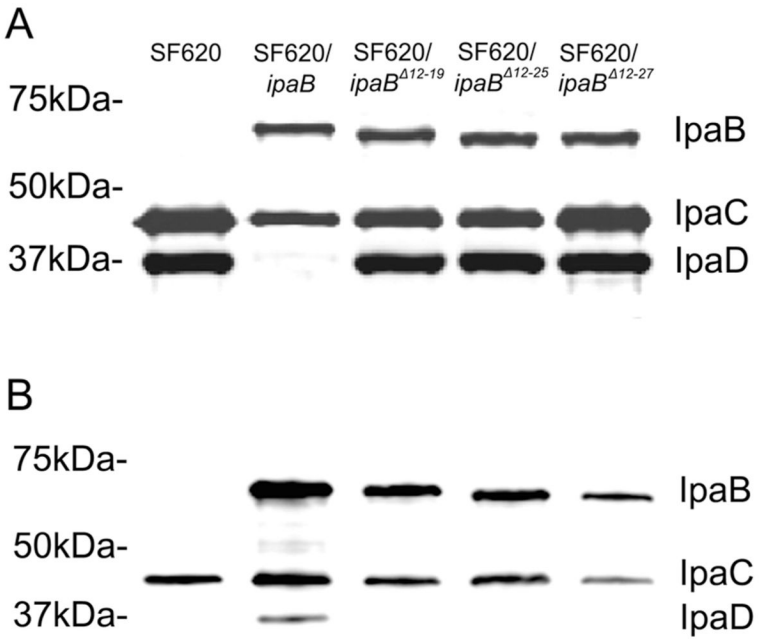

Figure 4. Identification of a region near the IpaB N-terminus necessary for proper *Shigella* T3SS secretion control

Western blots of IpaB, IpaC, and IpaD present in the supernatant **(A)** and the whole cell extracts **(B)** of overnight *S. flexneri* cultures.

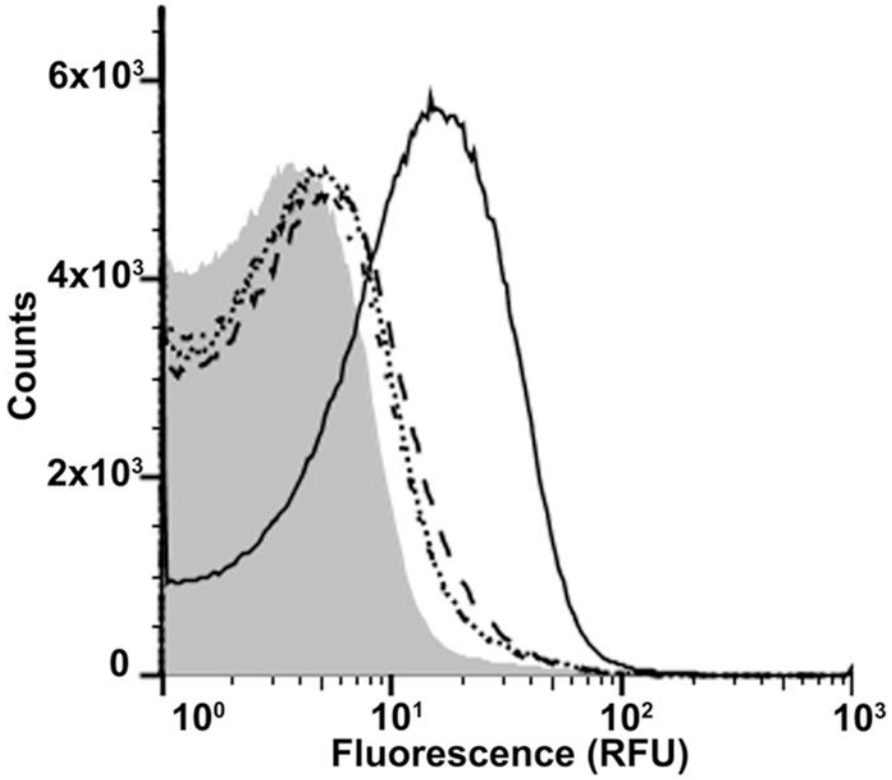

Figure 5. Identification of a region near the IpaB N-terminus necessary for optimal *Shigella* T3SA surface presentation

Fluorescence intensity histograms depicting DOC-induced levels of surface localization of IpaB for the SF620 (*ipaB* null) *S. flexneri* strain (shaded) and those complemented with wild-type IpaB (solid), IpaB$^{\Delta 12\text{-}19}$ (dashed), IpaB$^{\Delta 12\text{-}25}$ (dotted), and IpaB$^{\Delta 12\text{-}27}$ (dash-dot-dash). The decreased levels of the IpaB mutants compared to wild-type is consistent with in the *in vitro* experiments suggesting that this region near the N-terminus plays an important role in proper IpaD/IpaB interaction and T3SA localization.

Table 1

Calculated dissociation constants for the *Shigella* T3SS tip protein IpaD and N-terminal peptides of the translocator IpaB[a]

IpaB Peptide	T3SS Tip Protein	K_d (μM ± SD)
$IpaB^{1-226}$	IpaD	0.78 ± 0.43
$IpaB^{11-226}$	IpaD	0.39 ± 0.10
$IpaB^{28-226}$	IpaD	3.60 ± 0.37

[a]Apparent dissociation constants were calculated by fitting the polarization data points to a single site saturation model using SigmaPlot. (n = 3 independent measurements)

Table 2

FRET efficiencies characterizing the interaction between the *Shigella* T3SS proteins IpaD and IpaB[a]

Acceptor (Fluorescein) Location	FRET Efficiency (% ± SD)	
	No DOC	1 mM DOC
IpaB$^{11\text{-}226}$ S107C	−0.1 ± 0.0	33.1 ± 1.4
IpaB$^{11\text{-}226}$ S120C	8.1 ± 0.1	33.3 ± 0.5
IpaB$^{11\text{-}226}$ S128C	7.3 ± 0.1	21.4 ± 0.4
IpaB$^{11\text{-}226}$ S149C	8.0 ± 0.1	14.6 ± 0.2

[a]FRET efficiencies were measured between a coumarin donor probe located at the native cysteine (residue 322) in IpaD and a fluorescein acceptor bound to the single cysteine residues mutated into IpaB$^{11\text{-}226}$. Results are presented as %FRET efficiency ± standard deviation. (n = 3 independent measurements)

Table 3

Identifying a region near the IpaB N-terminus required for proper *Shigella flexneri* virulence phenotype

Bacterial Strain	Hemolysis[b] (% ± SD)	Relative Invasion[c] (% ± SD)	
		No DOC	1 mM DOC
ipaB null (SF620)	4.5 ± 0.3	0 ± 0	0 ± 0
SF620 + *ipaB*	100 ± 2.5	100 ± 4	288 ± 6
SF620 *ipaB*$^{\Delta 12\text{-}19}$	5.8 ± 0.3	15 ± 1	21 ± 1
SF620 *ipaB*$^{\Delta 12\text{-}25}$	10.1 ± 1.0	6 ± 1	7 ± 1
SF620 *ipaB*$^{\Delta 12\text{-}27}$	5.9 ± 0.4	1 ± 1	2 ± 1

[a] The ability of the *Shigella* mutants to lyse red blood cells was investigated by a hemolysis assay which spectrophotometrically quantifies the release of hemoglobin from erythrocytes following incubation with the *Shigella*.

[b] Results are presented as percent wild-type IpaB ± standard deviation (n = 3 independent measurements).

[c] Invasiveness was measured by a standard gentamicin protection assay (see methods) and the results are presented as the percent invasion by *S. flexneri* expressing wild-type IpaB in the absence of DOC. Experiments were repeated in triplicate.