The *Haemophilus influenzae* Hap autotransporter mediates microcolony formation and adherence to epithelial cells and extracellular matrix via binding regions in the C-terminal end of the passenger domain

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Summary

The pathogenesis of non-typable *Haemophilus influenzae* disease begins with colonization of the nasopharynx and is facilitated by bacterial adherence to respiratory mucosa. The *H. influenzae* Hap autotransporter is a non-pilus adhesin that promotes adherence to epithelial cells and selected extracellular matrix proteins and mediates bacterial aggregation and microcolony formation. In addition, Hap has serine protease activity. Hap contains a 110 kDa internal passenger domain called HapS and a 45 kDa C-terminal translocator domain called Hapb. In the present study, we sought to define the structural basis for Hap adhesive activities. Based on experiments using a panel of monoclonal antibodies against HapS, a deletion derivative lacking most of HapS and a purified fragment of HapS, we established that adherence to epithelial cells is mediated by sequences within the C-terminal 311 residues of HapS. In additional experiments, we discovered that bacterial aggregation is also mediated by sequences within the C-terminal 311 residues of HapS and occurs via HapS–HapS interaction between molecules on neighbouring organisms. Finally, we found that adherence to fibronectin, laminin and collagen IV is mediated in part by sequences within the C-terminal 511 residues of HapS and in full by sequences within the C-terminal 311 residues of HapS. Taken together, these results demonstrate that all Hap adhesive activities reside in the C-terminal portion of HapS. Coupled with earlier observations, the current results establish that HapS adhesive activities and HapS protease activity are contained in separate modules of the protein.

Introduction

Most bacterial infections begin with colonization of a particular mucosal surface. In general, the initial events of colonization involve bacterial attachment to host epithelium, a process that is mediated by specific interactions between surface-exposed bacterial proteins called adhesins and complementary receptors on epithelial cells. Non-typable *Haemophilus influenzae* is a non-encapsulated Gram-negative bacterium that is a common cause of human respiratory tract disease, including otitis media, sinusitis, bronchitis and pneumonia (Turk, 1984). The pathogenesis of non-typable *H. influenzae* disease begins with colonization of the nasopharynx and requires that organisms overcome mucociliary clearance and evade local immune mechanisms. To facilitate stable attachment to the respiratory epithelial surface, non-typable strains of *H. influenzae* elaborate a number of adhesins, including Hap, HMW1/HMW2, Hia, pili, P5 and lipooligosaccharide (Rao et al., 1999).

The *H. influenzae* Hap protein is a member of the autotransporter family of virulence factors common among Gram-negative pathogens (Henderson and Nataro, 2001) and consists of an N-terminal signal sequence, a 110 kDa internal passenger domain called HapS, and a 45 kDa C-terminal translocator domain called Hapb (St. Geme et al., 1994; Hendrixson et al., 1997). The signal sequence directs the precursor protein across the inner membrane, the translocator domain inserts into the outer membrane and is predicted to form a beta-barrel and a channel, and the passenger domain is extruded across the outer membrane and presented on the bacterial surface. Ultimately, autoproteolysis occurs, resulting in extracellular release of HapS and leaving HapS embedded in the outer membrane.
Initial studies demonstrated that Hap promotes adherence to cultured human respiratory epithelial cells (St. Geme et al., 1994). More recent evidence indicates that Hap promotes adherence to selected extracellular matrix proteins as well, including fibronectin, laminin and collagen IV (Fink et al., 2002). In addition, Hap facilitates bacterial aggregation and microcolony formation, a possible intermediate step in biofilm formation (Hendrixson and St. Geme, 1998). Based on studies examining purified HapS and bacteria expressing a series of Hap derivatives, it appears that all Hap adhesive activities reside within the HapS passenger domain (Hendrixson et al., 1998; Fink et al., 2002).

Beyond adhesive activity, HapS harbours serine protease activity and directs autoproteolytic cleavage at Leu1036–Asp1037, Leu1046–Thr1047, Phe1067–Ser1068 and Phe1077–Ala1078, resulting in HapS separation from Haps and release from the bacterial surface (Fink et al., 2001). Hap autoproteolysis occurs, at least in part, by an intermolecular mechanism with one Hap molecule cleaving neighbouring molecules (Fink et al., 2001). Sequence homology and site-directed mutagenesis established that Hap is a chymotrypsin (SA) clan serine protease with a catalytic triad consisting of H98, D140 and S243 (Fink et al., 2001). Based on comparison with other chymotrypsin clan members and molecular modelling, the protease domain corresponds to the N-terminal 300 amino acids of HapS (Fink et al., 2001). Mutation of H98, D140 or S243 eliminates Hap autoproteolysis and blocks release of HapS from the bacterial surface, resulting in increased Hap-mediated adherence and aggregation (Hendrixson et al., 1998; Fink et al., 2001; 2002).

In considering the paradox that HapS harbours adhesive activities and that HapS is ultimately released from the bacterial surface via autoproteolysis, it is notable that Hap autoproteolysis is inhibited by physiological concentrations of secretory leukocyte protease inhibitor (SLPI), a natural component of respiratory secretions (Hendrixson et al., 1998). Secretory leukocyte protease inhibitor is upregulated in the setting of inflammation and inhibits neutrophil elastase, suggesting a role in protecting the epithelium against the damaging effects of inflammation (Gauthier et al., 1982).

In the present study, we sought to define the domains in HapS responsible for adherence to epithelial cells, adherence to extracellular matrix proteins and formation of bacterial aggregates. We discovered that adherence to epithelial cells is mediated by sequences in the C-terminal 311 residues of the HapS passenger domain. Formation of bacterial aggregates is also mediated by sequences in the C-terminal 311 amino acids of HapS. Adherence to fibronectin, laminin and collagen IV involves sequences in the C-terminal 511 residues of HapS. These results extend our understanding of the structural organization of the Hap autotransporter and demonstrate that Hap serine protease activity and Hap adhesive activities are contained in separate modules of the protein.

**Results**

**Monoclonal antibodies reactive with the C-terminus of HapS inhibit Hap-mediated adherence.**

In our efforts to identify specific binding domains in the Hap adhesin, initially we sought to determine whether interactions between Hap and its cognate receptors on epithelial cells could be specifically inhibited by monoclonal antibodies directed against the HapS passenger domain. Eight different monoclonal antibodies collected from hybridoma culture supernatants were examined in 30-min adherence assays using *H. influenzae* strain DB117 expressing HapS243A, a derivative of Hap that fails to undergo autoproteolysis and therefore retains all of the HapS adhesive passenger domain on the bacterial cell surface. As shown in Fig. 1, this strain adheres very efficiently to Chang and A549 epithelial cells. Following preincubation with monoclonal antibody 314, adherence by DB117/pLS88:HapS243A to A549 cells was reduced by 50% and adherence to Chang cells was reduced by almost 70%. Pre-incubation with monoclonal antibodies 320 and 324 also resulted in markedly reduced adherence to both A549 cells and Chang cells (Fig. 1). In contrast, the remaining five anti-HapS monoclonal antibodies had no significant effect on adherence to either Chang cells or

![Fig. 1. Inhibition of adherence by DB117/pLS88:HapS243A with anti-HapS monoclonal antibodies. Adherence to Chang cells and A549 cells was calculated by dividing the number of adherent bacteria by the number of inoculated bacteria. Strains included DB117/pLS88 (vector control), DB117/pLS88:HapS243A treated with BHI alone (no antibody) and DB117/pLS88:HapS243A treated with BHI plus one of eight monoclonal antibodies. Bars represent the mean ± standard error of the mean of measurements made in triplicate from representative experiments.](image-url)
A549 cells. With this information in mind, we concluded that antibodies 314, 320 and 324 bind to Hap on the bacterial cell surface either at or nearby the regions responsible for Hap-mediated adherence.

To identify the Hap epitopes recognized by antibodies 314, 320 and 324, we generated a series of HapS243A in frame deletion derivatives spanning the Hap passenger domain (Hap residues 26 through 1036), then recovered outer membrane fractions from strain DB117 expressing these derivatives and performed immunoblot analysis. The in frame deletion derivatives included five 100-residue deletions encompassing Hap residues 126–725 and two series of 25- to 75-residue deletions, one encompassing Hap residues 26–125 and the other encompassing Hap residues 726–1036. These smaller deletions were necessitated by the instability of Hap derivatives lacking residues 26–125, 726–825, 826–925 or 926–1036. As shown in Fig. 2, all of the in frame deletion derivatives in the series were stably expressed in the outer membrane. Monoclonal antibodies 314, 320 and 324 recognized all of the deletion derivatives lacking sequence between Hap residues 26 and 775 but failed to recognize multiple derivatives containing 25-residue deletions spanning Hap residues 776–1036 (Fig. 2), suggesting that these antibodies interact with epitopes present at the C-terminal end of the Hap passenger domain.

The C-terminal 311 residues of Hap contain the domain responsible for adherence to epithelial cells

To further explore the involvement of the Hap C-terminal domain in Hap adhesive activity, we created an in frame deletion derivative lacking residues 26–725, retaining only the C-terminal one-third of the Hap passenger domain. This derivative, Hap26-725, was stably expressed in the outer membrane of DB117 and was recognized in immunoblots by monoclonal antibodies 314, 320 and 324 (Fig. 3), indicating the presence of the Hap epitopes interacting with these antibodies. Furthermore, in quantitative assays, adherence to A549 cells and Chang cells was similar for DB117 expressing Hap26-725 and DB117 expressing full-length HapS243A (Fig. 4), suggesting that the C-terminal 311 residues of Hap harbour the binding domain involved in Hap-mediated adherence to epithelial cells.

To obtain additional evidence that the C-terminal 311 residues of Hap are sufficient for adherence to epithelial cells, we examined binding by this region using paraformaldehyde-fixed Chang and A549 cells and both immunofluorescence microscopy and a cellular ELISA. As a first step, we generated a recombinant strain simultaneously expressing Hap26-725 and GP74.
expressing a 6 x His-tagged derivative of Hap\(\Delta26-725\) together with Hap\(\Delta1036-99\), a Hap mutant that facilitates release of the Hap\(S\) passenger domain from neighbouring precursor molecules via intermolecular hydrolysis of autoproteolytic cleavage sites (Fink et al., 2001). The Hap\(\Delta1036-99\) passenger domain lacks cleavage sites and therefore fails to undergo autoproteolysis and extracellular release. Using this strain, we purified the 6 x His-tagged Hap\(S\) C-terminal domain from the culture supernatant. As shown in Fig. 5A, Coomassie staining and immunoblot analysis after resolution of the purified sample by SDS-PAGE revealed four distinct species migrating at 44, 46, 50 and 52 kDa, representing release of passenger domain from 6 x His-tagged Hap\(\Delta26-725\) at each of the four autoproteolytic cleavage sites (the 50 kDa band is faint, reflecting inefficient cleavage at the quaternary site). In addition, there was a minor contaminating band migrating at 58 kDa (Fig. 5A). Confocal immunofluorescence microscopy of Chang and A549 epithelial cells incubated with the purified sample revealed a uniform punctate pattern of binding to both cell types, nearly identical to the pattern observed with purified full-length Hap\(S\) (Fig. 5B–5E), suggesting that the Hap\(S\) C-terminal domain and full-length Hap\(S\) may interact with the same cellular receptor. In control samples prepared with primary and secondary antisera but lacking purified protein, fluorescence was absent (not shown).

In cellular ELISAs, the purified 6 x His-tagged Hap\(S\) C-terminal domain bound to both A549 and Chang cells in a dose-dependent manner, similar to binding by purified full-length Hap\(S\) (Fig. 6). In contrast, a GST fusion protein containing a non-binding region of the H. influenzae Hia adhesin (Hia\(221-658\)) (St Geme and Cutter, 2000; Laarmann et al., 2002) demonstrated minimal binding, even at high concentrations (data not shown). Based on non-linear regression analysis using the GRAPHPAD PRISM Program (GraphPad Software), we generated binding curves and estimated dissociation constants (K_d) for each ELISA experiment. Using this approach, we found that binding to Chang and A549 cells by purified full-length Hap\(S\) was...
H. influenzae Hap binding domain

Fig. 6. Dose-dependent binding of purified HapS and 6×His-tagged HapS C-terminal domain to Chang cells and A549 cells. Binding of increasing concentrations of purified HapS to Chang cells (triangles) and A549 cells (squares) and binding of increasing concentrations of 6×His-tagged Hap726–1036 to Chang cells (diamonds) and A549 cells (circles). Binding was quantified by ELISA at an absorbance of 650 nm. Points represent the mean ± standard error of the mean of measurements made in triplicate. Binding curves were calculated by non-linear regression analysis using GRAPHPAD PRISM V3.0.

Fig. 7. Settling of cultures of H. influenzae strain DB117 expressing HapS243A or Hap in frame deletion derivatives.
A. Late exponential phase cultures of DB117/pLS88 (left) and DB117/pLS88:HapS243A (right) after incubation standing at room temperature for 4.5 h.
B. Cultures of DB117/pLS88 (vector), DB117/pLS88::HapS243A or DB117 expressing Hap in frame deletion derivatives were incubated standing at room temperature for 4 h, and bacterial aggregation was quantified by measuring absorbance at 600 nm.

high affinity, with $K_D$ values of approximately 30–35 nM. Binding by the 6×His-tagged HapS C-terminal domain was similar, with $K_D$ values of 50 nM and 70 nM respectively. The R-squared values for all of the binding curves were 0.99 or higher, indicating an excellent fit of the curves to the ELISA data. Differences in absolute signal intensity between the experiments involving full-length HapS and those involving the 6×His-tagged HapS C-terminal domain likely reflect the fact that polyclonal antiserum GP74 recognizes more epitopes on full-length protein than on the HapS C-terminal domain (rather than reflecting differences in binding affinities).

The C-terminal 311 residues of HapS contain the domain responsible for bacterial aggregation

Previous studies examining interactions between DB117/pHapS243A and epithelial cell monolayers revealed adherence as large clusters rather than single organisms (Hendrixson et al., 1998). The observed bacterial aggregation was presumed to arise from increased abundance of HapS on the bacterial cell surface. To address which regions of HapS might be responsible for Hap-mediated bacterial aggregation, we performed tube settling assays with cultures of bacteria expressing HapS243A in frame deletion derivatives. In these studies, cultures were grown to late exponential phase, and rates of bacterial aggregation were quantified by measuring culture absorbance at 600 nm as aggregates settled to the bottoms of the culture tubes. Cultures of DB117 expressing HapS243A rapidly lost turbidity as aggregates settled, whereas cultures of DB117 expressing vector alone remained turbid beyond 4 h (Fig. 7A). Six of the deletion derivatives lacking an individual 25-residue region within the C-terminal 311 residues of HapS promoted aggregation at relatively slow rates, compared with full-length HapS243A (Fig. 7B). All of the other derivatives, including those lacking 100-residue regions within the N-terminal 700 residues of HapS, promoted aggregation at rates equivalent to HapS243A (data not shown). With this information in mind, we compared DB117 expressing Hap26–725 and DB117 expressing HapS243A in a tube settling assay and observed comparable rates of settling (Fig. 7B), suggesting that the C-terminal 311 residues of HapS are sufficient to promote bacterial aggregation.
Hap-mediated bacterial aggregation occurs via interactions between HapS on neighbouring cells

Hap-mediated bacterial aggregation may occur via homooligomerization of HapS domains on neighbouring cells. Alternatively, HapS on one cell may interact with a heterologous structure on a neighbouring cell. To distinguish between these two possibilities, we prepared co-cultures of bacteria expressing HapΔ26-725 plus green fluorescent protein (GFP) and bacteria expressing either HapΔ26-725 alone or HapS alone. Bacterial aggregates from co-cultures grown to late-exponential phase and then incubated standing for 4 h were collected from the bottoms of culture tubes and examined by phase contrast and fluorescence microscopy. As shown in Fig. 8A–D, fluorescence microscopy revealed that aggregates from a culture of DB117 expressing HapΔ26-725 plus GFP were uniformly green, whereas aggregates from a co-culture of DB117 expressing HapΔ26-725 plus GFP and DB117 expressing HapΔ26-725 alone appeared variegated, indicating that both GFP-labelled and unlabelled bacteria participated in aggregate formation. In contrast, aggregates from a co-culture of DB117 expressing HapΔ26-725 plus GFP and DB117 expressing the HapS translocator domain alone were uniformly green (Fig. 8E and F), suggesting that only bacteria expressing the HapS C-terminal domain participated in aggregate formation. Consistent with these findings, aggregates from a co-culture of DB117/pHapΔ26-725 + pGFP and DB117/pGJB103 (empty vector) were also uniformly green (not shown).

The C-terminal 511 residues of HapS contain the domain responsible for adherence to fibronectin, laminin and collagen IV

Given that Hap also promotes bacterial adherence to several extracellular matrix proteins (Fink et al., 2002), we next examined whether the domain responsible for adherence to epithelial cells was sufficient for adherence to fibronectin, laminin and collagen IV as well. In contrast to observations with epithelial cells, adherence by DB117/pHapΔ26-725 to laminin and collagen IV in quantitative assays was only one-third to one-half as efficient as adherence by DB117 expressing HapS243A, and adherence by DB117/pHapΔ26-725 to fibronectin was only slightly greater than adherence by DB117 expressing empty vector (Fig. 9). Accordingly, we next examined...
an in-frame deletion derivative, HapΔ26-525, lacking only the N-terminal 500 residues of the HapS passenger domain. As shown in Fig. 9, DB117/pHapΔ26-525 adhered very efficiently to fibronectin, laminin, and collagen IV, comparable to DB117 expressing full-length HapS243A.

Discussion

The H. influenzae Hap autotransporter is a large protein that is synthesized as a precursor polypeptide with a 25-amino acid N-terminal signal sequence, a 110 kDa internal passenger domain, and a 45 kDa C-terminal translocator domain (St. Geme et al., 1994; Hendrixson et al., 1997). The signal sequence directs the protein across the cytoplasmic membrane and into the periplasm. Subsequently, the translocator domain inserts into the outer membrane and is presumed to form a pore, ultimately presenting the passenger domain on the surface of the organism. In previous work, we demonstrated that the HapS passenger domain contains adhesive activities and promotes intimate interaction with human epithelial cells and efficient adherence to selected extracellular matrix proteins (St. Geme et al., 1994; Hendrixson et al., 1998; Fink et al., 2002). In addition, we established that HapS facilitates bacterial aggregation and microcolony formation (Hendrixson et al., 1998).

In the current study, we obtained three lines of evidence that the binding domain responsible for adherence to Chang and A549 cells resides within the C-terminal one-third of HapS. First, using a panel of monoclonal antibodies raised against HapS, we identified three antibodies that were capable of partially blocking Hap-mediated adherence. Mapping studies revealed that all three of these antibodies recognized epitopes at the C-terminal end of HapS. Second, we observed that H. influenzae strain DB117 expressing a Hap derivative with a deletion of residues 26–525 was capable of full-level bacterial adherence to Chang cells and A549 cells, comparable to adherence by DB117 expressing HapS243A. Finally, we found that a His-tagged protein expressing HapΔ26-525 adhered as efficiently as bacteria expressing HapS243A, suggesting that the domain involved in Hap-mediated bacterial aggregation and microcolony formation resides in the same region as the domain responsible for adherence to epithelial cells. This region is nearly 80% identical at the amino-acid level in Hap proteins from seven diverse clinical isolates of non-typable H. influenzae, underscoring the idea that Hap-mediated bacterial adherence and aggregation play critical roles in H. influenzae infection. In experiments with GFP-labelled bacterial co-cultures, only bacteria expressing the HapS C-terminal domain were observed in aggregates, indicating that Hap-mediated bacterial aggregation occurs via interaction between HapS epitopes on neighbouring bacterial cells and not via interaction between Hap and a heterologous molecule. Hap oligomerization across two bacterial cell surfaces is reminiscent of the situation with the E. coli autotransporter Antigen 43, which promotes bacterial aggregation in liquid culture and participates in biofilm formation (Kjaergaard et al., 2000a; b). Although our data provide clear evidence that Hap-mediated bacterial aggregation involves HapS–HapS interaction and is determined by residues in the C-terminal 311-amino acids of HapS on neighbouring cells, it remains unclear whether the interacting surfaces on adjacent molecules are the same or different.

Our results with Hap-mediated adherence to fibronectin, laminin and collagen indicate that interactions between Hap and extracellular matrix proteins involve Hap residues beyond the C-terminal region responsible for adherence to epithelial cells. In experiments with fibronectin, we found that bacteria expressing HapΔ26-525 adhered as efficiently as bacteria expressing HapS243A, suggesting that the C-terminal half of HapS contains all fibronectin binding activity. In contrast, bacteria expressing HapΔ26-725 adhered minimally, raising the possibility that Hap may possess a single fibronectin-binding domain within the region bounded by residues 526–725. Alternatively, a single fibronectin-binding epitope may involve residues scattered throughout the C-terminal half of HapS, therefore requiring expression of this larger domain for proper folding.

In experiments with laminin and collagen IV, bacteria expressing HapΔ26-725 adhered approximately one-third to one-half as well as bacteria expressing HapS243A. Further analysis revealed that adherence by DB117...
expressing HapD26-525 was comparable to adherence by DB117/pHapS243A. Together these observations suggest that Hap-mediated interactions with laminin and collagen may involve two Hap binding domains that act cooperatively, including one between residues 526 and 725 and a second between residues 726 and 1036. With this possibility in mind, it is notable that several other bacterial adhesins employ multiple binding domains for maximal binding to a single extracellular matrix protein or for interactions with two distinct extracellular matrix proteins. Examples include FnbpA and FnbpB of Staphylococcus aureus, Protein F of Streptococcus pyogenes and YadA of Yersinia spp. (Ozeri et al., 1996; Tahir et al., 2000; Wann et al., 2000).

Based on examination of the Hap primary amino acid sequence, Hap binding activity is located adjacent to the outer membrane translocator domain, an interesting observation given that binding domains in other bacterial adhesins (including enteropathogenic E. coli Intimin, Yersinia Invasin, and uropathogenic E. coli FimH) are present at the tip of the adhesin, away from the bacterial cell wall, making them easily accessible to host cell receptor structures (Jones et al., 1995; Hamburger et al., 1999; Luo et al., 2000). Of note, Hap autoproteolysis involves cleavage of sites near the Hap N-terminus by the N-terminal protease domain of a neighbouring Hap molecule, suggesting that the Hap S N-terminus likely resides proximal to the bacterial cell surface. With this information in mind, we imagine that HapS surface expression may involve presentation of the C-terminal binding domain at the most distal portion of the folded protein. As shown in Fig. 10, the Hap N-terminus and a 40-residue intervening region containing the autoproteolytic cleavage sites may serve to extend the binding domain away from the bacterial surface. N-terminal to the adhesive region, Hap may fold back toward the cell surface, placing the protease domain in a position accessible to its target cleavage sites. Ongoing efforts to crystalize secreted HapS may validate this prediction.

In summary, the binding domain responsible for Hap-mediated adherence to epithelial cells resides within a 311-residue region at the C-terminus of HapS. Hap-mediated bacterial aggregation occurs via interaction between HapS epitopes on neighbouring bacterial cells and involves the same 311-residue region that directs adherence to epithelial cells. Hap-mediated adherence to fibronectin, laminin and collagen IV requires additional residues N-terminal to this region. These results extend our understanding of the structural organization of the Hap autotransporter. In particular, they demonstrate that the HapS passenger domain has a modular architecture, with serine protease activity contained in an N-terminal module and adhesive activities contained in adjacent C-terminal modules.

**Experimental procedures**

**Bacterial strains and plasmids**

The bacterial strains and plasmids used in this study are listed in Table 1. Haemophilus influenzae strains were grown in brain–heart infusion broth supplemented with NAD and hemin (BHIs) or on BHIs agar or chocolate agar as described previously (Anderson et al., 1972). These strains were stored at –80°C in brain–heart infusion broth with 20% glycerol. Escherichia coli strain DH5α was grown on Luria–Bertani agar or in LB broth. E. coli strains were stored at –80°C in LB broth with 50% glycerol. Antibiotic concentrations for H. influenzae included tetracycline (5 μg ml⁻¹) and kanamycin (50 μg ml⁻¹). Escherichia coli strains were grown with kanamycin at 50 μg ml⁻¹.

**Recombinant DNA methods**

The DNA ligations, restriction endonuclease digestions and gel

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Plasmids were introduced into E. coli strain DH5α by chemical transformation (Sambrook et al., 1989). Haemophilus influenzae strain DB117 was transformed using the MIV method of Herriott et al. (1970).

**Construction of Hap in frame deletion derivatives**

Deletion mutagenesis was performed using recombinant PCR technology and either Vent polymerase (New England Biolabs) or Expand polymerase (Roche). Initially, plasmid pHapΔ26-1036 was constructed by first amplifying two PCR fragments from pMLD100 (pUC19 containing a 6.7 kb insert with the hap gene from *H. influenzae* strain N187), one a 2.3 kb 5’ fragment containing the hap gene upstream region and sequence coding for the Hap signal peptidase and the other a 1.2 kb 3’ fragment containing sequence coding for the HapΔ translocator domain. The primers used to generate these fragments were designed such that the fragments overlapped at an 18-base region containing a BamHI site and an XbaI site separated by six bases. Additionally, PstI sites were incorporated into the primers corresponding to the fragment ends opposite the overlap region. The fragments were then combined in an equimolar ratio to serve as template in a recombinant PCR reaction to generate a 3.5 kb recombinant PCR product using the external primers from the initial PCR reactions plus a 1:100 dilution of an internal primer corresponding to the coding strand of the overlap region. The resulting recombinant PCR fragment, containing the *hap* upstream region and signal sequence fused in frame to HapΔ, was digested with PstI and ligated into PstI-digested pLS88.

Subsequent in frame deletion constructs were also generated by recombinant PCR, beginning with amplification from pHapS243A of initial 5’ and 3’ PCR fragments containing sequence coding for the Hap, passenger domain upstream and downstream, respectively, of the deleted region. The internal primers for these initial fragments were designed such that fragment pairs overlapped at 20-base regions, including the last 10 bases of the deleted region and the first 10 bases of the fragment to be deleted.
bases of the 5' fragment juxtaposed to the first 10 bases of the 3' fragment, thereby omitting the intervening sequence. External primers were designed to incorporate a BamHI site at the 5' end of the 5' fragment and an XbaI site at the 3' end of the 3' fragment. After generation of recombinant PCR products from each of the initial fragment pairs, the recombinant fragments were digested with BamHI and XbaI and then ligated into BamHI-XbaI-digested pHapΔ26-1036. For in-frame deletion derivatives pHapΔ26-50, pHapΔ26-525, pHapΔ26-725, pHapΔ26-725: N'-6×His, and pHapΔ1001-1036, one-step PCR reactions using pMLD100 as template generated inserts corresponding to Hap sequence outside the indicated deleted regions. The 5' and 3' primers for these reactions contained a BamHI site and XbaI site, respectively, with the 5' primer for pHapΔ26-725: N'-6×His also containing sequence coding for six histidine residues inserted in frame between the BamHI site and the ensuing hap sequence. Each of these PCR inserts was digested with BamHI and XbaI and then ligated into BamHI-XbaI-digested pHapΔ26-1036. For pHapΔ26-725: N'-6×His, the resulting plasmid was digested with PstI to liberate a 4.5-kb hap allele, which was then ligated into PstI-digested pGB103. To construct pSL88:HapS243A, a PCR fragment corresponding to the entire Hap passenger domain was amplified from pMLD100 and inserted into BamHI-XbaI-digested pHapΔ26-1036 as described above.

Plasmid pGFp was also constructed by recombinant PCR, beginning with amplification from pMLD100 of a 1.0 kb initial 5' fragment containing the upstream non-coding region of the hap gene and amplification from pFPV27 (Barker et al., 1998) of a 1.0 kb fragment containing a promoterless green fluorescent protein cassette. Internal primers were designed such that the initial fragments overlapped at an 18-base region, and external primers were designed incorporating PstI sites. After generation of a recombinant PCR product from the initial fragments as described above, the recombinant fragment was digested with PstI and ligated into PstI-digested pLS88.

**Anti-Hap₉ antibodies**

To generate monoclonal antibodies, mice were immunized at week 0 with 20 μg of adjuvant QS21 and 5 μg of Hap₉ purified from culture supernatants of *H. influenzae* strain DB117/pJS106 as previously described (Hendrixson et al., 1997). Mice were boosted at weeks 4, 6, and 11, and 16 with the same mixture and then boosted at week 20 with 20 μg of QS21, 20 μg of Hap₉ from DB117/pJS106, and 10 μg each of Hap₉ from strains P860295 and P861454. Mice were boosted once more with 20 μg of QS21 and 20 μg of Hap₉ from strain P860295, and hybridoma cell lines were prepared from spleens using standard protocols. Supernatants were collected from hybridoma cultures and either concentrated by centrifugation in spin concentrators or precipitated with 50% ammonium sulphate and dialysed extensively against phosphate-buffered saline (PBS). Working dilutions for the antibody preparations in all assays were 1:100 for antisera 314 and 1:50 for all other monoclonal sera.

**Antiserum GP74** is a guinea pig polyclonal antiserum that was raised against Hap₉ (Cutter et al., 2002).

**Quantitative adherence assays**

Adherence assays using A549 respiratory epithelial cells (ATCC CCL 185) and Chang conjunctival epithelial cells (Wong-Kilbourne derivative, clone 1–5c-4) were performed as described previously (St. Germe et al., 1993). A549 cells were maintained in minimum essential medium (MEM-Eagle) with 10% heat-inactivated fetal calf serum. Chang cells were maintained in MEM-Eagle with 10% heat-inactivated fetal calf serum and 1% non-essential amino acids. In assays examining adherence to extracellular matrix proteins, wells of 24-well tissue culture plates precoated with human plasma fibronectin, murine laminin or murine collagen IV (BD Biosciences) were rehydrated in 0.5 ml of minimum essential medium (Sigma) at 37°C for 1 h in a tissue culture incubator infused with 5% CO₂. To assess the ability of specific antibodies to inhibit Hap-mediated adherence, bacteria were grown to late-exponential phase, washed once in 1 ml of PBS, resuspended in BHI broth, and incubated with the relevant antibody for 1 h at room temperature. Samples were then washed once again with PBS, resuspended in PBS, and inoculated onto monolayers. Per cent adherence was calculated by dividing the number of adherent colony-forming units per well by the number of inoculated colony-forming units.

**Analysis of bacterial outer membrane fractions**

Bacteria were grown to an optical density at 600 nm (OD₆₀₀) of 0.8. Sarkosyl-insoluble outer membrane proteins were isolated by the method of Carlone et al. (1986) and resuspended in 25 μl of 10 mM Heps pH 7.4 plus 25 μl of 2× Laemmli buffer. Protein samples were resolved by SDS-PAGE using 7.5% SDS-polyacrylamide gels (Laemmli, 1970). To ensure that comparable amounts of protein were analysed, similar volumes from cultures of similar density were loaded into each lane. Resolved proteins were electrotransferred to a nitrocellulose membrane and then detected by immunoblot analysis using either antiserum GP74 diluted 1:2000 or anti-Hap₉ monoclonal antibodies at the previously indicated dilutions. An anti-guinea pig IgG antiserum (for GP74) or anti-mouse IgG antiserum (for monoclonal antibodies) conjugated to horseradish peroxidase (Sigma) was used as the secondary antibody, and detection of antibody binding was accomplished by incubation of the membrane in a chemiluminescent substrate solution (Pierce) and exposure to film.

**Purification of 6× His-tagged Hap₉ C-terminal domain (Hap726–1036)**

A 10 litre culture of DB117/pHapΔ26-725: N'-6×His + pHapΔ1001-99 was grown to late exponential phase (OD₆₀₀ = 0.9), and cells were pelleted by centrifugation at 10 000 g for 15 min. The supernatant was recovered and then concentrated to 500 ml by continuous recirculation across a 0.5 m² transmembrane filter with a 10 kDa molecular weight cut-off (Millipore). The concentrated supernatant was dialysated against 4 litres of 20 mM Tris, pH 8.0, 100 mM NaCl, while maintaining a sample volume of 500 ml. The sample was passed over a 1.5 ml bed of Talon beads (Clontech) using a gravity flow column, and the beads were then washed with 50 ml of 20 mM Tris, pH 8.0, 300 mM NaCl, 10 mM imidazole. Bound proteins were eluted from the Talon beads with 1.5 ml of 20 mM Tris, pH 8.0, 100 mM NaCl, 100 mM imidazole. Protein concentrations were determined by the Bio-Rad protein assay, and immunoblot analysis of SDS-PAGE resolved protein was accomplished using...
antiserum GP74 diluted 1:2000 and a secondary anti-guinea pig IgG antiserum conjugated to horseradish peroxidase.

Detection of protein binding by immunofluorescence microscopy

To visualize binding of purified Hap_S or 6×His-tagged Hap_C-terminal domain to epithelial cells, 1 ml volumes of A549 or Chang cells in suspensions with 1×10^6 cells ml^-1 were seeded onto glass cover slips in wells of a 24-well tissue culture plate and incubated for 18 h at 37°C in a tissue culture incubator. Cells were washed once with PBS, then fixed for 15 min with 50 μl of PBS plus 2.5% paraformaldehyde, 0.2% glutaraldehyde, followed by a 15 min incubation in 50 μl of PBS plus 10 mM ethanalamine. After blocking for 30 min in 200 μl of PBS plus 3% BSA (Sigma), fixed cells were incubated for 3 h at room temperature with 50 μl of purified full-length Hap_S or 6×His-tagged Hap_C-terminal domain diluted to 100 nM in PBS plus 0.3% BSA. Cells were then incubated with antiserum GP74 diluted 1:2500 in PBS plus 0.3% BSA followed by incubation with a secondary anti-guinea pig IgG antiserum conjugated to Cy2, diluted 1:100 in PBS plus 0.3% BSA. Samples were washed four times in PBS plus 0.1% Tween-20 (Sigma) after each incubation step. Bound protein was visualized by confocal laser scanning microscopy (TCS confocal imaging system, Leica).

ELISAs

To quantify the amount of purified Hap_S or 6×His-tagged Hap_C-terminal domain binding to epithelial cells, 200 μl volumes of A549 or Chang cells in suspensions with 1.8×10^6 cells ml^-1 were seeded into wells of a 96-well tissue culture plate and incubated for 18 h at 37°C in a tissue culture incubator. Cells were washed once with PBS, then fixed for 15 min with 50 μl of PBS plus 2.5% paraformaldehyde, 0.2% glutaraldehyde, followed by a 15 min incubation in 50 μl of PBS plus 10 mM ethanalamine. After blocking for 30 min in 200 μl of PBS plus 3% BSA (Sigma), 50 μl of purified full-length Hap_S or 6×His-tagged Hap_C-terminal domain, diluted to final concentrations of 0.064 nM, 0.32 nM, 0.64 nM, 0.96 nM, 3.2 nM, 1.6 nM, 8 nM, 40 nM, 200 nM and 1000 nM, was added to triplicate wells and incubated for 3 h at room temperature. Wells were then set standing at room temperature for 4.5 h. During this time, OD₆₀₀ readings were performed at 30 min intervals.

Quantitative aggregation assays

Bacteria were incubated to late exponential phase, and cultures were then set standing at room temperature for 4.5 h. During this time, OD₆₀₀ readings were performed at 30 min intervals.

Statistical analysis

Mean values were compared by using the two-tailed t-test. Significance was defined as P < 0.05.

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