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Shane E. Cotter
Washington University School of Medicine in St. Louis

Neeraj K. Surana
Washington University School of Medicine in St. Louis

Susan Grass
Duke University Medical Center

Joseph W. St. Geme III
Duke University Medical Center

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Journal of Bacteriology

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J. Bacteriol. **2006, 188(15):5400. DOI: 10.1128/JB.00164-06.**

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JOURNAL OF BACTERIOLOGY, Aug. 2006, p. 5400–5407 Vol. 188, No. 15
0021-9193/06/$08.00+0 doi:10.1128/JB.00164-06
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Trimeric Autotransporters Require Trimerization of the Passenger Domain for Stability and Adhesive Activity

Shane E. Cotter,[1] Neeraj K. Surana,[1] Susan Grass,[2] and Joseph W. St. Geme III[2]*

Edward Mallinckrodt Department of Pediatrics and Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, Missouri 63110,[1] and Departments of Pediatrics and Molecular Genetics and Microbiology, Duke University Medical Center, Children's Health Center, Durham, North Carolina 27710[2]

Received 30 January 2006/Accepted 12 May 2006

In recent years, structural studies have identified a number of bacterial, viral, and eukaryotic adhesive proteins that have a trimeric architecture. The prototype examples in bacteria are the *Haemophilus influenzae* Hia adhesin and the *Yersinia enterocolitica* YadA adhesin. Both Hia and YadA are members of the trimeric-autotransporter subfamily and are characterized by an internal passenger domain that harbors adhesive activity and a short C-terminal translocator domain that inserts into the outer membrane and facilitates delivery of the passenger domain to the bacterial surface. In this study, we examined the relationship between trimerization of the Hia and YadA passenger domains and the capacity for adhesive activity. We found that subunit-subunit interactions and stable trimerization are essential for native folding and stability and ultimately for full-level adhesive activity. These results raise the possibility that disruption of the trimeric architecture of trimeric autotransporters, and possibly other trimeric adhesins, may be an effective strategy to eliminate adhesive activity.

Microbial adherence to host tissues is an essential early step in the pathogeneses of infectious diseases (18). High-affinity adherence is important in allowing microorganisms to overcome normal host protective mechanisms associated with mechanical force, such as peristalsis, mucociliary clearance, and coughing. Typically, the process of adherence involves a specific interaction between a microbial surface protein, called an adhesin, and a complementary host cell receptor.

Autotransporter proteins are a large family of extracellular proteins that are present in a number of gram-negative pathogenic bacteria (16). These proteins are synthesized as precursor proteins with three functional domains, an N-terminal signal peptide, an internal passenger domain, and a C-terminal pore-forming translocator domain (17, 19, 27). The C-terminal translocator domain is embedded in the outer membrane and facilitates delivery of the internal passenger domain to the bacterial surface.

The trimeric autotransporters represent a subfamily of autotransporter proteins and are defined by the presence of a very short C-terminal translocator domain that forms highly stable trimers in the outer membrane (30, 39). Thus far, all characterized members of the trimeric-autotransporter subfamily have been found to possess adhesive activity, in most cases mediating bacterial adherence to eukaryotic cells (4, 6–8, 31, 35) or extracellular matrix proteins (25, 41) and in some cases resulting in binding of circulating factors, such as immunoglobulins or complement components (1, 13, 33, 34, 42).

The prototype members of the trimeric autotransporter subfamily are the *Haemophilus influenzae* Hia adhesin and the *Yersinia enterocolitica* YadA protein. Hia mediates high-affinity adherence to respiratory epithelial cells (23), and YadA is a virulence factor capable of mediating adherence to host cells and extracellular matrix proteins and involved in serum resistance (14). In experiments with Hia, the C-terminal 76 amino acids were capable of presenting a functional heterologous passenger domain on the bacterial surface (39). Similarly, in studies of YadA, the C-terminal 70 amino acids were sufficient for translocating an N-terminal FLAG epitope across the outer membrane (30). Further biochemical analysis established that the C termini of both Hia and YadA form heat-resistant, sodium dodecyl sulfate-resistant trimers in the outer membrane and that Hia requires formic acid denaturation for dissociation (30, 39). Consideration of this information in combination with secondary-structure predictions has led to the proposal that the C-terminal translocator domains of both Hia and YadA form 12-stranded pore-forming β-barrels containing four strands from each of three subunits (30, 39). The passenger domains of all three subunits are believed to be translocated to the bacterial cell surface through the trimeric pore (9).

Recent crystal structures of the Hia primary binding domain (HiaBD1) and the YadA collagen-binding domain have established that the passenger domains of these proteins are capable of trimerization independent of the translocator domain (28, 43). HiaBD1 is an intricately folded trimer with a large hydrophobic core and multiple subunit-subunit interactions. The adhesive activity of this domain maps to an acidic pocket that is formed by a single monomer and is present on all three faces of the trimer (three pockets per trimer). The YadA collagen-binding domain is a novel left-handed parallel β-roll with a trimeric architecture and potential to interact with three separate collagen fibers.

In the present study, we examined the relationship between trimerization and the capacity for adhesive activity in the Hia and YadA passenger domains. We found that subunit-subunit interactions and stable trimer formation are essential for na-

* Corresponding author. Mailing address: Department of Pediatrics, Duke University Medical Center, Children's Health Center, Room T901, DUMC 3352, Durham, NC 27710. Phone: (919) 681-4080. Fax: (919) 681-2714. E-mail: j.stgeme@duke.edu.

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tive folding and stability and ultimately for full-level adhesive activity. This study provides important insights into the structure and function of trimeric autotransporters and suggests that disruption of the trimeric architecture of these proteins may be an effective strategy to eliminate biological activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *E. coli* strains DH5α (Life Technologies), BL21(DE3), and XL-1 Blue have been described previously (32). *Escherichia coli* strain UT5600 is an OmpT$^-$ OmpP$^-$ derivative of strain RW193 (12).

Plasmid vectors used in this study include pACYC184 (New England BioLabs), pT7-7 (40), and pHAT10 (Clontech). The plasmid pMW10 (also referred to as pYadA) contains the *yadA* gene from *Yersinia enterocolitica* strain O:8 and was a gift from Virginia Miller (Washington University, St. Louis, MO). The plasmid pHMW8-7 (also referred to as pHia) contains the *hia* gene from *H. influenzae* strain 11 in pT7-7 (4). The plasmid pHMW8-7ΔBS2 (also referred to as pHiaΔBS2) is a derivative of pHMW8-7 with a deletion of the coding sequence for Hia residues 114 to 127, effectively disrupting HiaBD2 (23). The plasmid pNS1 is a derivative of pHAT10 that contains *hia* upstream sequence and coding sequence for the Hia signal peptide, the HAT epitope, and Hia residues 977 to 1098 (encompassing the Hia translocator domain) (10). The HAT epitope is a 19-amino-acid polyhistidine peptide that has affinity for nickel and is the target for commercially available antibodies (Clontech, Mountain View, CA).

E. coli strains were grown on Luria-Bertani (LB) agar or in LB broth and were stored at −80°C in LB broth with 30% glycerol. Plasmids were selected with 100 μg/ml of ampicillin and 30 μg/ml of chloramphenicol, as appropriate.

Recombinant DNA methods. DNA ligations, restriction endonuclease digestions, gel electrophoresis, and PCR were performed according to standard techniques (32). Plasmids were introduced into *E. coli* by electroporation (2). Nucleotide sequencing was performed using an ABI automotated sequencer and the Big Dye Terminator Premix-20 kit version 3.1 (Applied Biosystems/Perkin-Elmer).

Construction of plasmids used in this study. Derivatives of pHMW8-7ΔBS2 containing point mutations at residues involved in subunit-subunit interactions were generated using the QuikChange site-directed mutagenesis kit (Stratagene). All constructs were examined by nucleotide sequencing to ensure that the intended mutations were present and that the coding sequence was otherwise wild type.

The plasmid pACYC-HiaC′ encodes the Hia signal sequence and then the HAT epitope and Hia residues 977 to 1098, allowing expression of a HAT-tagged outer membrane protein consisting of Hia residues 977 to 1098. This plasmid was generated by digesting pNS1 with PvuII and BamHI, purifying the 0.6-kb fragment, and then ligating the 0.6-kb fragment into EcoRI-BamHI-digested pACYC184. The plasmid pACYC-YadA/C′ encodes the Hia signal sequence and then the HAT epitope and YadA residues 325 to 422, allowing expression of a HAT-tagged outer membrane protein consisting of YadA residues 325 to 422. In order to generate this plasmid, pACYC-HiaC′ was digested with SacI and EcoRI, releasing the fragment that encodes $Hia_{977–1098}$. The remaining plasmid backbone was ligated to a 0.3-kb SacI-EcoRI fragment that was amplified from pMW10 and encodes $YadA_{325–422}$.

Cell fractionation and protein analysis. Whole-cell sonicates were prepared by resuspending bacterial pellets in 10 mM HEPES (pH 7.4) and sonicating them to clarity. Outer membrane fractions were recovered on the basis of Sarkosyl insolubility as described by Carlone et al. (5). Unless otherwise noted, whole-cell sonicates and outer membrane fractions were treated with 95% formic acid to disrupt trimers, as described previously (38). Proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Western blots were performed with a guinea pig polyclonal antiserum raised against Hia residues 50 to 252 or a rabbit polyclonal antiserum raised against the HAT epitope (Clontech). An anti-guinea pig or an anti-rabbit immunoglobulin G antiserum conjugated to horseradish peroxidase (Sigma) was used as the secondary antibody. Proteins were visualized by incubation with a chemiluminescent substrate solution (Pierce) and exposure to film.

Flow cytometry. Flow cytometry was performed using a guinea pig polyclonal antiserum raised against Hia residues 221 to 658 and a Cy2-conjugated secondary antibody as described previously (23). The antiserum against $Hia_{221–658}$ reacts specifically with the Hia passenger domain.

Quantitative adherence assays. Chang epithelial cells were maintained in minimal essential medium supplemented with nonessential amino acids and 10% (vol/vol) fetal calf serum and were cultivated at 37°C in 7% CO_2. Adherence assays were performed as described previously (36, 37). Briefly, bacteria were inoculated into LB broth containing the appropriate antibiotics and allowed to grow to a density of $\sim 2 \times 10^9$ CFU per ml. Approximately 1.5×10^7 CFU was inoculated onto Chang cell monolayers in 24-well tissue culture plates, and the plates were gently centrifuged at $165 \times g$ for 5 min and then incubated at 37°C in 5% CO_2 for 25 min. Subsequently, the monolayers were rinsed four times with phosphate-buffered saline to remove nonadherent bacteria, treated with trypsin/EDTA, and resuspended in LB broth. Suspensions were plated on LB agar to yield the number of adherent CFU per monolayer. Adherence was calculated by dividing the number of adherent CFU per monolayer by the number of inoculated CFU per monolayer.

RESULTS

Point mutations that disrupt HiaBD1 subunit-subunit interactions result in increased susceptibility to degradation and loss of adhesive activity. In recent work, we demonstrated that each of the three acidic binding pockets in the HiaBD1 trimeric structure is formed by a single subunit (43). In the present study, we hypothesized that trimerization of HiaBD1 is necessary for proper folding of each subunit and is essential for adhesive activity. To examine this hypothesis, we began by introducing point mutations at subunit-subunit interfaces in the HiaBD1 hydrophobic core, replacing noncharged or hydrophobic residues with charged residues and intending to disrupt subunit-subunit interactions (Fig. 1A). Using a plasmid encoding HiaΔBS2 (an Hia derivative that lacks a functional secondary binding domain), we introduced mutations that changed the alanine at position 645 to aspartic acid (HiaΔBS2/A645D), the leucine at position 650 to aspartic acid (HiaΔBS2/L650D), or the phenylalanine at position 681 to glutamic acid (HiaΔBS2/F681E) and then transformed the mutant constructs into *E. coli* DH5α. As shown in Fig. 1B, the mutant proteins were not detectable in outer membrane preparations, suggesting proteolytic degradation due to global misfolding.

In an effort to circumvent proteolysis and examine the abilities of HiaΔBS2/A645D, HiaΔBS2/L650D, and HiaΔBS2/F681E to mediate bacterial adherence, we expressed these proteins in *E. coli* UT5600, a laboratory strain that lacks the OmpT and OmpP outer membrane proteases responsible for degrading misfolded outer membrane proteins (12). As controls, we expressed these proteins in *E. coli* RW193, the parent of UT5600 with functional OmpT and OmpP proteases. Western analysis and flow cytometry revealed appreciable levels of the mutant proteins in the outer membrane and on the surface of UT5600, comparable to levels of HiaΔBS2 (Fig. 2A and B). In contrast, the mutant proteins were absent from the bacterial outer membrane and the bacterial surface of *E. coli* RW193 (Fig. 2A and B) (indicating degradation by OmpT and/or OmpP). Interestingly, UT5600/pHiaΔBS2/A645D, UT5600/pHiaΔBS2/L650D, and UT5600/pHiaΔBS2/F681E were all nonadherent in assays with Chang epithelial cells (Fig. 2C), similar to *E. coli* harboring empty vector, suggesting that Hia lacking normal subunit-subunit interactions is misfolded and lacks adhesive activity.

Trimer formation is required for normal folding, stability, and adhesive activity of Hia and YadA. To extend our observations with point mutations at subunit-subunit interfaces, we sought more direct evidence that trimerization of the Hia passenger domain is required for normal folding, stability, and adhesive activity. With this goal in mind, we exploited the fact

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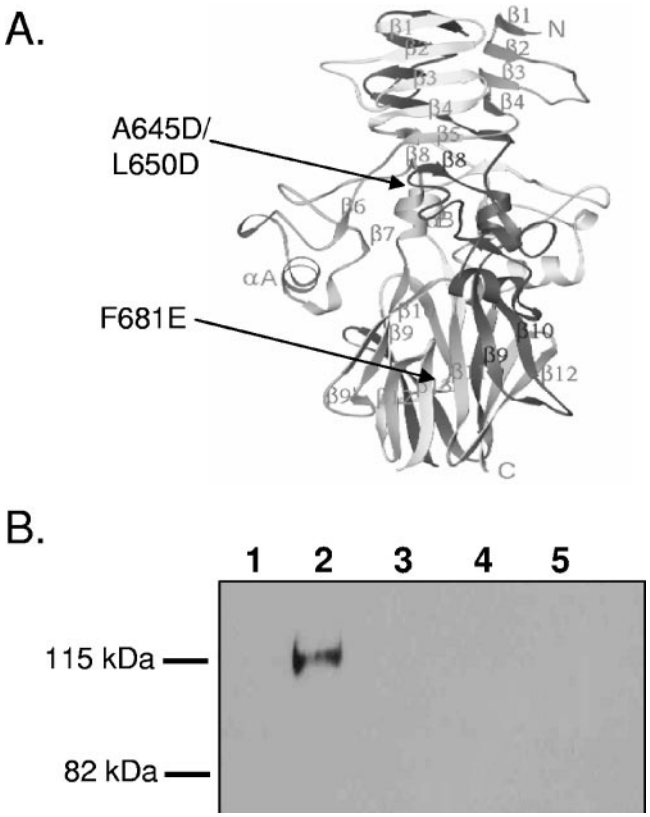

FIG. 1. Effects of point mutations at subunit-subunit interfaces on HiaBD1 stability and adhesive activity in *E. coli* DH5α. (A) Stereo ribbon diagram of the HiaBD1 trimer highlighting the locations of the three point mutations engineered to disrupt subunit-subunit interactions. Mutations A645D and L650D disrupt an internal α-helix, and mutation F681E lies deep in the hydrophobic core created by interdigitating β-strands from all three subunits. (B) Hia levels in outer membrane preparations, as assessed by Western analysis with an antiserum against Hia and a Cy2-conjugated secondary antibody. Samples were loaded as follows: lane 1, DH5α; lane 2, DH5α/pHiaΔBS2; lane 3, DH5α/pHiaΔBS2/A645D; lane 4, DH5α/pHiaΔBS2/L650D; lane 5, DH5α/pHiaΔBS2/F681E.

that the Hia C-terminal translocator domain undergoes trimerization. We reasoned that coexpression of full-length Hia with a construct encoding only the Hia C-terminal translocator domain (HiaC′) would lead to the generation of heterotrimers containing a trimeric translocator domain and either one or two full-length passenger domains (in addition to homotrimeric full-length Hia and homotrimeric HiaC′).

In anticipation of the possibility that heterotrimers might be misfolded and prone to degradation, we coexpressed full-length Hia and HiaC′ in both *E. coli* RW193 and *E. coli* UT5600 ($OmpT^-$ $OmpP^-$). As controls, we expressed full-length Hia with pACYC184 (empty vector) in strains RW193 and UT5600. As shown in Fig. 3A, Western analysis of outer membranes prepared from RW193/pHia+pACYC-HiaC′ (left, lane 3) revealed no detectable full-length Hia, presumably reflecting minimal formation of trimeric full-length Hia and efficient degradation of heterotrimers containing only one or two full-length Hia passenger domains. In contrast, examination of outer membranes prepared from UT5600/pHia+pACYC-HiaC′ (right, lane 3) demonstrated high levels of full-length Hia, comparable to levels in UT5600 expressing Hia alone. To confirm the presence of heterotrimers in UT5600/pHia+pACYC-HiaC′, we examined outer membranes preparations that had not been treated with formic acid. As shown in Fig. 3B, in the absence of formic acid denaturation, a small quantity of trimeric full-length Hia was present, but the predominant species containing full-length Hia were heterotrimers containing one or two full-length molecules (1× full-length Hia/2× HiaC′ or 2× full-length Hia/1× HiaC′).

To verify that Hia passenger domains present in heterotrimers were delivered to the bacterial cell surface, we performed flow cytometry with an antiserum against the Hia passenger domain. Consistent with the results of Western analysis of outer membrane preparations, surface-localized Hia was abundant in UT5600/pHia+pACYC-HiaC′ (Fig. 3C). Interestingly, UT5600/pHia+pACYC-HiaC′ exhibited slightly enhanced immunoreactivity compared to UT5600 expressing Hia alone, potentially reflecting increased exposure of epitopes recognized by the Hia antibody in a misfolded passenger domain.

To assess the adhesive activities of Hia passenger domains present on the bacterial surface in heterotrimers, we examined UT5600/pHia+pACYC-HiaC′ for adherence to Chang epithelial cells, using UT5600/pHia+pACYC184 (expressing wild-type Hia alone) as a control. As shown in Fig. 3D, adherence by UT5600/pHia+pACYC-HiaC′ was reduced ~60% compared to adherence by UT5600/pHia+pACYC184, suggesting that heterotrimers containing only one or two full-length Hia molecules possess minimal adhesive activity. In control experiments, we compared *E. coli* expressing wild-type Hia encoded by the low-copy-number plasmid pACYC184 and *E. coli* expressing wild-type Hia encoded by pHia and found marked differences in levels of Hia and comparable levels of adherence (data not shown), arguing that small differences in levels of full-length Hia between UT5600/pHia and UT5600/pHia+pACYC-HiaC′ do not account for the major differences in adherence.

To examine whether the results with Hia are applicable to the trimeric-autotransporter subfamily more generally, we coexpressed full-length YadA and the YadA C-terminal translocator domain (YadA/C′). As shown in Fig. 4A, full-length YadA was absent in outer membranes from RW193/pYadA+pACYC-YadA/C′ (left, lane 3) but was present at high levels in outer membranes from UT5600/pYadA+pACYC-YadA/C′ (right, lane 3). Furthermore, when outer membranes from UT5600/pYadA+pACYC-YadA/C′ were assessed by Western analysis after omission of formic acid denaturation, a banding pattern indicative of heterotrimers was observed (data not shown), similar to earlier studies by Roggenkamp et al. (30). Finally, adherence by UT5600/pYadA+pACYC-YadA/C′ was reduced ~70% compared to adherence by UT5600/pYadA+pACYC184 (Fig. 4B), supporting the conclusion that heterotrimers containing one or two full-length YadA molecules harbor minimal adhesive activity.

Together, these results provide strong evidence that trimerization of the passenger domain is necessary for full adhesive function of both Hia and YadA. In the absence of trimerization, the passenger domain is misfolded, eliminating adhesive activity and conferring susceptibility to degradation by OmpT and/or OmpP.

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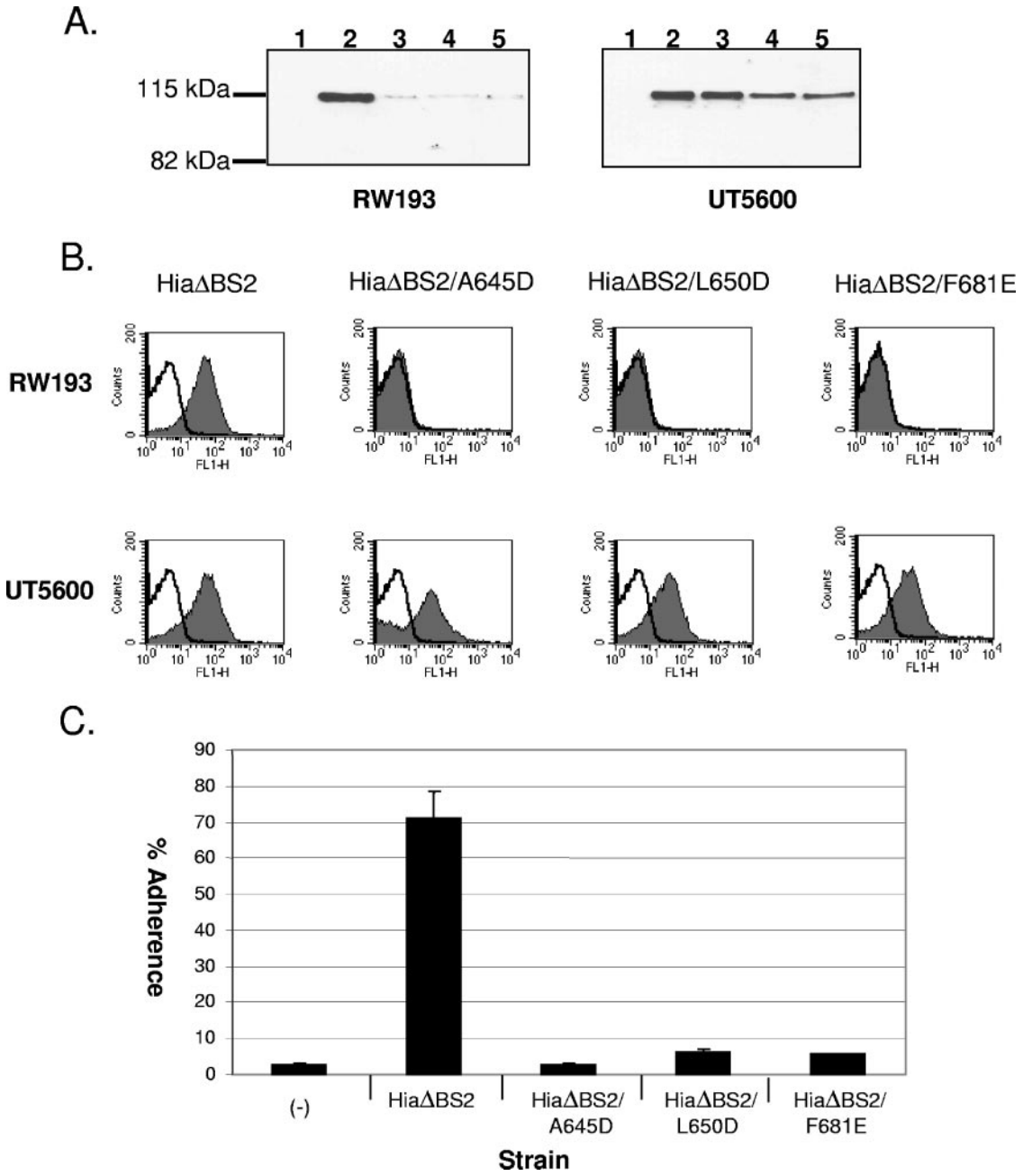

FIG. 2. Effects of point mutations at subunit-subunit interfaces on HiaBD1 stability and adhesive activity in *E. coli* RW193 and *E. coli* UT5600 ($OmpT^{-}$ $OmpP^{-}$). (A) Hia levels in outer membrane preparations from RW193 and UT5600, as assessed by Western analysis with an antiserum against Hia. Samples were loaded as follows: lanes 1, *E. coli* without plasmid; lanes 2, *E. coli*/pHiaΔBS2; lanes 3, *E. coli*/pHiaΔBS2/A645D; lanes 4, *E. coli*/pHiaΔBS2/L650D; lanes 5, *E. coli*/pHiaΔBS2/F681E. (B) Hia levels on the bacterial surfaces of RW193 and UT5600, as assessed by flow cytometry with an antiserum against Hia. *E. coli* without plasmid is indicated by the black line, and *E. coli* expressing the designated recombinant protein is indicated by the shaded curve. (C) Adherence to Chang epithelial cells by UT5600 derivatives. Adherence was calculated by determining the percentage of the inoculum that was adherent after a 30-min assay. The bars represent means plus standard errors of three measurements from a representative experiment. *E. coli* expressing vector alone (−) was used as a negative control.

The Hia and YadA C-terminal translocator domains do not inhibit each other. Given the structural similarities between the Hia and the YadA C-terminal translocators, we wondered whether the C termini of these heterologous proteins might be capable of stably interacting with each other, thereby abrogating adhesive activity. To address this possibility, we coexpressed the YadA C-terminal translocator domain with full-length Hia and the Hia C-terminal translocator domain with full-length YadA in *E. coli* DH5α (Fig. 5). YadA/C′ had a minimal effect on Hia protein levels (Fig. 5A, lane 4) and no significant effect on Hia-mediated adherence (data not shown). Similarly, HiaC′ had no appreciable effect on YadA protein

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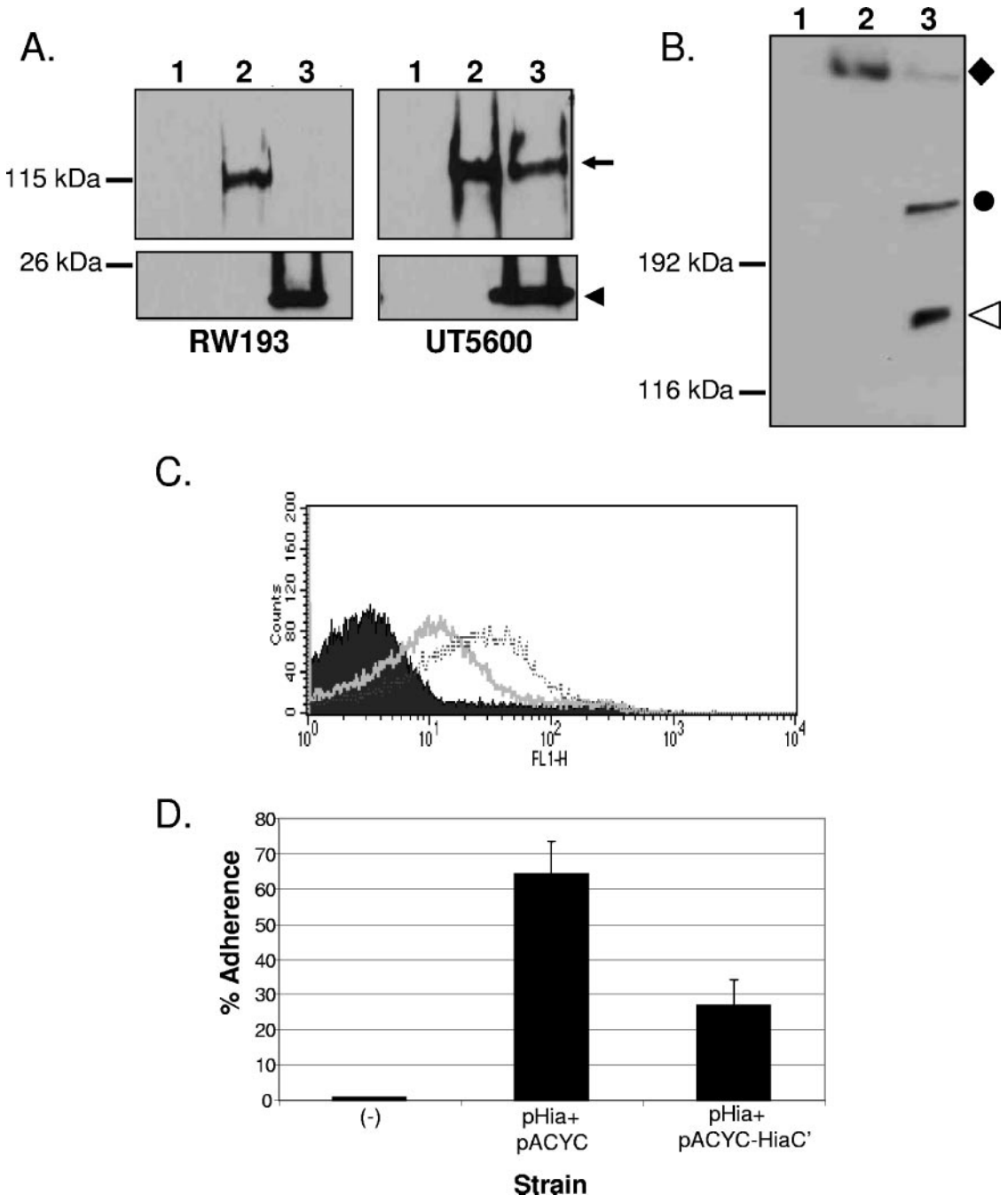

FIG. 3. Coexpression of HiaC′ and full-length Hia. (A) Full-length Hia (arrow) and HiaC′ (arrowhead) in outer membrane preparations from *E. coli* RW193 and *E. coli* UT5600 (an $OmpT^-$ $OmpP^-$ derivative of RW193) as visualized by Western analysis with an antibody specific to the Hia passenger domain (reactive with full-length Hia) and an anti-HAT antibody (reactive with HiaC′). After formic acid denaturation, monomeric Hia protein runs at ~115 kDa and monomeric HiaC′ protein migrates at ~13 kDa. Samples were loaded as follows: lanes 1, *E. coli* without plasmid; lanes 2, *E. coli*/pHia+pACYC184; lanes 3, *E. coli*/pHia+pACYC-HiaC′. (B) Hia heterotrimers in the outer membrane of *E. coli* strain UT5600/pHia+pACYC-HiaC′. Outer membrane fractions were recovered from UT5600, UT5600/pHia+pACYC184, and UT5600/pHia+pACYC-HiaC′ and suspended in Laemmli buffer, omitting formic acid denaturation. Subsequently, samples were boiled at 95°C for 10 min, resolved on a 4% to 15% SDS-polyacrylamide gel, and examined by Western analysis with an antiserum against $Hia_{50–252}$. Samples were loaded as follows: lane 1, UT5600; lane 2, UT5600/pHia+pACYC184; lane 3, UT5600/pHia+pACYC-HiaC′. The diamond corresponds to 3× full-length Hia (~345 kDa), the closed circle corresponds to 2× full-length Hia/1× HiaC′ (~245 kDa), and the open arrowhead corresponds to 1× full-length Hia/2× HiaC′ (~140 kDa). (C) Hia heterotrimers on the bacterial surface of *E. coli* UT5600 expressing Hia plus HiaC′, as assessed by flow cytometry with an antiserum against $Hia_{221–658}$ and a Cy2-conjugated secondary antibody. UT5600 ($OmpT^-$ $OmpP^-$) is depicted with a shaded curve (negative control), UT5600/pHia+pACYC184 is depicted with a solid gray line (positive control), and UT5600/pHia+pACYC-HiaC′ is depicted with a dotted black line. (D) Adherence to Chang epithelial cells by *E. coli* UT5600 expressing Hia or Hia plus HiaC′. Adherence was calculated by determining the percentage of the inoculum that was adherent after a 30-min assay. The bars represent means plus standard errors of three measurements from a representative experiment. UT5600 alone (−) was used as a negative control. The plasmid pACYC184 is abbreviated as pACYC.

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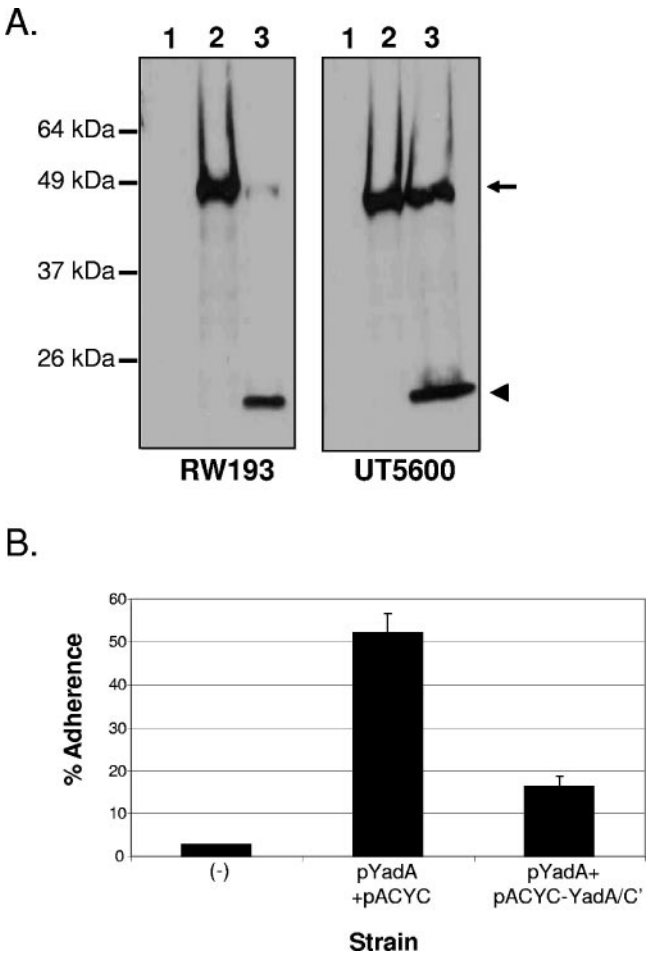

FIG. 4. Coexpression of YadA/C′ and full-length YadA. (A) Full-length YadA (arrow) and YadA/C′ (arrowhead) in outer membrane preparations from *E. coli* RW193 and *E. coli* UT5600 (an $OmpT^-$ $OmpP^-$ derivative of RW193), as visualized by Western analysis with an anti-HAT antibody. After formic acid denaturation, monomeric YadA protein migrates at ~45 kDa, and monomeric YadA/C′ protein migrates at ~11 kDa. Samples were loaded as follows: lanes 1, *E. coli* without plasmid; lanes 2, *E. coli*/pYadA+pACYC184; lanes 3, *E. coli*/pYadA+pACYC-YadA/C′. (B) Adherence to Chang epithelial cells by *E. coli* UT5600 expressing YadA or YadA plus YadA/C′. Adherence was calculated by determining the percentage of the inoculum that was adherent after a 30-min assay. The bars represent means plus standard errors of three measurements from a representative experiment. UT5600 alone (−) was used as a negative control. The plasmid pACYC184 is abbreviated as pACYC.

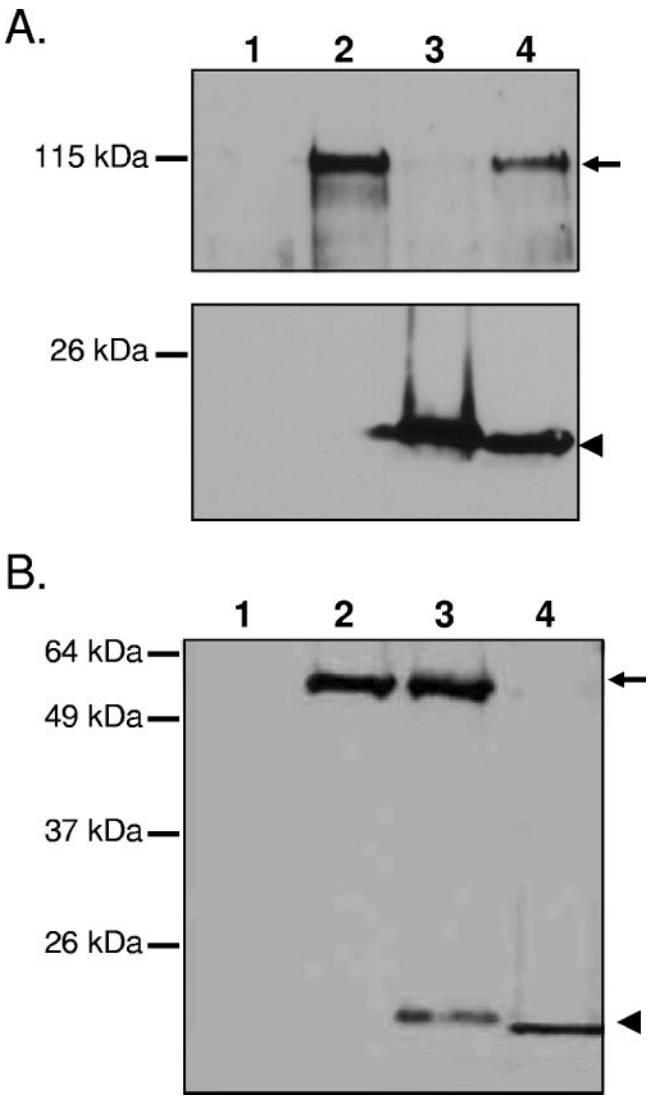

FIG. 5. Interchangeability of HiaC′ and YadA/C′. (A) Hia, Hia plus HiaC′, and Hia plus YadA/C′ protein levels in outer membrane preparations, as assessed by Western analysis with an antiserum against the Hia passenger domain (reactive with full-length Hia) (arrow) and an anti-HAT antibody that detects HiaC′ and YadA/C′ (arrowhead). Samples were loaded as follows: lane 1, DH5α; lane 2, DH5α/pHia+pACYC184; lane 3, DH5α/pHia+pACYC-HiaC′; lane 4, DH5α/pHia+pACYC-YadA/C′. (B) YadA alone, YadA plus HiaC′, and YadA plus YadA/C′ protein levels in outer membrane preparations as assessed by Western analysis with an anti-HAT antibody that detects full-length YadA (arrow), HiaC′ (arrowhead), and YadA/C′ (arrowhead). Samples were loaded as follows: lane 1, DH5α; lane 2, DH5α/pYadA+pACYC184; lane 3, DH5α/pYadA+pACYC-HiaC′; lane 4, DH5α/pYadA+pACYC-YadA/C′.

levels (Fig. 5B, lane 3) and no significant effect on YadA-mediated adhesive activity (data not shown).

DISCUSSION

In the present study, we examined whether subunit-subunit interactions and stable trimerization of the Hia and YadA passenger domains are important for Hia and YadA adhesive activities. Based on analysis of the HiaBD1 crystal structure, we were able to target specific residues in the hydrophobic core involved in subunit-subunit interactions, remote from the binding pocket. Insertion of charged residues in place of A645, L650, or F681 in the hydrophobic core disrupted overall protein conformation, resulting in a loss of adhesive activity. In addition, we were able to generate strains expressing Hia and YadA molecules with a trimeric translocator domain and zero, one, two, or three full-length passenger domains, exploiting the fact that the Hia and YadA C-terminal translocator domains undergo trimerization. These strains had decreased quantities of surface-localized trimeric passenger domains and markedly re-

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duced adhesive activities. Together, these data argue that subunit-subunit interactions and trimer formation are essential for normal folding and full-level adhesive activity of individual Hia and YadA passenger domains and potentially other trimeric autotransporters.

In considering our results with the A645D, L650D, and F681E mutations in Hia, it is notable that similar mutations in YadA have been examined. In particular, El Tahir et al. (15) identified a repeating NSVAIG-S motif in the head domain of YadA and replaced the small hydrophobic residues in this motif with either aspartic acid or glutamic acid. Based on the crystal structure of the YadA collagen binding domain, the NSVAIG-S motif forms the core of the YadA trimer, mediating hydrophobic interactions between β-rolls (28). Mutations in the NSVAIG-S motif disrupted subunit-subunit interactions and resulted in anomalous migration on an SDS-polyacrylamide gel electrophoresis gel and complete or almost complete loss of collagen binding (15), analogous to our observations with Hia.

In our experiments examining *E. coli* UT5600 expressing either Hia or YadA heterotrimers, we observed that bacterial adherence was decreased but not eliminated compared to control strains. Regarding the low-level adherence by these strains, it is possible that the misfolded monomeric and dimeric passenger domains present on the bacterial surface in heterotrimers retain some adhesive activity. Arguing against this possibility, UT5600/HiaΔBS2/A645D, UT5600/HiaΔBS2/L650D, and UT5600/HiaΔBS2/F681E (with mutations at subunit-subunit interfaces in Hia) all expressed misfolded passenger domains on the bacterial surface and were completely nonadherent, comparable to background. We postulate that the residual adherence by UT5600/pHia+pACYC-HiaC′ and UT5600/pYadA+pACYC-YadA/C′ is explained instead by the presence of small, but appreciable, quantities of wild-type homotrimeric protein on the bacterial surface, as demonstrated by Western analysis of outer membrane preparations. In support of this explanation, mutation of A645, L650, or F681 completely abrogated Hia-mediated adherence in a background devoid of wild-type homotrimeric protein.

In considering the stringent requirement for trimerization of Hia and YadA for full-scale adhesive activity, it is notable that a trimeric architecture with three identical faces provides the potential for three identical binding pockets and a multivalent interaction with the host cell surface (9). Compared with a monovalent interaction, multivalency results in increased avidity and a more stable interaction with higher affinity (9). This increase in avidity may be important in allowing organisms to overcome mechanical forces in the host, including mucociliary clearance and coughing in the respiratory tract and peristalsis in the intestinal tract. In this context, it is interesting that all characterized trimeric autotransporters have been found to possess adhesive activity, in the majority of cases mediating bacterial adherence to epithelial cells (4, 6–8, 31, 35) or to extracellular matrix proteins (25, 41).

The presence of a trimeric architecture and the capacity for multivalent high-affinity adherence has been observed in a number of viral and eukaryotic adhesive proteins, as well. One example is the adenovirus type 2 fiber, a homotrimer that contains three identical binding pockets and interacts with three separate host cell receptor molecules, referred to as coxsackievirus and adenovirus receptors. Based on studies using surface plasmon resonance to compare trivalent and monovalent interactions between the type 2 fiber and coxsackievirus and adenovirus receptors, the trivalent interaction results in a 25-fold increase in affinity (24).

Beyond the potential for multivalent high-affinity binding, homotrimeric viral proteins also exhibit resistance to proteases and detergents, with examples including the bacteriophage T4 gp9 and gp11 tail components, the bacteriophage P22 tailspike endorhamnosidase, and the adenovirus type 2 fiber (11, 21, 22, 26). The stability of these homotrimeric viral proteins is presumed to be critical for maintaining function as external virion proteins in the presence of proteases and denaturing conditions in nature. Along these lines, it is noteworthy that the three subunits in HiaBD1 are extensively intertwined, resulting in resistance to degradation by trypsin and to denaturation by SDS (43). We speculate that these properties may be applicable to all trimeric autotransporters.

Our results demonstrating the importance of trimerization for Hia and YadA adhesive activities raise the possibility that disruption of the trimeric architecture of Hia and YadA, and perhaps all trimeric autotransporters, may be a productive approach to eliminate adhesive activity. Using the Hia structure as a model, one approach would be to identify small molecules that interact with the hydrophobic core of the trimer and interfere with subunit-subunit interactions during the process of trimerization, analogous to the effect of point mutations at A645, L650, and F681. Additional crystal structures will be necessary to assess the likelihood that a given small molecule is capable of interfering with trimerization of multiple distinct trimeric autotransporters.

Coexpression of YadA/C′ with full-length Hia had minimal effect on Hia protein levels and no effect on Hia adhesive activity, and coexpression of HiaC′ with full-length YadA had no effect on YadA protein levels or YadA-mediated adherence. Together, these findings suggest that the YadA and Hia C-terminal translocator domains are unable to interact stably with each other. This specificity is somewhat surprising, considering the structural similarity between the C-terminal ends of Hia and YadA, with each predicted to form four transmembrane β-strands. However, it is possible that this specificity evolved to protect against hetero-oligomerization in bacteria that express more than one member of this subfamily, thereby preventing loss of passenger domain function (7, 29).

A prevailing hypothesis regarding autotransporter proteins is that the energy required for passenger domain translocation across the outer membrane is derived from the native folding of the passenger domain on the bacterial surface (20). Indeed, we have speculated previously that passenger domain translocation in trimeric autotransporters occurs in concert with trimerization (43). However, our findings in the present study argue against this hypothesis, at least for the trimeric-autotransporter subfamily. In particular, we were able to detect Hia and YadA heterotrimers with only one or two passenger domains on the bacterial surface, provided that the OmpT and OmpP outer membrane proteases were absent.

In conclusion, we have shown that trimerization of the Hia and YadA passenger domains is necessary for full-scale adhesive function. These are the first studies to define the relationship between trimer formation and function for members of the trimeric-autotransporter family. We speculate that our

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findings apply to all members of the growing trimeric-autotransporter subfamily, raising the possibility that disruption of trimerization may be a broadly effective strategy to abrogate the activities of trimeric autotransporters.

ACKNOWLEDGMENTS

This work was supported by NIH grant RO1-AI44167 to J.W.S., by NIH training grant T32-HL07873 to S.E.C., and by NIH training grant T32-AI07172 to N.K.S. S.E.C. and N.K.S. are members of the Medical Scientist Training Program at Washington University School of Medicine.

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