Sites in the CH3 Domain of Human IgA1 That Influence Sensitivity to Bacterial IgA1 Proteases

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The influence of regions, other than the hinge, on the susceptibility of human IgA1 to cleavage by diverse bacterial IgA1 proteases, was examined using IgA1 mutants bearing amino acid deletions, substitutions, and domain swaps. IgA1 lacking the tailpiece retained its susceptibility to cleavage by all of the IgA1 proteases. The domain swap molecule α1α2γ3, in which the CH3 domain of IgA1 was exchanged for that of human IgG1, was resistant to cleavage with the type 1 and 2 serine IgA1 proteases of Neisseria meningitidis, Neisseria gonorrhoeae, and Haemophilus influenzae, but remained sensitive to cleavage with the metallo-IgA1 proteases of Streptococcus pneumoniae, Streptococcus oralis, Streptococcus sanguis, and Streptococcus mitis. Substitution of the IgA1 Cα3 domain motif Pro440-Phe443 into the corresponding position in the Cγ3 domain of α1α2γ3 resulted in sensitivity to the type 2 IgA1 protease of N. meningitidis, indicating the possible requirement of these amino acids for sensitivity to this protease. For the H. influenzae type 2 protease, resistance of an IgA1 mutant in which the CH3 domain residues 399–409 were exchanged with those from IgG1, but sensitivity of mutant HuBov3 in which the Cα3 domain of bovine IgA replaces that of human IgA1, suggests that CH3 domain residues Glu403, Gln406, and Thr409 influence sensitivity to this enzyme. Hence, unlike the situation with the metallo-IgA1 proteases of Streptococcus spp., the sensitivity of human IgA1 to cleavage with the serum IgA1 proteases of Neisseria and Haemophilus involves their binding to different sites specifically in the CH3 domain. The Journal of Immunology, 2006, 177: 3913–3919.

IgA1 proteases are enzymes that specifically cleave human IgA1 (reviewed in Refs. 1–3). Most are produced by major bacterial pathogens that instigate infection at mucosal surfaces of the respiratory tract, such as Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis, or the genital tract such as Neisseria gonorrhoeae. IgA1 proteases represent a group of structurally diverse enzymes—metalloproteases, serine-type proteases, and cysteine-type proteases—with different enzymatic mechanisms. Evidence of in vivo production of IgA1 proteases in human infections (4–6), the association of recovery from infection with the development of Abs to IgA1 proteases (7–9), and the failure of related but nonpathogenic species of bacteria to produce IgA1 proteases (10, 11) point to IgA1 proteases being important virulence factors of the pathogens that produce them. Despite these persuasive indirect observations, the in vivo roles of IgA1 proteases in virulence are difficult to determine, because the substrate is restricted almost exclusively to (12–15) to IgA1 of humans, gorillas, and chimpanzees, and IgA of orangutans (16), and therefore convenient animal models are not available. However, IgA1 protease has been shown to compromise IgA-mediated killing of S. pneumoniae both in vitro and in vivo (17). S. pneumoniae-specific IgA1, when cleaved by IgA1 protease, loses its protective effects, and the resultant IgA fragments appear to enhance adherence of pneumococci to host cells (18).

The IgA1 hinge, an extended region that separates the Fab and Fc regions of the Ab and incorporates a duplication of an 8-aa sequence rich in proline, serine, and threonine, is the site of cleavage by all IgA1 proteases. IgA2 lacks this region and is thereby naturally resistant to IgA1 protease action. Although IgA1 proteases belong to very different families of enzymes, they are all postproline endopeptidases. They each cleave a specific peptide bond (either a Pro-Ser bond for type 1 enzymes or a Pro-Thr bond for type 2 enzymes) in only one of the duplicated halves of the IgA1 hinge.

The consequence of IgA1 cleavage is the generation of Fab and Fc fragments and thus the decoupling of recognition of Ags from mechanisms for their elimination. Moreover, the Fab formed may mask relevant epitopes from the immune system and thus prevent the binding of intact Abs of other isotypes (19, 20), activation of complement, and complement-mediated lysis. Some IgA1 proteases may have a role in virulence by mechanisms additional to or distinct from those arising through IgA1 cleavage (13, 15, 21).

It is becoming increasingly apparent that the sensitivity of human IgA1 to cleavage by IgA1 proteases is dependent not only on the presence of the hinge but also on structures elsewhere in IgA1. For example, it has been reported that structures, as yet undefined, in the IgA1 Fc region are required for cleavage by the type 1 IgA1 protease of H. influenzae and the type 2 IgA1 protease of N. gonorrhoeae (22). To understand more about the features of human IgA influencing its sensitivity to cleavage by bacterial IgA1 proteases, ultimately with a view to preparing IgA1 protease inhibitors, this study sought to localize structures in IgA1 outside the hinge that influence the sensitivity of human IgA1 to cleavage by IgA1 proteases. In anticipation of distinct structural requirements in terms of substrate recognition for different types of IgA1 protease, we tested a range of metallo-type and serine-type IgA1 proteases.

Materials and Methods

Primer

Primer BOVA3′SAL (5′-CCGGCCGTCGAGATCCACTATGTT-3′) contained a SalI restriction site (in italics) and bases complementary to the

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multiple cloning site (BanHI site underlined) of pBluescript SK vector. Primer HUMBOVDS1 (5'-TCTCCTACCAATCCGGAACGCTTTCC-3') incorporated bases from the 3' end of the CH2 exon of the human α1 gene (underlined) and bases from the 5' end of the CH3 exon of the bovine α gene (in italics). Primer HUMBOVDS2 was its complement.

Primer 399–409GAMS (5'-GTACCCTGACTTGGCCACCGGTGGCTTGAATGGCCACGATCCTTCA-3' underlined) and part of the IgG1 C-3 exon of human IgA1 (H9253) corresponded to the nucleotide sequence of the human IgA1 hinge (underlined) and part of the IgG1 H chain gene at 921–942. Primer AGS (5'-GGAGGGAGATGGGGTAGGTGGAGTTGAGGGAGATGGC-3') was its complement. Primer HUMBOVDS2 was its complement. Primer HUMBOVDS2 using a human IgA1 H chain expression vector, replacing the XhoI restriction site (underlined) and annealed to 100 nt downstream of the stop codon of human IgG1. Primer DS3 (5'-AGGCCTCCGAAATCTGATTTTCTCAGTGCCCAGGTAAGGTGGGAGATGGGGTAGGTGGAGTTGAGGGAGATGTCC-3') corresponded to the nucleotide sequence of the human IgA1 H chain were replaced by the corresponding amino acids in human IgG1. Primer 399–409GAMS is highlighted in gray. Bovine IgA amino acids differing from the corresponding amino acids in human IgA1 are underlined. The Pro440-Phe443 loop was included in the human IgA1-derived region substituted in CH3 domain of the parent Ig (25) (with C regions containing plasmid as template). The CH1 exon of bovine IgA was amplified from a pBluecript SK plasmid bearing the α-chain gene of bovine IgA (25) using primers HUMBOVDS1 and BOVA3'SAL. A second round of PCR overlap extension, with a wild-type IgA1 primer and HUMBOVDS2 using a human IgA1 α-chain-containing plasmid as template. The CH3 exon of bovine IgA was amplified from a pBluescript SK plasmid bearing the α-chain gene of bovine IgA2 (25) using primers HUMBOVDS1 and BOVA3'SAL. A second round of PCR overlap extension produced a fragment of ~2 kb. After cleanup with SalI and XhoI, it was ligated into an IgA1 H chain expression vector, replacing the wild-type sequence in this region.

In the IgA1 mutant 399–409GAM, amino acids Ala399 to Thr409 inclusive of the human IgA1 H chain were replaced by the corresponding amino acids from human IgG1 (Figs. 1 and 2). To generate the expression vector, internal mismatch primers 399–409GAMS and 399–409GAMAS, and the same 5' and 3' flanking primers as previously described (24) were used in PCR overlap extension, with a wild-type IgA1 α-chain-containing plasmid serving as template. The mutated PCR overlap product was ligated, as above, into unique XhoI and SalI restriction sites in the expression vector, replacing the wild-type sequence in this region.

As described previously, the preparation of recombinant IgA1 Abs (residues Pro220 to Ser240) was introduced into human IgG1, replacing the upper hinge region (residues Gln315 to Thr336) (25) inclusive (Eu numbering) (26) but retaining the inter-H chain disulfide bridges (Fig. 1). To generate the expression vector, PCR overlap extension was performed using internal primers AGS and AGAS, flanking primers DS3 and AG3', and a human IgG1 γ-chain-containing plasmid as template (25). The mutant overlap fragment generated was inserted as a BstEII-EcoRI fragment into an anti-NIP (3-nitro-4-hydroxy-5-iodophenylacetate) IgG1 H chain expression vector, replacing the wild-type sequence in this region (25).

In the TAHG-1 mutant, the hinge of human IgA1 (residues Pro223 to Ser240) was introduced into human IgG1, replacing the upper hinge region (residues Gln315 to Thr336) (25) inclusive (Eu numbering) (26) but retaining the inter-H chain disulfide bridges (Fig. 1). To generate the expression vector, PCR overlap extension was performed using internal primers AGS and AGAS, flanking primers DS3 and AG3', and a human IgG1 γ-chain-containing plasmid as template (25). The mutant overlap fragment generated was inserted as a BstEII-EcoRI fragment into an anti-NIP (3-nitro-4-hydroxy-5-iodophenylacetate) IgG1 H chain expression vector, replacing the wild-type sequence in this region (25).

We have described previously the preparation of the following mutant human IgA1 Abs: PTerm455, an IgA1 in which the 18-a C-terminal tailpiece is deleted (24) (Fig. 1); α1o2y3, a domain-swap Ab in which the CH3 domain of human IgA1 is exchanged for the CH3 domain of human IgG1 (25) (Figs. 1 and 2); α1o2y3PIALF, in which residues His535, Asn834, His835, and Tyr136 (Eu numbering) of the CH3 domain of the parent α1o2y3 construct are replaced by the Pro-Leu-Ala-Asp (PIALF) motif present at the equivalent position in the CH3 domain of human IgA1 (25) (Figs. 1 and 2); E125A, D255V, Q259R, and C311S featuring single amino acid substitutions in the CO2 domain (25, 28); and E437A and A442R with point mutations in the CH3 domain (25, 28).

**Microbial IgA1 proteases**

The bacterial strains used as sources of IgA1 proteases were as described previously (29), with the addition of *H. influenzae* strain R16, a producer of a type 1 enzyme. The culture conditions and preparation of the IgA1 proteases have been described previously (29).

**Digestion of recombinant Igs with IgA1 proteases and immunoblotting**

The digestion conditions, SDS-PAGE, and immunoblotting procedures were as described previously (29). All of the IgA1 protease preparations were initially calibrated with regard to activity on wild-type IgA1, and the optimal concentration of each enzyme was determined. Thereafter, for each enzyme, identical, optimal amounts were added to all digestion reactions. Following transfer to nitrocellulose, blots were blocked in 5% nonfat dried milk powder in PBS containing 0.1% Tween 20 (PBST), and then incubated in a 1/1000 dilution of HRP-labeled goat Ab to either human IgA1 or HUMBOVDS2 using a human IgA1 H chain expression vector, replacing the wild-type sequence in this region (25).

In each case, the derived plasmids were sequenced to confirm that the desired mutations had been incorporated and that no other base changes had occurred during the construction of the plasmids.
The cleavage of $\alpha$1$\alpha$2$\gamma$3, in which the C$\alpha$3 domain of human IgA had been replaced with the C$\gamma$3 domain of human IgG1, with the different bacterial IgA1 proteases was dependent on the type of protease used (see Table I). $\alpha$1$\alpha$2$\gamma$3 was resistant to cleavage with the type 1 and type 2 IgA1 proteases of $N$. gonorrhoeae, $N$. meningitidis, and $H$. influenzae, all of which cleave wild-type IgA1 at different positions in the C-terminal duplicated half of the hinge. By contrast, $\alpha$1$\alpha$2$\gamma$3 was cleaved by the IgA1 proteases of all of the strains of $S$. pneumoniae, Streptococcus oralis, Streptococcus sanguis, and Streptococcus mitis, which cleave wild-type IgA1 in the N-terminal half of the hinge (Fig. 4 and Table I).

The mutant $\alpha$1$\alpha$2$\gamma$3PLAF, in which four amino acids (residues His$^{433}$-Tyr$^{436}$) (Eu numbering) in an interdomain loop of the C$\gamma$3 domain of $\alpha$1$\alpha$2$\gamma$3 had been replaced with the corresponding residues from the C$\alpha$3 domain of human IgA1 (Pro$^{440}$-Phe$^{443}$) (Bur numbering), was found in general to be similar to $\alpha$1$\alpha$2$\gamma$3 in its sensitivity to cleavage with different IgA1 proteases (Fig. 4 and Table I). Thus, it was sensitive to cleavage with the IgA1 proteases of all the strains of $S$. pneumoniae, $S$. oralis, $S$. sanguis, and $S$. mitis (Fig. 4 and Table I), and resistant to cleavage with the type 1 and 2 IgA1 proteases of $N$. gonorrhoeae and $H$. influenzae, and the type 1 IgA1 protease of $N$. meningitidis (Fig. 4 and Table I). However, the type 2 IgA1 protease of $N$. meningitidis appeared to give partial cleavage of $\alpha$1$\alpha$2$\gamma$3PLAF but no cleavage of $\alpha$1$\alpha$2$\gamma$3 (Fig. 4).

These findings indicated that the sensitivity of human IgA1 to the type 1 and 2 IgA1 proteases of $N$. gonorrhoeae and $H$. influenzae and the type 1 IgA1 protease of $N$. meningitidis required not only the presence of the IgA1-specific hinge, but also sequences in the CH3 domain of IgA1 that could not be substituted by the CH3 domain of human IgG1. Furthermore, the sensitivity of IgA1 to cleavage by the type 2 IgA1 protease of $N$. meningitidis, unlike that of the other serine-type IgA1 proteases of Neisseria and Haemophilus, may be dependent on the presence of one or more of the amino acids in the Pro$^{440}$-Phe$^{443}$ (PLAF) interdomain loop of the C$\alpha$3 domain of human IgA1. In contrast, the IgA1 proteases of streptococcal species showed no requirement for the presence in human IgA1 of the C$\alpha$3 domain for cleavage to occur.

Analysis of the IgA1 mutants E254A, D255V, G259R, C311S, each with a single amino acid change in the C$\alpha$2 domain, and

![Table I. Sensitivity of different mutant Abs to bacterial IgA1 proteases](http://www.jimmunol.org/)

<table>
<thead>
<tr>
<th>Source of IgA1 Protease</th>
<th>PTerm455</th>
<th>TAHG-1</th>
<th>$\alpha$1$\alpha$2$\gamma$3</th>
<th>$\alpha$1$\alpha$2$\gamma$3PLAF</th>
<th>HuBova3</th>
<th>399-409GAM</th>
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<tr>
<td>$S$. pneumoniae SK690</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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</tr>
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<td>(+)</td>
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<td>+</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
<td>+</td>
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<td>$H$. influenzae type 1 H23 and R16</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>$H$. influenzae type 2 H15</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

+, Susceptible to cleavage by the protease; (+), partially sensitive to cleavage by the protease; 0, resistant to cleavage by the protease.

(α-Fab specific) (Kirkegaard & Perry Laboratories) or human IgG (γ-chain specific) (Sigma-Aldrich) in PBS for 2 h. After thorough washing, the membranes were developed in 10 ml of 50 mM Tris-HCl (pH 7.6) buffer containing 0.3 mg/ml nickel chloride, 10 mg diaminobenzidine, and 60 μl of 30% hydrogen peroxide. Some IgA1 molecules were heterogeneously glycosylated resulting in multiple H chain bands in immunoblots. Hence, some lanes contain Fab of more than one size, representing different glycoforms of the same cleavage product. The different IgA1 proteases cleave at a variety of previously defined sites in the hinge region. Those that cleave close to the CH1 domain generate Fab that run more quickly in SDS gels than those generated by IgA1 proteases that cleave closer to the Fc region, presumably in part as a result of the O-linked glycans that are attached to the intervening sequence. In addition, some Fab appear to be more readily detected by the anti-IgA Ab than others, possibly due to epitope loss upon protease treatment.

**Results**

When mutant PTerm455, which lacked the tailpiece, was incubated with the different bacterial IgA1 proteases, it was cleaved by all of them in a manner similar to that of wild-type IgA1 (see Fig. 3 and Table I). However, when mutant Ab TAHG-1, in which the hinge region of human IgA1 had been inserted into the corresponding position in human IgG1, was incubated with the different bacterial IgA1 proteases, it was found to be resistant to cleavage by all of them (Table I). Thus, the sensitivity of IgA1 to cleavage by the diverse bacterial IgA1 proteases was not dependent on the presence of the tailpiece in IgA1, but it was dependent on the presence of other elements in IgA1 (in addition to the hinge) that were not presented by IgG1.
FIGURE 4. Western blot analysis of the action of different IgA1 proteases on a1α2γ3 and a1α2γ3PLAF. After separation under reducing conditions, digests were probed with an anti-human α-chain-specific-HRP conjugate that bound to epitopes in the Fab region. a1α2γ3 (lanes 1–5) and a1α2γ3PLAF (lanes 6–10) were either untreated (lanes 1 and 6) or treated with the IgA1 protease of S. pneumoniae SK 690 (lanes 2 and 7), H. influenzae R 16 (type 1) (lanes 3 and 8), N. meningitidis HF13 (type 2) (lanes 4 and 9), or N. gonorrhoeae 6092 (type 1) (lanes 5 and 10). Both a1α2γ3 and a1α2γ3PLAF were cleaved by the IgA1 protease of S. pneumoniae but not by those of H. influenzae type 1 or N. gonorrhoeae type 1. a1α2γ3 was not cleaved by the type 2 IgA1 protease of N. meningitidis, whereas a1α2γ3PLAF was cleaved partially by this enzyme.

E437A and A442R, each with a point substitution in the Cα3 domain, showed that all remained sensitive to cleavage with the type 1 and 2 IgA1 proteases of N. meningitidis. N. gonorrhoeae, and H. influenzae (Table II). With the exception of the C311S mutation, all of these point substitutions lie at or near the Fc interdomain region, and hence are close to or, in the case of A442R, within the PLAF loop. Because this latter substitution produces a substantial change in the size and nature of the residue at position 442, it appears that the sensitivity of IgA1 to cleavage by the N. meningitidis type 2 protease depends on elements within the PLAF loop other than residue Ala442.

Domain-swap molecule HuBovα3, in which the Cα3 domain of human IgA1 was replaced by the Cα3 domain of bovine IgA, was cleaved by all the IgA1 proteases except for that of the type 1 enzyme of N. gonorrhoeae (Fig. 5 and Table I). This suggests that one or more of the 17 aa in the Cα3 domain of IgA1 that differ from those of the bovine IgA in the two positions (Fig. 2) may be required for the enzyme to cleave IgA1, and that this element(s) may not be required for cleavage by the other IgA1 proteases.

Mutant 399–409GAM, in which aa 399–409 of the Cα3 domain of human IgA1 were replaced with the equivalent region of the Cγ3 domain of human IgG1 (see Fig. 1), was cleaved by all the IgA1 proteases except for the type 2 enzyme of H. influenzae (Fig. 6 and Table I). This may indicate that one or more of the amino acids Ala399–Thr409 (see Fig. 2) in the CH3 domain of IgA1 are required for the activity of this enzyme but not for that of the other IgA1 proteases.

Table II. Sensitivity of IgA1 point mutants to cleavage by bacterial IgA1 proteases

<table>
<thead>
<tr>
<th>Source of IgA1 Protease</th>
<th>E254A</th>
<th>D255V</th>
<th>G259R</th>
<th>C311S</th>
<th>E437A</th>
<th>A442R</th>
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<td>S. pneumoniae SK690</td>
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<td>+</td>
<td>ND</td>
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</tr>
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</tr>
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</table>

* +, Susceptible to cleavage; ND, not determined.

Discussion

Despite recent advances (22, 29–31), not all the factors determining the sensitivity of human IgA1 to cleavage by bacterial IgA1 proteases are completely understood. The resistance of synthetic decapeptides with sequences mirroring parts of the human IgA hinge to cleavage by the type 2 IgA1 protease of N. gonorrhoeae (32) may be a consequence of the size of the artificial hinge being too small (29). Alternatively, or in addition, this resistance to cleavage may indicate that, as well as a requirement for a specific amino acid sequence of adequate size in the hinge, the presence of appropriate structural features in the IgA substrate outside the hinge are also necessary for cleavage by IgA1 proteases. Furthermore, sequential incubation of IgA1 with IgA1 proteases of different cleavage specificities revealed that remaining potential cleavage sites in the Fc fragments, but not those in Fab, could be further cleaved by a second IgA1 protease (33). This suggests that structural features in human IgA1 C-terminal to the cleavable peptide bond are particularly important in determining susceptibility to IgA1 protease activity. The purpose of the present study was to pinpoint regions within the Fc portion of the Ab capable of influencing the sensitivity of human IgA1 to cleavage by bacterial IgA1 proteases. Given the structural and mechanistic diversity of the IgA1 proteases and therefore the likely distinctions in substrate recognition, we chose to investigate this for a range of different IgA1 proteases.

The sensitivity of PTerm455, an IgA1 mutant devoid of the tailpiece, to cleavage with all of the IgA1 proteases indicated...
clearly that there was no requirement for the presence of the tailpiece in IgA1 for susceptibility to any of the IgA1 proteases. This was not unexpected because of the tailpiece’s location distal to the hinge and its relatively small size compared with the CH2 and CH3 domains.

An earlier study reported that a human IgA1-IgG2 domain swap Abs, featuring the Cc1, hinge, and Cc2 domain of IgA1 and the Cc3 domain of IgG2, was resistant to cleavage by the type 1 IgA1 protease of \( H. \) influenzae and the type 2 IgA1 protease of \( N. \) gonorrhoeae (22). In the present study, the observed resistance to cleavage by these enzymes of \( \alpha_1 \alpha_2 \gamma_3 \), a human IgA1-IgG1 domain swap, was consistent with this finding. In addition, we demonstrated that this molecule was also resistant to the type 1 IgA1 proteases of \( N. \) gonorrhoeae and \( N. \) meningitidis and the type 2 enzymes of \( H. \) influenzae and \( N. \) meningitidis. All of these IgA1 proteases cleave human IgA1 in the C-terminal half of the hinge. It would appear therefore that the ability of the serine IgA1 proteases of \( N. \) gonorrhoeae and \( H. \) influenzae to cleave IgA1 is dependent on the presence of particular amino acids or structures present in the CH3 domain of human IgA1 but not in the CH3 domain of human IgG. By contrast, all the metallo-IgA1 proteases of the different streptococci, which cleave wild-type IgA1 at the Pro227-Thr228 peptide bond in the N-terminal half of the IgA1 hinge, were able to cleave \( \alpha_1 \alpha_2 \gamma_3 \). Thus, either elements within the CH3 domain of human IgA1 are not required for cleavage of human IgA1 by the streptococcal IgA1 proteases, or structures contained within the Cγ3 domain can provide an adequate substitute for any requirements of this sort.

The reason for the resistance of \( \alpha_1 \alpha_2 \gamma_3 \) to cleavage by the IgA1 proteases of \( N. \) meningitidis and \( N. \) gonorrhoeae was examined further by determining their activity on the \( \alpha_1 \alpha_2 \gamma_3 \)PLAF mutant in which the PLAF loop from the Cc3 domain replaced residues His333, Asn334, His335, Tyr336 at the equivalent position in the Cγ3 domain of \( \alpha_1 \alpha_2 \gamma_3 \) (25). With one exception, all of the IgA1 proteases that failed to cleave \( \alpha_1 \alpha_2 \gamma_3 \) also failed to cleave \( \alpha_1 \alpha_2 \gamma_3 \)PLAF (Table 1). Thus, the PLAF loop at the interface of the Cc2 and Cc3 domains of IgA1 is not involved in interaction of the serine IgA1 proteases of \( N. \) meningitidis and \( N. \) gonorrhoeae with IgA1. The exception was the type 2 IgA1 protease of \( N. \) meningitidis, which appeared to partially cleave \( \alpha_1 \alpha_2 \gamma_3 \)PLAF. This finding suggests that, for this particular protease, the PLAF residues contribute in some way toward optimal binding of the enzyme to IgA1 substrates, thereby facilitating appropriate orientation or position to permit cleavage in the hinge region. The lack of impact of the A442R point mutation on IgA1 protease susceptibility indicates that residues Pro440, Leu441, and Phe443 probably play the major role in this respect. The PLAF loop, lying at the domain interface (Fig. 7), forms an important interaction surface on IgA1 Fc, having been previously shown to be critical for binding of not only FcoRI (CD89) (25, 28, 34, 35) but also streptococcal IgA-binding proteins (36) and staphylococcal toxin SSL7 (37). Its equivalent region on IgG is also involved in interaction with a variety of molecules and has been recognized as one of a limited number of regions on the Ig surface that is particularly suited to protein-protein interaction (38, 39). The loop is readily accessible and Fig. 7 illustrates how it might form an “anchor point” for the \( N. \) meningitidis type 2 IgA1 protease, assisting correct orientation to allow cleavage of the hinge above.

In an attempt to further define regions in the human IgA1 Cc3 domain critical for substrate recognition and cleavage by one or more of the IgA1 proteases of \( N. \) meningitidis, \( N. \) gonorrhoeae, and \( H. \) influenzae, we generated mutant 399–409GAM. This mutant is identical with human IgA1 except that residues 399–409 in the Cc3 domain have been replaced with the corresponding residues from human IgG1. The finding that the \( N. \) gonorrhoeae type 1 enzyme could partially cleave 399–409GAM but not HuBov3 suggests that this enzyme requires elements in the human Cc3 domain outside the 399–409 region that are present in human IgA1 but lacking in bovine IgA (see Fig. 2). Further experimentation will be required to define these elements more closely.

The finding that the type 2 IgA1 protease of \( H. \) influenzae failed to cleave 399–409GAM suggests that residues 399–409 (sequence Ala399-Ser400-Arg401-Gln402-Glu403-Pro404-Ser405-Gln406, Gly407-Thr408-Thr409) are necessary to allow cleavage by this enzyme. The Ab HuBov3 was cleaved by this protease and the only amino acids common to wild-type IgA1 and HuBov3 in this loop are Glu403, Gln406, and Thr409 (Fig. 2). These findings together may indicate that one or more of these amino acids or the entire
399–409 segment is required for the type 2 protease of *H. influenzae* to act. The 399–409 region lies within the upper central part of the Cα3 domain (Fig. 7), and in a side view of the Fc (Fig. 7B) is seen partially to protrude out from the surface of the domain. It is tempting to speculate that the *H. influenzae* type 2 protease may use this accessible protrusion as a means to stabilize its interaction with IgA1 and facilitate correct orientation for optimal cleavage of the Pro$^{399}$-Thr$^{409}$ peptide bond lying above it (see Fig. 7B). Interestingly, the same region on the Cα3 domain has been implicated in binding to the polymeric Ig receptor (40, 41). Taken together with the PLAF loop finding above, it appears that certain IgA1 proteases may take advantage of regions on the Fc region of IgA1 suited to protein-protein interaction that have been conserved because they form key interaction surfaces for host receptors.

In regard to the above conclusions, some possible caveats should be borne in mind. First, it is feasible that differential glycosylation profiles might impact on the susceptibility of substrates should be borne in mind. First, it is feasible that differential glycosylation patterns might influence susceptibility to cleavage by the streptococcal metallo-enzyme IgA1 proteases (30, 42). However, hinge glycosylation can influence susceptibility to cleavage by the lysosomal/phagosomal membrane protein h-lamp-1 is a target of the IgA1 protease of *Neisseria gonorrhoeae*. FEBS Lett. 405: 86–90. We do not consider it to be very likely, because our preliminary experiments on cleavage of wild-type IgA1 by IgA1 proteases from different strains have never revealed any interstrain differences.

An exciting aspect of our findings is that they point toward new possibilities for the design of both IgA1 protease inhibitors and therapeutic Abs. Before this study, peptide inhibitors modeled on the human Fc and its implications. J. Infect. Dis. 200: 143–149. Thus, we have revealed the possibility of engineering IgA1 molecules resistant to an IgA1 protease by virtue of mutations outside the hinge region. Therefore, that our conclusions may relate to IgA1 proteases from specific strains only. Although we cannot formally rule out this possibility, we do not consider it to be very likely, because our preliminary experiments on cleavage of wild-type IgA1 by IgA1 proteases from different strains have never revealed any interstrain differences.

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Disclosures

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References


37. Wines, B. D., N. Willoughby, J. D. Fraser, and P. M. Hogarth. 2006. A competitive mechanism for staphylococcal toxin SSL7 inhibiting the leukocyte IgA receptor, FcαRI, is revealed by SSL7 binding at the Cα2/Cα3 interface of IgA. *J. Biol. Chem.* 281: 1389–1393.


