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The Influences of Hinge Length and Composition on the Susceptibility of Human IgA to Cleavage by Diverse Bacterial IgA1 Proteases

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The influences of IgA hinge length and composition on its susceptibility to cleavage by bacterial IgA1 proteases were examined using a panel of IgA hinge mutants. The IgA1 proteases of *Streptococcus pneumoniae*, *Streptococcus sanguis* strains SK4 and SK49, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* cleaved IgA2-IgA1 half hinge, an Ab featuring half of the IgA1 hinge incorporated into the equivalent site in IgA1 protease-resistant IgA2, whereas those of *Streptococcus mitis*, *Streptococcus oralis*, and *S. sanguis* strain SK1 did not. Hinge length reduction by removal of two of the four C-terminal proline residues rendered IgA2-IgA1 half hinge resistant to all streptococcal IgA1 metalloproteinases but it remained sensitive to cleavage by the serine-type IgA1 proteases of *Neisseria* and *Haemophilus* spp. The four C-terminal proline residues could be substituted by alanine residues or transferred to the N-terminal extremity of the hinge without affect on the susceptibility of the Ab to cleavage by serine-type IgA1 proteases. However, their removal rendered the Ab resistant to cleavage by all the IgA1 proteases. We conclude that the serine-type IgA1 proteases of *Neisseria* and *Haemophilus* require the Fab and Fc regions to be separated by at least ten (or in the case of *N. gonorrhoeae* type I protease, nine) amino acids between Val222 and Cys241 (IgA1 numbering) for efficient access and cleavage. By contrast, the streptococcal IgA1 metalloproteinases require 12 or more appropriate amino acids between the Fab and Fc to maintain a minimum critical distance between the scissile bond and the start of the Fc. *The Journal of Immunology*, 2005, 174: 7792–7799.

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ome important pathogenic bacteria like, for example, the respiratory tract pathogens *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* and the genital tract pathogens *Neisseria gonorrhoeae* and *Ureaplasma urealyticum*, have the ability to initiate infection at mucosal surfaces of the body. This process is thought to be facilitated by the secretion of proteolytic enzymes, termed IgA1 proteases (see reviews Refs. 1–3), which specifically cleave the protective IgA1 in the hinge of the αH chain to generate Fab and Fc fragments. As a consequence, these invading pathogens escape IgA1-mediated agglutination and elimination mechanisms. Furthermore, epitopes on the pathogens are masked by the Fabs formed, thereby masking recognition of epitopes by other intact Igs (4, 5).

Additional evidence that IgA1 proteases are involved in virulence comes from observations that the amount of enzyme secreted is directly related to the pathogenicity of the isolate (6); strains of related species that do not produce IgA1 protease are nonpathogenic (7, 8); the products of IgA1 cleavage are detectable in the CSF, vaginal washings, and other body fluids of patients infected with an IgA1 protease-producing strain (9–11); and Ab to the IgA1 protease is found in convalescing patients recovering from infection with an IgA1 protease-producing pathogen (12–14). However, because the substrate for IgA1 proteases is restricted almost exclusively (15–17) to IgA1 from human, gorilla, chimpanzee, and the IgA of the orangutan (18), it is difficult to prove that IgA1 proteases are virulence factors in vivo. However, IgA1 protease has recently been shown to give in vivo and in vitro protection against IgA-mediated killing of *S. pneumoniae* (19). Moreover, IgA1 specific for *S. pneumoniae* has been shown to lose its protective effects when cleaved by IgA1 protease, with the resultant Fab actually enhancing adherence of pneumococci to host cells (20).

Although the IgA1 proteases produced by different bacteria belong to widely different families, namely, serine-, metallo-, and thiol-proteases, they all cleave human IgA1 only in the hinge. The IgA1 hinge comprises a duplication of a sequence of eight amino acids rich in proline, threonine, and serine (see Fig. 1). It is absent from human IgA2, which is thereby resistant to cleavage by the proteases. The IgA1 proteases are all prosynthetic endopeptidases and cleave at either a Pro-Ser (type 1 enzyme) or a Pro-Thr (type 2 enzyme) peptide bond. However, the enzymes are extremely specific in that the enzyme of a given bacterium cleaves a specific peptide bond in only one of the duplicated sequences of amino acids and not at the equivalent site in the other duplicate.

To understand more about the features of human IgA influencing such proteolytic cleavage, and to begin assessing the feasibility of preparing specific inhibitors for the IgA1 proteases, this study sought to examine the influence of variations in the structure and size of the IgA hinge on its sensitivity to cleavage. A series of IgA Abs with defined hinge mutations were constructed and tested for their susceptibility to cleavage by different IgA1 proteases.

Materials and Methods

Primers

Primers A1H6 and A2SEQ2, which anneal upstream of the CH1 exon and within the CH2 exon, respectively, of human IgA2m(1) have been previously described (21). Primer 4PDEL5 (5′-TGCTGCCACCCCCGACTGTCGGTGCAC-3′) contained the nucleotide sequence 701–727 of the CH2 exon of human IgA2m(1). Primer 4PDELAS (5′-AGATGGGGTAG-3′) corresponded to the sequence AGATGGG-3′, 5′-AGATGGGGTAG-3′...
that of part of the hinge of human IgA1 (in italics). Primer 4PRONS was contained the sequence of nucleotides 671–700 of human IgA2m(1) and (760 bp) comprising the 5′-BamHI fragment ligated into pBS2, replacing the original formed by inverted PCR (23), which was phosphorylated and self-ligated.

The Ab expression vectors constructed, the nomenclature of the Abs they formed hinge region of IgA1 was incorporated into the equivalent

Complementary to primer PTPSHINGE1. Primer A1H5 (5′-CACTCCAGTTCCCACCTCCCCCA-3′) contained the complement of primer 2PROADDS. Primer 4PRONS (5′-CTCCACCCCATCTCA-3′) contained the sequence of nucleotides 688–683 at the start of the CH2 exon of human IgA2m(1). Primer DELREP2 extended by three bases at the 5′ end. Primer DELREP1 (5′-TACCCCATCTGCTGCCGCTGGTAG-3′) contained the nucleotide sequence of part of the hinge of human IgA1 (in italics), and the nucleotide sequence 679–708 of the CH2 exon of human IgA2m(1) in which some C nucleotides had been substituted with G nucleotide (in bold) to code for alanine instead of proline. Primer 4ALREPS was the complement of primer 4ALREPS.

Primer 2PROADDS (5′-CACTCCCCCATCTCAACCATCGTGCCA-CCCGCA-3′) contained the nucleotide sequence of part of the hinge of human IgA1 (in italics) and that of nucleotides 689–694 (underlined) and 701–715 of the CH2 exon of human IgA2m(1). Primer 2PROADDS was the complement of primer 2PROADDS. Primer 4PRONS (5′-CTCTCGACCTGATTCCTCCTCCTTACCCCATCTCA-3′) contained the sequence of nucleotides 671–700 of human IgA1 (in italics) and that of part of the hinge of human IgA1 (in italics). Primer 4PRONS was the complement of primer 4PRONS. Primer PTPSHINGE1 (5′-CTCTC CACCTGATTCCTCCTCCTTACCCCATCTCA-3′) contained the sequence of nucleotides (in bold) coding for Pro, Thr, Ser, and Cys. Primer 4PRONAS (5′-CTTGTTCCCTCCTCCTCCTTACCCCATCTCA-3′) contained the complement of primer 2PROADDS. Primer 4PRONS and 4PRONAS as internal primers. Plasmids pBS13 and pBS15 were constructed in a similar way with the same flanking primers and 4ALREPS and 4ALREPAS as internal primers. Plasmids pBS13 and pBS15 were constructed in a similar way with the same flanking primers and with pBS5 DNA as template, and 2PROADDS and 2PROADAS as internal primers for pBS15, and 4PRONS and 4PRONAS as internal primers for pBS15. In each case, the ~915 bp fragment ligated into pBS2, replacing the original BanHI-Xhol fragment. Sequence analysis revealed that accidental deletion of an additional base had occurred 3′ to the intended deletion. This error was repaired through a further round of overlap PCR using primers DELREP1 and DELREP2 as internal primers and AIH6 and AIH5 as flanking primers and the same BanHI and Xhol sites for insertion of the corrected segment into the expression vector to generate pBS5.

Plasmid pBS14 was constructed by PCR overlap extension mutagenesis (24) using pBS2 as template and AIH6 and A2SEQ2 as flanking primers and 4ALREPS and 4ALREPAS as internal primers. Plasmids pBS13 and pBS15 were constructed in a similar way with the same flanking primers but with pBS5 DNA as template, and 2PROADDS and 2PROADAS as internal primers for pBS15, and 4PRONS and 4PRONAS as internal primers for pBS15. In each case, the ~915 bp fragment formed, which contained a mutated form of half the IgA hinge region, was cleaved with BanHI and Xhol and the product was ligated into the BanHI-Xhol cleaved site of the original IgA2m(1) expression vector (25), replacing the wild-type sequence in this region.

The Ab expression vector pBS26 was also constructed by PCR overlap extension mutagenesis with plasmid pBS1 (pSP73 containing a BanHI-Xhol fragment of the 5′ end of the human IgA2m(1) gene in its multiple cloning site) as template DNA and AIH6 and AIH5 as flanking primers and PTPSHINGE1 and PTPSHINGE2 as internal primers. The product of ~1100 bp was cleaved with BanHI and Xhol, and the ~760 bp product was ligated into the BanHI- and Xhol-cleaved site of the original IgA2m(1) expression vector (25), replacing the wild-type sequence in this region.

For all the constructed expression vectors, sequencing by an ABI 377 DNA sequencer confirmed that the base sequence around the half hinge of IgA1 had been modified as intended and that no PCR-generated errors had occurred in other coding regions.

Preparation of recombinant mutant Igs

Chinese hamster ovary CHO-K1 cells stably transfected previously with an appropriate mouse λ chain (25) were seeded in tissue culture-grade petri dishes and transfected with a constructed α chain expression vector using calcium phosphate as previously described (25). Positive transfectants were isolated by selection for the β-galactosidase and puromycin phosphoribosyltransferase selectable marker by growth in medium supplemented with hypoxanthine and thymidine (HT supplement; Invitrogen Life Technologies), xanthine (0.25 mg/ml), and mycophenolic acid (10 μg/ml). Several resistant colonies were picked, and cell lines producing the highest yields of IgA were identified by an ELISA measuring binding to the Ag NIP (3-nitro-5-hydroxy-4-isopropylphenylacetate) as previously described (25) before expansion into large cultures. Recombinant Abs were purified from the supernatants of the CHO-K1 transfected cultures by affinity chromatography on NIP-Sepharose as previously described (25). The purified Abs were supplemented with 0.1% sodium azide and stored in small aliquots at −20°C.

Microbial IgA1 proteases

The IgA1 proteases used were from: S. pneumoniae strain SK590; Streptococcus oralis strain SK10; Streptococcus sanguis strains SK1 (American Type Culture Collection 10556) (biovar 1), SK4 (biovar 2), and SK49 (biovar 4); Streptococcus mitis biovar 1 strains SK564, SK597, and SK599; N. meningitidis group B serotype 14 strain 3564 (type 1 enzyme) and group Y serotype 2c strain HF 13 (type 2 enzyme); N. gonorrhoeae serogroup W1 serovar 1A-2 strain 6092 (type 1 enzyme) and serogroup W1/III serovar 1B-6 strain 5489 (type 2 enzyme); and Haemophilus influenzae strains H23 (type 1 enzyme) and H15 (type 2 enzyme).

The streptococcal strains were cultured in 2TY broth (tryptone 1.6%, yeast extract 1%, sodium chloride 0.5% in distilled water, pH 7) at 37°C in air containing 5% CO2 and their IgA1 proteases concentrated and purified from the culture supernatants by fractional ammonium sulfate precipitation and subsequent dialysis against PBS (pH 7.2) containing 0.1% sodium azide. The other bacteria were cultured at 37°C in air containing 5% CO2 on dialysis tubing membranes on the surface of appropriate solid culture media as previously described (26) and their IgA1 proteases prepared similarly from the supernatants of the suspensions of the bacteria washed from the dialysis tubing membranes with PBS containing 0.1% sodium azide.

Digestion of rIgs with microbial IgA1 proteases and immunoblotting

Appropriate amounts of Ab and IgA1 protease in PBS (pH 7.2) containing 0.1% sodium azide in a final volume of 20 μl were incubated at 37°C for 30 min in 5% CO2 on dialysis tubing membranes on the surface of appropriate solid culture media as previously described (26) and their IgA1 proteases prepared similarly from the supernatants of the suspensions of the bacteria washed from the dialysis tubing membranes with PBS containing 0.1% sodium azide.

Results

Previous experiments showed that all the different bacterial IgA1 proteases cleaved recombinant wild-type human IgA1 to give fragments of mass consistent with cleavage in the hinge (22, 27). However, recombinant human IgA2 was resistant to cleavage with these enzymes. These findings indicated that the only proteolytic activity in the different enzyme preparations was that of an IgA1 protease.

A series of mutants were produced based on a hybrid IgA2/IgA1 half hinge Ab in which a sequence representing half of the duplicated hinge region of IgA1 was incorporated into the equivalent position in IgA2 (Fig. 1). IgA2-IgA1 half hinge was cleaved by the different IgA1 proteases of all the streptococcal strains except for those of S. oralis strain SK10, S. sanguis strain SK1, and S. mitis strains SK564, SK597, and SK599 (Fig. 2), which also failed to cleave the Abs hh4ProC, hh4ProC, hh4AlaC,
hhProN, and hhSTP derived from IgA2-IgA1 half hinge (Fig. 3 and Table I). The IgA1 proteases of the other streptococcal strains that cleaved the IgA2-IgA1 half hinge Ab were also unable to cleave Abs hhΔ4ProC, hhΔ2ProC, hh4ProN, and hhSTP but most gave partial cleavage of the hh4AlaC Ab. The results of incubation of some of these Abs with the protease of *S. pneumoniae* are shown in Fig. 4 as an example.

By contrast, the type 1 and type 2 IgA1 proteases from *H. influenzae, N. meningitidis*, and *N. gonorrhoeae* were much more active than the streptococcal IgA1 proteases on IgA2-IgA1 half hinge and most of the Abs derived from it. All these IgA1 proteases cleaved IgA2-IgA1 half hinge, hh4AlaC, hh4ProN, and hhΔ2ProC. However, hhΔ4ProC was resistant to cleavage by all the IgA1 proteases. The results of incubation of some of the Abs with the type 2 enzyme are shown in Fig. 5 as an example. Thus the effect of removing the four proline residues at the C terminus of the IgA1 half hinge, as in hhΔ4ProC, was to create an Ab that was resistant to all the proteases of all the strains tested. If only two of the proline residues were removed as in hhΔ2ProC, the Ab was resistant to cleavage with the streptococcal IgA1 proteases but remained sensitive to cleavage with the type 1 and 2 IgA1 proteases of *N. meningitidis, N. gonorrhoeae*, and *H. influenzae* (Fig. 6).

Replacement of the four proline residues at the C terminus of the IgA1 half hinge with alanine residues as in hh4AlaC, or their transfer to the N terminus of the IgA1 half hinge as in hh4ProN, resulted in the creation of Abs that were fully sensitive to the type 1 and type 2 IgA1 proteases of *N. meningitidis, N. gonorrhoeae*, and *H. influenzae* (Fig. 5 and Table I). Indeed, hh4ProN appeared as sensitive, if not more sensitive, to some of these IgA1 proteases than IgA2-IgA1 half hinge from which it was derived (Fig. 7 and Table I).

hhSTP in which the hinge region, taken to be the region lying between Val 222 and Cys 241 (wild-type IgA1 numbering), was shortened to only nine amino acids and was resistant to cleavage by all of the IgA1 proteases except for that of *N. gonorrhoeae* type 1 (Fig. 8 and Table I). Thus for this enzyme, a mutation that effectively represents the insertion of just four amino acids (Pro-Thr-Pro-Ser) into the hinge region of IgA2 is sufficient to render a previously resistant Ab sensitive to cleavage.
**Discussion**

A necessary requirement, although not the only one (28), for IgA sensitivity to cleavage by an IgA1 protease is the presence of a suitable amino acid sequence in the hinge. The only known IgA molecules to contain such a hinge sequence are the IgA1 isotypes of human, chimpanzee, and gorilla and the IgA of the orangutan (18). The hinge regions (lying between Val222 and Cys241 in human IgA1 or their equivalents) of these IgA1 protease-susceptible substrates are 16–18 aa long and are very similar in amino acid sequence, being particularly rich in proline, serine, and threonine (Table II). The hinge regions of the IgA2 isotypes in these species are much shorter being only five amino acids long between the equivalents of Val222 and Cys241 (human IgA1 numbering). These IgA1 protease-resistant IgA2 Abs share very similar amino acid sequences in the hinge, which are quite different from those of their IgA1 counterparts (see Table II).

IgA1 proteases normally cleave either a Pro-Ser peptide bond (type 1 enzymes) or a Pro-Thr peptide bond (type 2 enzymes). Although there are several representatives of such peptide bonds in the hinges of susceptible IgA, and the human and gorilla IgA1 hinges contain a tandem repeat of eight amino acids, each IgA1 protease exhibits a remarkable degree of selectivity for the particular peptide bond it preferentially cleaves in the wild-type Ab (Fig. 1).

In previous work (22) we created a hybrid Ab termed IgA2-IgA1 half hinge in which one of the eight residue repeats of the human IgA1 hinge was engineered into the hinge of IgA1 protease-resistant human IgA2. This hybrid Ab remained susceptible to nearly all of the different bacterial IgA1 proteases including those that cleave wild-type human IgA1 in the different duplicated halves of the hinge. This finding indicated that although the IgA1 proteases cleaved wild-type IgA1 at a specific peptide bond in only one of the duplicated half hinge regions, if the specific peptide bond is represented only once as in the hybrid Ab, most the enzymes were still able to cleave it. Thus for most IgA1 proteases,
half of the normal human IgA hinge was sufficient for recognition and cleavage by the proteases, and that if additional distal elements were required, the IgA2 framework represented an acceptable alternative to that of IgA1.

Further work with different mutants of the hybrid Ab has shown that IgA1 proteases can, if necessary, cleave alternative peptide bonds to the ones normally cleaved. Thus half hinge mutants lacking a Pro-Ser peptide bond can be cleaved by type 1 IgA1 proteases and those lacking a Pro-Thr peptide bond can be cleaved by type 2 IgA1 proteases (21). Furthermore, the substitution of proline for serine at the C-terminal end of the half hinge, thereby creating a contiguous sequence of six proline residues, caused a significant increase in the resistance of the Ab to cleavage despite the presence in the hinge of potentially cleavable Pro-Ser and Pro-Thr peptide bonds (21).

In the present study we aimed to determine the minimum size requirements of the hinge necessary to permit IgA1 protease cleavage. We also sought to determine the influence on protease sensitivity of the distance between susceptible peptide bonds in the hinge and the Fc and Fab regions of the Ab, through mutation and repositioning of up to four proline residues at the different ends of the hinge.

All the IgA1 protease preparations cleaved wild-type recombinant human IgA1 to yield Fab and Fc fragments whereas recombinant human IgA2, which lacks the hinge of IgA1, was resistant to cleavage. This confirmed that all the proteolytic activity in the enzyme preparations was that of the IgA1 protease and arose through cleavage of the hinge of IgA1. Although the precise site of cleavage by each IgA1 protease in the hinges of the different mutant Abs was not determined, the masses of the cleavage products formed were consistent with cleavage occurring only in the hinge.

**Cleavage by streptococcal IgA1 proteases**

Despite all the IgA1 proteases of the different streptococcal species being zinc metalloproteases and cleaving the same peptide bond in human IgA1 (29–32), they showed different activities on the IgA2-IgA1 half hinge Ab. Supporting and extending our earlier observations (22), we found that the IgA1 proteases of *S. sanguis*, and some of the strains of *S. sanguis*, cleaved the hybrid whereas those of *S. oralis*, *S. mitis* biovar 1 strains, and *S. sanguis* strain SK1 did not. The failure of the latter group to cleave the hybrid Ab (and its derivatives) may indicate that these enzymes require structures outside the half hinge for substrate recognition and that the IgA2 framework does not serve as an acceptable alternative to IgA1 for provision of these elements.

The finding that the IgA1 protease of *S. sanguis* strain SK1, unlike that of the other *S. sanguis* strains tested, failed to cleave the IgA2-IgA1 half hinge Ab may indicate that the C terminus of the protease plays some role in the recognition or cleavage because

**Table II. IgA1 protease sensitivity of certain mammalian IgA Abs**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Hinge Region Sequence</th>
<th>Susceptibility to IgA1 Proteases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IgA1</td>
<td>VPSTPPTPSPSTPPTPSPS</td>
<td>S</td>
</tr>
<tr>
<td>Chimpanzee IgA1</td>
<td>GSPTPCPTPSTPSTPSKPS</td>
<td>S</td>
</tr>
<tr>
<td>Gorilla IgA1</td>
<td>VPSTPPTPSPSTPPTPSPS</td>
<td>S</td>
</tr>
<tr>
<td>Orangutan IgA</td>
<td>VPPPTTPTPSTPSPS</td>
<td>S</td>
</tr>
<tr>
<td>Human IgA2</td>
<td>VPPPPP</td>
<td>R</td>
</tr>
<tr>
<td>Chimpanzee IgA2</td>
<td>VPPPPP</td>
<td>R</td>
</tr>
<tr>
<td>Gorilla IgA2</td>
<td>VPPPPP</td>
<td>R</td>
</tr>
</tbody>
</table>

*The amino acid sequence of the hinges of some IgA1 protease sensitive (S) and resistant (R) mammalian IgA molecules. Sequences are taken from the translations of nucleic acid sequences using the following accession numbers: J00220 (human IgA1), J00221 (human IgA2), X3702 (chimpanzee IgA1), X3706 (chimpanzee IgA2), X15045 (gorilla IgA1), X3707 (gorilla IgA2), and X53704 (orangutan IgA). Sequences shown begin at the first residue encoded by the hinge-Cys2 exon (Val272 in human IgA1) and end at the residue preceding the first Cys residue encoded by this exon (Cys291 in human IgA1), except in orangutan IgA, which because of an extra Cys residue within the hinge, the sequence ends at the residue preceding the second Cys residue of this exon.*
this region of protease of the S. sanguis strain SK1 is different from the regions of the other streptococcal species, which share marked similarities (32). It is also interesting to note that the amino acid sequence in the N-terminal third of streptococcal IgA1 proteases, unlike the remainder of the enzyme, is very variable. It contains many repeat sequences that vary in number, size, and nucleotide sequence. These repeats are not essential for enzyme activity but are thought to contribute to antigenic diversity (33). The IgA1 proteases of S. mitis strains are known to display extensive antigenic variation but overall are more closely related to the IgA1 proteases of S. oralis than to those of the other streptococci (32).

Thus it is plausible that the S. mitis and S. oralis IgA1 proteases interact with the substrate IgA2-IgA1 half hinge Ab in a different way than do the proteases of the other streptococcal species and are, thereby, unable to cleave it. For these reasons, it was not particularly surprising to find that the IgA1 proteases of those streptococcal species and strains unable to cleave the IgA2-IgA1 half hinge hybrid Ab (S. oralis, S. sanguis SK1, and S. mitis biovar 1) were also unable to cleave hinges of the same or shorter size present in hh4ProC, hh2ΔProC, hh4AlaC, hh4ProN, and hhΔSTP, which all derived from the half-hinge hybrid (see Table I).

Although the IgA1 proteases of S. pneumoniae and the S. sanguis SK4 and SK49 strains were able to cleave the IgA2-IgA1 half hinge hybrid, none of them were able to cleave hh4ΔProC or hh2ΔProC, which lacked four and two proline residues, respectively, from the C-terminal end of the hinge region. The resistance of the hh4ΔProC and hh2ΔProC Abs to these proteases might be explained on the basis that in these Abs the hinge region was either too small or conformationally constrained to allow optimal access by the protease, or in some other way the activity of the protease on the hinge region was restricted. However, when the size of the hinge was restored to approximately the same as that in the IgA2-IgA1 half hinge Ab by the engineering of four alanine residues at the C terminus, as in hh4AlaC, the proteases of S. pneumoniae and the S. sanguis SK4 could gain access and the hinge was cleaved. Thus for cleavage by these two enzymes, it appears that there is a requirement for some form of “spacer” between the scissile bond and the Fc region of the Ab. If we assume that these enzymes cleave at their preferred Pro-Thr bond in the available hinge region, the requirement would seem to be for a spacer of at least six amino acids between this Thr and Cys\textsuperscript{241} at the start of the Fc. The size of the spacer appears to be more important than its precise sequence because alanine residues are able to substitute for proline residues. Available evidence (34) indicates that residue Cys\textsuperscript{241} (considered the first residue of the CH2 domain in our study) on one H chain forms a disulfide bridge to its counterpart on the other H chain, and therefore represents a point of close approach between the two H chains. One might speculate that this interchain tether will tend to restrict movement of the two chains relative to each other immediately upstream. Thus, in hh4ΔProC and hh2ΔProC, it is likely that access to the scissile bond in the hinge region of one H chain may be sterically obstructed by the other H chain or by other parts of the Fc region. Only when sufficient residues separate the cleavable portions of the hinge region from Cys\textsuperscript{241} can the scissile bonds, or the hinge as a whole, adopt suitable conformations and/or become suitably accessible for cleavage to occur. Interestingly, introduction of additional residues at the N-terminal end of the half hinge region, as in hh4ProN, did not help to overcome the restriction placed on cleavage by the loss of four residues C-terminal to the hinge region because none of the streptococcal proteases could cleave this mutant Ab (Table I).

Cleavage by IgA1 proteases of Haemophilus and Neisseria

In contrast to the streptococcal IgA1 proteases, those from H. influenzae, N. meningitidis, and N. gonorrhoeae were much more active on the IgA2-IgA1 half hinge Ab and on many of the Abs it derived. This activity may be a consequence of the nature of the enzymes because although streptococcal IgA1 proteases are metalloproteases (31, 32), those of Haemophilus and Neisseria are serine proteases, which have smaller active sites (35).

The sensitivity of the IgA2-IgA1 half hinge Ab to the type 1 and type 2 IgA1 proteases of N. meningitidis, N. gonorrhoeae, and H. influenzae was dramatically altered to resistance to all these IgA1 proteases by the removal of the four proline residues immediately C-terminal to the half hinge as in hhΔProC. However, the sensitivity of the IgA1 mutants derived from the IgA2-IgA1 half hinge Ab to cleavage by these IgA1 proteases was dependent neither on the presence specifically of these four proline residues, nor their position, because when the prolines were substituted by four alanine residues as in hh4AlaC, or when the four proline residues were moved to a position at the N-terminal end of the hinge as in hh4ProN, the resultant Abs were sensitive to cleavage with these IgA1 proteases. It appeared therefore that the four proline residues acted as a spacer, in this case, to separate the CH1 of the Fab region from the CH2 domain of the Fc region and permit successful access of the serine-type IgA1 proteases of Neisseria and Haemophilus and interaction with the hinge substrate. Unlike the streptococcal IgA1 proteases, the requirement for such a spacer by the proteases of Haemophilus and Neisseria spp. does not appear to have a strict positional constraint, as it can function on either the N-terminal or C-terminal side of the half hinge. The finding that hh2ΔProC was also sensitive to the type 1 and type 2 IgA1 proteases of N. meningitidis, N. gonorrhoeae, and H. influenzae suggests that even a short spacer is sufficient to allow access and cleavage by these serine-type IgA1 proteases. Thus a total of 10 or more residues between Val\textsuperscript{222} and Cys\textsuperscript{241} (IgA1 numbering), serving to separate the globular domains of the Fab and Fc regions, appears to be necessary for this group of enzymes to recognize and act upon a hinge region.

The recombinant Ab with the shortest hinge, hhΔSTP, contained an insertion of just four amino acids, Pro-Thr-Pro-Ser, into the hinge position in IgA2. As was expected, it was resistant to cleavage by all the streptococcal IgA1 proteases that had been unable to cleave hhΔProC, which had a slightly longer hinge. Of all the IgA1 proteases that cleaved hhΔProC, only that of N. gonorrhoeae type 1 was also able to cleave the even shorter hinge of Ab hhΔSTP. Thus for this enzyme, a separation of just nine amino acids between Val\textsuperscript{222} and Cys\textsuperscript{241} (IgA1 numbering) is sufficient to facilitate access and allow cleavage of an appropriately placed hinge region scissile bond.

It is not understood why the related type 1 IgA1 protease of N. meningitidis, which cleaves the same Pro-Ser peptide bond in wild-type human IgA1 as the N. gonorrhoeae type 1 enzyme, failed to cleave hhΔSTP. Perhaps the distance separating the Fab and Fc is simply too short to allow access, to allow appropriate orientation for interaction, or both.

It was interesting to note that those IgA1 proteases that cleave toward the C-terminal end of the hinge region in wild-type IgA1 were the most able to cleave those mutant half hinge Abs with very short hinges. This would appear to be in keeping with the proposal that amino acid sequences or structures outside the hinge and C-terminal to it may also be influential in determining the sensitivity of the molecule to cleavage by IgA1 proteases (28).
Disclosures

The authors have no financial conflict of interest.

References


