Combination of Two Separate Binding Domains Defines Stoichiometry between Type III Secretion System Chaperone IpgC and Translocator Protein IpaB

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Type III secretion systems (TTSSs) utilized by enteropathogenic bacteria require the presence of small, acidic virulence-associated chaperones for effective host cell infection. We adopted a combination of biochemical and cellular techniques to define the chaperone binding domains (CBDs) in the translocators IpaB and IpaC associated with the chaperone IpgC from Shigella flexneri. We identified a novel CBD in IpaB and furthermore precisely mapped the boundaries of the CBDs in both translocator proteins. In IpaC a single binding domain associates with IpgC. In IpaB, we show that the binding of the newly characterized CBD is essential in maintaining the tertiary arrangement of chaperone-translocator complex. This hitherto unknown function is reflected in the co-crystal structure as well, with an IpgC dimer bound to an IpaB fragment comprising both CBDs. Moreover, in the absence of this novel CBD the IpaB/IpgC complex aggregates. This dual-recognition of a domain in the protein by the chaperone in facilitating the correct chaperone-substrate organization describes a new function for the TTSS-associated chaperone-substrate complexes.

Shigella species cause bacillary dysentery, a global human health problem with an estimated 165 million infections and 1.1 million deaths annually (1). Shigella, like many Gram-negative pathogens infecting both plants and humans, uses a type three secretion system (TTSS) to deliver virulence factors into host cells (2, 3). A TTSS requires cooperation of specific chaperones for proper assembly and operation. The cytosolic chaperones of TTSS share similar physico-chemical properties, although they differ in their amino acid sequence and substrate specificity. They are small, typically less than 20 kDa, possess an acidic pI, and can bind to single or multiple substrates. From the co-crystal structures of chaperone-substrate complexes available so far the chaperone binding domain (CBD) in substrate molecules associates with the chaperone in an extended manner. Moreover, TTSS-associated chaperones are frequently encoded in the same operon as their substrates.

Based on substrate binding characteristics, chaperones can be grouped into three classes (4). The crystal structures of representative proteins from each class alone and with their cognate substrates are now available. Structures of chaperones of class IA that bind to one or class IB that can bind to two different effectors in complex with the CBD of substrates reveal a generalized mode of association. The structures show the CBD wrapped around the chaperone in an elongated non-globular fashion (5–8). The structure of class III chaperones, such as FlIS of flagellar export system, in complex with the CBD reveals a mode of substrate binding similar to class I chaperones (9). However, class I chaperones display a homodimeric structure with a conserved α-β fold, whereas FlIS from class III exists as monomer and is entirely α-helical. Unlike class I and III, the class II chaperones are composed of tetratricopeptide repeats, motifs adopting a helix-turn-helix conformation, and form an asymmetric dimer (10–12). Class II chaperones can bind independently (13) or together (14) to two different translocators proposed to form a pore in the host cell membrane. IpgC, a class II chaperone from Shigella flexneri, recognizes a sequence motif in the IpaB class of translocators, which is encapsulated by the cleft of the chaperone in an extended conformation (11). It is suggested that the unfolded conformation of the substrates in complex with the class I chaperones likely primes the effectors to the secretion apparatus (5, 6). For class II and III chaperones, substrate binding is suggested to prevent premature self-association of the substrates (9, 13). Apart from the role in targeting effectors to secretion apparatus (15, 16), stabilization and maintaining the substrates in a secretion-competent state (13, 14, 17), some chaperones are reported to have a regulatory role in the transcription of genes encoding components of TTSS (18–22). Whereas chaperones are implicated in imparting temporal hierarchy to secretion (6), their role in effector folding remains unanswered (23). Moreover, it is unclear how the substrate binding vis-à-vis chaperones drives a particular arrangement.

In S. flexneri, the components of the TTSS are encoded by mxi-spa operon of the 31-kb “entry region” present on a 220

The abbreviations used are: TTSS, Type III secretion system; CBD, chaperone binding domain; ITC, isothermal titration calorimetry; MALLS, multi-angle laser light scattering; SEC, size exclusion chromatography; Bpa, p-benzoyl phenylalanine.
IpgC Translocator Organization

kb virulence plasmid (24, 25, 26). The ipa operon of the entry region encodes the chaperone IpgC and the associated translocator proteins IpaB and IpaC. IpgC binds independently to IpaB and IpaC (13). IpaB and IpaC are essential for invasion of epithelial cells, membrane lysis of the phagocytic vacuole, contact hemolysis and macrophage cell death (27–29). By two-hybrid selection in yeast (Y2H), the CBD in IpaB and IpaC were mapped to residues 58–72 and 73–122 respectively (30). We have recently shown that the CBD in IpaB starts at residue 51, based on proteolytic cleavage on IpgC-complexed IpaB (11). In IpaC, a region between residues 50–80 was mapped by fluorescence polarization and fluorescence resonance energy transfer (FRET) (31) as the CBD.

Given the unclear and overlapping nature of CBDs obtained by different techniques, we took a biochemical approach to precisely map the boundaries of the CBDs in IpaB and IpaC. We identified a novel CBD in IpaB which plays a critical role in defining the proper stoichiometry of the IpaB/IpgC complex. We corroborated the stoichiometry defining role for the CBD in IpaB by determining the co-crystal structure of IpaB/IpgC complex, wherein one IpaB peptide binds to an IpgC dimer. This ternary arrangement is reflected in solution as well. In the case of IpaC, we mapped the CBD to a single domain.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Cell Culture—The S. flexneri wild-type strain M90T (serotype 5A), BS176, and SF620 (ΔipaB) have been described (24, 28). SF620 was transformed with plasmid pUC19 (39) or with plasmids carrying either wild-type or mutant ipaB. The Escherichia coli strains DH5α and BL21(DE3) were used for construction of the plasmid and gene expression, respectively. E. coli was grown in Luria-Bertani (LB) and S. flexneri was grown at 37 °C in tryptic soy broth (TSB) supplemented with ampicillin (50 μg/ml), kanamycin (30 μg/ml), tetracycline (10 μg/ml), streptomycin (50 μg/ml) when necessary.

Construction of Plasmids—Supplemental Table S1 lists the plasmids used in this study. The insert carried by each plasmid and the mutations performed were confirmed by sequencing. Site-directed mutations and internal deletions in ipaB and ipaC were created using either pMK101 or pMK151 and pMK202 respectively as templates. Mutations were produced employing two-stage PCR protocol using the QuikChange™ site-directed mutagenesis kit (Stratagene) as described previously (38). For generating plpBPS (IpaB having PreScission cleavage site in place of residues 51–72), first, ipaB comprising amino acids 73–580 was cloned in the sites of HindIII and XhoI of pET21a; next, ipaB comprising amino acids 1–50 was cloned in the sites of NdeI and SacI; finally, dsDNA obtained by annealing complementary ssDNA comprising PreScission cleavage site flanked by 4 amino acid linker on either side and having 5’ SacI and 3’ HindIII was cloned into the respective sites to create plpBPS.

Purification of His-tagged Proteins—Protein (co-)expression was induced using 0.5 mM IPTG in E. coli BL21(DE3) transformants for 3 h, and pellets were subsequently lysed by French press. Protein complexes from the lysates were purified utilizing His6 tag on IpgC by either Ni-NTA-agarose (Qiagen) or HisTrap HP column (GE Healthcare) following manufacturer’s protocol. The buffer was finally exchanged to 1× PBS for photocrosslinking experiments. For co-crystallization, the His-tagged IpgC and His-tagged IpaB16–72 was purified using Superdex 200 in 20 mM HEPES (pH 7.4), 150 mM NaCl. IpgC and IpaB16–72 was mixed in molar ratio of 2:1. Co-crystallization was set up at a final concentration of 15 mg/ml.

Photocrosslinking—Plasmid pSup-BpaRS-6TRN (a kind gift from Dr. Peter G. Schultz) (33), having a chloramphenicol resistance was co-transformed with either pMKbxxx (ipaB) or pMKcxxx (ipaC) genes along with 6× histidine-tagged ipgC gene (pMK001) having ampicillin and kanamycin resistance respectively into BL21(DE3). Bacteria were incubated at 37 °C in LB containing 100 μg/ml ampicillin, 50 μg/ml chloramphenicol, 30 μg of kanamycin, and 1 mM Bpa. At A600 = 0.6, expression was induced by the addition of 0.5 mM IPTG and incubated for 4 h. Cells were harvested by centrifugation and lysed with French press, and protein complex was purified exploiting the His tag in IpgC. The protein complex was exchanged to 1× PBS. Purified protein concentration was measured by the Bradford method. Crosslinking reactions were performed in a 24-well microtiter plate (Nunclon, Nalge Nunc, Denmark) by using 300 μl of protein complex on ice. Samples were irradiated at 365 nm by using a UV Stratalinker 2400 (Stratagene, 15 Watts) for 0, 15, and 30 min. Samples were removed from the wells and boiled with SDS loading buffer before resolution of products by SDS–PAGE on a 12% gel.

Limited Proteolysis—For limited proteolysis purified IpaB/IpgC and IpaC/IpgC complexes were digested with thermolysin and trypsin on ice and at 25 °C with protein at a concentration of 1 mg/ml in 20 mM HEPES (pH 7.4), 125 mM NaCl and trypsin on ice and at 25 °C with protein at a concentration of 1 mg/ml in 20 mM HEPES (pH 7.4), 125 mM NaCl for digestion with thermolysin and similar buffer at pH 8.0 in case of digestion with trypsin. The enzyme-substrate ratio was 1:100 (by weight) in each case. At different time intervals, aliquots (15 μl) were sampled and boiled immediately in 10 μl of 2× SDS sample buffer for 5 min to stop the proteolysis and stored at −20 °C. The protein samples were analyzed on 16.5% criterion peptide gel (Bio-Rad).

Secretion Assay—Secretion assay was performed as described (13). Cultures of exponentially growing Shigella were standardized by measuring the A600 and harvested. Crude bacterial extracts were obtained from the pellets, and proteins of filtered (0.2-μm pore size) culture supernatants were precipitated with 10% trichloroacetic acid. Protein expression and secretion was analyzed in basal conditions from pellets and supernatants, respectively of cultures grown without specific inducers. Protein samples were analyzed by 12% SDS–PAGE. Immunoblotting procedures were carried out with antibodies as applicable.

Virulence Assays—Infections of HeLa cells were performed as previously described (39) using a multiplicity of infection of 100. Briefly, HeLa cells infected for 20 min were incubated in the presence of gentamicin (100 μg/ml) for an additional 1 and 2 h. Intracellular bacteria were determined after lysing the infected cells, plating dilutions of the lysates, and counting.
the CFU. The standard error was calculated based on at least three independent determinations done in triplicates.

Isothermal Titration Calorimetry (ITC)—Titration experiments were carried out using a VP-ITC isothermal titration microcalorimeter (MicroCal, Northampton, MA). IpgC titrations were performed by injecting consecutive 12-µl aliquots of 1 mM IpgC or 2 mM IpgCdm into 1.4 ml of Strep-IpaB16–72 and Strep-tag solution (0.04 mM) in the sample cell in separate experiments. Heats of injection were corrected by subtraction of heats of dilution generated by injecting 1 mM IpgC or 2 mM IpgCdm into the sample cell containing buffer (20 mM HEPES pH 7.5, 150 mM NaCl). Synthetic Strep-tag peptide was dissolved in and the proteins dialyzed against 20 mM HEPES pH 7.5, 150 mM NaCl. Binding stoichiometry, enthalpy, and equilibrium association constants were determined by fitting the corrected data to one set of sites model equation using the evaluation software provided by the manufacturer.

Multi-angle Laser Light Scattering (MALLS)—For mass determination a combined setup consisting of size exclusion chromatography (SEC) and subsequent online detection by UV absorption, (three angle) static laser light scattering and differential refractive index measurement was used as described earlier (11). SEC was performed with a Tricorn Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 20 mM HEPES (pH 7.5), 150 mM NaCl. For static light scattering and differential refractive index measurements a linear coupled miniDAWN™ Tristar (Wyatt Technology) system and a differential refractive index detector (RI-101, Shodex), respectively, was used. All calculations were done with the software ASTRA (Wyatt Technology). Each experiment was repeated at least in triplicate.

Crystallization and Data Collection—IpaB16–72/IpgC co-crystals were grown at 18 °C by hanging drop vapor diffusion against 0.1 M HEPES (pH 7.5) and 0.8 M potassium sodium tartrate tetrahydrate. Crystals belong to space group P3,21, with two IpgC molecules and one IpaB peptide in the asymmetric unit (solvent content 65%). Diffraction data were collected using crystals flash-frozen in crystallization buffer containing 30% (v/v) glycerol, at the wavelength of 0.91841 Å in BESSY (BL14.2), Berlin, at 2.65 Å resolution. Indexing, integration, merging and scaling were done using the program XDS (40).

Crystal Structure Determination—The co-crystal IpaB16–72/IpgC belongs to the same crystal form as the apo IpgC structure, which we already solved (PDB ID 3GYZ). Cycles of manual building and refinement using Coot (41) and CNS version 1.21 (42) led to the final structure, which includes residues 8–151 of IpgC copy A and 9–151 of copy B, residues 64–70 of IpaB peptide bound to the IpgC copy B, 12 waters, 2 glycerol molecules, and 1 imidazole. A final TLS refinement was performed with REFMAC version 5.5.0072 (43), defining two groups for each IpgC molecule including residues 8–32 and 33–151, respectively, and adding another TLS groups for the IpaB peptide.

RESULTS

IpaB Yields a Stable Core whereas IpaC Is Susceptible to Proteolytic Cleavage—To investigate the role of the chaperone IpgC in the expression and stability of IpaB and IpaC, we heterologously expressed these proteins alone or in various combinations in E. coli (Fig. 1A). Individually, expression of ipaB led to death of E. coli as evidenced by decreasing cell density (Fig. 1B), and this correlates with low protein levels of IpaB evaluated by immunodetection assays (32). IpaC, though expressed in significant amounts, remained mostly insoluble when standard expression conditions were employed (32). Although heterologous co-expression of the ipaB/ipaC could
be observed (Fig. 1A), the complex aggregated during purification. Cytosolic expression of *ipaB* and enhanced solubility of IpaC was achieved when each was co-expressed with *ipgC*, indicating the chaperones role in maintaining stability, inhibiting unspecific aggregation of substrates and toxicity to *E. coli*. This prompted us to probe the role of IpgC in conferring conformational stability to IpaB and IpaC by performing limited proteolytic cleavage on the complex of IpaB or IpaC with IpgC.

The co-purified complexes of IpaB/IpgC and IpaC/IpgC were subjected to limited proteolysis combined with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and/or tandem MS (MS/MS) and Edman sequencing. Treatment of the purified IpaB/IpgC complex with a narrow-specificity protease, trypsin, yielded a stable 50 kDa core of IpaB (Fig. 1C) comprising residues from 16–482 (supplemental Fig. S1). This indicates that the chaperone-bound IpaB restricts access to trypsin beyond Lys-15. We had earlier reported proteolytic cleavage of IpaB/IpgC complex with a broad-specificity protease, thermolysin, in which the IpaB core had an N terminus at Ile-51 (11). Whereas the latter result is consistent with reported CBD in IpaB as residue 58–72 from Y2H analysis (30), the difference in the cleavage specificity of these two proteases is interesting considering the possibility of a putative additional or longer CBD. The stable core of IpgC had the N terminus at Leu-3 and except for the amino acid regions 61–83 and 117–140, the entire IpgC could be identified by MS. Similar treatment of the IpaC-IpgC complex with either trypsin (Fig. 1D) or thermolysin (supplemental Fig. S2) resulted in rapid and complete degradation of IpaC. This implies IpaC, unlike IpaB, is susceptible to proteolytic degradation and therefore is not stably folded, even in the presence of IpgC.

**IpgC Binding Regions in IpaB and IpaC**—To characterize the putative chaperone binding region in IpaC, we carried out tryptic digestion of the IpaC/IpgC complex for a time period sufficient to obtain fragmented IpaC. After 15 min on ice, we quenched the proteolysis by adding the trypsin inhibitor phenylmethylsulfonyl fluoride (PMSF) and immediately sub-quenched the proteolysis by adding the trypsin inhibitor and preservation of IpgC (supplemental Fig. S3). The MALDI-MS analysis of the eluate yielded five fragments of IpaC covering the different putative chaperone binding regions (see above) individually or in combination (Fig. 2). We co-expressed and purified these *ipaC* constructs with His-tagged IpgC. We first investigated the binding of N-terminal IpaC1–126 and C-terminal IpaC174–360 moieties to IpgC separately. Though both constructs were soluble, we observed that only the N-terminal moiety was bound to IpgC (Fig. 2, B and C). Next, we tested the binding of the construct IpaC126–363, which, could not be co-purified with IpgC. Thus, the fragment which included four of the five putative chaperone binding domains did not bind to IpgC. Therefore, we tested IpaC1–126 that comprises the remaining putative CBD spanning residues 47–75. This construct exhibited low solubility, hence its association to IpgC could not be validated. Therefore, we examined the constructs addressing this region i.e. IpaC1–75 and IpaC75–126. The former overlaps a region, IpaC50–80, previously characterized by fluorescence polarization and FRET (31), while the latter contains IpaC73–122, identified by Y2H analysis (30) as the regions interacting with IpgC. Both fragments were soluble and co-purified with IpgC. The construct IpaC76–363 that included the putative CBD identified by Page et al. (30) also co-purified with IpgC.

Thus, from limited proteolysis coupled with SEC and subsequent pull-down experiments, we assigned the CBD in IpaC to be within the N-terminal region 1–126. To map precisely the boundaries of CBD, we employed a technique based on Ryu and Schultz (33) to site-specifically introduce artificial photoexcitable amino acid *i.e.* p-benzoyl phenylalanine (Bpa). This method works by introducing an amber mutation at a specific position in the gene. Then the gene is co-expressed with an amber suppressor tRNA and an aminoacyl-tRNA synthetase for Bpa. Co-expression of the above in medium supplemented with Bpa results in incorporation of Bpa at the amber site in the modified protein. The involvement of the specific amino acid position in intermolecular association could be assessed by UV crosslinking (365 nm) and observing the formation of covalent bond as evidenced by an upward shift in SDS-PAGE gel. We generated IpaC mutants containing Bpa individually at the amino acid positions-29, 33, 36, 42, 50, 60, 64, 68, 73, 80, 84, 88, 99, 116, 120, 127, 146, and 154 to be co-expressed with *ipgC*. Each of these IpaC mutants was co-purified with IpgC, UV crosslinked for two different time intervals and subjected to SDS-PAGE to test for the formation of an intermolecular covalent bond (Fig. 3, A and B). A covalent complex between IpaC and IpgC upon UV excitation was observed for Bpa at positions 36, 42, 50, 60, 64, and 68 in the translocator. A weak coupling was observed for IpaC having Bpa at position 80 after extended exposure. It is possible that the region IpaC75–126 associated with IpgC in pull-down assays because it is enriched with hydrophobic residues. Hence, using a combination of different techniques we could define the CBD in IpaC to be between amino acids 33 and 73 with the region IpaC75–126 interacting, presumably, hydrophobically.

Combining our observations from limited proteolysis and Y2H analysis (30) we defined positions for site-specific photocrosslinking to precisely map the boundaries of CBD in IpaB. We generated mutations of *ipaB* for introducing Bpa individually at positions 45, 48, 51, 55, 59, 62, 65, 68, 74, and 79 to be co-expressed and co-purified with *ipgC*. IpaB containing Bpa at positions 51, 55, 62, 65, 68 formed a complex with IpgC upon UV excitation. This result maps the CBD in IpaB to be between residues 48 and 74 (Fig. 3C).
CBD Mutant IpaB<sub>51–72</sub> Exhibits Wild-type Phenotype—To determine the effect of the CBD, IpaB<sub>51–72</sub>, on TTS, we generated N-terminal (IpaB/H9004<sub>1–50</sub>, IpaB/H9004<sub>1–72</sub>) and internal (IpaB/H9004<sub>51–72</sub>) deletions. All mutants were tested for their ability to complement a <i>S. flexneri</i> non-polar ipaB deletion mutant (ΔipaB) for invasion of HeLa cells using a gentamicin protection assay (Fig. 4A). We found N-terminal truncations, IpaB<sub>51–72</sub> unable to restore invasion, whereas IpaB<sub>1–126</sub> showed invasiveness similar to wild-type <i>S. flexneri</i>. Next, we analyzed mutant IpaB expression and secretion in the bacterial extracts and culture supernatants by immunoblotting (Fig. 4B). ΔipaB strains complemented with ipaB<sup>wt</sup> or ipaB<sub>51–72</sub> produced and secreted the translocator protein. In contrast, IpaB<sub>51–72</sub>
IpaB is localized to the bacterial cytoplasmic membrane. The production of IpaB, which is essential for the secretion of IpaC, is detected in cellular extract but not secreted, while the production of IpaB [H9004] 1–72 was severely abrogated. To further decipher the effect of the CBD, IpaB [H9004] 1–72, on interactions with IpgC, the IpaB mutants described above were heterologously co-expressed with IpgC and tested for their ability to associate with IpgC. E. coli co-expressing IpaB [H9004] 1–72/IpgC exhibited a phenotype similar to heterologous expression of IpaBwt alone leading to cell death upon induction of gene expression (data not shown). Moreover, the co-purification of IpaB [H9004] 1–72 and IpgC could not be detected on SDS-PAGE (Fig. 4C). Lack of co-purification with IpgC due to reduced expression or non-stability of IpaB [H9004] 1–72 implies no interaction of residues downstream of 72 with IpgC. To our surprise, both IpaB [H9004] 1–50 and IpaB [H9004] 51–72 could be co-purified with IpgC. Co-expression and co-purification of IpaB [H9004] 51–72 or IpaB [H9004] 1–50 but not of IpaB [H9004] 1–72 with IpgC (Fig. 4C) indicated the presence of an additional CBD upstream to residue 51 in IpaB. Furthermore, results from the secretion and invasion assays corroborate the functional influence of this region in imparting stability to IpaB through binding to IpgC.

A Novel CBD in IpaB Is between Residues 15 and 45—To rule out possible structural rearrangement around the CBD contributing to the association of the mutant, IpaB [H9004] 51–72 with IpgC, we constructed a chimeric IpaB, IpaBPS. In IpaBPS amino acids 51–72 were replaced by a PreScission cleavage site (34) and flanked by a 4 amino acids linker on either side to disrupt the local secondary structure. Indeed, this construct co-purified with IpgC, further supporting the existence of an additional CBD within N terminus 50 residues in IpaB ((lane Ni-NTA, Fig. 4D)). Cleavage of IpaBPS with PreScission protease was inefficient and led to precipitation of IpaBPS/IpgC complex, signifying stability imparting role of N-terminal region in IpaB.

Using site-specific photocrosslinking we proceeded to precisely screen the additional chaperone binding site upstream to residue 50 in IpaB. We generated ipaB constructs having Bpa at positions 10, 15, 18, 22, and 35 and co-expressed each with IpgC. The purified complex showed intermolecular covalent binding for Bpa, upon subjection to UV light at positions 18, 22, and 35 (Fig. 4E), corroborating the presence of an ad-
ditional binding site within amino acids 15 to 45. This region in IpaB will be referred as CBD1 to distinguish it from the downstream CBD, IpaB51–72, which will be henceforth called CBD2.

We next analyzed if the N terminus of CBD1 overlaps with the secretion signal in IpaB. For this purpose we complemented ΔipaB (SF620) for epithelial cell invasion and secretion. Ĉ, co-expression and co-purification of IpaB mutants as indicated with IpgC. Samples were from pull down of corresponding IpaB mutant with His6 IpgC. IpaB51–72/IpgC was additionally subjected to SEC. ĉ, co-purification of IpaB with IpgC. The chimeric IpaBPS, in which residues 51–72 are replaced with a PreScission cleavage site, associated with IpgC. Cleavage with PreScission protease was inefficient and led to precipitation. Ĝ, photoreactive Bpa is introduced into IpaB at positions indicated. The complex of IpaB/IpgC is denoted with an asterisk. Ĝ, expression and secretion of wild-type and mutated IpaB were analyzed by immunoblot. DnaK was used as an indicator for bacterial membrane integrity and as a loading control. Although IpaB was detected in all bacterial extracts only culture supernatants of ΔipaB complemented with ipaBWT or ipaBAS–SS indicated IpaB secretion.

**CBD1 Defines the Ternary Arrangement of IpaB/IpgC Complex**—To investigate the influence of CBD1 on the architecture of the substrate-chaperone complex, we subjected the IpaB51–72/IpgC complex to SEC (Fig. 5A). Remarkably, in the absence of CBD1, IpaB51–50/IpgC forms soluble aggregate of higher molecular weight, while the wild-type IpaBWT/IpgC complex elutes as ternary complex of stoichiometry 1:2, (Fig. 5B) as confirmed by MALLS.

We had earlier reported heterotetrameric architecture of IpaB51–72/IpgC complex with two IpaB peptides interacting with one IpgC dimer in the co-crystal structure (11). To address the influence of CBD1 in the ternary arrangement found by MALLS, we solved the crystal structure of IpgC complexed with a fragment of IpaB encompassing both CBD’s i.e. residues 16–72 (Table 1, supplemental Fig. S5; purification scheme of IpaB16–72). A notable difference from the earlier reported heterotetrameric IpaB51–72/IpgC co-crystal structure is the presence of a single IpaB peptide bound to the IpgC dimer in IpaB16–72/IpgC co-crystal (Fig. 5C). The crystallographic asymmetric unit contains the same dimer as the apo IpgC structure (11). The apo and IpaB16–72 complexed IpgC
dimers superimpose well with a Cα root mean-square deviation of 0.4 Å. Seven residues covering Ile64 to Pro70 in IpaB were traceable in the electron density in the substrate binding cleft of one subunit in IpgC dimer, with a conformation similar to what is found in the IpaB51–72/IpgC complex (Cα root-mean-square deviation of 1.17 Å). The peptide is oriented
antiparallel to the helix H2 of IpgC with a solvent surface area of 450 Å² buried between the peptide and IpgC. When the peptide-bound subunits of the IpaB16–72/IpgC and IpaB51–72/IpgC complexes are superimposed, IpaB residues Ile64 and Ala69 are the farthest apart, with a Cα distance of 3.2 and 2.0 Å, respectively. As in the IpaB51–72/IpgC complex, extensive interactions mediate binding of the peptide, with key residues Pro65, Leu67, and Pro70 occupying the pockets P1, P2, and P3 presented by the cleft of IpgC (11). However, no electron density was observed in the cleft of the other subunit. Although the lack of electron density for residues 16–63 limits structural interpretation, the influence of CBD1 in the ternary arrangement is likely. Furthermore, we corroborated ternary arrangement by subjecting the IpaB/IpgC complex to native PAGE (Fig. 5D).

We used isothermal titration calorimetry to directly measure the binding of purified Strep-IpaB16–72 to dimeric IpgC or monomeric IpgC (IpgC(Dm), (11)) (Fig. 5E, left and right). An equilibrium dissociation constant (Kd) of 15.2 ± 0.6 μM and 146 ± 5 μM was observed for Strep-IpaB16–72 to dimeric IpgC and IpgC(Dm), respectively. Furthermore, to check the effect of Strep-tag, either forms of IpgC were titrated into a solution of Strep-tag. The contribution from Strep-tag was found to be negligible (supplemental Fig. S6, A and B).

**DISCUSSION**

In the cytoplasm of *Shigella*, IpgC has been shown to interact with IpaB and IpaC independently (13). Inactivation of *ipgC* led to loss of virulence in HeLa cells, possibly due to decreased stability of IpaB and IpaC (13). We and others have shown that heterologous expression of *ipaB* leads to the death of *E. coli* and IpaB is observable only by immunoblot while IpaC accumulates in inclusion bodies (32). However, improved expression of *ipaB* and better solubility of IpaC is achieved in *E. coli* when co-expressed with *ipgC*.

In this report we characterized the IpgC binding regions in IpaB and IpaC to gain insights into the molecular function, including the protective role, of IpgC. Proteolysis as a protein footprinting tool in mapping the putative CBD of IpaC in IpaC/IpgC did not yield any conclusive leads, as the effector was degraded completely. We adopted an improved approach to map the CBD in IpaC using limited proteolysis followed by SEC. Based on MS analysis of the eluted fractions and pull-down experiments we assigned the CBD to the N-terminal 126 residues in IpaC. This region comprises the fragments previously identified as the CBD in IpaC by biochemical and genetic approaches i.e. residues 50–80 and 73–122 (30, 31). We mapped the boundaries of the CBD to residues 36–68 using site-specific photocrosslinking. Furthermore, the region IpaC79–126 associated with IpgC in pull-down. A weak complex of IpgC with residue 80 in IpaC was formed, suggesting an overlap of reported CBDs. In the light of this it remains to be seen how these regions in IpaC interact with IpgC by high resolution techniques such as x-ray crystallography or NMR to gain insights into the exact nature and the region(s) interacting.

We have shown that the protease resistant core of IpaB in the IpaB/IpgC complex starts at amino acid 51. Furthermore, we precisely mapped the boundaries of this CBD to residues 51–72 in contact with IpgC using site-specific photocrosslinking (33). This data is in agreement with the Y2H assignment of CBD as IpaB58–72 (30). Importantly, using deletion mutants in combination with photocrosslinking technique we identified an additional CBD in IpaB (CBD1) between residues 15 and 45. This novel CBD has important implications in maintaining the exact stoichiometry of IpaB/IpgC complex. This is the first time to our knowledge that the photocrosslinking technique has been successfully applied in mapping protein-protein interacting regions. Previous studies using the site specific crosslinking simply showed whether protein-protein interactions exist. Our results define the interactions necessary for ternary arrangement of IpaB/IpgC complex. We propose a 1:2 IpaB/IpgC stoichiometry (see working model, Fig. 6A) based on several lines of evidence. MALLS analysis of IpaB/IpgC showed a mass of 100 kDa, suggesting a substrate/chaperone stoichiometry of 1:2. Previously we have shown that in vivo and in vitro IpgC is a biological dimer and the stability of IpaB depended on the dimeric state of IpgC (11). Ternary stoichiometry was corroborated by native gel-electrophoresis. Moreover, excess IpgC in the IpaB bound or unbound state migrated as a dimer, indicating that indeed IpgC is a dimer (11) both in the complexed and apo form (11). We presented the IpaB16–72/IpgC co-crystal structure, which may mimic the physiological interaction of the IpaB fragment encompassing entire region in IpaB i.e. CBD 1 and 2 that associates with IpgC. Here, the crystallographic asymmetric unit consisted of an IpaB bound to an IpgC dimer. The newly identified CBD1

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**TABLE 1**

Data collection and refinement statistics for IpaB16–72/IpgC

| Data collection | Space group |
|-----------------|-------------|
| Cell dimensions | P3,21 |
| a, c (Å)        | 113.72, 76.37 |
| Resolution (Å)  | 40-2.65 (2.80-2.65) |
| Rmerge or Rmerge | 0.069 (0.845) |
| I / σ(I)        | 26.38 (3.40) |
| Completeness (%)| 92.6 (90.4) |
| Redundancy      | 17.3 (13.6) |

**Refinement**

| Resolution (Å)  | 35.60-2.65 (2.72-2.65) |
| No. reflections | 15633 (1109) |
| Rmerge or Rmerge | 0.233 (0.454/0.512) |
| B-factors |
| Protein (IpgC) | 77.80 |
| Protein (IpaB16–72) | 141.87 |
| Ligand/ion | 99.13 |
| Water | 59.66 |

**R.m.s. deviations**

| Bond lengths (Å) | 0.021 |
| Bond angles (°)  | 1.894 |

**Ramachandran plot**

| Residues in most favored regions | 86.7% |
| Residues in additionally allowed regions | 12.9% |
| Residues in generously allowed regions | 0.4% |
| Residues in disallowed regions | 0.0% |

*Values in parentheses are for highest-resolution shell.

*Rmerge* is calculated using 5% of reflections randomly chosen.
In summary, we have mapped the CBDs in IpaB and IpaC. In IpaB, we have mapped an additional novel CBD which, in conjunction with IpgC, is essential in maintaining the stoichiometry of IpaB/IpgC complex. We have reported the three-dimensional structure of IpaB/IpgC complex, which reveals its physiological state. The interaction of the additionally characterized CBD in IpaB with IpgC may explain how it blocks the putative substrate binding site in IpgC dimer. The binding to the chaperone immediately after the signal sequence of IpaB in conferring a selective advantage in the spatial regulation of translocator secretion needs to be addressed.

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