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Structure and function of the *Haemophilus influenzae* autotransporters

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Autotransporters are a large class of proteins that are found in the outer membrane of Gram-negative bacteria and are almost universally implicated in virulence. These proteins consist of a C-terminal β-domain that is embedded in the outer membrane and an N-terminal domain that is exposed on the bacterial surface and is endowed with effector function. In this article, we review and compare the structural and functional characteristics of the *Haemophilus influenzae* IgA1 protease and Hap monomeric autotransporters and the *H. influenzae* Hia and Hsf trimeric autotransporters. All of these proteins play a role in colonization of the upper respiratory tract and in the pathogenesis of *H. influenzae* disease.

Keywords: *Haemophilus influenzae*, autotransporter, adhesin, serine protease

INTRODUCTION

Autotransporters are a large family of proteins that are inserted into the outer membrane in Gram-negative bacteria and have a variety of effector functions mediated by an extracellular domain, including adherence, invasion, protease activity, and cytotoxicity, among others (Henderson and Nataro, 2001; Pallen et al., 2003). All autotransporters share a common domain structure consisting of an N-terminal signal sequence, a C-terminal β-barrel (translocator) domain, and an internal passenger domain (Desvaux et al., 2004). In the first step of secretion, the cleavable signal sequence directs the nascent protein to the Sec machinery, which facilitates translocation across the inner membrane. Subsequently, the β-barrel domain targets the protein to the outer membrane, where it inserts and facilitates presentation of the internal passenger domain on the bacterial surface.

There are two forms of autotransporter proteins, namely monomeric and trimeric. Monomeric autotransporters consist of a single polypeptide that contains a C-terminal β-barrel domain that is approximately 300 residues in length and folds into 12 β-strands (Klauser et al., 1993; Maurer et al., 1999; Oomen et al., 2004). In contrast, trimeric autotransporters have a short β-barrel domain that is about 70 residues in length and forms four β-strands. Because of the shortened β-barrel domain in trimeric autotransporters, trimerization must occur to form a functional, full-size β-barrel with 12 β-strands (Roggenkamp et al., 2003; Surana et al., 2004).

Initially, it was believed that all the components necessary for successful folding and insertion of autotransporters into the outer membrane were contained in a single polypeptide and that translocation of the passenger domain across the outer membrane occurred without the aid of accessory proteins. However, recent experimental evidence has established that the term "autotransporter" is a misnomer, as outer membrane localization and passenger domain surface presentation require the *Bam*A integral outer membrane protein (Voulhoux et al., 2003; Jain and Goldberg, 2007; Lehr et al., 2010). Understanding the mechanism of outer membrane insertion and passenger domain surface presentation via the Bam complex is still limited, and a number of models exist to explain this process.

Folding of the β-barrel domain occurs as a result of interactions with the Bam complex, with at least partial folding of some translocator domains occurring in the periplasm (Ieva et al., 2008). Regarding transport of the passenger domain across the outer membrane, multiple mechanisms have been proposed. One possibility is that threading of the passenger domain occurs in an N- to C-terminal direction. However, deletions in the N-terminal region of autotransporters do not inhibit translocation, making this scenario unlikely (Bernstein, 2007). Another possibility is that the C-terminal end of the passenger domain forms a hairpin within the translocator pore as an initial step. According to this model, movement through the pore occurs in a C-terminal to N-terminal direction, with folding of the passenger domain providing the energy needed for secretion. In support of this model, there is evidence that the C-terminal end passes through the β-barrel pore as an initial step in passenger domain secretion and that folding of this region facilitates translocation of the rest of the protein (Junker et al., 2009; Peterson et al., 2010). The passenger domain may be partially folded while in the periplasm, requiring addition space in the β-barrel pore during secretion (Skillman et al., 2005). To accommodate a monomeric passenger domain with some degree of tertiary structure, the β-barrel pore may be held in an open conformation by the Bam complex until the passenger domain is fully translocated. In support of this model, several members of the Bam complex have been shown to

interact with autotransporter β-barrel and passenger domains at multiple locations along the protein, presumably aiding in protein folding and translocation (Bernstein, 2007; Peterson et al., 2010; Ieva et al., 2011). In the case of trimeric autotransporters, three passenger domains must be secreted and trimerized. It is difficult to envision how three passenger domains could fit through a single β-barrel pore. Translocation of one monomer at a time through the pore would require folding and trimerization to occur as a last step, which would not supply energy for the translocation process. Secretion of a trimeric passenger domain through the small β-barrel pore could be explained if the Bam complex holds the trimeric β-barrel pore in an open configuration, as in the proposed monomeric secretion model, creating enough space for the secretion of all three subunits.

IgA1 PROTEASE

IDENTIFICATION

IgA1 protease was initially identified in several *Neisseria* and *Streptococcus* species as a secreted bacterial protein able to cleave human immunoglobulin A1 (IgA1), the IgA subclass that predominates in the nasopharyngeal cavity (Mehta et al., 1973; Plaut et al., 1975, 1977). IgA antibody protects mucus membranes by agglutinating invading bacteria, thus inhibiting bacterial interaction with the epithelial surface, and by binding to and neutralizing microbial enzymes and toxins (Kett et al., 1986). IgA cleavage eliminates these protective mechanisms, presumably enhancing bacterial colonization. Based on the observation that only pathogenic bacteria produce IgA1 proteases, it seems likely that these proteins are virulence factors; however, because the IgA1 proteases are specific for human, chimpanzee, and gorilla IgA1, there is no animal model to directly test their pathogenic role (Mulks and Plaut, 1978; Kilian et al., 1979). *Haemophilus influenzae* IgA1 protease shares significant homology with the *Neisseria* IgA1 proteases and has the characteristic autotransporter extended N-terminal signal sequence, a C-terminal β-barrel domain called Iga_{β}, and an internal passenger domain called Iga_{P} (**Figure 1**; Pohlner et al., 1987). The *iga* gene from *H. influenzae* type b strain HK368 spans 4,646 bp and encodes a protein with a predicted molecular mass of 169 kDa, while *iga* in the prototypic laboratory strain Rd is slightly larger, spanning 5,082 bp and encoding a protein with a predicted molecular mass greater than 180 kDa. *H. influenzae* IgA1 protease undergoes autoproteolytic cleavage, resulting in release of the passenger domain from the membrane-embedded β-barrel domain (Grundy et al., 1987; Poulsen et al., 1989; Plaut et al., 2000). Most *H. influenzae* strains are capable of cleaving serum IgA1 and, to a lesser extent, the dimeric secretory form of IgA1, including encapsulated meningitis isolates, non-typeable otitis media isolates, and nasopharyngeal isolates from healthy individuals (Kilian et al., 1979). As a reflection of the clonal population structure of encapsulated isolates of *H. influenzae,* typeable strains have similar IgA1 proteases with similar cleavage capabilities. In contrast, non-typeable *H. influenzae* (NTHi) IgA1 proteases are highly variable antigenically, presumably due to horizontal gene transfer and recombination between multiple colonizing strains to avoid immune detection (Musser et al., 1986; Lomholt et al., 1993). This variability leads to strain to strain differences in protease activity, with disease isolates from NTHi having a higher level of activity than colonizing isolates of NTHi (Vitovski et al., 2002).

STRUCTURE

The crystal structure of the IgA1 protease passenger domain reveals an N-terminal trypsin/chymotrypsin-like protease domain and a β-helical spine as the major structural components (**Figure 2A**; Johnson et al., 2009). The spine has a core of hydrophobic residues and an exterior of stacked serine, threonine, and asparagine residues and serves to extend the N-terminal domain away from the bacterial membrane. The N-terminal domain has a globular structure with unique loops in

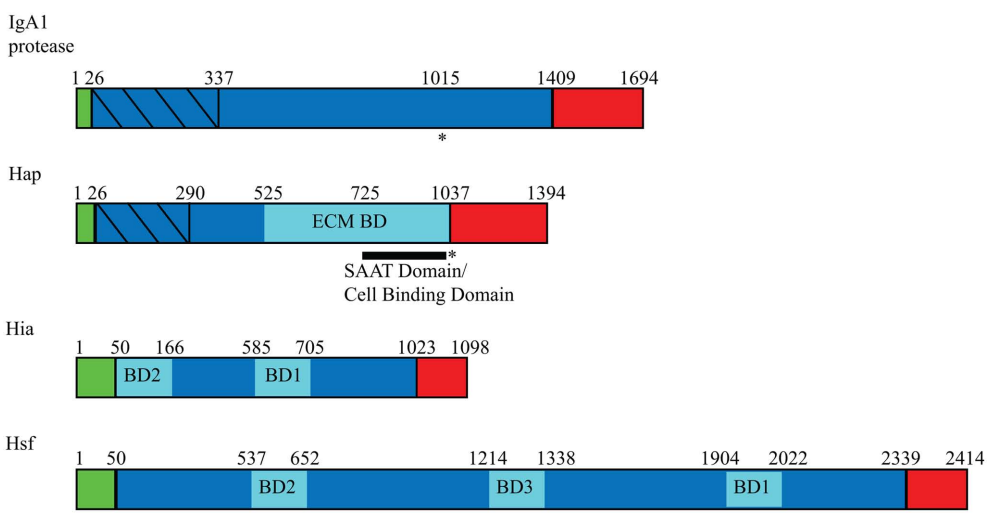

FIGURE 1 | Domain arrangement of *Haemophilus influenzae* autotransporters. Autotransporters contain three general domains: a signal peptide (green), a passenger domain (blue), and an outer membrane β-barrel domain (red). Within the passenger domains, binding domains are represented in pale blue, and protease domains are represented with diagonal lines. Asterisks (*) indicate regions of autoproteolysis, and SAAT refers to self-associating autotransporter.

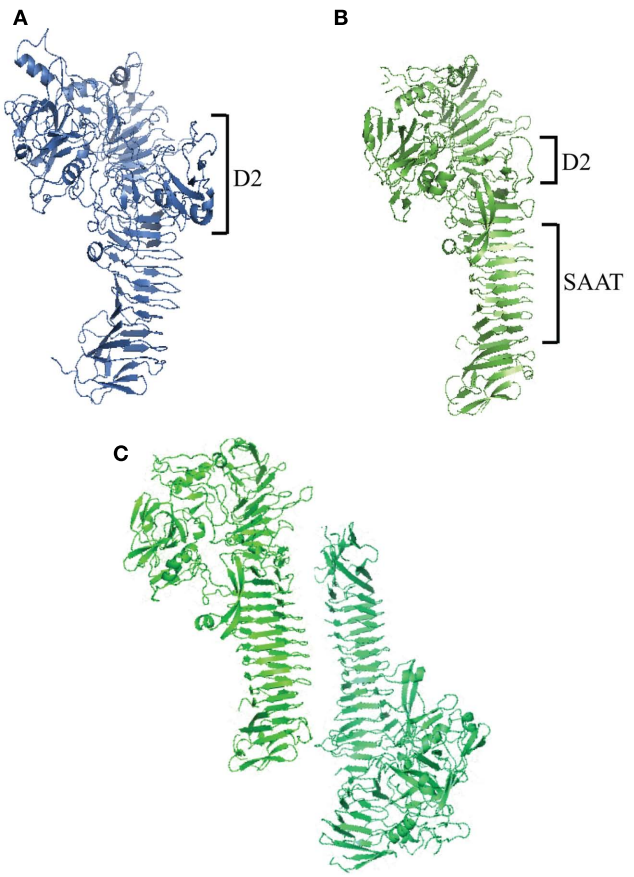

FIGURE 2 | *Haemophilus influenzae* autotransporter structures. (A) Crystal structure of IgA1 protease passenger domain (IgA_p) with an N-terminal globular protease domain and a larger Domain 2 (D2) compared to Hap_s. **(B)** Crystal structure of Hap passenger domain (Hap_s) with an N-terminal globular protease domain and a C-terminal SAAT β-helical domain. **(C)** Hap dimer highlighting Hap–Hap interactions via the SAAT domain.

the chymotrypsin fold that are important for substrate selection and a catalytic triad that is responsible for protease activity (Perona and Craik, 1995). The active site of the protease domain is shallow, creating a perfect environment for proline residues typically found at IgA1 protease cleavage sites. Of note, a small domain (Domain 2) protrudes from the stalk of the β-helix, giving the protein a Y-shaped structure. Computational docking studies of the crystal structure of human IgA1 with IgA1 protease have shown that the IgA1 Fc domain binds in a valley formed by Domain 2 and the protease domain, implicating this small region in substrate recognition (Johnson et al., 2009).

PROTEASE ACTIVITY

The *H. influenzae* IgA1 protease is a serine protease that mediates autoproteolytic cleavage near the junction of the N-terminal passenger domain and the C-terminal β-barrel domain and cleavage of the hinge region of human IgA1. The N-terminal protease domain contains the sequence GDSGSPLF, where **S** is the active-site serine characteristic of serine proteases (Bachovchin et al., 1990). Autoproteolytic cleavage occurs at a proline-rich sequence in the C-terminal region of the passenger domain, releasing the extracellular domain from the bacterial surface. Within this sequence, there are four sites that have the potential for cleavage (Poulsen et al., 1989). A similar proline-rich sequence exists in the IgA1 hinge region. Cleavage of IgA1 at the hinge region separates the Fab regions from the Fc region. The IgA1 Fab regions bind antigens, while the IgA1 Fc region interacts with receptors on mucins and various immune cells (Mestecky and McGhee, 1987; Phalipon et al., 2002). When cleaved, Fab fragments can still bind surface antigens, masking epitopes, and preventing recognition of these epitopes by uncleaved antibodies. In this way, cleavage by IgA1 protease may facilitate bacterial survival on mucosal surfaces (Mansa and Kilian, 1986; Kilian et al., 1988).

IgA1 proteases are classified as type 1 or type 2 and are distinguished by the bond that they cleave in the IgA1 hinge region. Type 1 proteases cleave at the proline–serine bond between residues 231 and 232, and type 2 proteases cleave at the proline–threonine bond between residues 235 and 236 (Kilian et al., 1983). In encapsulated *H. influenzae* strains, the enzyme type is correlated with the polysaccharide capsule serotype. Type 1 IgA1 proteases are produced by serotypes a, b, d, and f, while type 2 IgA1 proteases are produced by serotype c and e strains. Non-typeable strains can have either or both enzyme types (Mulks et al., 1982).

Hap

IDENTIFICATION

Hap is a 155-kDa monomeric autotransporter that was identified when a genomic library of the clinical *H. influenzae* isolate N187 was screened for factors that promote interaction with respiratory epithelial cells (**Figure 1**; St. Geme et al., 1994). A clone expressing a secreted high-molecular mass protein demonstrated adherence and low-level invasion in assays with Chang epithelial cells. This transformant contained a 4182-bp gene that was named *hap* for *Haemophilus* adherence and penetration and encoded a predicted protein with homology to serine-type IgA1 proteases from *H. influenzae* strains and *N. gonorrhoeae* strains. The Hap passenger domain harbors an adhesive region that promotes adherence to human epithelial cell, extracellular matrix (ECM) proteins, and neighboring Hap-expressing bacteria and a serine protease domain that mediates autoproteolytic cleavage.

STRUCTURE

Hap is a unique protein that has characteristics of both self-associating autotransporters (SAATs) that promote bacterial aggregation and serine protease autotransporters and may be an evolutionary link between the two autotransporter types (Hendrixson and St. Geme, 1998; Klemm et al., 2006). In light of this observation, efforts were made to understand the structure of the Hap passenger domain (Hap_s) in detail. Initially, electron microscopy of purified Hap_s revealed an arrowhead shape similar to the crystal structure of *E. coli* hemoglobin protease (Hbp), another serine protease autotransporter (Otto et al., 2005; Unzai et al., 2005). Crystallization of Hap_s revealed a structure similar to the structures of Hbp and IgA1 protease, with an N-terminal globular serine protease domain and a C-terminal β-spine (**Figure 2B**; Meng et al., 2011). Hap_s also has four smaller domains that may

play structural roles. Domain 2 (D2) is similar to IgA1 protease D2 and forms a loose hairpin structure that protrudes away from the molecule. Compared to Hap D2, IgA1 protease D2 is much larger and has a branched, Y-shaped morphology that could inhibit self-association of the C-terminal β-spine by hindering close association of nearby IgA1 protease molecules.

As in IgA1 protease, the Hap_s globular domain has a V-shaped substrate-binding pocket with a catalytic triad located in the center that mediates protease activity. As predicted by mutational analysis, the shallow depth of the binding pocket suggests that a leucine at the P1 position of the cleavage site is the preferred amino acid for efficient substrate recognition; however, a nearby disordered loop could explain the ability of Hap_s to cleave peptides with larger side chains such as the phenylalanine side chain at the P1 position (Kenjale et al., 2009).

The Hap_s crystal structure revealed a multimer, reflecting the activity of the SAAT domain, which lies C-terminal to the serine protease domain. The SAAT domain consists of a 7-turn β-helix with a triangular prism morphology. The core of this structure consists of hydrophobic and aromatic residues, while the exterior is made up of hydrophilic residues at the edges and stacked asparagine and aspartic acid ladders. The β-helix region is straighter and more ordered compared to other autotransporters (e.g., IgA1 protease), allowing for the close association of Hap proteins on neighboring bacteria that is necessary for oligomerization. The shape of this region taken together with the Y-structure and the short β-spine of IgA1 protease explains the differences in self-association between Hap and IgA1 protease, which are structurally similar otherwise. Hap_s molecules pack against each other along the SAAT domain in a tail-to-tail configuration (**Figure 2C**). Higher order Hap multimers could form via associations at this interface, overcoming repulsive forces between adjacent cells to form microcolonies. Disruptions in this region truncate the edges of interaction and lead to a decrease in Hap-mediated bacterial aggregation, implicating this prism structure in microcolony formation (Meng et al., 2011).

PROTEASE ACTIVITY

A region of the Hap passenger domain with homology to IgA1 protease was found to have a similar catalytic site comprising the sequence GD**S**GSPLF, where **S** is the active-site serine (Brenner, 1988; St. Geme et al., 1994). In spite of the same active site, Hap is unable to cleave human IgA1 and instead mediates autoproteolytic cleavage at four C-terminal sites (St. Geme et al., 1994; Hendrixson et al., 1997). This cleavage releases Hap_s from Hap_β and the bacterial cell surface. The catalytic region responsible for proteolysis is consistent with that of typical SA (chymotrypsin) clan serine proteases and consists of amino acids H98, N140, and S243 (Fink et al., 2001). Mutation of these amino acids individually to alanine abolishes protease activity and results in accumulation of full-length Hap in the bacterial outer membrane. Mutations in the proposed substrate-binding groove have identified amino acids N274, L263, R264, and E265 as important for mediating interactions between the catalytic site and its substrate (Kenjale et al., 2009). The catalytic site is responsible for cleavage of the passenger domain predominantly between residues L1036 and N1037 (P1 and P1′); however, when this bond is unavailable, other cleavage sites are recognized at positions L1046-T1047, F1077-A1078, and F1067-S1068, the secondary, tertiary, and quaternary sites, respectively (Hendrixson et al., 1997; Kenjale et al., 2009). The consensus sequence for cleavage at these sites is (Q/R)(A/S)X(L/F) in the P4–P1 positions. Further analysis of the cleavage sites revealed more efficient cleavage at the primary and secondary sites compared to the tertiary and quaternary sites, due to a preference for leucine rather than phenylalanine at the P1 position.

ADHERENCE

In general, *H. influenzae* preferentially binds to non-ciliated cells, areas of damaged epithelium, and mucus in the respiratory tract. The C-terminal 311 amino acids of Hap_s mediate bacterial binding to cultured epithelial cells by interacting with an unknown receptor (Fink et al., 2003). Additionally, the C-terminal 511 amino acids of Hap_s facilitate *H. influenzae* binding to several ECM proteins, including fibronectin, laminin, and collagen IV (Fink et al., 2002). Inhibition of Hap serine protease activity results in an increase in Hap-mediated adherence to epithelial cells and ECM proteins, reflecting retention of the Hap passenger domain on the bacterial surface (Fink et al., 2002, 2003).

Haemophilus influenzae interactions with epithelial cells and the ECM are enhanced by bacterial autoaggregation and microcolony formation, which are mediated by a region within Hap_s. Aggregation occurs due to intercellular multimerization of neighboring Hap molecules at the SAAT domain. This interaction generates van der Waals forces that mediate stable cell–cell interactions (Meng et al., 2011). Like the *E. coli* autotransporter Antigen 43, this aggregation can be appreciated in liquid culture and may have a role in biofilm formation, potentially facilitating *H. influenzae* persistence in middle ear effusions and in the lower respiratory tract in patients with cystic fibrosis or chronic obstructive pulmonary disease (COPD; Kjaergaard et al., 2000a,b; Fink et al., 2003).

Hap autoproteolytic activity is inhibited by secretory leukocyte protease inhibitor (SLPI), a small protein found in human upper respiratory secretions that is upregulated by TNFα and IL-1β (Hendrixson and St. Geme, 1998). SLPI is believed to protect the respiratory epithelium from injury due to neutrophil elastase and other proteases involved in acute inflammation (Gauthier et al., 1982). Similar to mutations in the Hap catalytic triad, inhibition of Hap autoproteolysis by SLPI results in increased Hap-mediated bacterial adherence to respiratory epithelial cells and ECM proteins, bacterial autoaggregation, and bacterial microcolony formation because of the retention of Hap_s on the bacterial surface (Hendrixson and St. Geme, 1998). During natural infection, inhibition of Hap autoproteolysis presumably facilitates *H. influenzae* colonization of the respiratory mucosa, while release of Hap_s may result in dispersal and migration from the site of infection.

INVASION

Haemophilus influenzae has been observed inside cells in adenoidal tissue obtained from patients with otitis media and in bronchial cells from individuals with COPD, potentially representing a mechanism to evade local immune mechanisms (Forsgren et al., 1994; Moller et al., 1998). Invasion has also been shown *in vitro* and is mediated at least in part by Hap (St. Geme et al., 1994). *H. influenzae* invasion into epithelial cells is dependent on actin

and microtubules and begins with the formation of lamellipodia around the invading bacterium (St. Geme and Falkow, 1990; Ketterer et al., 1999). While invasion occurs at low levels, it is possible that an intracellular population of organisms accounts for the recurrence of *H. influenzae* infection in patients with recurrent otitis media or COPD.

Hia AND Hsf

IDENTIFICATION

Hia and Hsf are homologous trimeric autotransporters found in non-typeable and encapsulated strains of *H. influenzae*, respectively (**Figure 1**; St. Geme and Cutter, 2000; Cotter et al., 2005). Hia was first identified in strain 11, a strain lacking HMW1/HMW2-like proteins but still able to adhere efficiently to epithelial cells (Barenkamp and St. Geme, 1996). A recombinant phage library of strain 11 was constructed and was screened for reactivity with convalescent serum from a child naturally infected with strain 11, resulting in recovery of a clone expressing a high-molecular mass protein important for adherence to epithelial cells. The 3294-bp open reading frame encodes a protein that was named Hia for *H. influenzae* adhesin. The predicted molecular mass of Hia from strain 11 is 114.1 kDa, but the protein trimerizes, causing it to migrate on SDS-PAGE gels at a size greater than 300 kDa. The *hia* gene is found in about 25% of non-typeable strains, corresponding to nearly all strains that lack HMW1/HMW2-like proteins (Barenkamp and St. Geme, 1996). Shortly after the discovery of *hia*, a similar locus ubiquitous in encapsulated *H. influenzae* strains was characterized and was designated *hsf*, for *Haemophilus* surface fibrils, reflecting the fact that this gene encodes short thin surface fibrils. The *hsf* gene from a prototypic type b strain encodes a protein with a predicted molecular mass of greater than 230 kDa and promotes adherence to human respiratory epithelial cells (St. Geme and Cutter, 1995). Hia and Hsf are 81% similar and 72% identical, with the regions of greatest homology at their N- and C-terminal ends.

STRUCTURE

The Hia passenger domain consists of repeating domains that are structurally similar, including five Trp-ring domains connected by two IsNeck domains and two KG domains (**Figure 3**; Meng et al., 2008). Hsf has a similar architecture, but is extended with 14 Trp-ring domains, three IsNeck domains, and four KG domains, among others. This extension may be needed for Hsf to extend past the capsule in encapsulated strains. The crystal structures of three different fragments of the Hia passenger domain reveal a trimeric architecture in all cases. The trimers are held together by a hydrophobic core that consists primarily of phenylalanine and tryptophan residues. Both Hia and Hsf have two binding domains located at opposite ends of the passenger domain that mediate adherence to epithelial cells (Laarmann et al., 2002; Cotter et al., 2005). These binding domains are always found in regions of the passenger domain where an IsNeck domain is immediately followed by a Trp-ring domain. The binding domains contain an acidic pocket that is necessary for adherence. A single trimeric binding domain contains three identical binding pockets, possibly allowing the protein to interact with three separate receptor molecules or with related domains on one receptor molecule (Yeo et al., 2004).

The Hia passenger domain is connected to the C-terminal β-barrel domain by a Neck domain (Meng et al., 2008). Like other trimeric autotransporters, Hia contains a relatively short β-barrel translocator domain (Surana et al., 2004). Analysis of the Hia β-barrel domain has shown that it is a trimer that consists of 12 transmembrane β-strands, including four strands from each monomer (**Figure 3**). The outer surface of the barrel is hydrophobic, facilitating its insertion into the outer membrane. The translocator domain has a central channel that is 1.8 nm and is filled with three N-terminal α-helices that are essential for stability of the trimer. Interestingly, this region contains a ring of basic residues that may coordinate the trimerization of the N-terminal passenger domain (Meng et al., 2006). The Hia β-barrel trimer has a structure that is

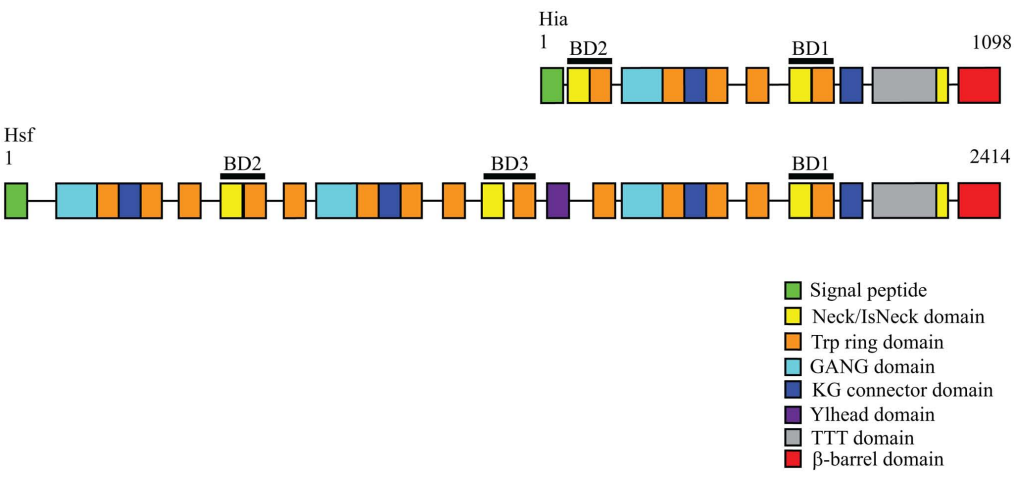

FIGURE 3 | Domain arrangements of Hia and Hsf trimeric autotransporters. The Hia passenger domain is characterized by a repetitive architecture consisting of multiple domain types. Hsf consists of a similar but extended domain arrangement compared to Hia. Domain arrangements were obtained from the daTAA server (http://toolkit.tuebingen.mpg.de/dataa).

A

Autotransporter	Type	Adhesive activity	SAAT activity	Protease activity
IgA1 protease	Monomeric	No	No	Yes
Hap	Monomeric	Yes - cells, ECM	Yes	Yes
Hia/Hsf	Trimeric	Yes - cells	No	No

B

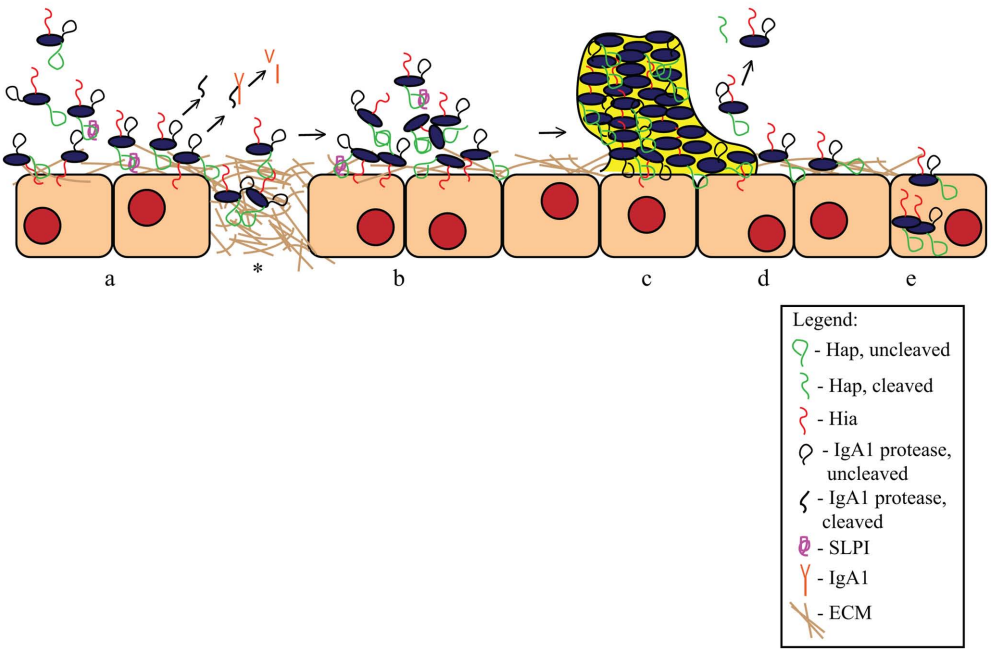

FIGURE 4 | *Haemophilus influenzae* autotransporter proteins. (A) Summary of key features of *H. influenzae* autotransporter proteins. **(B)** Schematic of the role of *H. influenzae* autotransporter proteins in the pathogenesis of disease. (a) Hap and Hia facilitate *H. influenzae* attachment to epithelial cells. Hap also promotes adherence to the ECM on the cell layer and at areas of damaged epithelium (*). Hap autoproteolysis is inhibited by SLPI, resulting in the retention of Hap_s on the bacterial surface. IgA1 protease is released from the bacterial surface and cleaves human IgA1. (b) Once bacteria have adhered, Hap–Hap interactions lead to microcolony formation. (c) Microcolony formation leads to the establishment of a biofilm that is resistant to host immune factors. (d) In the absence of SLPI, Hap autoproteolytic activity allows some bacteria to disperse, presumably to new sites of infection. (e) Hap-mediated attachment promotes bacterial invasion into epithelial cells, potentially providing a protected niche, and allowing bacterial evasion of local immune mechanisms.

similar to the C-terminal domain of monomeric autotransporters, with the same number of β-strands, the same angle of the strands relative to the axis of the barrel, and similar pore sizes. These structural similarities suggest a common mechanism for translocation among all autotransporters.

ADHERENCE

The Hia and Hsf proteins contribute to *H. influenzae* adherence to epithelial cells. With both Hia and Hsf, the passenger domain contains highly homologous binding domains that mediate the adhesive activity (**Figure 1**; St. Geme et al., 1996; Laarmann et al., 2002; Cotter et al., 2005). The Hia passenger domain harbors two binding domains that must trimerize for full adhesive activity, resulting in three identical binding pockets (Yeo et al., 2004; Cotter et al., 2006). Both binding domains interact with the same host receptor, but with differing affinities. The primary binding domain of Hia (HiaBD1) is located adjacent to the C-terminal translocator domain. HiaBD1 lies closer to the bacterial surface than the secondary binding domain (HiaBD2) and has greater affinity for the yet unknown host receptor (Laarmann et al., 2002). The core of the HiaBD1 binding pocket is comprised of an aspartic acid, an alanine, and a valine, while the HiaBD2 pocket is comprised of a glutamine, an alanine, and a valine. Therefore, the different binding affinities of HiaBD1 and HiaBD2 appear to be due to differences in the aspartic acid and glutamine side chains (Yeo et al., 2004). One model of Hia binding proposes that the distal

HiaBD2 is responsible for the initial interaction with the receptor, bringing the host cell within reach of the bacteria. The higher affinity HiaBD1 may then competitively displace HiaBD2 from the cellular receptor, resulting in more intimate bacterial attachment to the epithelial cell layer. Unlike Hap, the passenger domains of Hia and Hsf are not cleaved from the C-terminus and remain cell associated, enhancing adherence to host cells (St. Geme and Cutter, 2000).

The Hsf passenger domain contains three regions homologous to the Hia binding domains; however, only two have acidic binding pockets that mediate adhesive activity. Similar to Hia, both binding domains recognize the same receptor, with the proximal Hsf binding domain (HsfBD1) having a higher affinity for the host cell receptor than does the distal Hsf binding domain (HsfBD2; Cotter et al., 2005; Radin et al., 2009). The key contact residues in the HsfBD1 and HsfBD2 binding pockets are identical except for an aspartic acid in HsfBD1 and a glutamic acid in HsfBD2, accounting for the different binding affinities (Radin et al., 2009). The Hia binding model can be applied to Hsf, where HsfBD2 initiates binding to the cellular receptor and HsfBD1 later displaces HsfBD2, bringing the bacteria into closer contact with the host cell. For Hsf to interact with the host, the passenger domain must extend past the polysaccharide capsule. The intervening region between HsfBD1 and HsfBD2 is three times longer than the sequence between HiaBD1 and HiaBD2 and may be necessary for this extension. The third region of homology to the Hia binding domains is called HsfBD3 and is located in this intervening region. While HsfBD3 is structurally similar to the other binding domains, it lacks an acidic binding pocket and does not mediate adherence to epithelial cells (Cotter et al., 2005). Instead, HsfBD3 may play a role in stabilizing the passenger domain or may mediate adherence to yet untested cell types or other host molecules.

CONCLUDING REMARKS

The *H. influenzae* IgA1 protease, Hap, Hia, and Hsf autotransporters are virulence factors that promote colonization and persistence in the human host. Adherence to the respiratory epithelium, microcolony formation leading to biofilm formation, and protease activity facilitating bacterial spread and immune evasion are important pathogenic mechanisms that are mediated by these proteins. The mechanisms by which these virulence factors may work together to establish a *H. influenzae* infection are outlined in **Figure 4**. Hia-mediated adherence to epithelial cells and Hap-mediated adherence to both epithelial cells and the ECM may be responsible for initial contact with the host, while IgA1 protease cleaves IgA1, protecting the bacteria from the innate immune response. As infection progresses, Hap-Hap interactions result in microcolony formation and may eventually lead to biofilm formation, another mechanism of immune evasion. Because of Hap autoproteolytic activity, some bacteria may be released from the biofilm to colonize elsewhere. Finally, bacterial invasion mediated by Hap can lead to an intracellular bacterial reservoir that may be responsible for the recurrent infections seen in COPD and otitis media. Further examination of these proteins will aid in understanding *H. influenzae* disease progression and may facilitate development of novel antimicrobials.

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