Structural basis for host recognition by the *Haemophilus influenzae* Hia autotransporter

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Haemophilus influenzae is an important human pathogen that initiates infection by colonizing the upper respiratory tract. The *H. influenzae* Hia autotransporter is an adhesive protein that promotes adherence to respiratory epithelial cells. Hia adhesive activity resides in two homologous binding domains, called HiaBD1 and HiaBD2. These domains interact with the same host cell receptor, but bind with different affinities. In this report, we describe the crystal structure of the high-affinity HiaBD1 binding domain, which has a novel trimeric architecture with three-fold symmetry and a mushroom shape. The subunit constituents of the trimer are extensively intertwined. The receptor-binding pocket is formed by an acidic patch that is present on all three faces of the trimer, providing potential for a multivalent interaction with the host cell surface, analogous to observations with the trimeric tumor necrosis factor superfamily of proteins. Hia is a novel example of a bacterial trimeric adhesin and may be the prototype member of a large family of bacterial virulence proteins with a similar architecture.

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Introduction

Adherence to host tissues is a fundamental early step in the pathogenesis of most bacterial infections. The process of adherence requires specialized bacterial proteins called adhesins, which recognize specific host cell receptors and mediate stable interaction with the host cell surface (Hultgren *et al*, 1993; St Geme, 1997a).

In order to mediate interaction with host cells, adhesins must be presented on the bacterial surface in an active binding conformation. In Gram-negative bacteria, surface localization requires that the protein be translocated across the inner and outer membranes. In general, surface localization occurs via one of five different secretion pathways, distinguished at least in part by the mechanism of translocation across the outer membrane and designated types I–V (Stathopoulos *et al*, 2000; Thanassi and Hultgren, 2000).

Proteins secreted by the type V pathway are referred to as autotransporters (Henderson *et al*, 2000). Typically, autotransporters are expressed as precursor proteins with three basic functional domains, including an N-terminal signal peptide, an internal passenger domain, and a C-terminal translocator domain (β domain) (Henderson *et al*, 1998). The signal peptide directs export of the precursor protein across the inner membrane via the Sec machinery and is then cleaved by signal peptidase I. Subsequently, the β domain inserts into the outer membrane and forms a β -barrel structure with a central channel, allowing extrusion of the passenger domain across the membrane (Shannon and Fernandez, 1999; Veiga *et al*, 2002). Once on the surface of the organism, the passenger domain is usually cleaved from the translocator domain and released extracellularly. In some cases, the passenger domain is cleaved but remains cell associated.

Haemophilus influenzae is a human-specific pathogen and a major source of morbidity worldwide, producing systemic diseases such as meningitis and sepsis and localized respiratory tract diseases such as otitis media, bronchitis, and pneumonia (Turk, 1984; St Geme, 1993; Moxon and Murphy, 2000). The pathogenesis of *H. influenzae* disease begins with colonization of the nasopharynx, and adherence to the respiratory epithelium represents an essential early step in the colonization process (Murphy *et al*, 1987; St Geme, 1997b). A high-molecular-mass protein called Hia is the predominant adhesin in a subset of nonencapsulated strains (Barenkamp and St Geme, 1996; St Geme *et al*, 1998). A homolog of Hia called Hsf is ubiquitous in encapsulated isolates and is the major nonpilus adhesin in these strains (St Geme *et al*, 1996).

In previous work, we established that Hia is a member of the autotransporter family (Figure 1) (St Geme and Cutter, 2000). In addition, we found that Hia remains uncleaved at the C terminus and fully cell associated, an unusual fate for autotransporters (St Geme and Cutter, 2000). More recently, we discovered that Hia is an example of a variant subfamily of autotransporters characterized by an unusually small

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Figure 1 Schematic diagram of the primary sequence of Hia. The Hia domain organization is indicated: signal peptide (SP; residues 1–49), passenger domain (residues 50–1022), and β domain (residues 1023–1098). The black boxes in the β domain reflect predicted β strands. HiaBD1 (residues 549–705) and HiaBD2 (residues 50–374) are indicated by green boxes. The structure-based minimal Hia binding domains are indicated by blue boxes: HiaBDp (primary) and HiaBDs (secondary). The domains with homology to β 8 strand and α B helix of Domain 2 and all of Domain 3 in HiaBD1 are designated D3a, D3b, D3c, and D3d and are indicated by clear blue boxes (D3b and D3c) or black lines (D3a and D3d).

translocator domain that undergoes trimer formation (Surana *et al*, 2004). Examination of a series of GST–Hia fusion proteins and Hia deletion derivatives demonstrated that the Hia passenger domain contains two homologous binding domains, called HiaBD1 and HiaBD2 (Laarmann *et al*, 2002). These two binding domains recognize the same host cell receptor but bind with differing affinities. HiaBD1 is defined by residues 541–714 and has a K_d of 0.05–0.1 nM, while HiaBD2 is defined by amino acids 50–374 and has a K_d of 1–2 nM. The identity of the host cell receptor is not known.

In this study, we report that the HiaBD1 binding domain has a novel trimeric architecture with three-fold symmetry. Using site-directed mutagenesis, we show that the HiaBD1 receptor-binding domain is located in an acidic pocket present on all three faces of the trimer, suggesting potential for a multivalent interaction with the host cell surface, analogous to observations with the trimeric tumor necrosis factor superfamily of proteins. Hia is a novel example of a bacterial trimeric adhesin and may be the prototype member of a large subfamily of autotransporter proteins with a similar trimeric architecture.

Results and discussion

Structure determination

In earlier work, we discovered that the high-affinity binding activity of Hia resides in the region defined by amino acids 541–714, referred to as HiaBD1. As judged by size-exclusion chromatography, purified HiaBD1 (Hia_{541–714}) forms stable oligomers (likely globular hexamers or elongated trimers), mimicking purified Hia_{50–779} (full-length passenger domain). Native HiaBD1 crystallized in the monoclinic space group P2₁

with two trimers per asymmetric unit and diffracted to a resolution of 2.1 Å. Given that HiaBD1 contains no methionine residues, use of the multiple anomalous dispersion (MAD) phasing method at and around the selenium edge was not possible. An exhaustive search for heavy metal derivatives was performed and was unsuccessful. Ultimately, site-directed mutagenesis was performed, and two methionines were introduced into HiaBD1 at sites that do not disturb adhesive function (data not shown). Subsequently, selenomethionine-substituted HiaBD1 (SeMetHiaBD1) crystals were generated, allowing phasing using the MAD method. The SeMetHiaBD1 protein crystallized in the trigonal space group P321 with two molecules per asymmetric unit and diffracted to a resolution of 2.7 Å. The expected four selenium sites in the asymmetric unit were located using the programs SOLVE, SnB, and CNS. Experimental phases followed by solvent flipping (CNS) provided an electron density map of excellent quality (Figure 2A). The model was built using the program O and was first refined against the SeMetHiaBD1 data to a resolution of 3.0 Å. The refined model was then positioned into the native data by molecular replacement and was refined against the native data to a resolution of 2.1 Å. The final model has an R/R_{free} of 21.7/25.4% (Table I). The present model contains residues 548–705 of chain A, residues 549–705 of chains B, E, and F, residues 550–706 of chain C, residues 549–706 of chain D, and 488 water molecules.

Molecular architectures of the HiaBD1 subunit and of the functional trimer

The HiaBD1 subunit contains three well-defined structural domains, designated Domain 1 (residues 548–585), Domain 2

Figure 2 Structure of the Hia monomer. (A) Representative region (α B helix and β 9 strand) of the experimental phased electron density at 3 Å resolution. The electron density results from a map calculated using MAD phases after solvent flipping and is contoured at a 1.2σ level. The final refined model is shown in stick representation and is color coded with yellow for carbon, blue for nitrogen, and red for oxygen. (B) Topology diagram of the HiaBD1 subunit. β strands and helices (α and 3_{10} helices) are represented as arrows and cylinders, respectively. Secondary structural elements are labeled from β 1 to β 13 for β strands (indicated as arrows), α A and α B for α helices, and $3_{10}\alpha$ and $3_{10}\beta$ for 3_{10} helices. N and C denote the N and C termini of HiaBD1, respectively. The β strands provided from adjacent chain monomers to complete the β sheets of the green subunit of Figure 3A are shown using the same color coding used for the other subunits in Figure 3A, that is, yellow and red. (C) Sequence alignment of HiaBD1 (residues 541–714) from nontypable *H. influenzae* strain 11 with the corresponding region of the Hsf protein from type b strain C54. Identical, strongly conserved, and conserved residues are indicated by star, double dot, and dot symbols, respectively. Secondary structural elements of HiaBD1 are shown at the top of the sequences with β strands and helices indicated as arrows and cylinders, respectively. Amino-acid numbering at the top refers to HiaBD1 of strain 11. Residues involved in subunit–subunit interactions are shown in colored boxes; for example, residues of the green subunit involved in intermolecular contacts with the yellow and red subunits are indicated in yellow and red boxes, respectively, while residues of the green subunit interacting with both subunits are indicated in green. (D) Stereo ribbon presentation of the HiaBD1 monomer structure (Carson, 1997). β strands and helices are represented with arrows and thick helices, respectively. Secondary structures are labeled as in panel C. N and C denote the N and C termini of HiaBD1, respectively.

(residues 586–653), and Domain 3 (residues 654–705) (Figure 2B–D). Submission of the coordinates of the full-length HiaBD1 and/or individual domains to the DALI server revealed no known structures with significant similarity, indicating a novel protein fold.

Domain 1 is a four-stranded antiparallel β sheet, which forms a slightly concave sheet structure. One side of the sheet

is composed of hydrophobic residues, while the other side consists primarily of polar residues. Domain 2 is a globular domain with mixed structure, containing four short β strands ($\beta 5$ – $\beta 8$) and four helices (αA – B and $3_{10}a$ – b). This domain protrudes laterally and forms a knob. Domain 3 is an all β domain and consists of five long β strands ($\beta 9$ – $\beta 13$) and one short β strand ($\beta 9'$). These strands are twisted and segregate

Table 1 Crystallographic data and summary of refinement

Data set	Native HiaBD1 (APS)	SeMet HiaBD1 (SRS)		
		λ_1 peak	λ_2 inflection	λ_3 remote
<i>Data collection</i>				
Space group	P2 ₁	P321		
Cell parameters <i>a</i> , <i>b</i> , <i>c</i> (Å)	83.58, 86.20, 89.12 ($\beta = 99.08^\circ$)	84.61, 84.61, 154.25		
Wavelength (Å)	0.99187	0.9800	0.9805	0.9686
Resolution (Å)	50–2.1 (2.18–2.10)	50–2.7 (2.8–2.7)		
Reflections (total/unique)	354 239/72 387	129 143 (34 060)	125 426 (34 080)	129 436 (34 067)
Completeness (%) ^a	99.2 (98.0)	99.9 (100)	99.9 (99.9)	100 (100)
<i>I</i> / σ (<i>I</i>) ^a	25.05 (2.92)	13.87 (2.73)	12.98 (2.62)	13.00 (2.60)
<i>R</i> _{sym} ^{a,b}	4.4 (27.1)	8.0 (52.2)	8.5 (54.0)	8.4 (55.9)
<i>Refinement</i>				
Resolution (Å)	50–2.1			
$ F /\sigma(F)$	> 0			
Reflections (working/test)	66 679/3587			
Completeness (%) (overall/outer shell)	96.1/90.5			
Total number of atoms	7362			
Protein molecules	6			
Water molecules	488			
<i>R</i> _{cryst} / <i>R</i> _{free}	0.217/0.254			
Average <i>B</i> factors (Å ²) (main/side chains)	35.2/36.2			
r.m.s. deviations				
Bonds (Å)	0.009			
Angles (deg)	1.5			
<i>B</i> values (Å ²) (main/side chain)	1.004/1.619			

^aNumbers in parentheses indicate values in the highest resolution shell (2.13–2.1 for native HiaBD1 and 2.8–2.7 for SeMet HiaBD1).

^b $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum I$, where *I* is the observed intensity and $\langle I \rangle$ is the average intensity for symmetry-related reflections.

into three distinct subdomains, including a β hairpin formed by the antiparallel $\beta 9/\beta 9'$ and $\beta 10$ strands, a connector formed by the $\beta 11$ strand, and a β hairpin formed by the antiparallel $\beta 12$ and $\beta 13$ strands. There are very few macromolecular contacts between these subdomains, and Domain 3 appears to hold together via association with other HiaBD1 subunits (Figure 2B).

As shown in Figure 3A and B, the crystal structure of purified HiaBD1 reveals that individual subunits are assembled into a trimer, forming a mushroom-shaped structure with a broad stem at the N-terminal end and an elongated cap at the C-terminal end. The trimer has dimensions of 80 Å in length and 45 Å in width. At least three pieces of evidence argue that the trimer detected by crystallography represents the physiologic state of HiaBD1. First, in other work, we have discovered that the Hia C-terminal translocator domain of Hia forms a stable trimer in the bacterial outer membrane (Surana *et al*, 2004). Second, based on size-exclusion chromatography, purified HiaBD1 and SeMetHiaBD1 form oligomers in solution. Third, while HiaBD1 and SeMetHiaBD1 crystallize in different space groups, in both crystal forms the arrangement of the protein subunits is very similar, with head-to-head trimers.

All three domains in HiaBD1 participate in the trimer interface (Figures 2C and 3C). The total buried area between subunits within the trimer is approximately 15 240 Å², with Domain 1 contributing 2580 Å², Domain 2 contributing 4440 Å², and Domain 3 contributing 8220 Å². Of the available solvent-accessible surface area, approximately 25% of Domain 1, 24% of Domain 2, and 49% of Domain 3 is used for trimer formation (Figure 3C), suggesting that trimer formation may be required for protein stability. The Domain 1 β strands in one subunit interact with the corre-

sponding β strands in both neighboring subunits to form an almost perfect triangle (the stem of the mushroom). The Domain 2 $\beta 5$ strand in one subunit forms a small β sheet together with the Domain 1 $\beta 4$ strand in a second subunit and the Domain 2 $\beta 8$ strand in a third subunit, while the αB helix in one subunit interacts with the αB helix in the other subunits to form a tight hydrophobic core (Figure 3C). The Domain 3 β strands in one subunit interdigitate with the Domain 3 β strands in the other two subunits, resulting in extensive subunit–subunit interactions and a very compact structure (Figures 2B and 3C). Each of the Domain 3 subdomains from one subunit contributes to a separate side of the three-sided tapering mushroom cap. In addition, the Domain 3 region holds the trimer together through an extensive hydrophobic/aromatic core consisting primarily of phenylalanine and tryptophan residues.

In considering the surface of HiaBD1, several structural features are notable. The concave β sheet of Domain 1 forms a shallow groove that runs parallel to the three-fold axis of the trimer (Figure 3B). Domain 2 forms a knob that extends laterally from the structure, into the solvent *in vitro* and into the extracellular milieu *in vivo* (Figure 3A and B). This knob consists of the αA helix, the $3_{10}A$ helix, and the loop structures between $\beta 6$ and $\beta 8$ (Figure 2D). Domains 2 and 3 form a major groove that runs diagonal to the three-fold axis of the trimer, just underneath the knob (Figure 3B). This groove is lined with charged residues, including R651, K661, R674, K682, R697, and R698 (Figure 3B).

Structural relationship of HiaBD1 and HiaBD2 and identification of repeating structural units

The Hia passenger domain contains a secondary binding domain defined by residues 50–374 and designated HiaBD2.

Figure 3 Structure of the HiaBD1 trimer. (A) Stereo ribbon diagram (Carson, 1997) of the HiaBD1 trimer. The three subunits are shown in green, yellow, and red. The subunit in green is the same orientation as in Figure 2D. For clarity, only selected secondary structural elements are labeled. (B) Surface electrostatic potential of the HiaBD1 trimer. The surface was contoured and displayed using the program GRASP (Nicholls *et al*, 1991). Color coding is according to charge, with blue for the most positive regions and red for most negative regions and linear interpolation in between. In the left panel, the orientation of the trimer is the same as in panel A, while in the right panel, the trimer has been rotated 60° along the vertical axis. Charged residues located in the groove under the knob are labeled. (C) Surface presentation of one HiaBD1 monomer (the green subunit in panel A), with the second and the third subunits shown as red and yellow ribbons. The two views are the same as in panel B. Residues of the first subunit (green) involved in intermolecular interactions with the second subunit (yellow) or the third subunit (red) are mapped on the surface of the first monomer and are indicated in yellow and red, respectively. The residues interacting with both subunits are mapped on the surface of the first subunit in green.

HiaBD1 and HiaBD2 share sequence homology and interact with the same host cell receptor. With our knowledge of the structure of HiaBD1, we re-examined an alignment of the amino-acid sequences of HiaBD1 and HiaBD2. As shown in Figure 4A, residues 585–705 of HiaBD1 (Domains 2 and 3) share striking homology with residues 50–166 of HiaBD2, with 44% identity and 76% similarity overall. This high level of homology suggests that the 585–706 region of HiaBD1 (HiaBDp in Figure 1) and the 50–166 region of HiaBD2 (HiaBDs in Figure 1) have very similar structures.

Interestingly, sequence homology between HiaBD1 and the remainder of the Hia passenger domain is not limited to amino acids 50–166. Additional analysis revealed that residues 641–705 encompassing the β 8 strand and the α B helix of Domain 2 and all of Domain 3 could be aligned with two other stretches of 67 and 64 residues within the Hia passenger domain, namely residues 250–316 (D3b) and 359–422 (D3c)

(Figures 1 and 4B). Among the 67 residues in D3b, 18 (27%) are identical and 43 (63%) are conserved relative to the corresponding residues in Domains 2 and 3 of HiaBD1. Similarly, among the 64 amino acids in D3c, 11 (19%) are identical and 42 (65%) are conserved relative to the corresponding amino acids in HiaBD1. Search of the Swiss-Prot data base using the motif [LVMI]-X(3)-G-W-X(7,10)-[TN]-X(6,9)-V-X(5)-V-X-F-X-[GSA] returns 80 hits belonging to 42 sequences, 38 of which are Hia, Hsf, or NhhA sequences from different strains. Both Hsf from *H. influenzae* and NhhA from *Neisseria meningitidis* are known close homologs of Hia. Extending this motif to include residues in the β 12 and β 13 strands of Domain 3 results in a marked decrease in the number of hits and restricts them to Hia, Hsf, and NhhA sequences. Thus, the D3 homology is restricted to Hia, Hsf, and NhhA.

These observations indicate that the Hia passenger domain has a modular structure with repeating Domain 3-like regions.

A

B

Figure 4 Modular architecture of the Hia passenger domain. (A) Location of identical, conserved, and nonconserved residues between HiaBD1 and HiaBD2 on the surface of HiaBD1. A sequence alignment is provided at the bottom of the panel. Color coding is indicated. (B) Structure-based sequence alignment of Hia residues 641–705 with homologous regions elsewhere in the Hia passenger domain. Corresponding amino-acid numbers are indicated in the left column. Identical and conserved residues throughout the sequences are highlighted in green and pale green, respectively.

Identification of the receptor-binding region

In an effort to identify the receptor-binding region of the HiaBD1 structure, we began by generating a GST fusion protein that contains Hia_{581–714}, which encompasses Domains 2 and 3 and lacks all but five residues in Domain 1. Following cleavage of the GST moiety, Hia_{581–714} was purified and examined for adherence activity. Based on size-exclusion chromatography, Hia_{581–714} formed oligomers in solution, analogous to purified HiaBD1 and Hia_{50–779}. In assays assessing cell binding activity and capacity to inhibit adherence by bacteria expressing wild-type Hia, Hia_{581–714} was indistinguishable from HiaBD1 (data not shown). Thus, Domain 1 is not required for HiaBD1-mediated adherence.

With this information in mind, we focused primarily on Domains 2 and 3 and generated site-specific mutations in a series of surface-exposed residues (Table II and Figure 5A). We concentrated on residues lining potential binding pockets and introduced the mutations into pHMW8-7ΔBS2, then expressed the mutant proteins in *Escherichia coli*. Western analysis of outer membrane preparations confirmed that the mutant proteins were present in the bacterial outer membrane at wild-type levels (data not shown). Examination of bacterial adherence revealed that the D618K, A620R, and

V656R mutations resulted in complete abrogation of Hia-mediated bacterial adherence. As depicted in Figure 5A, residues D618 and A620 are located on the underside of the Domain 2 knob, forming a roof over an acidic patch containing E668 and E678. Residue V656 is adjacent to the same acidic patch.

To extend these results, we produced GST fusions of the mutated HiaBD1 proteins. After cleavage of the GST moiety, the HiaBD1 derivatives were purified and then examined for their capacity to inhibit Hia-mediated adherence. Consistent with previous results, purified wild-type HiaBD1 was capable of complete inhibition (<5% of adherence observed when monolayers were untreated) (Figure 5B). In contrast, purified HiaBD1/D618K, HiaBD1/A620R, and HiaBD1/V656R had minimal effect on Hia-mediated adherence (70–100% of adherence observed when monolayers were untreated) (Figure 5B). Interestingly, purified HiaBD1/N617R, HiaBD1/E668K, and HiaBD1/E678A also had minimal effect on Hia-mediated adherence (Figure 5B), suggesting that N617, E668, and E678 also contribute to the Hia receptor-binding region. Control proteins including HiaBD1/K597E, HiaBD1/D600R, HiaBD1/L606K, HiaBD1/A610R, and HiaBD1/K630D all behaved like wild-type HiaBD1 (Figure 5B).

Table II Adherence activity of HiaBD1 point mutants

Mutation	Adherence	Mutation	Adherence
Wild type	+	K597A	+
N564D	+	K597E	+
A568E	+	D600R	+
N602A	+	L606K	+
D611A	+	A610R	+
D619A	+	N617R	+
D628A	+	D618K	—
D641A	+	A620R	—
K661A	+	K622E	+
E668A	+	K630E	+
H670A	+	Q635A	+
R674A	+	N642A	+
K682A	+	V656R	—
N685D	+	E668K	+
N695A	+	R674D	+
R697A	+	E678A	+
R698A	+	F681E	—
V592W	+	R698D	+

Adherence was measured in quantitative assays examining *E. coli* BL21(DE3) expressing the HiaBD1 derivatives in the context of pHMW8-7ABS2. In all cases, HiaBD1 derivatives were examined in triplicate in at least three different assays. In each panel, mutations are listed in the left column, and adherence activity is listed in the right column. (+) denotes intact adherence (>60% of adherence by BL21(DE3)/pHMW8-7ABS2), and (—) denotes absence of adherence (<5% of adherence by BL21(DE3)/pHMW8-7ABS2).

To confirm that the loss of adhesive activity of HiaBD1/D618K, HiaBD1/A620R, HiaBD1/V656R, HiaBD1/N617R, HiaBD1/E668K, and HiaBD1/E678A was not due to global changes in protein folding and disruption of the trimer, we assessed susceptibility to limited trypsin proteolysis. As shown in Figure 5C, wild-type HiaBD1 was completely resistant to trypsin in a 30 min assay using 15 µg of purified HiaBD1 and 250 ng of trypsin, indicating a stable structure. Similarly, all of the adhesive and nonadhesive HiaBD1 derivatives were resistant to trypsin treatment (Figure 5C). As a control, we introduced a mutation at F681, which lies within the β 11 strand in Domain 3 and is involved in subunit-subunit interactions with both of the other subunits in the trimer. As predicted, purified HiaBD1/F681E was completely degraded by trypsin, suggesting an unstable trimer and an unfolded protein (Figure 5C). Similarly, a derivative of HiaBD1 with a deletion of residues 672–682 in the core (HiaBD1 Δ 6) was completely degraded by trypsin (Figure 5C).

Together, these results indicate that the HiaBD1 receptor-binding domain is an acidic pocket formed by N617, D618, A620, V656, E668, and E678. The entire binding pocket is contained within a single monomer, and thus the trimer has three identical binding sites related by a 120° rotation.

Conclusion

Adherence to host tissues is a fundamental step in the pathogenesis of most infectious diseases and is mediated by specialized microbial factors called adhesins. In this study, we have characterized the structure of the HiaBD1 binding domain of the *H. influenzae* Hia adhesin, an autotransporter protein that mediates efficient adherence to the respiratory epithelium and facilitates *H. influenzae* colonization of the upper respiratory tract. HiaBD1 has a novel trimeric archi-

tecture with three-fold symmetry and three identical receptor-binding pockets.

The HiaBD1 subunit fold is inherently unstable by itself and is stabilized by trimerization, with each domain in each subunit lending complementary secondary structures to the other subunits. Most striking is the extensive interdigitation between Domain 3 β strands, an arrangement that appears to be repeated four times along the Hia passenger domain (designated D3a, D3b, D3c, and D3d in Figure 1). Moreover, mutations in the core of the trimer interface such as F681E destabilize the trimer and eliminate adhesive activity. Thus, trimerization appears to be an essential structural feature of a functional Hia passenger domain.

In most autotransporters, the C-terminal translocator domain is approximately 300 residues in length and forms a β barrel with 12–14 transmembrane β strands. However, the Hia C-terminal translocator domain is much smaller, consisting of only 76 amino acids (Surana *et al*, 2004). Similarly, the YadA autotransporter expressed by *Yersinia* spp has a C-terminal translocator domain that contains only 70 amino acids (Roggenkamp *et al*, 2003). Hia and YadA appear to define a new subfamily of autotransporters with a distinctive C-terminal translocator domain that trimerizes. Based on homology analysis, at least 28 proteins belong to this subfamily, including proteins expressed by *Salmonella enteritidis*, *N. meningitidis*, *Moraxella catarrhalis*, and pathogenic *E. coli*, among other pathogens (Table III). Members of this subfamily may share a similar Hia-like architecture.

The fact that both the translocator domain and the passenger domain are trimeric in this class of trimeric autotransporters has implications regarding the mechanism of translocation of the passenger domain. Indeed, three passenger domains must translocate through a central trimeric β -domain pore to form an extensively intertwined trimer. We would like to propose that, to produce a functional trimeric adhesion domain, the passenger domain of each subunit must be translocated in a concerted fashion and that as these domains are extruded from the pore, folding and trimerization must occur coincidentally, perhaps providing the driving force that pulls the unfolded passenger domain across the pore.

Site-directed mutagenesis established that residues N617, D618, A620, V656, E668, and E678 contribute to the receptor-binding pocket of HiaBD1. Mutations of D618, A620, and V656 abrogated adhesive activity in purified HiaBD1 and also in full-length Hia expressed on the surface of bacteria. In contrast, mutations of N617, E668, and E678 abolished adhesive activity in purified HiaBD1 but had little effect on Hia expressed by bacteria. Residues D618, A620, and V656 form the rim of the binding pocket, while residues N617, E668, and E678 lie behind the rim or at the base of the pocket. One possibility is that N617, E668, and E678 are sites of secondary contact with the Hia receptor; as a consequence, mutations of these residues may result in only a modest decrease in HiaBD1 binding affinity, an effect that can be overcome by the cooperative effect of multiple molecules of Hia on the surface of an organism, at least under the conditions of our adherence assay. Based on alignment of the amino-acid sequences of Hia proteins from nine different strains of *H. influenzae*, it appears that the HiaBD1 receptor-binding pocket is highly conserved, with absolute conservation of D618, A620, V656, E668, and E678. Comparison

A

B

C

Figure 5 Identification of the receptor-binding region of HiaBD1. (A) Left: Location of the residues mutated in this study (see Table II). Mutations in residues in cyan do not affect adherence, while mutations in residues in magenta result in reduced adhesive activity. Right: Same as on the left but rotated ~ 50 degrees on the vertical axis, providing a clear view of the binding site. (B) Inhibition of Hia-mediated adherence by purified derivatives of HiaBD1. Chang epithelial cells were preincubated with 100 nM purified protein containing the indicated point mutation for 1.5 h. Subsequently, $1-2 \times 10^7$ CFU of BL21(DE3)/Hia were inoculated onto monolayers, and adherence was measured in a 30-minute assay. Adherence values were normalized to adherence by BL21(DE3)/Hia to monolayers that were not pretreated with purified protein (designated 'no protein', defined as 100%). Wild-type HiaBD1 (designated 'WT') was used as a control and inhibited adherence by $\sim 95\%$. (C) Stability of the HiaBD1 trimer. Normal folding of HiaBD1 derivatives was assessed by measuring susceptibility to treatment with trypsin for 30 min. Samples designated (-) were incubated in buffer without trypsin, and samples designated (+) were incubated in buffer with 250 ng of trypsin. BS6 is a derivative of HiaBD1 with deletion of residues 672–682.

of the HiaBD1 and HiaBD2 primary and secondary binding domains reveals absolute conservation of A620, V656, E668, and E678. In contrast, HiaBD1 has an aspartic acid at position 618, while HiaBD2 has a glutamine at the corresponding

position, perhaps accounting in part for the differences in HiaBD1 and HiaBD2 binding affinity.

The observation that a single HiaBD1 trimer contains three identical receptor-binding pockets is reminiscent of the tumor

Table III Proteins identified as having a C terminus similar to the Hia C terminus

Gene	Description	Organism	Accession
OMP100	Unknown	<i>Actinomyces actinomycetemcomitans</i>	BAB86905
Aa32-1-2	Putative adhesin/invasin	<i>A. actinomycetemcomitans</i>	AAL91673
EibE	Immunoglobulin-binding protein	<i>E. coli</i>	AAF63045
EibD	Immunoglobulin-binding protein	<i>E. coli</i>	AAF63040
EibC	Immunoglobulin-binding protein	<i>E. coli</i>	AAF63035
BMEI1872	Cell-surface protein	<i>B. melitensis</i>	NP_540789
EibE	Immunoglobulin-binding protein	<i>B. melitensis</i>	NP_539123
EibF	Immunoglobulin-binding protein	<i>E. coli</i>	AAL31493
Z5029	Putative adhesin	<i>E. coli</i>	NP_290185
Z0639	Unknown	<i>E. coli</i>	NP_286235
FN1499	Cell-surface protein	<i>Fusobacterium nucleatum</i>	NP_602326
DsrA	Serum resistance, adhesin	<i>Haemophilus ducreyi</i>	AAF37814
Hia	Adhesin	<i>H. influenzae</i>	AAC43721
Hsf	Adhesin	<i>H. influenzae</i>	AAC44560
UspA2	Serum resistance	<i>M. catarrhalis</i>	AAL76059
UspA2H	Adhesin	<i>M. catarrhalis</i>	AAF40121
NadA	Putative adhesin/invasin	<i>N. meningitidis</i>	AAM53100
NhhA	Putative adhesin	<i>N. meningitidis</i>	AAK68871
Hsf	putative adhesin	<i>Pasteurella multocida</i>	NP_245651
EibA	Immunoglobulin-binding protein	<i>E. coli</i>	AAF63234
RSp1620	Putative hemagglutinin-related protein	<i>Ralstonia solanacearum</i>	NP_523179
SapB	Putative autotransporter	<i>Salmonella enterica</i>	NP_458235
STM3691	Putative inner membrane protein	<i>Salmonella typhimurium</i>	NP_462591
ORF1	Unknown	<i>Xanthomonas campestris</i>	AAF63394
XadA	Putative adhesin	<i>Xanthomonas oryzae</i>	AAG01335
XF1981	Surface protein	<i>Xylella fastidiosa</i>	NP_299263
XF1529	Surface protein	<i>X. fastidiosa</i>	NP_298818
YadA	Adhesin	<i>Yersinia enterocolitica</i>	NP_052433

necrosis factor (TNF) superfamily of proteins, which play a critical role in several fundamental cellular processes, including apoptosis, survival, differentiation, and proliferation (Aggarwal, 2003). TNF α , TNF β , and all other members of the TNF family are trimeric proteins with three spatially distinct but equivalent receptor-binding sites that recognize members of the TNF receptor superfamily (Locksley *et al*, 2001). In general, each trimer interacts with three receptor monomers in a final 3:3 complex (Banner *et al*, 1993; Locksley *et al*, 2001). In the case of Hia, it is possible that a single HiaBD1 trimer interacts with three separate receptor molecules or with three related domains on one receptor. A multivalent interaction with the host cell surface increases avidity and may stabilize bacterial adherence, allowing organisms to overcome physical forces in the respiratory tract such as coughing, sneezing, and mucociliary activity.

Based on comparison of the Hia sequences from diverse strains, the most variable portion of the HiaBD1 structure is the knob that protrudes laterally on all three faces (residues 599–619 and 631–636). This observation suggests that the knob may be a major target of the host immune response, perhaps in part because of its overt accessibility to the immune system. Accordingly, it is possible that strain-to-strain antigenic variation in this region of Hia has occurred to evade the immune response, analogous to the hypervariable regions that protrude from the *N. gonorrhoeae* pilin protein (Parge *et al*, 1995).

Hia is a major nonpilus adhesin of nonencapsulated *H. influenzae* (Barenkamp and St Geme, 1996; St Geme *et al*, 1998). A homolog of Hia called Hsf is ubiquitous in encapsulated isolates of *H. influenzae* and is the major nonpilus adhesin in these strains (St Geme *et al*, 1996). The HiaBD1 and HiaBD2 domains are highly conserved in Hsf.

Thus, the receptor-binding pocket identified in HiaBD1 represents an attractive template for design of binding inhibitors that target encapsulated *H. influenzae* and an important subset of nonencapsulated *H. influenzae*. More broadly, knowledge of the HiaBD1 structure may facilitate development of novel antimicrobials with activity against other pathogens that express homotrimeric autotransporters, perhaps by disrupting trimerization.

Experimental procedures

Bacterial strains and plasmids

E. coli strains included BL21 (DE3), XL-1 Blue, and DL41, and have been described previously (Sambrook and Russell, 2001).

The plasmid pHMW8-7 contains wild-type *hia* from *H. influenzae* strain 11 in pT7-7. The plasmid pHMW8-7 Δ BS2 has been described previously and is a derivative of pHMW8-7 with a deletion of coding sequence for Hia residues 114–127, effectively eliminating the adhesive function of the HiaBD2 domain. The plasmid pGEX6P-1WT1BD has been described previously and contains coding sequence for Hia residues 541–714 cloned into the *Bam*H1/*Eco*RI sites of pGEX6P-1 (Amersham), allowing production of GST–HiaBD1. The plasmid pGEX6P-HiaBD1 (I560M/I657M) is a derivative of pGEX6P-1WT1BD and encodes a GST fusion protein containing HiaBD1 with methionine residues inserted in place of I560 and I657. pGEX6P-HiaBD1 (I560M/I657M) was produced for crystallization to determine the structure of HiaBD1 using the multiwavelength anomalous diffraction (MAD) method. Adhesive activity of HiaBD1 (I560M/I657M) is similar to that of wild-type HiaBD1 (data not shown),

indicating that mutations of I560 and I657 to methionine do not affect receptor binding.

Recombinant DNA methods

DNA ligations, restriction endonuclease digestions, gel electrophoresis, and polymerase chain reactions were performed according to standard techniques. Plasmids were introduced into *E. coli* by electroporation (Ausubel *et al*, 1994).

Site-directed mutagenesis reactions were performed using standard protocols and Stratagene site-directed mutagenesis kits. QuikChange was used for mutations in pGEX6P-1WT1BD, while QuikChange XL was used for mutations in pHMW8-7ΔBS2. Mutants plasmids were initially transformed in *E. coli* XL-1 Blue, and nucleotide sequencing was performed. Plasmid clones with the correct nucleotide sequence were then transformed into *E. coli* BL21 (DE3) and *E. coli* DL41.

Protein production and purification

In order to produce native HiaBD1 and most HiaBD1 mutants, pGEX-6P-1WT1BD derivatives were introduced into *E. coli* BL21(DE3), and the resulting strains were incubated at 37°C in LB medium containing 100 µg/ml ampicillin. In order to produce selenomethionine-substituted HiaBD1 (SeMetHiaBD1), the plasmid pGEX6P-HiaBD1(I560M/I657M) was introduced into *E. coli* DL41, and the resulting strain was incubated at 37°C in LeMaster medium containing selenomethionine and 100 µg/ml ampicillin (Lemaster and Richards, 1985). All cultures were incubated to an OD₆₀₀ = 0.4–0.5, then supplemented with isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.1 mM and shifted to 30°C. After incubation for another 4–5 h, bacteria were harvested by centrifugation at 6600 g, then resuspended in 25 ml lysis buffer (5 mM EDTA, 1 mM Pefabloc SC, 1:100 protease inhibitor mix (Roche Molecular Biochemicals) in PBS) and lysed using a French pressure cell. Cell fragments were removed by centrifugation at 10 000 g, Triton X-100 was added to a final concentration of 1%, and GST fusion proteins were isolated by affinity chromatography using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). Following extensive washing with Tris-buffered saline (TBS), the HiaBD1 portion of fusion proteins was cleaved from GST by adding 80 U/ml PreScission protease (Amersham Pharmacia Biotech) and 1 mM dithiothreitol to the beads and incubating overnight at 4°C. The HiaBD1 protein was separated from the GST moiety by centrifugation at 1000 g for 5 min. At this step, isolated proteins were analyzed for purity by SDS–polyacrylamide gel electrophoresis and staining with Coomassie blue. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad #500-0006). For crystallization, native HiaBD1 and SeMetHiaBD1 were further purified using a gel filtration column (Sephacryl S300 26/60 column, Amersham Pharmacia Biotech) equilibrated in a buffer containing 20 mM HEPES (pH 7.0), 100 mM NaCl, 1 mM DTT, and 10% glycerol. This step was necessary to produce crystals. The highly pure fractions (about 95%) from a single peak were combined and concentrated to approximately 10 mg/ml.

Crystallization of HiaBD1 and data collection

The native HiaBD1 crystals were grown at room temperature by vapor diffusion in hanging drops against a reservoir solution containing 25% PEG 4000, 0.2 M MgCl₂, and with

a 100 mM buffer ranging from pH 3.8 to 10. In the acidic condition, crystals formed as well-shaped triangular plates, but these crystals did not diffract well. When using Tris–HCl (pH 8.5), thin plate crystals were formed within 18 h and grew for a week. Single crystals (typical dimensions 0.3 × 0.4 × 0.05 mm) were cryoprotected gradually to a final solution containing 30% PEG 4000, 0.2 M MgCl₂, 100 mM Tris–HCl (pH 8.5), and 25% glycerol, and the crystals were flash-frozen in liquid nitrogen. Crystals were in monoclinic space group P2₁ with cell dimension $a = 83.58$ Å, $b = 86.2$ Å, $c = 89.12$ Å, and $\beta = 99.08^\circ$. Crystals contained six molecules per asymmetric unit and diffracted to a resolution of 2.8 Å in the laboratory and 2.1 Å at the synchrotron (APS, beamline 19BM). Crystals of selenomethionyl HiaBD1 (SeMetHiaBD1) were grown under the same conditions as the native crystals. However, these crystals were not stable. With further extensive screening efforts, crystals of selenomethionyl HiaBD1 were grown in a solution containing 1.4 M Na-citrate and 100 mM HEPES (pH 7). Long hexagonal needle or hexagonal tube crystals formed within 24 h and grew for a week. Single crystals (typical dimensions 0.1 × 0.6 × 0.025 mm) were cryoprotected in a solution containing 1.45 M Na-citrate and 25% glycerol, and the crystals were flash-frozen in liquid nitrogen. Crystals were in trigonal space group P321 with cell dimension $a = b = 84.61$ and $c = 154.25$. Crystals contained two molecules per asymmetric unit, with solvent contents of 72%, and diffracted to a resolution of 2.7 Å at the synchrotron (SRS Daresbury, beamline 14-2).

Structure determination and refinement

The SeMetHiaBD1 monomer contains two methionines. Accordingly, four selenium sites were expected in the asymmetric unit. The four sites were found using the programs SOLVE (Terwilliger and Berendzen, 1999), SnB2.2 (Smith *et al*, 1998), and CNS (Brünger *et al*, 1998), and were then employed in phasing using the MAD phasing method followed by density modification (solvent flipping), as implemented by the program CNS. The resulting 3.0 Å electron density map was readily interpretable, into which approximately 60% of the secondary structural elements could be built as poly-ala fragments using the program O (Jones *et al*, 1991). The complete asymmetric unit was generated using NCS operators derived from Se sites. From this partial model, an NCS mask was constructed using the program MAMA (Kleywegt and Jones, 1999). Several cycles of combining calculated phases from the minimized partial model with MAD experimental phases followed by NCS averaging and solvent flipping (program CNS) resulted in electron density maps that allowed assignment of the remaining poly-ala chain. As phases continued to improve, side chains were gradually introduced. Most of the side chains could also be placed unambiguously into density. The resulting atomic model was refined (program CNS) against 3.0 Å data using conjugate gradient minimization. A random 5% of the data were removed prior to refinement and constituted the test set for crossvalidation. When R/R_{free} reached 0.30/0.32, the model was used as the search model to solve the native crystal structure using the molecular replacement method (Navaza and Saludjian, 1997). For molecular replacement, we used only the model from residues 580–704, since the density and the model of this portion were better defined than those of residues 550–579. The final model was refined

against the 2.1 Å resolution native dataset. During the final rounds of refinement, solvent molecules (water) were added. The final model, with R/R_{free} factors of 21.7/25.4, includes 1370 atoms of residues 548–705 of chain A, 549–705 of chains B, E, and F, 550–706 of chain C, 549–706 of chain D, and 488 water molecules in the asymmetric unit (see Table 1 for refinement statistics). The model has been deposited in the pdb (entry code 1S7M).

Quantitative adherence assays

Chang epithelial cells (Wong–Kilbourne derivative, clone 1-5c-4, human conjunctiva; ATCC CCL20.2) were maintained in minimal essential medium (MEM) supplemented with non-essential amino acids and 10% (vol/vol) fetal calf serum (FCS) and were cultivated at 37°C in 5% CO₂. Adherence assays were performed as described previously (St Geme *et al*, 1993). Briefly, cells were seeded into 24-well tissue culture plates at a density of 1.8×10^5 cells/well and were incubated overnight. Bacteria were inoculated into broth and allowed to grow to a density of $\sim 2 \times 10^9$ colony-forming units (CFU) per ml. Approximately $1\text{--}2 \times 10^7$ CFU were inoculated into each well, and plates were gently centrifuged at 165 g for 5 min. After incubation at 37°C in 5% CO₂ for 25 min, monolayers were rinsed with PBS to remove nonadherent bacteria, treated with trypsin–EDTA, then resuspended in BHI broth. Suspensions were plated on 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.

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