The Haemophilus influenzae Hia Autotransporter Contains an Unusually Short Trimeric Translocator Domain*

Received for publication, October 20, 2003, and in revised form, January 14, 2004.
Published, JBC Papers in Press, January 15, 2004, DOI 10.1074/jbc.M311496200

Neeraj K. Surana‡§, David Cutter‡, Stephen J. Barenkamp‡, and Joseph W. St. Geme III‡¶

From the ‡Edward Mallinckrodt Department of Pediatrics and Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110 and the ¶Department of Pediatrics, St. Louis University School of Medicine, St. Louis, Missouri 63104

Gram-negative bacterial autotransporter proteins are a growing group of virulence factors that are characterized by their ability to cross the outer membrane without the help of accessory proteins. A conserved C-terminal β-domain is critical for targeting of autotransporters to the outer membrane and for translocation of the N-terminal “passenger” domain to the bacterial surface. We have demonstrated previously that the Haemophilus influenzae Hia adhesin belongs to the autotransporter family, with translocator activity residing in the C-terminal 319 residues. To further insight into the mechanism of autotransporter protein translocation, we performed a structure-function analysis on Hia. In initial experiments, we generated a series of in-frame deletions and a set of chimeric proteins containing varying regions of the Hia C terminus fused to a heterologous passenger domain and discovered that the final 76 residues of Hia are both necessary and sufficient for translocation. Analysis by flow cytometry revealed that the region N-terminal to this shortened translocator domain is surface localized, further suggesting that this region is not involved in β-barrel formation or in translocation of the passenger domain. Western analysis demonstrated that the translocation-competent regions of the C terminus migrated at masses consistent with trimers, suggesting that the Hia C terminus oligomerizes. Furthermore, fusion proteins containing a heterologous passenger domain demonstrated that similarly small C-terminal regions of Yersinia sp. YadA and Neisseria meningitidis NhhA are translocation-competent. These data provide experimental support for a unique subclass of autotransporters characterized by a short trimeric translocator domain.

Secretion of proteins in Gram-negative bacteria is complicated by the presence of two membranes in the cell envelope. To overcome this barrier, organisms have evolved a variety of protein secretion systems that differ in part, by the mechanism of translocation across the outer membrane. In some cases, translocation across the outer membrane is accomplished via a channel that spans the cytoplasmic membrane, periplasm, and outer membrane, allowing translocation of the protein directly from the cytoplasm to the extracellular milieu. In other cases, the polypeptide is transported across the cytoplasmic membrane and into the periplasm via the Sec apparatus and then is translocated across the outer membrane by a pore-forming complex. The individual pathways of this latter class of protein secretion systems are defined by the nature of the pore formed in the outer membrane. For example, the secretin proteins of the Type II secretion pathway form very large ring-shaped structures, with 10–14 subunits giving rise to a pore with an external diameter of 14–20 nm and an internal diameter of 5–10 nm (1). The PapC usher required for the assembly of P pilus on uropathogenic Escherichia coli forms a hexameric ring, with an external diameter of ~12 nm and an internal diameter of ~2 nm (2). Autotransporter proteins, named for their ability to traverse the outer membrane without the assistance of accessory proteins, have long been thought to be translocated through a monomeric pore. However, recent evidence presented by Veiga et al. (3) suggests that the pore formed by the Neisseria gonorrhoeae IgA1 protease is oligomeric, with at least six subunits, and has an external diameter of ~9 nm and an internal diameter of ~2 nm.

Autotransporters are a rapidly growing family of proteins that are being recognized increasingly as virulence factors in pathogenic bacteria. These proteins are present in diverse organisms and mediate a wide array of functions, including proteolysis, adherence, invasion, and cytotoxicity (4). Members of the autotransporter family are characterized by three domains. An N-terminal signal sequence directs the polypeptide through the Type II secretion pathway form very large ring-shaped structures, with 10–14 subunits giving rise to a pore with an external diameter of 14–20 nm and an internal diameter of 5–10 nm (1). The PapC usher required for the assembly of P pilus on uropathogenic Escherichia coli forms a hexameric ring, with an external diameter of ~12 nm and an internal diameter of ~2 nm (2). Autotransporter proteins, named for their ability to traverse the outer membrane without the assistance of accessory proteins, have long been thought to be translocated through a monomeric pore. However, recent evidence presented by Veiga et al. (3) suggests that the pore formed by the Neisseria gonorrhoeae IgA1 protease is oligomeric, with at least six subunits, and has an external diameter of ~9 nm and an internal diameter of ~2 nm.

Autotransporters are a rapidly growing family of proteins that are being recognized increasingly as virulence factors in pathogenic bacteria. These proteins are present in diverse organisms and mediate a wide array of functions, including proteolysis, adherence, invasion, and cytotoxicity (4). Members of the autotransporter family are characterized by three domains. An N-terminal signal sequence directs the polypeptide through the Sec apparatus and into the periplasm. A C-terminal β-domain then integrates into the outer membrane and forms a pore, allowing the internal “functional” domain, referred to as the passenger domain, to be presented on the bacterial surface (4). In the vast majority of cases, the passenger domain is cleaved from the β-domain and then either released into the extracellular medium or non-covalently tethered to the bacterial surface. Given the vast differences in passenger domain functions, the defining characteristic of an autotransporter lies in the ability of the β-domain to translocate the passenger domain. The distinctive nature of this translocation process has resulted in a great deal of study. Deletion analyses performed with a number of autotransporters have shown that the β-domain is generally ~300 residues in length (5–7). Secondary structure analyses of the β-domain of autotransporters predict that this region has an α-helix transmembrane strand followed by 14 transmembrane β-strands, which are believed to form a β-barrel (6–9). In contrast, recent evidence has shown that the trimer-forming C-terminal 70 residues of Yersinia sp. YadA are capable of translocating an N-terminal epitope tag to the surface, leading the authors to suggest that...
YadA belongs to a distinct subfamily of oligomeric autotransporters (10).

In a previous work (11), we demonstrated that the Haemophilus influenzae Hia adhesin is an autotransporter protein, establishing that residues 780–1098 harbor translocator activity. More recently, we discovered that Hia contains two distinct binding domains, both of which mediate attachment to epithelial cells via the same host cell receptor, although with differing affinities (12). The primary high-affinity binding domain resides within residues 541–714, whereas the secondary binding domain is contained in residues 50–374 and binds to host cells with 20-fold decreased affinity. In the present study, we examined the Hia β-domain in greater detail. Using a series of deletion mutants and chimeric proteins, we found that the C-terminal 76 residues are both necessary and sufficient for translocation of a passenger domain. In addition, we discovered that the Hia C terminus oligomerizes, forming a trimer. Further analyses revealed that the C termini of YadA and Neisseria meningitidis NhaA are sufficient for surface localization of foreign polypeptides. Our results extend previous work performed with YadA and clearly demonstrate the existence of a subfamily of autotransporter proteins, typified by a short translocator domain that undergoes trimer formation to form a β-barrel.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—Non-typeable H. influenzae (NTHi) strain 11 is the strain from which hia was originally cloned and has been described previously (13). NTHi strain 32 was recovered from the middle ear fluid of a child with acute otitis media. Laboratory strains employed in these studies included H. influenzae strain DB117 (rec+/+) (14), E. coli strain DH5α (Invitrogen), and E. coli BL21(DE3) (15). N. meningitidis strain BB-1148 is a serotype C strain and was a generous gift from Carl Frasch (Food and Drug Administration, Bethesda, MD).

The plasmid pGJB103 containing the hap gene from H. influenzae strain N187 with a point mutation at the codon encoding the active-site serine (9). The plasmid pDH106 contains the coding sequence for the Hap passenger domain (residues 1–1054) with an engineered Sall site at the 5′ end. The plasmid pHMW8-7 contains hia from H. influenzae strain 11 in pT7-7, and pHMW8-6 contains hia interrupted with a kanamycin cassette (13). The plasmid pHMW32-1 contains hia from NTHi strain 32 cloned into the Sall and EcoRi sites of pG7-T7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid pHAT10 encodes a novel polyhistidine binding domain, with six histidine residues spread throughout 19 EcoRI sites of pT7-7. The plasmid PHMW8-7 was used as a template to amplify the coding sequence for Hia residues 780–1098, 977–1098, 1023–1098, and 1047–1098 with an engineered Sall site at the 5′ end and an engineered BamHI site at the 3′ end. These PCR products were digested with Sall and BamHI and then ligated into Sall-BamHI-digested pHD106, generating pUC19::Hap::Hia780–1098, pUC19::Hap::Hia977–1098, pUC19::Hap::Hia1023–1098, and pUC19::Hap::Hia1047–1098. These plasmids were introduced into E. coli BL21(DE3) (15).

To generate plasmids encoding the HapS-Hia chimeras, pGJB103, generating pHpS::HiaSS. Transformants with the proper orientation of the insert were identified by nucleotide sequencing to ensure that the inserts were in-frame and that all PCR products were free of mutations. The fragment was ligated into a HindIII-digested alkaline phosphatase-rec1 DB117 (NTHi) strain 11 is the strain from which hia was originally cloned and has been described previously (13). NTHi strain 32 was recovered from the middle ear fluid of a child with acute otitis media. Laboratory strains employed in these studies included H. influenzae strain DB117 (rec+/+) (14), E. coli strain DH5α (Invitrogen), and E. coli BL21(DE3) (15). N. meningitidis strain BB-1148 is a serotype C strain and was a generous gift from Carl Frasch (Food and Drug Administration, Bethesda, MD).

The plasmid pGJB103 containing the hap gene from H. influenzae strain N187 with a point mutation at the codon encoding the active-site serine (9). The plasmid pDH106 contains the coding sequence for the Hap passenger domain (residues 1–1054) with an engineered Sall site at the 5′ end. The plasmid pHMW8-7 contains hia from H. influenzae strain 11 in pT7-7, and pHMW8-6 contains hia interrupted with a kanamycin cassette (13). The plasmid pHMW32-1 contains hia from NTHi strain 32 cloned into the Sall and EcoRi sites of pG7-T7. The plasmid pHAT10 encodes a novel polyhistidine epitope fused to varying Hia C-terminal regions, a fragment containing the hia promoter and coding sequence for the Hia signal sequence was amplified by PCR, engineering HindIII sites on both ends. After digestion with HindIII, the fragment was ligated into a HindIII-digested alkaline phosphatase-treated pHAT10, generating pHAT::HiaSS. Transformants with the proper orientation of the insert were identified by nucleotide sequencing, pUC19::Hap::Hia780–1098, pUC19::Hap::Hia977–1098, pUC19::Hap::Hia1023–1098, and pUC19::Hap::Hia1047–1098 were digested with Sall and BamHI to liberate inserts encoding Hia C-terminal regions. These inserts were then ligated into Sall-BamHI-digested pHAT::HiaSSS, generating pHAT::Hia780–1098, pHAT::Hia977–1098, pHAT::Hia1023–1098, and pHAT::Hia1047–1098. The plasmid pHMW8-7 was used as a template to amplify the coding sequence for Hia residues 820–1098, 853–1098, 872–1098, 889–1098, 911–1098, 928–1098, 948–1098, and 990–1098 with an engineered Sall site at the 5′ end and an engineered BamHI site at the 3′ end. PCR products were digested with Sall and BamHI and ligated into Salt-Sall-digested pHAT::HiaSS, generating pHAT::Hia780–998, pHAT::Hia853–1098, pHAT::Hia872–1098, pHAT::Hia911–1098, pHAT::Hia928–1098, pHAT::Hia948–1098, and pHAT::Hia990–1098. All constructs were examined by nucleotide sequencing to ensure that the inserts were in-frame and that all PCR products were free of mutations.

Cell Fractionation and Western Immunoblotting—Whole-cell sonicates were prepared by resuspending bacterial pellets in 10 ml HEPES (pH 7.4) and sonicating to clarify. Outer membrane proteins were recovered on the basis of Sarkosyl insolubility as described by Carline et al. (21). Unless otherwise noted, whole-cell sonicates and outer membrane fractions were treated with 95% formic acid as described previously (22). Where indicated, bacteria were incubated with cross-linkers (dithio bis(succinimidyl propionate) or 3,3′-dithiobis(succinimidyl propionate), Pierce) according to the manufacturer’s instructions prior to preparation of outer membrane proteins. Proteins were resolved by SDS-PAGE using 7.5–15% polyacrylamide gels. To ensure that comparable amounts of protein were analyzed, similar volumes from cultures of similar density were loaded into each lane. Western blots were performed with a guinea pig polyclonal antiserum raised against Hia residues 50–252, a guinea pig polyclonal antiserum raised against Hap, or a rabbit polyclonal antiserum raised against the HAT epitope (Clontech). An anti-guinea pig or anti-rabbit IgG antiserum conjugated to horseradish peroxidase (Sigma) was used as the secondary antibody. Detection of antibody reactivity was accomplished by incubation of the membrane in 14-aminobenzoic acid and exposure to film.
bacteria was fixed for 30 min with 4% paraformaldehyde phosphate-buffered saline, washed once with Tris-buffered saline, and resuspended in 0.5 ml of Tris-buffered saline containing 50 mM EDTA, 0.1% bovine serum albumin, and the appropriate primary antibody diluted 1:1000. Samples were washed twice with phosphate-buffered saline and resuspended in 0.25 ml of Tris-buffered saline containing 50 mM EDTA, 0.1% bovine serum albumin, and Cy2-conjugated secondary antibody (Jackson ImmunoResearch; 1:100). Incubations with the primary and secondary antibodies were for 1 h each. After incubation with the secondary antibody, samples were washed twice with phosphate-buffered saline, and surface-localized protein was measured by flow cytometry on a FACS Calibur (BD Biosciences). Data were analyzed with Cellquest software (BD Biosciences).

**Quantitative Adherence Assays—**Adherence assays were performed with Chang epithelial cells (Wong-Kilbourne derivative, clone 1-5c-4, human conjunctiva, ATCC CCL20.2) or with A549 respiratory epithelial cells (ATCC CCL185) as described previously (23). Percent adherence was calculated by dividing the number of adherent colony-forming units/monolayer by the number of colony-forming units inoculated.

**Nucleotide Sequencing and DNA and Protein Sequence Analysis—**Nucleotide sequencing was performed using an Applied Biosystems automated sequencer and the BigDye Terminator Premix-20 kit (Applied Biosystems/PerkinElmer Life Sciences). Double-stranded plasmid DNA was used as template, and sequencing was carried out along both strands. Amino acid sequence alignments were performed using ClustalW at EBI (ebi.ac.uk/clustalw/).

**Nucleotide Sequence Accession Numbers—**The nucleotide sequence for *hia* from NTHi strain 32 has been deposited with GenBank™ and assigned the accession number AY443498.

**RESULTS**

**The C-terminal 76 Residues of Hia Are Necessary and Sufficient for Translocator Activity—**In initial studies, we compared the full-length Hia sequences from 10 different clinical isolates of NTHi and found significant overall similarity throughout the entire protein sequence. However, the protein expressed by strain 32 (Hia32) was found to be exceptional, lacking the sequence corresponding to residues 590–976 of Hia from the prototypic strain 11 (Hia11) (Fig. 1A). Interestingly, as shown in Fig. 1B, this truncation results in loss of the N-terminal seven β-strands of the predicted β-domain. Examination of outer membrane fractions by immunoblotting and surface-localized protein by flow cytometry revealed appreciable amounts of Hia in the outer membrane and on the surface of strain 32 (data not shown). Comparison of wild-type strain 32 and an isogenic Hia-deficient mutant (32hia) revealed that Hia32 was capable of mediating high levels of adherence to Chang cells, comparable with adherence by Hia11 (Fig. 1C). Consistent with this result, *E. coli* DH5α expressing Hia32 was highly adherent (Fig. 1C).

With this information in mind, we hypothesized that the functional translocator domain of Hia from other isolates may also be smaller than recognized previously. To explore this possibility, we constructed a series of in-frame deletions within the Hia11 C terminus, allowing the examination of progressively shorter regions of the Hia C terminus for the ability to translocate the Hia passenger domain. Western analysis demonstrated that all deletion mutants except for HiaΔ780–1046 were properly inserted into the outer membrane (Fig. 2A). Flow cytometry analysis using an antisemur raised against residues 221–658 of Hia11 revealed that full-length Hia, HiaΔ780–976, and HiaΔ780–1022 were all surface-localized, whereas the passenger domain in HiaΔ780–1046 was absent from the bacterial surface (Fig. 2B). Consistent with these flow cytometry data, in adherence assays with Chang cells, *E. coli* BL21(DE3) expressing HiaΔ780–976 or HiaΔ780–1022 was capable of wild-type levels of adherence. In contrast, BL21(DE3) expressing HiaΔ780–1046 was non-adherent, indistinguishable from BL21(DE3) harboring vector alone (Fig. 2C).

To extend these results, we generated fusions between the passenger domain of the *H. influenzae* Hap adhesin (HapP) and regions of the Hia C terminus comparable with those examined in the Hia deletion mutants. Hap has been shown previously to be an autotransporter that promotes microcolony formation and mediates adherence to cultured epithelial cells and extracellular matrix proteins (9, 24). After translocation of the pas-
senger domain (HapB) to the bacterial surface, Hap undergoes an autoproteolytic cleavage event that releases HapS into the extracellular milieu. Mutating the active site serine to an alanine (HapS243A) prevents this cleavage event, locking the adhesin in a fully cell-associated form and thus enhancing adhesive properties (9). HapS243A was used as a positive control in these experiments, as the HapS-Hia fusions lack the cleavage sites normally present in Hap. Western analysis of outer membrane proteins prepared from H. influenzae DB117 expressing HapS243A, HapS-Hia1023–1098, HapS-Hia977–1098, HapS-Hia1023–1098 or HapS-Hia1047–1098 revealed that all the chimeras except HapS-Hia1047–1098 were present in the outer membrane, albeit at reduced levels compared with HapS243A (Fig. 3A). Furthermore, flow cytometry analysis confirmed the presence of HapS on the bacterial surface for all constructs except HapS-Hia1047–1098 (Fig. 3B). Consistent with these data, all the chimeras except HapS-Hia1047–1098 were capable of promoting appreciable bacterial adherence to A549 cells, although to a lesser level than HapS243A (Fig. 3C). Considered together, these results indicate that the C-terminal 76 residues of Hia are both necessary and sufficient for translocation of a passenger domain.

Given that the translocator domain is much smaller than previously believed, we elected to explore further the role of residues 780–1022. Specifically, we wondered whether residues 780–1022 form transmembrane β-strands, as suggested by secondary structure predictions and depicted in Fig. 1B. One possibility is that these residues form additional transmembrane β-strands that are dispensable for translocator activity but are important in other undetermined ways. Alternatively, this region may be completely surface-localized, thus explaining why it is not required for translocator activity. To discriminate between these possibilities, we generated constructs consisting of the Hia signal sequence and the HAT epitope fused upstream of progressively shorter regions of the Hia C terminus. The arrowheads in Fig. 1B identify the starting residues for these constructs, which address every predicted periplasmic and extracellular loop. Western analysis of outer membrane proteins prepared from bacteria expressing these constructs confirmed proper localization of all proteins except Hia1047–1098, which was not present in the outer membrane (data not shown). Furthermore, flow cytometry analysis revealed that the HAT epitope was surface localized in all cases except Hia1047–1098 (data not shown). These data indicate that residues 780–1022 are surface localized and do not form transmembrane β-strands. Additionally, residues 1023–1046 are required for either Hia insertion into the outer membrane or Hia stability in the outer membrane.

The Hia C Terminus Undergoes Oligomerization—In considering the finding that the C-terminal 76 residues of Hia are able to translocate a passenger domain to the bacterial surface, we wondered about the mechanism by which this limited sequence might form a channel large enough to allow transit of the passenger domain. Studies performed with other outer membrane proteins have revealed that all form β-barrel structures with an even number of β-strands (25). Furthermore, the smallest β-barrels recognized to date are OmpA and OmpX, both consisting of eight β-strands (25). However, based on our secondary structure predictions of the Hia C terminus, Hia1023–1098 contains a maximum of only five β-strands (Fig. 1B). In this context, it is notable that when we have performed immunoblots on outer membrane proteins recovered from bacteria expressing wild-type Hia, we have observed higher order structures and have speculated that these may represent multimers (13). Accordingly, we hypothesized that the Hia C terminus undergoes oligomerization, incorporating a larger number of β-strands and forming a more typical β-barrel.

To test this hypothesis, we isolated outer membrane proteins from E. coli DH5α expressing HAT-tagged Hia1023–1098, Fe-
solved these proteins under standard denaturing conditions, and examined them by Western analysis. As shown in Fig. 4 (left lane), Hia1023–1098 migrated at a molecular mass consistent with a trimer. In contrast, when treated with formic acid, the sample migrated at its predicted molecular mass (Fig. 4, right lane). Samples resolved by native gel electrophoresis or prepared in the presence of a cross-linker did not reveal complexes larger than a trimer (data not shown). These data demonstrate that Hia1023–1098 forms a trimer in the outer membrane.

The C Termini of YadA and NhhA Harbor Translocator Activity—Based on the fact that an epitope tag placed directly upstream of the C-terminal 70 residues of YadA is surface-localized, Roggenkamp et al. (10) speculated that YadA defines a subfamily of oligomeric autotransporters. To extend these findings and assess whether the YadA C terminus truly harbors translocator activity and can present a functional polypeptide on the bacterial surface, we created a HapS-YadA325–422 chimera. In addition, to establish more definitively a subfamily of trimeric autotransporters with a short translocator domain, we examined the C terminus of N. meningitidis NhhA for translocator activity, generating a HapS-NhhA473–591 fusion protein. NhhA is an outer membrane protein that was first discovered upon sequencing the genome of strain MC58 and contains a C terminus that is highly conserved among diverse strains and serogroups and shares significant homology with the Hia and YadA C termini (26, 27). Adherence assays showed that both HapS-YadA325–422 and HapS-NhhA473–591 behaved like HapS-Hia1023–1098 and promoted attachment to A549 cells, indicating that both YadA and NhhA are autotransporters with short translocator domains (Fig. 5).

DISCUSSION

In earlier work (11), we demonstrated that Hia belongs to the autotransporter family and established that Hia translocator activity resides within residues 780–1098. In the present study, we discovered that the Hia translocator domain is much smaller than recognized previously. In particular, the final 76 residues are sufficient to translocate both the Hia passenger domain and a heterologous protein, reflecting the capacity to form a trimer.

Examination of the Yersinia sp. YadA and NhhA revealed that the C-terminal 98 amino acids of YadA and the C-terminal 119 amino acids of NhhA have translocator activity. In recent work, Roggenkamp et al. (10) demonstrated that the final 70 residues of YadA are sufficient for outer membrane localization and that epitopes N-terminal to this membrane anchor region are surface-localized, leading the authors to suggest that YadA belongs to a novel subfamily of autotransporters. Our results with HapS fusions extend the work of Roggenkamp et al. (10) and provide clear evidence that Hia, YadA, and NhhA belong to the same subfamily of autotransporter proteins with a small translocator domain.

Beyond possessing small translocator domains, Hia, YadA, and NhhA are unusual among autotransporters in several re-
H. influenzae DB117 expressing the indicated proteins. NhhA as autotransporters. Adherence to A549 epithelial cells by H. influenzae DB117 expressing the indicated proteins. Bars represent the means ± S.E.

spect. For example, these three proteins lack the consensus sequence that defines the N-terminal 20 residues in almost all other known autotransporters (8). In addition, Hia, YadA, and NhhA lack the intramolecular chaperone domain required for proper folding of Bordetella pertussis BrkA and conserved in a diverse set of autotransporters (28). Finally, Hia and YadA are two of very few autotransporters wherein the passenger domain remains covalently attached to the β-domain (there are currently no experimental data regarding this possibility for NhhA) (11, 29). In this context, it is notable that all proteins with a C terminus similar to Hia, YadA, and NhhA (defined by the conserved domain pfam03895) lack the consensus C-terminal sequence and the intramolecular chaperone domain. In addition, among the few proteins that have an Hia-like C terminus and have been characterized, the passenger domain uniformly remains covalently attached to the putative β-domain. Together these observations provide further evidence that Hia-like autotransporters represent a distinct subfamily of autotransporter proteins.

Given the small size of the Hia translocator domain and previous work suggesting multimerization of the N. gonorrhoeae IgA1 protease β-domain and the YadA C terminus (3, 10), we wondered whether the Hia translocator domain multimerizes. Indeed, we found that Hia1023–1098 exists in the outer membrane as a stable complex with a molecular mass consistent with a trimer. This observation is consistent with our recent finding that the primary binding domain of Hia also forms a trimer (40). Interestingly, Hia1047–1098 lacks translocator activity and fails to multimerize or insert into the outer membrane. The faint band observed in the outer membrane for Hia1047–1098 in Fig. 2A may be because of trimerization of the passenger domain, partially compensating for the inability of Hia1047–1098 to oligomerize or insert into the outer membrane. An alternative explanation for the faint band is that Hia1047–1098 is partially degraded in the periplasm.

Earlier studies suggested that YadA oligomerizes via a coiled coil domain (29); however, Hia1023–1098 has no predicted coiled coil region and must therefore oligomerize via a different mechanism. It is possible that residues 1023–1046 of Hia are critical for multimerization and that oligomerization is a prerequisite for insertion into the outer membrane, perhaps explaining why Hia1047–1098 is not localized to the outer membrane. Based on the correlation between multimerization and insertion into the outer membrane of Hia, we propose that the translocator domain multimerizes in the periplasm and inserts into the outer membrane as a trimer. Of note, porin subunits oligomerize in the periplasm and are unable to insert into the outer membrane as monomers (25).

Considering the constraints governing β-barrel formation (25), it is likely that Hia1023–1098 contains four, rather than the predicted five, β-strands resulting in an even number of total β-strands for the trimeric structure. Virtually all crystallized β-barrel proteins with 12 or fewer β-strands are unable to form a pore and have a shear number no larger than 12 (30). The one exception is TolC, which forms a 12-stranded β-barrel with a central pore and has an unusually large shear number of 20, a critical determinant of pore formation (30, 31). Hia1023–1098 is predicted to form a pore and therefore may have a similarly large shear number. Furthermore, each Hia1023–1098 monomer probably contributes its β-strands to a common β-barrel, similar to TolC and α-hemolysin (31, 32).

Given the limited size of the Hia translocator domain, it is interesting to consider the state of the passenger domain during translocation. It is currently unclear whether secretion of autotransporter proteins occurs in a folded or unfolded state. Early studies of autotransporters performed using cholera toxin B or PhoA as heterologous passenger domains suggested that the passenger domain must be unfolded during translocation (7, 11, 33). However, more recent evidence indicates that at least some passenger domains may fold in the periplasm and remain folded during the translocation process (34, 35). The recently determined crystal structure of the Hia primary binding domain reveals that it has a diameter of 4.5 nm (40), significantly larger than the ~2-nm pore size of classic autotransporter β-domains (3, 36). Moreover, it is difficult to envisage how the ~33-kDa Hia1023–1098 β-barrel forms a pore with a 4.5-nm diameter, indirectly suggesting that the passenger domain must fold on the surface of the organism. It is interesting to speculate that concerted folding and trimer formation of the passenger domain on the bacterial surface may provide the driving force for translocation.

In the course of our experiments, we observed that bacteria expressing a fusion protein consisting of Hap2 and the C terminus of Hia, Yersinia sp. YadA, or N. meningitidis NhhA were not able to adhere to epithelial cells as well as bacteria expressing HapS243A. This finding parallels observations with other chimeric autotransporter proteins (12, 16) and raises the possibility that a specific relationship exists between an autotransporter β-domain and its native passenger domain. This relationship may depend on the oligomeric state and pore size of the β-domain as well as the overall structure of the passenger domain, leading to more efficient translocation of a homologous rather than a heterologous peptide.

The finding that residues 780–1022 of Hia are not involved in the translocation process helps to resolve an apparent paradox related to Hia adhesive activity. As described in an earlier publication (12), we found that the Hia primary binding domain resides within residues 541–714, originally suggesting juxtaposition to the outer membrane and potential interference by other bacterial surface structures. Instead, residues 780–1022 are surface-localized, raising the possibility that this sequence serves as a spacer region, effectively distancing the primary binding domain from the bacterial surface and enhancing the likelihood of interaction with the host cell receptor.

It is intriguing to note that Hia32 contains only one binding domain, as identified by sequence homology. In contrast, Hia111 and the Hia sequences from other strains all contain two binding domains (12). Although sequence alignments cannot predict whether the Hia32 binding domain is functionally more similar to the Hia111 primary or secondary binding domain, the high levels of adherence attained with Hia32 suggest that its
binding domain is analogous to the Hia$_{11}$ primary binding domain. Further investigation is needed to address the precise nature of the Hia$_{11}$ binding domain as well as the role of the Hia$_{11}$ secondary binding domain.

To summarize, Hia, YadA, and NhhA appear to define a unique subfamily of autotransporters characterized by a short trimeric translocator domain. An improved understanding of structure-function relationships in these unusual translocator domains may provide general insights into bacterial protein secretion systems.

Acknowledgments—We thank Sven Laarmann for general discussions and Shane Cotter for critical reading of the manuscript.

REFERENCES

1. Stathopoulos, C., Hendrixson, D. R., Thanassi, D. G., Hultgren, S. J., St. Geme, J. W., III, and Curtiss, R., III (2000) Microbes Infect. 2, 1061–1072
2. Thanassi, D. G., Saulino, E. T., Lombardo, M. J., Roth, R., Heuser, J., and Hultgren, S. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3146–3151
3. Veiga, E., Sugawara, E., Nikiado, H., de Lorenzo, V., and Fernandez, L. A. (2002) EMBO J. 21, 2122–2131
4. Henderson, I. R., Navarro-Garcia, F., and Nataro, J. P. (1998) Trends Microbiol. 6, 370–378
5. Klaus, T., Kramer, J., Otzelberger, K., Pohliner, J., and Meyer, T. F. (1993) J. Mol. Biol. 24, 799–803
6. Maurer, J., Jose, J., and Meyer, T. F. (1999) J. Bacteriol. 181, 7014–7020
7. Suzuki, T., Lett, M. C., and Sasakawa, C. (1995) J. Biol. Chem. 270, 30874–30880
8. Loveless, B. J., and Safer, M. H., Jr. (1997) Mol. Membr. Biol. 14, 113–123
9. Hendrixson, D. R., de la Morena, M. L., Stathopoulos, C., and St. Geme, J. W., III (1997) Mol. Microbiol. 26, 505–518
10. Roggenkamp, A., Ackermann, N., Jacobi, C. A., Truelisch, K., Hoffmann, H., and Hessemann, J. (2000) J. Bacteriol. 183, 3735–3744
11. St. Geme, J. W., III, and Curtiss, R. (2000) J. Bacteriol. 182, 6005–6013
12. Laarmann, S., Cutter, D., Juehne, T., Barenkamp, S., and St. Geme, J. W., III (2002) Mol. Microbiol. 46, 731–743
13. Barenkamp, S. J., and St. Geme, J. W., III (1996) Mol. Microbiol. 19, 1215–1223
14. Setlow, J. K., Brown, D. C., Boling, M. E., Mattingly, A., and Gordon, M. P. (1968) J. Bacteriol. 95, 546–558
15. Studier, F. W., and Moffatt, B. A. (1986) J. Mol. Biol. 189, 113–130
16. Hendrixson, D. R., and St. Geme, J. W., III (1998) Mol. Cell 2, 841–850
17. Anderson, P., Johnston, R. B., Jr., and Smith, D. H. (1972) J. Clin. Invest. 51, 31–38
18. Sambrook, J., and Russel, D. W. (2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988) Nucleic Acids Res. 16, 6127–6145
20. Herriott, R. M., Meyer, E. M., and Vogt, M. (1970) J. Bacteriol. 101, 517–524
21. Carlone, G. M., Thomas, M. L., Rumschlag, H. S., and Sotnik, F. O. (1986) J. Clin. Microbiol. 24, 330–332
22. St. Geme, J. W., III, Kumar, V. V., Cutter, D., and Barenkamp, S. J. (1998) Infect. Immun. 66, 364–368
23. St. Geme, J. W., III, Falkow, S., and Barenkamp, S. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2875–2879
24. Fink, D. L., Buscher, A. Z., Green, B., Fernsten, P., and St. Geme, J. W., III (2003) Cell Microbiol. 5, 175–186
25. Schulz, G. E. (2000) Curr. Opin. Struct. Biol. 10, 443–447
26. Peak, I. R., Srikhanta, Y., Dieckelmann, M., Moxon, E. R., and Jennings, M. P. (2000) FEMS Immunol. Med. Microbiol. 28, 329–334
27. Pizza, M., Scarlato, V., Massignani, V., Giuliani, M. M., Arico, B., Cemanducci, M., Jennings, G. T., Baldi, L., Bartoloni, E., Capechi, B., Galeotti, C. L., Luzzi, E., Manetti, R., Marchetti, E., Mora, M., Nuti, S., Ratti, G., Santini, L., Savino, S., Scarselli, M., Sterni, E., Zuo, P., Broeker, M., Hundt, E., Knapp, B., Blair, E., Mason, T., Tettelin, H., Hood, D. W., Jeffries, A. C., Saunders, N. J., Granoff, D. M., Venter, J. C., Moxon, E. R., Grandi, G., and Rappuoli, R. (2000) Science 287, 1816–1820
28. Oliver, D. C., Huang, G., Nodel, E., Pleasance, S., and Fernandez, R. C. (2003) Mol. Microbiol. 47, 1367–1383
29. Hoiczyk, E., Roggenkamp, A., Reichenbecher, M., Lupas, A., and Hessemann, J. (2000) EMBO J. 19, 5898–5900
30. Schulz, G. E. (2002) Biochim. Biophys. Acta 1565, 308–317
31. Kiselev, N., Sharet, J. M., Mianski, E., and Luiss, H. (2000) Nature 405, 914–919
32. Song, L., Hobaugh, M. R., Shustak, C., Cheley, S., Bayley, H., and Gouaux, J. E. (1996) Science 274, 1559–1566
33. Klausner, T., Pohliner, J., and Meyer, T. F. (1992) EMBO J. 11, 2327–2335
34. Brandon, L. D., and Goldberg, M. B. (2001) J. Bacteriol. 183, 951–958
35. Veiga, E., de Lorenzo, V., and Fernandez, L. A. (1999) Mol. Microbiol. 33, 1232–1243
36. Shannon, J. L., and Fernandez, R. C. (1999) J. Bacteriol. 181, 5838–5842
37. Rost, B., and Sander, C. (1993) J. Mol. Biol. 232, 584–599
38. Altschul, S. F., and Lipman, D. J. (1997) J. Mol. Biol. 26, 367–384
39. Schirmer, T., and Cowan, S. W. (1993) Protein Sci. 2, 1361–1363
40. Yeo, H.-J., Cotter, S. E., Laarmann, S., Juehne, T., St. Geme, J. W., III, and Waksman, G. (2004) EMBO J., in press
The *Haemophilus influenzae* Hia Autotransporter Contains an Unusually Short Trimeric Translocator Domain
Neeraj K. Surana, David Cutter, Stephen J. Barenkamp and Joseph W. St. Geme III

*J. Biol. Chem. 2004, 279:14679-14685.*
doi: 10.1074/jbc.M311496200 originally published online January 15, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311496200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 16 of which can be accessed free at
http://www.jbc.org/content/279/15/14679.full.html#ref-list-1