Oligomeric states of the *Shigella* translocator protein IpaB provide structural insights into formation of the type III secretion translocon

Nicholas E. Dickenson,¹ Shyamal P. Choudhari,¹ Philip R. Adam,¹
Ryan M. Kramer,² Sangeeta B. Joshi,² C. Russell Middaugh,²
Wendy L. Picking,¹ and William D. Picking^{1*}

¹Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, Oklahoma 74078

²Department of Pharmaceutical Chemistry, Macromolecule and Vaccine Stabilization Center, University of Kansas, Lawrence, Kansas 66047

Received 4 October 2012; Revised 10 February 2013; Accepted 22 February 2013

DOI: 10.1002/pro.2245

Published online 4 March 2013 proteinscience.org

Abstract: The *Shigella flexneri* Type III secretion system (T3SS) senses contact with human intestinal cells and injects effector proteins that promote pathogen entry as the first step in causing life threatening bacillary dysentery (shigellosis). The *Shigella* Type III secretion apparatus (T3SA) consists of an anchoring basal body, an exposed needle, and a temporally assembled tip complex. Exposure to environmental small molecules recruits IpaB, the first hydrophobic translocator protein, to the maturing tip complex. IpaB then senses contact with a host cell membrane, forming the translocon pore through which effectors are delivered to the host cytoplasm. Within the bacterium, IpaB exists as a heterodimer with its chaperone IpgC; however, IpaB's structural state following secretion is unknown due to difficulties isolating stable protein. We have overcome this by coexpressing the IpaB/IpgC heterodimer and isolating IpaB by incubating the complex in mild detergents. Interestingly, preparation of IpaB with *n*-octyl-oligo-oxyethylene (OPOE) results in the assembly of discrete oligomers while purification in *N,N*-dimethyldodecylamine *N*-oxide (LDAO) maintains IpaB as a monomer. In this study, we demonstrate that IpaB tetramers penetrate phospholipid membranes to allow a size-dependent release of small molecules, suggesting the formation of discrete pores. Monomeric IpaB also interacts with liposomes but fails to disrupt them. From these and additional findings, we propose

Abbreviations: AUC, analytical ultracentrifugation; CD, circular dichroism; CMC, critical micelle concentration; DGS-NTA, 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid)succinyl]; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DSP, dithiobis[succinimidyl propionate]; FM, fluorescein maleimide; FRET, F—rster resonance energy transfer; Ipa, invasion plasmid antigen; Ipg, invasion plasmid gene; LDAO, *N,N*-dimethyldodecylamine *N*-oxide; Mxi, major exporter of Ipas; OPOE, *n*-Octyl-oligo-oxyethylene; SATA, *N*-succinimidyl-S-acetylthioacetate; SEC, size exclusion chromatography; SRB, sulforhodamine B; *T*_m, thermal unfolding midpoint; TCEP, (tris (2-carboxyethyl)phosphine; TRITC DHPE, (*N*-(6-tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine); T3SS, type-III secretion system; T3SA, type III secretion apparatus.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: NIH; Grant numbers: R01 AI067858R21 AI090149R01 AI099489; Grant sponsor: OCAST; Grant number: HR10-128S.

*Correspondence to: William D. Picking, Department of Microbiology and Molecular Genetics, Oklahoma State University, 307 Life Science East, Stillwater, OK 74078. E-mail: william.picking@okstate.edu

that IpaB can exist as a tetramer having inherent flexibility, which allows it to cooperatively interact with and insert into host cell membranes. This event may then lay the foundation for formation of the *Shigella* T3SS translocon pore.

Keywords: *Shigella*; type III secretion; IpaB; translocon

Introduction

Shigella spp. are gram-negative, nonmotile, enteric pathogens that are commonly transmitted by the fecal-oral route.¹ This mode of transmission and the fact that as few as 10–100 organisms are sufficient to cause an infection makes regional epidemics in the developing world common with up to 90 million cases of shigellosis reported annually.¹ Severe dehydration, ulceration of the colon, and other complications caused by *Shigella* infections contribute to 1.1 million deaths annually with about two-thirds being children.²

The ability of *Shigella* to colonize a human host requires its Type III secretion system (T3SS). The T3SS is an essential virulence system that translocates bacterial effector proteins into host cells to alter normal cell functions for the benefit of the pathogen.^{3,4} Many important pathogens employ T3SSs as virulence mechanisms and while there are distinct pathogen-specific differences in T3SS effector functions, the Type III secretion apparatus (T3SA) maintains significant structural homology across diverse species boundaries.³ In all cases, the T3SA is anchored in the bacterial envelope by a basal body, which supports an external needle composed of many copies of a small, polymerized needle protein terminating at a needle tip complex that contributes to secretion control.⁵ *Shigella* is currently the only pathogen for which each step in the T3SA needle tip complex maturation can be artificially induced to mimic natural maturation.^{6–8} As a first step of *Shigella* T3SA maturation, IpaD forms a pentameric structure at the tip of the nascent MxiH needle.⁹ In its capacity as an environmental sensor, IpaD detects small molecules such as deoxycholate (DOC), promoting a conformational change in IpaD that results in the recruitment of IpaB, the first translocator, to the maturing T3SA needle tip.^{7,10–12} IpaB then interacts with cholesterol and sphingolipid rich host cell membranes, which can be mimicked using defined liposomes.⁸ The interaction of IpaB with host cells or cholesterol/sphingolipid rich liposomes recruits IpaC, the second translocator,^{8,13} to assemble the final T3SA needle tip complex, which results in formation of the translocon pore.¹⁴ This event completes the unidirectional conduit between *Shigella* and host cell cytoplasm and the concomitant full induction of effector protein translocation although whether this occurs as a single step or stepwise process that requires the T3SA is not entirely clear.¹⁵ Thus, the *Shigella* T3SA provides the best model available for

studying the temporal assembly of the T3SA needle tip complex and the molecular events leading to induction of Type III secretion.¹⁶ The *Shigella* T3SA also provides a needed model for characterizing each of the three tip proteins with respect to their interaction as homo and heteropolymers.

Within the *Shigella* cytoplasm, IpaB is chaperoned by IpgC to prevent premature interaction with IpaD and IpaC in addition to interaction with the bacterial cell membrane.¹⁷ We have recently solved the structure of a stable N-terminal core of IpaB (residues 74–224) at a 2.1 Å resolution.¹⁸ A prominent feature of the IpaB N-terminal domain is an elongated coiled-coil. Using this structure, we investigated the interaction between the soluble N-terminal domain of IpaB and IpgC.¹⁹ In this work we identified two distinct chaperone binding domains near the extreme N-terminus of IpaB. These sites promote the formation of a heterodimer of IpaB:IpgC. With respect to IpaB's function after secretion, the coiled-coil may serve as scaffolding for an IpaB homopolymer that associates with the elongated open structure of IpaD at the tip of the T3SA needle. This study explores the nature of IpaB oligomerization as an initial step toward understanding IpaB behavior following its secretion.

When coexpressed with IpgC in *E. coli*, IpaB is readily purified as part of a heterodimer, which can be separated by incubation with mild, nonionic detergents.¹⁷ Separation of IpaB from IpgC with 0.5% *n*-octyl-oligo-oxyethylene (OPOE) results in self assembly of IpaB into discrete oligomers while separation with 0.05% *N,N*-dimethyldodecylamine *N*-oxide (LDAO) maintains IpaB in a monomeric state. Here we define the biochemical and biophysical characteristics of the “membrane active” recombinant IpaB oligomer, comparing it to the “membrane inactive” monomer, to investigate the initial step of T3SA translocon pore formation *in vitro*. These results provide a significant step forward in our understanding of IpaB's role in translocon formation and host cell interaction.

Results

IpaB purifies in discrete oligomeric states

Because soluble IpaB is not stably expressed in the absence of IpgC, we express recombinant His-tagged IpaB in the presence of IpgC. After purification of the IpaB/IpgC complex, IpgC was removed by the

Figure 1. IpaB forms oligomers in OPOE and monomers in LDAO. (A) After separation from IpgC, the purified IpaB was run over a Superdex 200 10/300 size exclusion column in PBS containing OPOE (*) or LDAO (▼). The IpaB in OPOE consistently eluted earlier than the IpaB in LDAO, suggesting that the protein in OPOE forms higher order oligomers as compared to the protein in LDAO. (B) IpaB prepared in the presence of 0.5% OPOE and 0.05% LDAO migrate at their expected IpaB molecular mass by SDS–PAGE (first two lanes). When IpaB prepared in OPOE was crosslinked with DSP and examined by SDS–PAGE, a higher order structure in the 290 to greater than 500 kDa range is seen in the absence of reducing agent (TCEP) and the monomeric form is regained after reduction with TCEP (middle two lanes marked by an asterisk, respectively). When the crosslinking was performed for IpaB prepared in LDAO, a monomer is observed with or without TCEP (last two lanes marked by two asterisks). (C) IpaB prepared in OPOE (*) or LDAO (▼) were crosslinked with DSP and then analyzed by SEC. The crosslinked IpaB oligomers (in OPOE) eluted at ~9 mL and migrated as an extremely high molecular weight species while the crosslinked IpaB purified and maintained in LDAO eluted from the SEC column at ~11.5 mL.

addition of 0.5% OPOE, a nonionic detergent.¹⁷ To further purify the isolated protein, IpaB in 0.5% OPOE (OPOE-IpaB) was then passed over a SEC column to give a stable, soluble protein preparation that existed as a multimeric complex.²⁰ While OPOE is a chemically heterogeneous detergent with a broad range of molecular sizes (see Supporting Information Fig. S1), LDAO is a single chemical species that was also found to efficiently disrupt the IpaB/IpgC complex. Interestingly, IpaB prepared with 0.05% LDAO (LDAO-IpaB) eluted from SEC significantly later than OPOE-IpaB [Fig. 1(A)]. When the SEC-purified IpaB fractions were analyzed by SDS–PAGE, the protein migrated at 62 kDa, the monomeric size of IpaB, regardless of the detergent used during purification [Fig. 1(B), lanes 1 and 2]. Two very distinct products were visualized; however, when OPOE-IpaB or LDAO-IpaB were subjected to crosslinking and analyzed by SDS–PAGE [Fig. 1(B), lanes 3 and 5]. Crosslinked OPOE-IpaB migrated as an extremely high molecular weight species whereas crosslinked LDAO-IpaB migrated as a monomer. When these crosslinked products were again passed through the SEC, the OPOE-IpaB eluted at ~9 mL while LDAO-IpaB eluted at ~11.5 mL [Fig. 1(C)]. Crosslinking with DSP caused the OPOE-IpaB oligomer to elute slightly earlier than in the absence of crosslinking. Otherwise they maintained nearly identical elution profiles with the exception of the appearance of a shoulder at the void volume of the crosslinked OPOE-IpaB [Fig. 1(C)]. SDS–PAGE identified this shoulder as large,

and therefore probably nonspecifically crosslinked IpaB complexes (data not shown).

It is worth noting that removal of OPOE from the isolated IpaB by buffer exchange followed by filtration to remove the largely aggregated protein resulted in the appearance of a putative trimeric IpaB species (identified by crosslinking, data not shown). This species appears to be similar to that described by Hume *et al.* in which the protein was expressed independently of IpgC and purified from the bacterial membrane fractions using OPOE.²¹ CD spectroscopy measurements of the protein in which the OPOE had been removed following isolation from its chaperone indicated that IpaB purified in this manner had reduced α -helical content within its secondary structure and possessed reduced stability to thermal unfolding. Because of these difficulties and the fact that a similar trimeric species had been previously described as unable to disrupt phospholipid membranes,²¹ it was not examined further in this study.

On the basis of the crystal structure of its N-terminal region, IpaB is a highly elongated protein preventing the use of SEC to accurately determine the stoichiometry of the putative IpaB oligomer. Therefore, analytical ultracentrifugation (AUC), which relies on the protein sedimentation coefficient to determine the molecular mass, was employed. OPOE-IpaB and LDAO-IpaB were subjected to crosslinking, dialyzed into PBS and analyzed by AUC (see Supporting Information Fig. S2). A single dominant peak with a calculated molecular weight

Figure 2. Characterization of the IpaB oligomeric and monomeric states by analytical ultracentrifugation (AUC). IpaB monomer in 0.05% LDAO and IpaB oligomer in 0.5% OPOE were chemically crosslinked with DSP as described in Methods. The crosslinked proteins were thoroughly dialyzed into PBS lacking any detergent. Sedimentation velocity AUC radial absorbance scans were obtained for each IpaB sample (see Supporting Information Fig. S2) and fit with the SEDFIT continuous sedimentation coefficient distribution method for both LDAO-IpaB (A) and OPOE-IpaB (B). The SEDFIT results were then used to calculate the species molecular weights in both conditions and found that IpaB in OPOE forms multiple oligomeric species with the two most predominant at 265 and 477 kDa (B) while IpaB in LDAO results in nearly exclusive formation of a monomeric species with a calculated molecular weight of 64.7 kDa (A).

of 64.7 kDa was detected in the sample containing LDAO-IpaB [Fig. 2(A)], indicating this sample possessed monomeric IpaB. It is important to note that precise molecular mass determinations from observed AUC sedimentation coefficients require knowledge of the unique partial specific volume (V_{bar}) for the protein in question.²² A V_{bar} value of 0.8 mL/g was experimentally determined [Eq. (1)] for the crosslinked LDAO-IpaB that was dialyzed into PBS and resulted in the calculated molecular weight of 64.7. While 0.8 mL/g is somewhat higher than the predicted V_{bar} value of 0.7447 mL/g estimated based on the IpaB primary sequence, it likely results from the additional molecular volume of the covalently bound DSP crosslinker. This value was used in all molecular weight determinations. AUC identified two major IpaB species within the population of crosslinked OPOE-IpaB [Fig. 2(B) and Supporting Information Fig. S2]. The calculated molecu-

lar weights of 265 and 477 kDa are indicative of a tetramer, which appears to be the dominant species, and an octamer. Identification of these oligomeric states following purification of recombinant IpaB in 0.5% OPOE and 0.05% LDAO now allows biochemical and biophysical characterization of this translocator protein as it is proposed to exist following maturation of the *Shigella* T3SA needle tip complex.

Effects of detergent conditions on IpaB secondary structure and stability

CD spectroscopy was used to probe the secondary structure content and stability of IpaB under native and crosslinked conditions in both LDAO and OPOE. In the presence of each detergent, the spectra exhibit defined minima at 208 and 222 nm, characteristic of highly α -helical secondary structure. Chemical crosslinking of OPOE-IpaB or LDAO-IpaB had little effect on the observed CD spectra [Fig. 3(A,B)]. Further analysis of the spectra using DichroWeb,²³ to predict secondary structure content indicated that IpaB in either detergent possesses similar secondary structure content with predominantly helical characteristics (Supporting Information Table S1). Crosslinking with DSP did not significantly change these predicted secondary structures, which returned to essentially native levels upon cleavage of the DSP with the reducing agent TCEP (Supporting Information Table S1).

OPOE-IpaB and LDAO-IpaB before and after crosslinking were subjected to thermal unfolding using CD spectroscopy to determine their thermal transition temperatures (T_m), defined as the inflection point of each transition. OPOE-IpaB and LDAO-IpaB both exhibited sharp T_m s but at significantly different temperatures [Fig. 3(C,D)]. The oligomeric OPOE-IpaB exhibited a T_m of $57.1 \pm 1.3^\circ\text{C}$, nearly 9°C higher than the $48.8 \pm 0.1^\circ\text{C}$ of the monomer in LDAO. Not surprisingly, crosslinking of the oligomer and monomer resulted in broader transitions at higher temperatures with crosslinking presumably stabilizing local secondary structure. Cleavage of the DSP resulted in the reappearance of a sharp transition and reduction of the T_m s to native levels [Fig. 3(C,D), Supporting Information Table S1]. Together, these data indicate that although OPOE and LDAO result in distinct IpaB stoichiometries, the secondary structures of the monomer and oligomer remain largely unchanged. Furthermore, production of the IpaB oligomers by OPOE results in a more stable state of IpaB relative to the monomeric protein in LDAO, as might be expected for an evolved quaternary structure. Finally, the use of DSP as a chemical crosslinking agent produced only subtle structural changes but higher T_m s, which were reversible, demonstrating that the reaction and subsequent modifications to IpaB had no lasting consequences on its structure or stability.

Figure 3. IpaB prepared in OPOE or LDAO have similar secondary structure content and thermal stability. (A) The CD spectra for IpaB prepared in OPOE is shown before (■) and after (●) crosslinking with DSP, both indicating predominant α -helical content. (B) The CD spectra for IpaB prepared in LDAO is shown before (+) and after (×) crosslinking with DSP, both again indicating significant α -helical content. (C) Thermal unfolding of IpaB prepared in OPOE is shown based on the CD signal α -helical minimum at 222 nm with the signal plotted from 10 to 90°C. Native IpaB (■), IpaB crosslinked with DSP (●), and the latter after reduction with TCEP (▲) are shown. (D) Thermal unfolding of IpaB prepared in LDAO is shown based on the CD signal α -helical minimum at 222 nm with the signal plotted from 10 to 90°C. Native IpaB (+), IpaB crosslinked with DSP (*), and crosslinked IpaB after reduction with TCEP (x) are shown.

IpaB oligomers disrupt phospholipid vesicles

A key function of IpaB is insertion into host cell membranes while remaining within the context of the T3SA tip complex to lay the foundation for translocon formation. Accordingly, the influence of the IpaB oligomeric state on its ability to disrupt phospholipid bilayers was investigated by monitoring the release of sulforhodamine B (SRB) from defined phospholipid vesicles. The high concentration of SRB in the liposomes results in autoquenching that is relieved when the dye is released.^{24–26} The oligomeric OPOE-IpaB quickly and efficiently disrupts the phospholipid bilayers, thereby relieving SRB auto-quenching [Fig. 4(A)]. Monomeric LDAO-IpaB, however, exhibited a very low level of SRB release that was similar to PBS alone. PBS containing either LDAO or OPOE did not result in appreciable dye release (<6%) [Fig. 4(A)]. Only minor SRB release was also seen for the IpaB/IpgC complex ($11.2 \pm 1.8\%$) as previously reported [Fig. 4(B)].¹⁷ We also tested the N-terminal fragment, IpaB_{28–226}

which can be expressed in the absence of IpgC and/or detergents due to the lack of the putative hydrophobic regions presumably involved in IpaB-membrane interaction. Although this fragment forms homodimers in solution, it did not elicit substantial SRB release [$2.4 \pm 0.1\%$ in Fig. 4(B)]. In light of the recent discovery of large IpaB complexes capable of membrane disruption in low concentrations of LDAO,²⁷ the oligomeric state of IpaB and its ability to disrupt membranes was tested as a function of LDAO concentration (Fig. 5). Dialyzing the protein into progressively lower concentrations of detergent resulted in the gradual formation of higher order species (>500 kDa) consistent with what was seen previously by Senerovic *et al.*²⁷ SRB release assays showed that the ability of IpaB to disrupt liposomes was directly related to the formation of the high order species, however, none approached the efficiency of the IpaB tetramer in OPOE [Fig. 5(B)], suggesting that while the removal of LDAO promoted the formation of membrane active IpaB

Figure 4. Oligomeric IpaB promotes release of SRB from liposomes. (A) SRB release from liposomes results in a relief of autoquenching, which is measured as an increase in rhodamine fluorescence emission. Both detergents (OPOE, dotted line, and LDAO, long dashed line) did not result in a time-dependent release of any SRB. IpaB in OPOE led to significant SRB release (solid line) while IpaB in LDAO did not give rise to SRB release (short dashed line). (B) The addition of Triton X-100 at the end of each assay resulted in 100% SRB release and allowed quantification (as percentage of total dye release) caused by each protein. The samples tested are listed along the X-axis and included buffer (PBS), OPOE, or LDAO in PBS, the IpaB/IpgC heterodimer in PBS, the IpaB N-terminal domain (IpaB²⁸⁻²²⁶), IpaB in OPOE with or without crosslinking with DSP, and IpaB in LDAO with or without crosslinking. Additionally, IpaB in OPOE and LDAO were tested after reaction with *N*-succinimidyl-S-acetylthioacetate (SATA), which mimics the reactions occurring with DSP but without the result of crosslinking.

oligomers, they do not represent the most efficient form and likely are not the stoichiometry that would exist at the T3SA needle tip just prior to translocon formation. They may, however, be responsible for other aspects of IpaB effector functions *in vivo*.

Crosslinked OPOE-IpaB and LDAO-IpaB were also tested for their ability to disrupt liposomes [Fig. 4(B)]. While there was a slight increase in SRB

release by the crosslinked IpaB monomer, SRB release from the crosslinked IpaB oligomer was reduced by more than 2.5-fold. In an effort to restore membrane disruption, the crosslinked oligomer was treated with TCEP after crosslinking but prior to addition to liposomes. Interestingly, reduction did not restore the ability of the IpaB oligomer to release SRB (data not shown). To mimic the chemical modifications caused by DSP without

Figure 5. Reducing LDAO concentration results in the formation of membrane active IpaB oligomers. IpaB was dialyzed against decreasing concentrations of LDAO following purification in 0.05%. (A) The species were crosslinked and separated on 4–20% acrylamide SDS–PAGE gradient gels, identifying the formation of higher order oligomers as the detergent concentration is decreased. Most notable is the appearance of a >500 kDa oligomer beginning at 0.01% LDAO. Cleavage of the crosslinker with the addition of DTT returned the IpaB to a monomer, as expected (right). (B) A liposome disruption assay (SRB release) was carried out as described in Methods section to characterize several of the IpaB fractions above. The appearance of the high molecular weight species driven by the decrease in detergent concentration favors liposome disruption; however, it remains far less efficient than the tetrameric form in 0.5% OPOE. Detergent only controls resulted in minimal SRB release as in Figure 4 (removed for clarity).

crosslinking, N-succinimidyl-S-acetylthioacetate (SATA) was added to modify primary amines. SATA modification decreased SRB release by both the monomeric and oligomeric species, even when compared to the DSP crosslinked species, suggesting that the primary amines of exposed lysine side chains play an important role in membrane interaction and/or disruption. Together, these results show that the IpaB oligomer disrupts phospholipid bilayers while monomeric IpaB does not. Moreover, chemical crosslinking and/or the modification of primary amines on the membrane active oligomeric species significantly reduce the ability of IpaB to disrupt liposomes, suggesting that protein flexibility and exposed primary amines may play a critical role in IpaB-mediated membrane effects.

Quantifying IpaB interaction with phospholipid vesicles through liposome flotation and FRET analyses

A liposome flotation assay was used to determine whether the lack of phospholipid membrane disruption by LDAO-IpaB or the crosslinked IpaB species was due to loss of the ability for the protein to interact with membranes. Each IpaB species was incubated with defined liposomes and subjected to sucrose gradient ultra-centrifugation. In this assay, the denser proteins remain in the bottom fractions unless they interact with the more buoyant liposomes, which cause them to migrate to the top lipid-containing fractions (see Supporting Information Fig. S3). In the absence of lipids, IpaB remains in the bottom fraction. In contrast, ~77% of both the oligomeric and monomeric populations of IpaB migrate to the top with the liposomes demonstrating that both protein states interact with liposomes (Fig. 6). As expected, the N-terminal IpaB^{28–226} fragment, IpgC, and the IpaB/IpgC complex were almost entirely localized to the bottom fraction since they cannot interact with liposomes. The inclusion of 10 mol% of the His6-binding DGS-NTA lipid into the liposomes resulted in the migration of IpaB/IpgC to the top through a forced interaction of the NTA lipids and the His6 tag on IpaB, thus validating the assay (Fig. 6).

Chemically crosslinking the IpaB multimer with DSP and covalent linkage of SATA to available primary amines caused a marked decrease in SRB release and thus these were also investigated for their ability to interact with liposomes. Crosslinking of the IpaB oligomer or monomer resulted in a significant shift of the protein from the top liposome-rich fraction to the bottom fraction, suggesting that crosslinking prevented both species from interacting with the liposomes (Fig. 6). In contrast, treatment with SATA only slightly reduced the amount of protein found in the liposome-rich top layer. Thus, although the IpaB multimer is able to efficiently dis-

rupt liposomes while the monomer is not, both states are equally capable of interacting with membranes. Therefore, because crosslinking prevented interaction with the liposomes, it is not only oligomerization but also perhaps some aspect of protein flexibility that is required for liposome disruption.

Because Förster resonance energy transfer (FRET) provides a sensitive spectroscopic ruler with Å-level resolution,^{28,29} it was also used to probe for protein/liposome interactions. By covalently linking a fluorescein FRET donor to the native cysteine of IpaB and including a TRITC acceptor probe conjugated to the polar head group of the phospholipid DHPE in the liposomes, energy transfer between the FRET pair could be examined to provide confirmation of the IpaB-liposome interaction. FRET efficiency was determined by measuring donor fluorescence emission in the presence and absence of the FRET acceptor for multimeric OPOE-IpaB, monomeric LDAO-IpaB, and the heterodimeric IpaB/IpgC complex (Supporting Information Fig. S4). The multimeric and monomeric forms of IpaB both appeared to interact with liposomes based on their FRET efficiencies (Supporting Information Table S2). The donor-labeled IpaB/IpgC complex served as a negative control, resulting in minimal energy transfer. These results are in agreement with the flotation assay, suggesting that the heterodimeric IpaB/IpgC complex is prevented from interacting with liposome phospholipid membranes although both monomeric and multimeric IpaB are capable of interaction. Inclusion of 10 mol% DGS-NTA lipids as a positive control for interaction gave rise to similar efficiencies of ~50% in all cases, including for the IpaB/IpgC complex (Supporting Information Table S2). The specificity and sensitivity of this technique make it valuable in confirming findings from the liposome flotation assay.

IpaB oligomers have pore-like properties after insertion into phospholipid bilayers

We have demonstrated that both monomeric and multimeric IpaB interact with phospholipid vesicles but efficient membrane disruption is only achieved with the multimeric form. We have proposed that, as a part of the T3SA needle tip complex, IpaB senses host cell membrane contact and then inserts to become an integral component of the translocon pore. Thus, to determine whether the recombinant, multimeric OPOE-IpaB has pore-like properties, a fluorescent dextran release assay was employed. Liposomes containing fluorescein-labeled 3, 10, 40, and 70 kDa dextrans were used to provide insight into the mechanism of membrane disruption by the defined IpaB multimers. Fluorescence kinetic curves for the release of fluorescein-labeled dextrans following addition of 15 nM OPOE-IpaB show a maximal release of the 3 kDa dextran of $42.3 \pm 3.4\%$ (Fig. 7

Figure 6. Liposome flotation indicates that IpaB prepared in OPOE or LDAO can associate with liposomes. Liposome flotation was carried out as described in Methods section and the presence of protein in top, middle, and bottom fractions of the sucrose gradient was determined by SDS-PAGE and quantified by performing densitometry of the gels after Coomassie blue staining. Lipids migrate to the top of the gradient along with any proteins associated with the lipids. Proteins not associating with the lipids remain at the bottom. (A) Representative gels identifying the location of the protein within the sucrose gradient. (B) The quantitative densitometry results compare the location of all conditions tested. The black bars are the relative amounts of protein found in the top (lipid) fraction, the medium gray bar is the relative amount of protein found in the middle fractions, and the light gray bar is the relative amount of protein found at the bottom of the gradient. The total amount of protein for each treatment totals to 100%. IpaB migrated to the top of the gradient (lipid fraction) whether prepared in OPOE or LDAO. In the absence of lipids, the IpaB remains in the bottom fractions to indicate that the detergent had no effect on the protein's migration. The IpaB N-terminal domain (IpaB²⁸⁻²²⁶) and the IpaB/IpgC heterodimer both also remain in the bottom fraction. As an internal control, the IpaB/IpgC complex did migrate to the top fraction if NTA-lipids are included in the lipid preparation by virtue of a His6 tag on IpaB. The different treatments for IpaB are as listed along the X-axis.

and Table I). Similar kinetics is seen with the 10 and 40 kDa dextrans with releases of $31.0 \pm 2.8\%$, $30.0 \pm 1.2\%$, respectively. Dextran release, however, is slowed greatly for the 70 kDa dextrans with only $12.0 \pm 0.2\%$ release (Fig. 7 and Table I). Although the random and heterogeneous distribution of dextran shapes versus their mass makes it difficult to determine a precise size cutoff for dextran release, DLS measurements of the dextran solutions indicate that IpaB multimers form pore-like structures with effective hydrodynamic diameter cutoffs between 3 and 5 nm (Fig. 7 and Table I). Similar values have been determined for the MxiH needle channel^{5,30} as well as for the channel of the IpaD pentamer⁹ and support the idea that the IpaB oligomer represents a membrane active structure with pore-like properties. Perhaps this is the scaffold for the translocon pore that forms at the interface between the *Shigella* T3SA and the host cell membrane.

Discussion

S. flexneri is the causative agent for shigellosis, a severe form of dysentery that is prevalent in the developing world. The T3SA is a common nanomachine among the *Shigella* spp. as well as many other

gram negative pathogens.² An understanding of its interaction with the host cell could promote development of antimicrobial agents to block this step. We have previously demonstrated that IpaB is the T3SA protein that senses the host cell membrane to promote Type III secretion induction.⁸ We have also demonstrated that IpaB can be stably expressed in as a recombinant complex with IpgC and this heterodimer can then be separated into its component proteins using OPOE with the resulting IpaB being able to promote small molecule release from liposomes.¹⁷ In this study we have now demonstrated that IpaB can adopt a monomeric or oligomeric state depending upon the detergent used in its preparation. On the basis of SEC and crosslinking analyses we show that IpaB in 0.5% OPOE oligomerizes while IpaB in 0.05% LDAO maintains a monomeric state. AUC analysis of the crosslinked products indicated that IpaB in OPOE forms a homotetramer with a molecular weight of ~ 265 kDa, while in LDAO it remains monomeric at 64.7 kDa.

Both detergents were used at just above their critical micelle concentrations (CMCs) and were found to stabilize IpaB and prevent precipitation presumably through stabilization of their

Figure 7. Release of fluorescent dextrans from liposomes identifies the functional hydrodynamic cutoff of IpaB pores. The liposome release assay was modified to monitor the release of fluorescein-labeled dextrans of different sizes from liposomes. Liposomes were prepared to contain fluorescein-labeled 3, 10, 40, and 70 kDa dextrans and time-based release was monitored. The 3 kDa dextran release is shown by the solid line, 10 kDa dextran release is shown by the short dashed line, 40 kDa dextran release is shown by the long dashed line, and 70 kDa dextran release is shown with the dashed-dotted line. Quantification of release was then determined after addition of Triton X-100 which resulted in 100% dextran release.

hydrophobic domains.^{31,32} It is not entirely clear, however, why IpaB behaves differently in these two detergents. LDAO is a small (230 Da) zwitterionic surfactant possessing a polar head group with distinct positively and negatively charged regions. In contrast, OPOE is a nonionic detergent with a heterogeneous size distribution ranging from about 162.3 to 450.6 Da. It contains a single polar hydroxyl moiety in the head group with dispersed ether linkages throughout the structure (Supporting Information Fig. 1). IpaB is rich in lysine and has an overall pI of about 8.2. The zwitterionic nature of LDAO may interfere with IpaB oligomerization by disrupting exposed charged residues. In fact, high salt concentrations effectively prevent IpaB oligomerization by presumably buffering key surface charges and preventing electrostatic interactions involved in

oligomer formation (data not shown). Conversely, OPOE is less likely to disrupt charge interactions while still stabilizing the protein's hydrophobic domains. Although both detergents are used just above their CMCs, neither disrupts liposomes.

While the secondary structures of OPOE-IpaB and LDAO-IpaB are similar and relatively unaffected by crosslinking, the thermal stability of oligomeric IpaB was significantly higher than that of the monomer in LDAO. Such a stabilization effect might be expected upon oligomerization of the monomer into a multimer, but it further suggests a physiological relevance for this discrete oligomerization beyond random protein–protein interactions involving IpaB's hydrophobic regions. Furthermore, the IpaB monomer did not disrupt liposomes while the oligomer did, however, both the monomer and multimer were found to associate with membranes. Liposome disruptions by the multimer and liposome association by both species were greatly diminished when the proteins were subjected to crosslinking. Since, chemical crosslinking would result in a more rigid protein structure; the decrease in liposome disruption/association by the crosslinked proteins implicates protein conformational dynamics as having a role in membrane interaction. Furthermore, a decrease in liposome disruption was also seen following modification of the primary amines of the oligomer by SATA, which had no impact on membrane association. Thus, while the monomer and oligomer can associate with liposomes in an interaction that may require protein flexibility, the disruption of liposomes involves both global protein dynamics of the multimer as well as an interaction requiring accessible lysine side chains. By examining fluorescent dextran release, we also demonstrated that liposome disruption is caused by formation of a defined pore that allows dextrans with a hydrodynamic diameter of $\leq 3\text{--}5$ nm to be readily released.

During the preparation of this manuscript, a study by Senerovic *et al.* reported on the identification of very large IpaB oligomers when the concentration of LDAO was reduced to 0.0005% (~ 100 -fold less than the CMC).²⁷ They found that these oligomers not only disrupted liposomes, but led to an ion

Table I. IpaB-Mediated Dextran Release from Phospholipid Vesicles

Dextran	Molecular weight (kDa) ^a	Hydrodynamic diameter (nm) ^b	Dextran release (%) ^c
D3-Fluorescein	1.5–2	2.1 $\pm$ 0.2	42.3 $\pm$ 3.4
D10-Fluorescein	9–11	2.3 $\pm$ 0.3	31.0 $\pm$ 2.8
D40-Fluorescein	35–50	3.2 $\pm$ 0.2	30.0 $\pm$ 1.2
D70-Fluorescein	60–90	5.5 $\pm$ 0.5	12.0 $\pm$ 0.2

^a Molecular weight range provided by the manufacturer.

^b Hydrodynamic diameter determined by DLS. ($n = 30$ measurements spanning three independent samples).

^c Release of fluorescent dextrans was monitored following introduction of 15 nM IpaB oligomer in 0.5% OPOE. Release was quantified as a percent of the total fluorescence following complete liposome lysis with Triton X-100. ($n = 3$ independent measurements).

imbalance in exposed eukaryotic cells resulting in phagosomal lysis and Caspase-1 mediated apoptosis. We have also observed the formation of large (>500 kDa) complexes upon dialyzing the LDAO concentration well below its CMC [Fig. 5(A)]. In contrast to what we observed upon removal of OPOE, far-UV circular dichroism (CD) measurements suggested that the protein secondary structure remained relatively unchanged and that the secondary structure thermal stabilities were surprisingly unaffected under these conditions as well (data not shown), suggesting that the protein integrity remained intact at these low detergent (LDAO) concentrations. Several of these IpaB populations dialyzed into PBS containing decreasing LDAO concentrations were characterized for their abilities to disrupt liposomes (SRB release assay) and were compared to the defined tetramer in 0.5% OPOE. An increase in liposome disruption efficiency directly correlated to the formation of high molecular weight oligomers through detergent removal; however, they were only about 50% as efficient as an equivalent concentration of IpaB in OPOE [Fig. 5(B)]. These results, together with the sheer physical size constraints at the tip of the T3SA, suggest that the tetramer described in this study would more likely represent the form of the protein associated with the T3SA tip where IpaB is responsible for initial host cell contact and formation of the translocon prepore. It is important to realize, however, that the roles of IpaB can be divided into two broad categories—structural and effector. While the described tetramer most likely fulfills the former, the stoichiometry of the secreted IpaB responsible for the latter may differ greatly, perhaps leading to the diversity of functions observed for IpaB. With this in mind, it may prove interesting to compare the identified oligomeric states of IpaB for their abilities to elicit physiological responses through selective ion channel formation *in vivo*.

From the data presented here, we propose a mechanism for the interaction and disruption of phospholipid membranes by recombinant IpaB as it would appear at the T3SA needle tip (Fig. 8). IpaB self assembles in the presence of 0.5% OPOE to form oligomers that associate with and insert into membranes to produce pores of ~3–5 nm. This size is in agreement with the diameter of the pentameric IpaD channel as well as the MxiH needle channel.^{5,9,13} Therefore, we believe the IpaB oligomer (tetramer) is an extension of the IpaD multimer (pentamer) that forms atop the nascent T3SA to allow interaction with host cell membranes as the initial step in translocon formation. One of the most interesting results of this study was the suggestion that membrane interaction require IpaB flexibility or some form of structural dynamics as well as the charged residues of the protein. Thus, one can spec-

Figure 8. Model for IpaB association with and formation of pores in model membranes. IpaB coexpressed with IpgC forms a heterodimer that does not interact with phospholipid membranes. In the presence of OPOE or LDAO, the IpaB/IpgC complex dissociates with IpgC forming a homodimer and IpaB either becoming an oligomer (tetramer) in the presence of OPOE or a monomer in the presence of LDAO. Both forms of IpaB are able to associate with phospholipid membranes; however, only the oligomeric form is able to insert to form pore-like structures. Crosslinking with DSP greatly reduced the interaction of each with the membrane. SATA treatment does not block this association, although, it does prevent either protein from forming the pore-like structure in the membrane.

ulate that upon contact with the host cell, IpaB undergoes a conformational change as it inserts into the membrane with charged lysine groups interacting with the head groups of the membrane lipids. Meanwhile, it is possible that a second population of IpaB is secreted following translocon formation, which may form very large complexes that are responsible for IpaB's contributions to vacuolar escape and Caspase-1 activation. Future studies will require that we establish the oligomeric state of IpaB as it exists while sitting at the T3SA needle tip as was recently done for IpaD. However, the findings presented here provide significant new insight into the functional state(s) of IpaB prior to *Shigella* contact with host cells and together with previous

studies^{21,27} may indicate that IpaB can exist within the context of at least three multimeric complexes—as a heterodimer with IpgC while waiting to be secreted, as a tetramer at the T3SA needle tip where it is primed to become the major translocon component, and as a larger complex within host cells where it carries out IpaB-specific effector functions.

Materials and Methods

Materials

Escherichia coli strains, protein expression plasmids, and Clonables 2X Ligation Premix were from Novagen (Madison, WI). Restriction enzymes were from New England Biolabs (Ipswich, MA). Iminodiacetic acid-Sepharose, SRB, and LDAO were from Sigma Chemical (St. Louis, MO). OPOE was purchased from Enzo Life sciences (Farmingdale, NY). Triton X-100, dithiobis[succinimidyl propionate] (DSP), and SATA were from Fisher Scientific (Pittsburgh, PA). TRITC-labeled DHPE (*N*-(6-tetramethylrhodamine-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine) and fluorescein-conjugated dextrans were from Invitrogen. (Grand Island, NY). All other phospholipids and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). All other solutions and chemicals were of reagent grade.

Protein expression and purification

The purification of IpaB and IpgC has been described previously.^{17,20} Briefly, the IpaB:IpgC complex was purified from the cytoplasm of BL21(DE3) *E. coli* using standard Ni²⁺-chelation and hydrophobic interaction chromatography. The protein complex was again bound to a Ni²⁺-charged iminodiacetic acid Sepharose column and the IpgC released by incubating overnight in either 0.5% OPOE or 0.05% LDAO in binding buffer. The column was washed to remove the IpgC. IpaB was then eluted in the presence of the appropriate detergent. To remove remaining contaminants, IpaB was passed over a HiLoad 16/60 Superdex 200 Prep Grade size exclusion column. After a buffer exchange into 10 mM sodium phosphate (pH 7.2)/150 mM NaCl (PBS) containing either 0.5% OPOE or 0.05% LDAO, IpaB was stored at 4°C for not more than two weeks.

Chemical crosslinking

The oligomeric states of recombinant IpaB were investigated employing chemical crosslinking. The protein concentrations were adjusted to 10 μM in PBS pH 7.4 containing 0.5% OPOE or 0.05% LDAO and covalently crosslinked by the addition of 168 μM DSP, which is a primary amine reactive, thiol-containing, homobifunctional crosslinker (see Supporting Information Fig. S1 for chemical structures).¹⁷ SATA served as a control under identical conditions to mimic the covalent linkage to exposed primary

amines without crosslinking (Supporting Information Fig. S1). The reaction was allowed to proceed for 30 min at room temperature before quenching with 3× SDS-PAGE sample buffer for direct visualization by SDS-PAGE or 70 mM Tris-HCl pH 7.4 for purification on an ÄKTA FPLC equipped with a Superdex 200 10/300 GL size exclusion column (GE Healthcare Life Sciences). For SDS-PAGE analysis, the reactions were split into two aliquots with one receiving 10 mM tris (2-carboxyethyl)phosphine (TCEP) to cleave the DSP crosslinker and boiled. The products were separated on 4–20% acrylamide gradient SDS-PAGE gels (BioRad) with unstained HiMark molecular mass ladder (Invitrogen). The gels were then stained with Oriole UV-fluorescent gel stain (BioRad).

Analytical ultracentrifugation

Sedimentation velocity experiments were conducted using a Beckman Proteomlab XL-I analytical ultracentrifuge equipped with scanning UV/visible optics.³³ An-60 Ti four-hole rotor and cells with 12 mm charcoal centerpieces and quartz windows were used. The IpaB samples were prepared in OPOE or LDAO and crosslinked using DSP. The detergent was then removed by dialysis and samples were analyzed at 30,000 rpm and 20°C scanning continuously until complete sedimentation was achieved. The data were then analyzed using a continuous *c*(s) distribution and SEDFIT version 13.0b software.²² Partial specific volumes were calculated using SEDNTERP version 20120618 using the equation:

$$\bar{V} = \left(\frac{1}{\rho_0} \right) \left\{ 1 - \left[\frac{(\rho - \rho_0)}{C} \right] \right\} \quad (1)$$

where $\bar{V}$ is the partial specific volume, ρ and ρ_0 are the densities of the solution and buffer, respectively, and C is the concentration of protein.³⁴

Far-UV circular dichroism spectroscopy

Far-UV CD spectra were collected with a Jasco Model J-815 spectropolarimeter equipped with a Peltier temperature controller (Jasco, Easton, MD). Spectra were acquired using a 0.1 cm path length quartz cuvette at 10°C with a spectral resolution of 0.2 nm, a scanning rate of 50 nm/min, and a 2 s data integration time. All spectra are an average of three measurements. Secondary structure thermal stability was monitored at 222 nm over a temperature range from 10 to 90°C. Data were acquired every 2.5°C and the temperature ramp rate was 15°C/h. The protein concentration was 0.3–0.5 mg/mL in phosphate citrate buffer pH 7.4 for all proteins. The buffers contained appropriate detergents and/or reducing agent as dictated by the experiment and are described in each figure/table legend. CD signals were converted to mean residue molar ellipticities

and secondary structure content was determined using the DichroWeb²³ software package CDSSTR.³⁵ The thermal transitions were determined by plotting the second derivative of the thermal unfolding curves and identifying the inflection point of each transition (OriginPro 8.6.0).

Preparation of phospholipid vesicles

The phospholipids DOPC (dioleoylphosphatidylcholine) and DOPG (dioleoylphosphatidylglycerol), the nickel salt of the phospholipid analog DGS-NTA (1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl]) and cholesterol were made to 25 mg/mL in chloroform. The fluorescently labeled phospholipid TRITC DHPE (Supporting Information Fig. S1) was dissolved in chloroform at 1 mg/mL. The stock solutions were combined and dried under nitrogen followed by vacuum for 3 h to create lipid films of 66.8, 23.5, and 9.7 mol% DOPC, DOPG, and cholesterol, respectively, with 10 mol% DGS-NTA and/or 1 mol% TRITC DHPE added where appropriate. Lipid films were hydrated in PBS alone or in water containing 100 mM SRB for liposome flotation assays³⁶ and SRB release assays,^{25,26} respectively. Liposomes used for dextran release assays were hydrated with 3 mM, 1 mM, 125 μ M, or 71.5 μ M solutions of fluorescein-labeled 3, 10, 40, or 70 kDa anionic dextrans, respectively. The hydrated films were sonicated briefly and extruded through a 100 nm pore size membrane 10 times at 42°C. Vesicles hydrated in PBS alone were used directly. Excess dye was separated from the SRB liposomes by gel filtration using Sephadex G-50 equilibrated with PBS. Unincorporated fluorescent dextrans were separated from the liposomes using Sepharose CL-6B-200. All liposomes were verified by DLS to be monodisperse with hydrodynamic diameters of ~100 nm prior to storage at 4°C and were used within one week.

Time-based fluorophore release from lipid vesicles

SRB release from phospholipid vesicles was monitored at 20°C using a FluoroMax-4 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ). After an initial baseline of fluorescence from the liposomes was established, protein was added to a final concentration of 300 nM and SRB release detected as an increase in fluorescence intensity over time.²⁶ Addition of 0.1% Triton X-100 resulted in complete fluorophore release and represented the 100% fluorescence intensity standard for each condition. Buffer only, buffer containing the appropriate detergents, IpgC, or IpaB/IpgC were used as negative controls for liposome disruption, as appropriate. Release of 3, 10, 40, and 70 kDa fluorescein-labeled dextrans from lipid vesicles was monitored using a modified protocol described previously³⁷ following the addition of

15 nM oligomeric IpaB. It is important to note that all of these release assays are strongly dependent on protein and liposome concentration²⁵ as well as the final concentration of detergent. Proper controls preceded each assay to ensure that the liposomes were not compromised and that minimal lysis was incurred in the absence of protein.

Liposome flotation assays

Liposomes were incubated with protein samples for 30 min in PBS at 2.5 mg/mL and 3 μ M concentrations, respectively. The protein/liposome mixture was adjusted to a final concentration of 30% sucrose. This solution (150 μ L) was transferred to an 11 mm ultracentrifuge tube, overlaid with 1 mL 22.5% sucrose in PBS and 100 μ L PBS alone, and centrifuged at 240,000g for 1 h at 4°C.³⁶ Top, middle, and bottom gradient fractions were analyzed by SDS-PAGE, stained with Oriole UV-fluorescent protein stain and quantified using the densitometry software of AlphaImager (ProteinSimple, Santa Clara, CA).

Förster resonance energy transfer (FRET) between IpaB and liposomes

To label the native Cys309 with fluorescein maleimide (FM), IpaB in 0.05% LDAO, IpaB in 0.5% OPOE, and IpaB/IpgC were dialyzed overnight in 50 mM HEPES (pH 7.0), 150 mM NaCl, and 5 mM TCEP with a final step of 1 mM TCEP just prior to labeling. FM was dissolved in a minimal volume of dimethylsulfoxide (DMSO) and added at a 10-fold molar excess to a 2 mg/mL solution of protein. The reactions were stirred under nitrogen for 2 h and the free dye removed using a G-50 gel filtration column equilibrated with PBS with the appropriate detergent.

Lipid vesicles were constructed to include 65.5 mol% DOPC, 23.5 mol% DOPG, 10 mol% cholesterol, and 1 mol% DHPE. A TRITC conjugated DHPE was substituted to provide a FRET acceptor probe where appropriate. The His6-binding lipid analog DGS-NTA (10 mol%) was also included to serve as a positive control for lipid interaction (Supporting Information Fig. S1). FM-labeled protein (100 nM) was incubated for 5 min at room temperature with ~20 molar excess of liposomes with integrated TRITC acceptor. The fluorescence emission spectra (500–560 nm) were obtained at 20°C using a FluoroMax-4 spectrofluorometer with excitation of the fluorescein donor at 485 nm.¹² The excitation and emission slit widths were 2.5 nm and the integration time was 0.1 s/nm. Energy transfer efficiencies were determined for each protein/liposome condition by comparing the peak donor emission intensities in the presence of liposomes containing the acceptor probe to the intensities of the donor in the presence of liposomes lacking the TRITC acceptor probe on the DHPE.²⁸ The percent energy transfer was calculated

by:

$$E = \left[1 - \left(\frac{F_{DA}}{F_D} \right) \right] * 100 \quad (2)$$

where E is the observed energy transfer efficiency, F_{DA} is the peak intensity of the donor emission in the presence of the TRITC acceptor, and F_D is the peak intensity of the donor emission in the absence of the TRITC acceptor probe.

References

- DuPont HL, Levine MM, Hornick RB, Formal SB (1989) Inoculum size in shigellosis and implications for expected mode of transmission. *J Infect Dis* 159: 1126–1128.
- Kotloff KL, Winickoff JP, Ivanoff B, Clemens JD, Swerdlow DL, Sansonetti PJ, Adak GK, Levine MM (1999) Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. *Bull World Health Organ* 77:651–666.
- Schroeder GN, Hilbi H (2008) Molecular pathogenesis of *Shigella* spp.: controlling host cell signaling, invasion, and death by type III secretion. *Clin Microbiol Rev* 21:134–156.
- Cornelis GR (2010) The type III secretion injectisome, a complex nanomachine for intracellular “toxin” delivery. *Biol Chem* 391:745–751.
- Blocker A, Jouihri N, Larquet E, Gounon P, Ebel F, Parsot C, Sansonetti P, Allaoui A (2001) Structure and composition of the *Shigella flexneri* “needle complex,” a part of its type III secretin. *Mol Microbiol* 39:652–663.
- Espina M, Olive AJ, Kenjale R, Moore DS, Ausar SF, Kaminski RW, Oaks EV, Middaugh CR, Picking WD, Picking WL (2006) IpaD localizes to the tip of the Type III secretion system needle of *Shigella flexneri*. *Infect Immun* 74:4391–4400.
- Olive AJ, Kenjale R, Espina M, Moore DS, Picking WL, Picking WD (2007) 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* 75:2626–2629.
- Epler CR, Dickenson NE, Olive AJ, Picking WL, Picking WD (2009) Liposomes recruit IpaC to the *Shigella flexneri* type III secretion apparatus needle as a final step in secretion induction. *Infect Immun* 77: 2754–2761.
- Epler CR, Dickenson NE, Bullitt E, Picking WL (2012) Ultrastructural analysis of IpaD at the tip of the nascent MxiH type III secretion apparatus of *Shigella flexneri*. *J Mol Biol* 420:29–39.
- Stensrud KF, Adam PR, La Mar CD, Olive AJ, Lushington GH, Sudharsan R, Shelton NL, Givens RS, Picking WL, Picking WD (2008) Deoxycholate interacts with IpaD of *Shigella flexneri* in inducing the recruitment of IpaB to the type III secretion apparatus needle tip. *J Biol Chem* 283:18646–18654.
- Barta ML, Guragain M, Adam P, Dickenson NE, Patil M, Geisbrecht BV, Picking WL, Picking WD (2012) Identification of the bile salt binding site on IpaD from *Shigella flexneri* and the influence of ligand binding on IpaD structure. *Proteins* 80:935–945.
- Dickenson NE, Zhang L, Epler CR, Adam PR, Picking WL, Picking WD (2011) Conformational changes in IpaD from *Shigella flexneri* upon binding bile salts provide insight into the second step of Type III secretion. *Biochemistry* 50:172–180.
- Blocker A, Gounon P, Larquet E, Niebuhr K, Cabiaux V, Parsot C, Sansonetti P (1999) The tripartite type III secretin of *Shigella flexneri* inserts IpaB and IpaC into host membranes. *J Cell Biol* 147:683–693.
- Mueller CA, Broz P, Cornelis GR (2008) The type III secretion system tip complex and translocon. *Mol Microbiol* 68:1085–1095.
- Akopyan K, Edgren T, Wang-Edgren H, Rosqvist R, Fahlgren A, Wolf-Watz H, Fallman M (2011) Translocation of surface-localized effectors in type III secretion. *Proc Natl Acad Sci U S A* 108:1639–1644.
- Picking WL, Picking WD (2009) Early steps in inducing Type III secretion in *Shigella*. *Microbes Infect* 4: 554–559.
- Birket SE, Harrington AT, Espina M, Smith ND, Terry CM, Darboe N, Markham AP, Middaugh CR, Picking WL, Picking WD (2007) Preparation and characterization of translocator/chaperone complexes and their component proteins from *Shigella flexneri*. *Biochemistry* 46:8128–8137.
- Barta ML, Dickenson NE, Patil M, Keightley A, Wyckoff GJ, Picking WD, Picking WL, Geisbrecht BV (2012) The structures of coiled-coil domains from type III secretion system translocators reveal homology to pore-forming toxins. *J Mol Biol* 417:395–405.
- Adam PR, Patil MK, Dickenson NE, Choudhary S, Barta M, Geisbrecht BV, Picking WL, Picking WD (2012) Binding affects the tertiary and quaternary structures of the *Shigella* translocator protein IpaB and its chaperone IpgC. *Biochemistry* 51:4062–4071.
- Martinez-Becerra FJ, Kissmann JM, Diaz-McNair J, Choudhary SP, Quick AM, Mellado-Sanchez G, Clements JD, Pasetti MF, Picking WL (2012) Broadly protective *Shigella* vaccine based on type III secretion apparatus proteins. *Infect Immun* 80:1222–1231.
- Hume PJ, McGhie EJ, Hayward RD, Koronakis V (2003) The purified *Shigella* IpaB and *Salmonella* SipB translocators share biochemical properties and membrane topology. *Mol Microbiol* 49:425–439.
- Schuck P (2000) Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. *Biophys J* 78:1606–1619.
- Whitmore L, Wallace BA (2004) DICHROWEB: an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Res* 32:W668–W673.
- Mach H, Middaugh CR (1995) Interaction of partially structured states of acidic fibroblast growth factor with phospholipid membranes. *Biochemistry* 34:9913–9920.
- De Geyter C, Wattiez R, Sansonetti P, Falmagne P, Ruyschaert JM, Parsot C, Cabiaux V (2000) Characterization of the interaction of IpaB and IpaD, proteins required for entry of *Shigella flexneri* into epithelial cells, with a lipid membrane. *Eur J Biochem* 267: 5769–5776.
- Harrington A, Darboe N, Kenjale R, Picking WL, Middaugh CR, Birket S, Picking WD (2006) Characterization of the interaction of single tryptophan containing mutants of IpaC from *Shigella flexneri* with phospholipid membranes. *Biochemistry* 45:626–636.
- Senerovic L, Tsunoda SP, Goosmann C, Brinkmann V, Zychlinsky A, Meissner F, Kolbe M (2012) Spontaneous formation of IpaB ion channels in host cell membranes reveals how *Shigella* induces pyroptosis in macrophages. *Cell Death Dis* 3:e384.
- Lakowicz JR (1983) Principles of Fluorescence Spectroscopy. New York: Plenum Press.

29. Dickenson NE, Picking WD (2012) Förster resonance energy transfer (FRET) as a tool for dissecting the molecular mechanisms for maturation of the *Shigella* Type III secretion needle tip complex. *Intl J Mol Sci* 13:15137–15161.
30. Fujii T, Cheung M, Blanco A, Kato T, Blocker AJ, Namba K (2012) Structure of a type III secretion needle at 7-Å resolution provides insights into its assembly and signaling mechanisms. *Proc Natl Acad Sci U S A* 109:4461–4466.
31. Prive GG (2007) Detergents for the stabilization and crystallization of membrane proteins. *Methods* 41:388–397.
32. Rosenbusch JP (1990) The critical role of detergents in the crystallization of membrane proteins. *J Struct Biol* 104:134–138.
33. Markham AP, Jaafar ZA, Kemege KE, Middaugh CR, Hefty PS (2009) Biophysical characterization of *Chlamydia trachomatis* CT584 supports its potential role as a Type III secretion needle tip protein. *Biochemistry* 48:10353–10361.
34. Prakash V (1982) Partial specific volumes and interactions with solvent components of alpha-globulin from *sesamum-indicum* L in urea and guanidine-hydrochloride. *J Bioscience* 4:347–359.
35. Johnson WC (1999) Analyzing protein circular dichroism spectra for accurate secondary structures. *Proteins* 35:307–312.
36. Hyland C, Vuillard L, Hughes C, Koronakis V (2001) Membrane interaction of *Escherichia coli* hemolysin: flotation and insertion-dependent labeling by phospholipid vesicles. *J Bacteriol* 183:5364–5370.
37. Faudry E, Vernier G, Neumann E, Forge V, Attree I (2006) Synergistic pore formation by Type III toxin translocators of *Pseudomonas aeruginosa*. *Biochemistry* 45:8117–8123.