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Infection and Immunity

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Structural Determinants of Autoproteolysis of the Haemophilus influenzae Hap Autotransporter

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Haemophilus influenzae is a gram-negative bacterium that initiates infection by colonizing the upper respiratory tract. The H. influenzae Hap autotransporter protein mediates adherence, invasion, and microcolony formation in assays with respiratory epithelial cells and presumably facilitates colonization. The serine protease activity of Hap is associated with autoproteolytic cleavage and extracellular release of the HapS passenger domain, leaving the HapH C-terminal domain embedded in the outer membrane. Cleavage occurs most efficiently at the LN1036-37 peptide bond and to a lesser extent at three other sites. In this study, we utilized site-directed mutagenesis, homology modeling, and assays with a peptide library to characterize the structural determinants of Hap proteolytic activity and cleavage specificity. In addition, we used homology modeling to predict the S1, S2, and S4 subsite residues of the Hap substrate groove. Our results indicate that the P1 and P2 positions at the Hap cleavage sites are critical for cleavage, with leucine preferred over larger hydrophobic residues or other amino acids in these positions. The substrate groove is formed by L263 and N274 at the S1 subsite, R264 at the S2 subsite, and E265 at the S4 subsite. This information may facilitate design of approaches to block Hap activity and interfere with H. influenzae colonization.

Haemophilus influenzae is a gram-negative bacterium that typically colonizes the nasopharynxes of children and adults. In addition, this organism is an important cause of localized respiratory tract and invasive disease. Nonecapsulated strains cause otitis media, sinusitis, conjunctivitis, and exacerbations of respiratory symptoms in individuals with underlying lung disease, bronchiectasis, and cystic fibrosis (21, 29). Encapsulated strains are an important cause of bacteremic diseases, including sepsis and meningitis (29). Colonization of the upper respiratory tract represents an early step in the pathogenesis of H. influenzae disease and requires adherence to respiratory epithelium (19). Adherence is facilitated by a number of adhesins, including Hap, Hia, Hsf, HMW1/HMW2, P5, pili, and lipoooligosaccharide (2, 18, 21, 26, 27).

The Hap adhesin is ubiquitous among isolates of H. influenzae and is a member of the autotransporter family of virulence factors that have been recognized among many gram-negative bacteria (10). Autotransporters are synthesized as precursor proteins with three functional regions, namely, an N-terminal signal sequence, an internal passenger domain, and a C-terminal β-barrel domain (11). The signal sequence targets the precursor protein to the inner membrane and is then cleaved. The C-terminal β-barrel domain inserts into the outer membrane and facilitates presentation of the passenger domain on the bacterial cell surface. Depending upon the protein, the passenger domain remains covalently attached to the β-barrel domain, is cleaved but remains loosely attached to the β-barrel domain, or is cleaved and released entirely from the cell surface (10–12). Although diverse autotransporters share a similar structural organization and a common secretion mechanism, they vary widely in function, possibly reflecting adaptations to particular bacterial pathogenic niches. Autotransporters may function as adhesins mediating tissue tropism, as proteases involved in tissue degradation, as toxins causing host tissue damage, or as mediators of serum resistance (11).

Hap is synthesized as a 155-kDa prepolytein encompassing a 110-kDa passenger domain, HapS, and a 45-kDa β-barrel domain, HapH. The HapS passenger domain harbors adhesive activity that has been shown to promote interactions with human respiratory cells, as well as with extracellular matrix proteins such as fibronectin, laminin, and collagen IV (7). HapS is also responsible for bacterial aggregation via Hap-Hap interactions, contributing to microcolony formation (5). Adherence to epithelial cells and bacterial aggregation are mediated by the C-terminal 311 amino acids of HapS, whereas interaction with extracellular matrix proteins is mediated by the C-terminal 511 amino acids of HapS (7).

Beyond possessing adhesive activities, the HapS passenger domain functions as a protease that directs the autoproteolytic cleavage of HapS from HapH, resulting in release of HapS from the bacterial cell surface (6). Hap autoproteolysis has been determined to occur at least partly through intermolecular...
cleavage on the surface of the bacterium and involves a catalytic triad consisting of residues His98, Asp140, and Ser243. Ser243 is part of the GD\textsubscript{S}GS motif that is characteristic of many serine proteases (6, 13). In wild-type Hap, cleavage occurs most abundantly at the L1036-N1037 peptide bond, which is referred to as the primary cleavage site (13). Site-directed mutagenesis of this site and N-terminal sequencing of the resulting cleaved Hap fragments has identified three additional cleavage sites, including L1046-T1047, F1077-A1078, and F1067-S1068, termed the secondary, tertiary, and quaternary cleavage sites, respectively (see Table 2) (6). Alignment of the amino acid sequences at these cleavage sites has revealed a consensus target sequence motif that consists of (Q/R)(A/S)X(L/F) at the P4 through P1 positions (see Table 2) (6).

Hap protease activity can be blocked by selected serine protease inhibitors, including secretory leukocyte protease inhibitor, a component of human respiratory secretions (14). This inhibition results in accumulation of full-length Hap in the outer membrane, with Hap\textsubscript{S} on the bacterial surface. Similar to the effect of serine protease inhibitors, mutation of the active site serine in the Hap protease domain causes retention of Hap\textsubscript{S} on the bacterial surface, increased adherence to epithelial cells, increased adherence to extracellular matrix proteins, increased bacterial aggregation, and increased microcolony formation, suggesting that Hap protease activity might play a regulatory role in bacterial adherence. Indeed, autoproteolytic cleavage of Hap results in reduced adhesive activity (5).

In the present study, we sought to further elucidate the structural determinants of Hap proteolytic activity. We used site-directed mutagenesis and in vitro assays with a synthetic peptide library to characterize Hap cleavage site specificity, and we used homology modeling and site-directed mutagenesis to define the Hap substrate groove. We found that the two residues immediately N-terminal to Hap cleavage sites are critical for cleavage, with leucine preferred over larger hydrophobic residues or other amino acids in these positions. In addition, we defined the residues that likely form the Hap substrate groove.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in the present study are listed in Table 1. Plasmid constructs encoding hap were expressed in H. influenzae strain DB117, a rec-1 derivative of...
TABLE 2. Alignment of sequences at the Hap cleavage sites

| Cleavage site | Sequence alignment*
|---------------|-----------------------------
| Primary 1036-37 | D Q S L L N A L E A |
| Secondary 1046-47 | K Q A E L T A E T O |
| Tertiary 1077-78 | D Q S L F A L E A A |
| Quaternary 1067-68 | K R A V F S D P L L |

* The peptide bond where cleavage occurs is between the P1 and P1' columns. According to nomenclature by Schechter and Berger (22), the residues N terminal to the cleavage site are labeled P1, P2, ..., Pn in the N-terminal direction, and the residues C-terminal to the cleavage site are labeled P1', P2', ..., Pn'.

Pertactin protein.

Bordetella pertussis

Electron microscopy of purified Hap5. In order to study the architecture of Hap5, we began by purifying Hap5 from culture supernatants of H. influenzae strain DB117 expressing wild-type Hap. As shown in Fig. 1A, the sedimentation coefficient of purified Hap5, as estimated by glycerol gradient sedimentation, was found to be 5.5 S. This S value of Hap5, together with its known molecular weight, gives rise to an Smasm/S value of 1.53, indicating that Hap5 has an elongated shape (Fig. 1A) (23). To further investigate the structure of Hap5, we performed rotary shadowing electron microscopy. As shown in Fig. 1B, this technique revealed structures that were ~15 nm in length and resembled very short arrowheads (Fig. 1B, middle panel). Similar arrowhead-shaped structures were also visible with negative staining transmission electron microscopy (Fig. 1B, left panels). The arrowhead shape of Hap5 resembled the crystal structure of E. coli hemoglobin protease (Hbp, Fig. 1B, right panel), a Hap homolog with heme-binding and serine protease activities that is expressed by clinical isolates associated with intra-abdominal infections (20, 30). Alignment of the amino acid sequences of Hap5 and the protease domain of Hbp revealed an identity of 41% and a similarity of 54%, as highlighted in Fig. 2A.

Homology based modeling of the Hap5 structure. Sequence alignment using the Pfam server (http://pfam.sanger.ac.uk) suggested that Hap5 consists of an N-terminal domain (residues 70 to 550) sharing 43% sequence identity with Hbp and a C-terminal domain (residues 851 to 980) sharing 43% sequence identity with the Bordetella pertussis Pertactin protein. To gain further insight into the structure of Hap5, we modeled residues 70 to 550 containing the protease domain based on the crystal structure of Hbp (PDB code 1WXR) and residues 851 to 980 based on the crystal structure of Pertactin (PDB code 1DAB) using Swissmodel (http://swissmodel.expasy.org) and 3Djigsaw (http://bmm.cancerresearchuk.org) homology modeling servers (Fig. 2B). Based on the Hbp-like shape of Hap5 observed by electron microscopy, we modeled the region between the protease and Pertactin-like domains on the crystal structure of Hbp, assuming that Hap5L550 shares the same β-helix fold as observed in the Hbp crystal structure and other
conventional autotransporters (31). Hap551-850 was predicted by the PredictProtein server to be a domain consisting only of H9252 strands (http://www.predictprotein.org/).

Using the homology model of Hap S, we predicted the Hap substrate groove. First, we aligned Hap 63-287 with the published crystal structures of alpha-chymotrypsin (PDB code 2CHA) and beta-trypsin (PDB code 5PTP). The structures of Hap63-287, alpha-chymotrypsin, and beta-trypsin are very similar, with a root mean square deviation in C\(^{\alpha}\) positions of 1.0 Å between Hap 63-287 and alpha-chymotrypsin and 2.2 Å between Hap63-287 and beta-trypsin (Fig. 3A). Next, we inserted the inhibitor p-sulfinotoluene in these aligned structures to highlight the catalytic triad and to predict the S subsite residues of HapS (Fig. 3A). The coordinates of inhibitor p-sulfinotoluene were extracted from the superimposed alpha-chymotrypsin structure (2CHA) and merged into the homology model of Hap63-287. By nomenclature, binding sites on a protease are called subsites (S residues), and each subsite residue interacts with a residue of the substrate (called P residues) (22). The predicted S1 subsite contains S243, N274, L263, and K240; the predicted S2 subsite contains H98, R264, and Y137; and the predicted S4 subsite contains E265 (Fig. 3B).

Mutagenesis analysis of the predicted substrate groove. To assess the accuracy of the predicted substrate groove, we performed site-directed mutagenesis, changing each of the predicted subsite residues individually and assessing the effect of each mutation on Hap autoproteolytic activity (Table 3). Wild-type residues were changed to amino acids that differ in terms of polarity, charge, or length of side chain. For the predicted S1 residues, we changed K240 from a charged amino acid to a nonpolar amino acid (K240A), L263 from a nonpolar amino acid to a charged polar amino acid (L263R), and N274 from an uncharged polar amino acid to a charged polar amino acid (N274R). For the predicted S2 subsite, we changed Y137 from an aromatic amino acid to a nonpolar amino acid (Y137A) and R264 from a charged polar amino acid to a nonpolar amino acid (R264A). For the predicted S4 subsite, we changed E265 from a charged polar amino acid to nonpolar amino acid (E265A) or an aromatic amino acid (E265W). In Western blot analysis of outer membrane preparations from strain DB117 expressing wild-type Hap, a 45-kDa band corresponding to Hap\(_{551-850}\) is apparent, representing cleavage at the primary cleavage site (Fig. 4A). By comparison, the N274R and L263R mutations at the predicted S1 subsite disrupted autoproteolysis, resulting in accumulation of the 155-kDa Hap precursor (Fig. 4A). Similarly, the R264A mutation at the predicted S2 subsite and the E265W mutation at the predicted S4 subsite also disrupted autoproteolysis. The E265A mutation had only a modest effect on autoproteolysis, as evidenced by partial accumulation of Hap precursor in the outer membrane. In contrast, the Y137A and K240A mutations had no effect on autoproteolysis, suggesting that Y137 and K240 may not be essential for mediating interactions between Hap\(_{551-850}\) and its substrate (Fig. 4A). Taken together, these mutagenesis data provide further support that residues N274, L263, R264, and E265 may indeed form the substrate binding groove in Hap\(_{551-850}\) predicted by our homology model (Fig. 3B).

Analysis of Hap cleavage specificity. In earlier studies we found that mutation of the P1 residue of the Hap cleavage site disrupted cleavage (13). To extend these studies, we studied Hap...
cleavage specificity by systematically mutating the P1 to P4 residues at cleavage sites and analyzing the effect of these mutations on autoproteolysis (Table 3). The P1 residue at the primary (L1036-N1037) and secondary (L1046-T1047) sites is a leucine. In contrast, the P1 residue at the tertiary (F1077-A1078) and quaternary (F1067-S1068) sites is a phenylalanine (Table 2). To address the possibility that leucine at the P1 position is preferred over the bulkier phenylalanine, leucine in the P1 position at the primary cleavage site was mutated to phenylalanine (L1036F). In addition, the bulky phenylalanine in the P1 position at both the tertiary and quaternary cleavage sites was mutated to leucine (F1077L and F1067L, respectively). As shown in Fig. 4B, the previously characterized L1036S mutation (6) that changes the P1 residue at the primary cleavage site to a serine resulted in loss of cleavage at the primary cleavage site and enhanced cleavage at the secondary, tertiary, and quaternary cleavage sites. The L1036F mutation in the present study resulted in slightly diminished cleavage at the primary cleavage site and enhanced cleavage at the secondary cleavage site (Fig. 4B). The F1077L mutation resulted in slightly increased cleavage at the tertiary cleavage site (Fig. 4B). When the L1036S and the F1077L mutations or the L1036F and the F1077L mutations were combined, cleavage at the tertiary site was markedly increased, whereas cleavage at the primary, secondary, and tertiary sites was markedly diminished (Fig. 4B). The F1067L mutation resulted in a marked increase in cleavage at the quaternary site (Fig. 4B). Taken together, these findings suggest that cleavage after leucine in the P1 position is more efficient than cleavage after the bulkier phenylalanine in that position.

The P2 residue at the primary and tertiary cleavage sites is leucine, while the P2 residue at the secondary cleavage site is glutamic acid and at the quaternary cleavage site is valine. To

FIG. 2. (A) Sequence alignment between Hap and Hbp serine protease domains. Invariant residues are indicated by an asterisk. The conserved serine protease motif containing the catalytic serine is underlined. (B) Homology model of HapS. Hap351-550 (red and salmon) was modeled based on the Hbp structure (PDB code 1WXR). The region highlighted in red corresponds to the serine protease domain. Hap551-850 (yellow) is modeled based on the Pertactin structure (PDB code 1DAB). Hap851-980 (green) is predicted to be a β domain and is built on the crystal structure of Hbp based on the appearance of purified HapS by electron microscopy.
determine whether leucine is preferred in the P2 position, we initially changed the leucine at the primary cleavage site to polar residues serine (L1035S) and threonine (L1035T). The L1035S mutation resulted in diminished cleavage at the primary site and enhanced cleavage at the secondary, tertiary, and quaternary sites. The L1035T mutation virtually eliminated all cleavage, resulting in accumulation of the Hap precursor (Fig. 4B). These data indicate that the P2 position is important for substrate recognition. In order to further test the P2 position, we changed the P2 leucine at the primary cleavage site to a glutamic acid (L1035E), which is also present in the P2 position at the secondary cleavage site. The L1035E mutation showed wild-type cleavage (Fig. 4B). Next, we changed the glutamic acid in the P2 position at the secondary cleavage site to leucine (E1045L). The E1045L mutation resulted in prominent cleavage at the secondary cleavage site and almost no cleavage at the primary, tertiary, and quaternary cleavage sites (Fig. 4C). To further assess cleavage preference at the P2 position, we mutated the valine in the P2 position at the quaternary cleavage site to leucine (V1066L). As shown in Fig. 4C, the V1066L mutation resulted in enhanced...

FIG. 3. (A) Stereo ribbon diagram of the superimposed Hap serine protease domain (salmon) and the p-sulfinotoluene bound catalytic alpha chymotrypsin (blue). The inhibitor, p-sulfinotoluene, and the adjacent catalytic triad of Hap H98, D140, and S243 are shown in stick. (B) Putative Hap substrate-binding groove. The catalytic triad and the residues that have been tested to be important for Hap autoproteolysis are labeled. L263 and N274 are predicted S1 residues, R264 is a predicted S2 residue, and E265 is a predicted S4 residue. Also labeled are residues K240 and Y137, which had no effect on autoproteolysis when mutated.
cleavage at the quaternary cleavage site. These observations indicate that the P2 residue is important in Hap cleavage specificity and suggest that leucine and glutamic acid are the preferred residues in this position for Hap proteolysis.

The P3 residue at all four cleavage sites is either a serine or an alanine. To test the importance of the P3 residue in Hap proteolysis, we mutated the serine to a charged polar glutamic acid (S1034E), a much bulkier residue. We also changed the serine to a charged polar glutamic acid (S1034F), a much bulkier residue. Both of these mutations had no effect on autoproteolysis (Fig. 4C).

We also investigated the importance of the P4 residues in Hap cleavage specificity. However, the mutations N1037R, A1038S, L1039S, and E1040S at the P1, P2, P3, and P4 positions, respectively, had no effect on autoproteolysis (Fig. 4C), suggesting that this position does not significantly influence Hap cleavage specificity.

The P4 residue is glutamine at the primary, secondary, and tertiary cleavage sites and arginine at the quaternary site. To test the importance of the P4 residue in Hap proteolysis, we mutated the glutamine to arginine (Q1033A), and to glutamic acid, a charged polar residue (Q1033E). These mutations did not have any effect on autoproteolysis (Fig. 4C), suggesting that the P4 position is not an important determinant of Hap proteolysis.

We also investigated the importance of the P5 residues in Hap cleavage specificity. However, the mutations N1037R, A1038S, L1039S, and E1040S at the P1', P2', P3', and P4' positions, respectively, had no effect on autoproteolysis (Fig. 4C).

**TABLE 3. Autoproteolysis phenotypes of Hap mutant derivatives**

<table>
<thead>
<tr>
<th>Wild type or mutation</th>
<th>Cleavage site or subsite</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hap wild type</td>
<td>NA</td>
<td>1°, 2°, and 3° cleavage</td>
</tr>
<tr>
<td>HapL1036S</td>
<td>P1 (1°)</td>
<td>No 1° cleavage; 2°, 3°, and 4° cleavage</td>
</tr>
<tr>
<td>HapL1036F</td>
<td>P1 (1°)</td>
<td>1° cleavage slightly diminished, enhanced secondary cleavage</td>
</tr>
<tr>
<td>HapF1077L</td>
<td>P1 (3°)</td>
<td>Slightly enhanced 3° cleavage</td>
</tr>
<tr>
<td>HapF1077L/L1036S</td>
<td>P1 (3°) + P1 (1°)</td>
<td>Enhanced 3° cleavage</td>
</tr>
<tr>
<td>HapF1077L/L1036F</td>
<td>P1 (3°) + P1 (1°)</td>
<td>Enhanced 3° cleavage</td>
</tr>
<tr>
<td>Hap F1067L</td>
<td>P1 (4°)</td>
<td>Enhanced 4° cleavage, some 1° and 3° cleavage</td>
</tr>
<tr>
<td>HapL1035S</td>
<td>P2 (1°)</td>
<td>Very little 1° cleavage, enhanced 2° cleavage</td>
</tr>
<tr>
<td>HapL1035T</td>
<td>P2 (1°)</td>
<td>Minimal autoproteolysis, accumulation of Hap precursor</td>
</tr>
<tr>
<td>HapL1035E</td>
<td>P2 (1°)</td>
<td>No effect on autoproteolysis</td>
</tr>
<tr>
<td>HapE1045L</td>
<td>P2 (2°)</td>
<td>Enhanced 2° cleavage</td>
</tr>
<tr>
<td>HapE1045A</td>
<td>P2 (2°)</td>
<td>Enhanced 2° cleavage</td>
</tr>
<tr>
<td>HapV1066L</td>
<td>P2 (3°)</td>
<td>Enhanced 4°, some 1° and 3° cleavage</td>
</tr>
<tr>
<td>HapS1034F</td>
<td>P3 (1°)</td>
<td>No effect on autoproteolysis</td>
</tr>
<tr>
<td>HapS1034E</td>
<td>P3 (1°)</td>
<td>No effect on autoproteolysis</td>
</tr>
<tr>
<td>HapQ1033A</td>
<td>P4 (1°)</td>
<td>No effect on autoproteolysis</td>
</tr>
<tr>
<td>HapQ1033E</td>
<td>P4 (1°)</td>
<td>No effect on autoproteolysis</td>
</tr>
<tr>
<td>HapN1037R</td>
<td>P1' (1°)</td>
<td>No effect on autoproteolysis</td>
</tr>
<tr>
<td>HapA1038S</td>
<td>P2' (1°)</td>
<td>No effect on autoproteolysis</td>
</tr>
<tr>
<td>HapL1039S</td>
<td>P3' (1°)</td>
<td>No effect on autoproteolysis</td>
</tr>
<tr>
<td>HapE1040S</td>
<td>P4' (1°)</td>
<td>No effect on autoproteolysis</td>
</tr>
<tr>
<td>HapK240A</td>
<td>S1</td>
<td>No effect on autoproteolysis</td>
</tr>
<tr>
<td>HapL263R</td>
<td>S1</td>
<td>Diminished autoproteolysis, accumulation of Hap precursor</td>
</tr>
<tr>
<td>HapN274R</td>
<td>S2</td>
<td>Diminished autoproteolysis, accumulation of Hap precursor</td>
</tr>
<tr>
<td>HapR264A</td>
<td>S2</td>
<td>Diminished autoproteolysis, accumulation of Hap precursor</td>
</tr>
<tr>
<td>HapY137A</td>
<td>S2</td>
<td>Diminished autoproteolysis, accumulation of Hap precursor</td>
</tr>
<tr>
<td>HapE265A</td>
<td>S4</td>
<td>Diminished autoproteolysis, accumulation of Hap precursor</td>
</tr>
<tr>
<td>HapE265W</td>
<td>S4</td>
<td>Diminished autoproteolysis, accumulation of Hap precursor</td>
</tr>
</tbody>
</table>

*All Hap derivatives were expressed in H. influenzae strain DB117.

Numbers in parentheses indicate the primary (1°), secondary (2°), tertiary (3°), or quaternary (4°) cleavage sites. NA, not applicable.

*Descriptions are based on visual inspection of the blots in triplicate.*

**Discussion**

In this study, we have investigated the Hap cleavage specificity and substrate groove in detail. Our results demonstrate that the P1 and P2 residues are important determinants of Hap cleavage specificity, that small hydrophobic amino acids are favored in the P1 and P2 positions strongly favored proteolytic cleavage. Arginine at P1 and glutamic acid at P2 seemed to favor cleavage to a lesser extent. Glutamic acid seems to be preferred at the P3 position. A number of amino acids had selectivity ratios greater than 1 at the P4 position, although no strong preference was evident for any particular amino acid.
nine, with all of these residues fitting within the deep hydrophobic pocket of the protein (16, 17). However, chymotrypsin can also cleave after leucine, methionine, and histidine in the P1 position, although with lower efficiency (15, 16). In contrast, elastase prefers small hydrophobic residues such as alanine in the P1 position, reflecting a smaller S1 subsite pocket (24). In the present study, we demonstrated that Hap cleaves with high preference for leucine over phenylalanine in the P1 position, suggesting that the S1 subsite pocket in Hap is relatively shallow and accommodates small hydrophobic residues better than bulky residues. This conclusion is consistent with the relative inefficiency of cleavage at the tertiary and quaternary sites in wild-type Hap, where the P1 residue is phenylalanine.

In chymotrypsin, proteolysis is also influenced by interaction between the P2 residue and the S2 subsite pocket (3). In this context, it is noteworthy that our work supports an important role for the P2 residue in Hap cleavage specificity, with leucine or glutamic acid favored over other amino acids. Interestingly, insertion of leucine in the P2 position at the secondary cleavage site (HapE1045L) results in a preference for this cleavage site to the exclusion of other cleavage sites. It is possible that these mutations result in local steric hindrance that causes increased binding of the mutant substrate and more efficient cleavage at the secondary cleavage site. However, without a high-resolution crystal structure, we cannot explain the apparent preference of the secondary cleavage site over the primary cleavage site in this mutant. We also found that insertion of glutamic acid at the P2 position (L1035E) of the primary cleavage site had no significant effect on autoproteolysis. Consistent with this finding, glutamic acid is the P2 residue at the native secondary cleavage site (E1045).

Hap cleavage of a random peptide library revealed high preference for leucine at the P1 and P2 positions, agreeing with our mutagenesis results. Interestingly, phenylalanine was not selected at either of these positions, despite the fact that cleavage occurs naturally at F1067 and F1077 in wild-type Hap (albeit to a lesser extent than with leucine in the P1 position). In considering this information, it is possible that the S1 and S2 subsites in the Hap proteolytic pocket have reduced affinity for phenylalanine in the context of linear peptides compared to native folded protein. Based on selectivity ratios, arginine at the P1 position and glutamic acid at the P2 position seem to be the favored amino acids after leucine. Serine and threonine have the lowest selectivity ratio at the P1 and P2 positions, indicating that they have an inhibitory effect on proteolytic cleavage, in agreement with our mutagenesis data (L1036S, L1035S, and L1035T). Further analysis of the peptide library cleavage assay revealed that glutamic acid was selected at the P3 position and that a number of amino acids were selected at the P4 position.

Considered together, our mutational analysis and our peptide library cleavage results indicate that the P1 and P2 residues are important determinants of Hap cleavage specificity. Previously, we reported a consensus cleavage site sequence based only on alignment of the primary, secondary, tertiary, and quaternary cleavage sites (6). In the present study, mutational analysis reveals a target motif that consists of XX(L/E/V)(L/E) and the peptide library cleavage assay reveals a target motif of XE(L/E)(L/R) at the P4 through P1 positions for the Hap protease. The difference in results between mutational

FIG. 4. Western blots of outer membrane proteins purified from Hap mutant derivatives. The presence of Hap in the outer membrane was detected using antibody Rab290 raised against the C-terminal Hap fragment encompassing residues 996 to 1395. (A) Mutagenesis analysis of predicted Hap substrate binding groove. (B and C) Mutagenesis analysis of Hap cleavage specificity. Full-length Hap is seen at 155 kDa, and Hap* species are seen at 45 to 39 kDa. The different Hap* derivatives result from cleavage at the different cleavage sites and are labeled “1” for primary cleavage (45 kDa), “2” for secondary cleavage (43 kDa), “3” for tertiary cleavage (39 kDa), and “4” for quaternary cleavage (41 kDa). Immunoblots shown here are representative of blots performed in triplicate. Assessment of cleavage at the various cleavage sites was performed by manual inspection.

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analysis and peptide library cleavage may be due to the intrinsic difference between cleavage of full-length folded protein and linear peptides. Mutations introduced at the P1/H11032 residues of the primary cleavage site had no effect on autoproteolysis, indicating that these residues do not significantly affect proteolytic cleavage.

In an effort to define the Hap substrate binding groove, we developed a homology model based on the crystal structures of the E. coli Hbp and the B. pertussis Pertactin autotransporters. Guided by this model, we performed site-directed mutagenesis, changing residues at the predicted S1, S2, and S4 subsites. Our results demonstrated that L263, N274, R264, and E265 are critical for efficient autoproteolysis, supporting a role for these residues in forming the Hap substrate groove and interacting intimately with substrate residues. Mutations at Y137 and K240 that are located at the rim of S1 and S2 subsites in our model did not affect autoproteolysis.

The implication of the Hap serine protease domain in the pathogenesis of H. influenzae disease could be twofold. First, autoproteolytic release of HapS from the bacterial surface may allow the organism to modulate interactions with host tissues during various stages of colonization and infection. Second, the secreted Hap protease domain may have activity against host substrates such as extracellular matrix proteins or immune factors. We have previously determined that Hap can bind to various extracellular matrix proteins such as fibronectin, collagen IV, and laminin (7). Further investigation is needed to determine whether Hap protease activity might facilitate spread to deeper tissues or promote persistence in the face of the host immune response.

In conclusion, in this study we have provided important insights into the structural determinants of H. influenzae Hap proteolytic activity. Complete knowledge of the Hap substrate groove and cleavage specificity may facilitate the design of therapeutic agents that block Hap proteolytic activity and thereby attenuate H. influenzae disease.

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