Cleavage of a Recombinant Human Immunoglobulin A2 (IgA2)-IgA1 Hybrid Antibody by Certain Bacterial IgA1 Proteases

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To understand more about the factors influencing the cleavage of immunoglobulin A1 (IgA1) by microbial IgA1 proteases, a recombinant human IgA2/IgA1 hybrid molecule was generated. In the hybrid, termed IgA2/A1 half hinge, a seven-amino-acid sequence corresponding to one half of the duplicated sequence making up the IgA1 hinge was incorporated into the equivalent site in IgA2. Insertion of the IgA1 half hinge into IgA2 did not affect antigen binding capacity or the functional activity of the hybrid molecule, as judged by its ability to bind to IgA Fcε receptors and trigger respiratory bursts in neutrophils. Although the IgA2/A1 hybrid contained only half of the IgA1 hinge, it was found to be cleaved by a variety of different bacterial IgA1 proteases, including representatives of those that cleave IgA1 in the different duplicated halves of the hinge, namely, those of Prevotella melaninogenica, Streptococcus pneumoniae, S. sanguis, Neisseria meningitidis types 1 and 2, N. gonorrhoeae types 1 and 2, and Haemophilus influenzae type 2. Thus, for these enzymes the recognition site for IgA1 cleavage is contained within half of the IgA1 hinge region; additional distal elements, if required, are provided by either an IgA1 or an IgA2 framework. In contrast, the IgA2/A1 hybrid appeared to be resistant to cleavage with S. oralis and some H. influenzae type 1 IgA1 proteases, suggesting these enzymes require additional determinants for efficient substrate recognition.

Secretory IgA (S-IgA) protects mucous membranes from attack by pathogenic microorganisms. It acts by neutralizing toxins, enzymes, and viruses, agglutinating bacteria, and preventing bacterial adhesion to mucous membranes by blocking receptors and, by virtue of its hydrophilic nature, causing repelling interactions with the mucosal epithelium (16, 18, 38, 40).

The ability of S-IgA to carry out its defensive effector functions is dependent on its structural integrity. The physicochemical nature of S-IgA renders it resistant to most types of proteolytic attack (20). However, a few pathogenic bacteria such as Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitidis, N. gonorrhoeae, and Prevotella melaninogena, which cause infections at mucous membranes leading to diseases like pneumonia, meningitis, gonorrhea, and periodontitis, produce a variety of enzymes called IgA1 proteases (for reviews, see references 15, 26, and 35). They are so named because they cleave only human IgA1 and not the IgA2 isotype. These enzymes may be important virulence factors because they produce in vivo (3, 14), convalescing patients from infections with these bacteria have neutralizing antibodies to the enzymes (5, 9, 11), and the related but nonpathogenic species of these bacteria do not produce them (26). Moreover, some may have a role in virulence by mechanisms additional to or distinct from that arising through IgA1 cleavage (19, 30).

IgA2 is resistant to IgA1 proteases because it lacks a sequence of 16 amino acids which is present in the hinge region of IgA1 and which is the cleavage site for all IgA1 proteases. The sequence, which is rich in proline, threonine, and serine, is unusual in that it contains a repeat of two identical and contiguous sequences each of eight amino acids. Although the IgA1 proteases belong to widely different families, i.e., serine proteases, metalloproteases, and thiol proteases, they are all highly specific post-proline endopeptidases. The IgA1 proteases of the different bacteria always cleave at either Pro-Ser (type 1 enzymes) or Pro-Thr (type 2 enzymes) peptide bonds. However, they are extremely specific in that one enzyme of a given organism cleaves the specific peptide bond in only one of the duplicated eight amino acid sequences and not at the equivalent site in the other duplicated eight-amino-acid sequence (Fig. 1).

To understand more about the factors influencing the cleavage of IgA by microbial IgA1 proteases, a recombinant hybrid IgA molecule was constructed such that an amino acid sequence representing half of the duplicated hinge region of IgA1 was incorporated into the equivalent position in IgA2. The functional activity and sensitivity of the recombinant hybrid IgA2/A1 molecule to a variety of microbial IgA1 proteases were then determined.

MATERIALS AND METHODS

Primers. Primer AIH6 (5’ CCGCCCGGCCGATCCGGTCTCAAACCGAGGC 3’) contained a BamHI site (italics) and annealed about 150 bp upstream of the CH1 exon of human IgA2m(1). Primer AIH2 (5’ AGATGGGCTAGGGTGGAG TTGAGGGAACCTGAGTG 3’) contained nucleotides complementary to nucleotides 675 to 688 of the coding strand for the CH1 region of human IgA2m(1) and (in italics) nucleotides complementary to nucleotides 667 to 687 of human IgA1, coding for half of the hinge region. Primer AIH3 (5’ TCAACTCCACCT ACCCCATCCTCAACCTCCCATTG 3’) contained (in italics) the nucleotides coding for half the hinge region, i.e., nucleotides 667 to 687, of human IgA1, followed by nucleotides 689 to 702 of the coding strand for the CH2 region of human IgA2m(1). Primer AIH5 (5’ CCACCCTGACTTGA 3’) was comple-
saturating amounts (250 μg/ml) for 1 to 2 h at room temperature. The cells were processed as described previously (27) from heparinized blood taken from healthy volunteers. Rosetting of sensitized erythrocytes to neutrophils was performed in V-bottomed microtiter plates as described previously (39). After addition of acridine orange solution (6 μg/ml, final concentration) to stain nucleated cells, the cells were resuspended and examined by UV microscopy for rosetting. A rosette was defined as a fluorescent neutrophil with three or more erythrocytes attached.

Chemiluminescence assay of respiratory bursts. Wells of a chemiluminescence microtiter plate (Dynatech, Billinghamurst, Sussex, United Kingdom) were coated with 150 μg of NIP-BSA/ml in coating buffer (0.1 M sodium carbonate buffer [pH 9.6]) and incubated overnight at 4°C. After three washes with PBS, 150 μl of diluted antibody (30 μg/ml) was added in triplicate to the wells and left overnight at 4°C. After three washes in PBS, 100 μl of luminol (67 μM/ml in Hanks’ balanced salt solution [HBSS]) containing 20 mM HEPES buffer and 0.1% [wt/vol] ovalbumin-free BSA [HBSS-BSA]) was added to each well. Following the addition of 50 μl of neutrophils (10⁶/ml in HBSS-BSA) to each well, the plate was transferred to a Microtiter LB96P luminometer, and chemiluminescence was measured at regular intervals for 1 h.

Microbial IgA1 proteases. The IgA1 proteases used were from S. pneumoniae type 23 strain 3626, S. orta, NCTC 11427, S. pyogenes biovar 2 strain SK4, H. influenzae HK368, R11, R12, R14, R20, R25, and R27 (all type 1 enzyme), H. influenzae 110022H and R4 (both type 2 enzyme), N. meningitidis group B serotype 14 strain 3564 (type 2 enzyme), N. gonorrhoeae STR 208 (type 2 enzyme), P. melaninogenica ATCC 25845. The enzymes from S. pneumoniae SK4, H. influenzae HK368, and P. melaninogenica ATCC 25845 were pure; the others were partially purified and either concentrated from liquid culture supernatants or prepared as previously described (34) from the bacteria grown on dialysis tubing covering appropriate culture media, blood agar, heated blood agar, or modified New York City agar for 3 days at 37°C in 5% CO₂.

The enzyme preparations were stored at −20°C.

Digestion of recombinant IgA preparations with microbial IgA1 proteases and immunoblotting. Initial preliminary experiments determined the appropriate volume of protease to antibody to use to permit assessment of cleavage by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Such volumes of recombinant IgA1 or IgA2 or hybrid IgA2/A1 protease preparations were added to PBS (pH 7.2) containing 0.1% sodium azide to give a total volume of 20 μl. In the case of P. melaninogenica protease, the buffer used was 0.1 M sodium phosphate (pH 5.5) containing 0.1 M EDTA and 0.1 M dithiothreitol. The reaction mixtures were incubated at 37°C for 2 h prior to analysis on SDS–10% polyacrylamide gels under reducing and nonreducing conditions. The proteins were then transferred to nitrocellulose membranes, which were then blocked by agitation for 30 min in 5% nonfat dried milk powder in PBS. After thorough washing in PBS, the membranes were immersed in horseradish peroxidase-labeled antibody, either sheep anti-human IgA Fc antibody (Sigma) or goat anti-human IgA (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) or sheep anti-mouse L-chain antibody (Nordic Immunological Laboratories, Tilburg, the Netherlands) diluted 1:1,000 in PBS containing 0.1% Tween 20 (PBST) and agitated for 2 h at room temperature. After washing with PBST, the examination was to be made for binding of biotinylated lectins, the nitrocellulose membranes were blocked by immersion in 1% BSA in PBST and agitation for 30 min. After incubation with the biotinylated lectin (Vector Laboratories, Peterborough, United Kingdom) for 1 to 2 h at room temperature and thorough washing in PBS, the membranes were incubated with 1 μg of streptavidin-labeled horseradish peroxidase per ml in PBS for 30 min at room temperature.

In all instances, after thorough washing in PBS, the membranes were developed in 10 ml of 50 mM Tris-HCl (pH 7.6) buffer containing 0.3 mg of nickel chloride per ml, 10 mg of dianisobenzidine, and 60 μl of 30% hydrogen peroxide.

RESULTS

Expression of IgA2/A1 half hinge in CHO-K1 cells. DNA sequence analysis of the IgA2/A1 half hinge expression vector confirmed that nucleotides 667 to 687 of α₁ had been correctly incorporated between nucleotides 688 and 689 of α₁ and that no misincorporations had occurred during PCR amplification. Analysis of the IgA2/A1 half hinge antibody expressed in CHO-K1 cells showed that in the reduced form, the hybrid α₂/α₁ chain appeared as two glycoprotein bands of 68 and 63 kDa (Fig. 2). These differed only in the extent of N-glycosylation, for after incubation with recombinant peptide-N-glycosidase F (Glyko, Upper Heyford, Nr. Bicester, United King-
FIG. 2. Western blot analysis of proteins separated under reducing conditions of recombinant wild-type IgA1 (lanes 1 and 4), recombinant hybrid IgA2/A1 half hinge (lanes 2 and 5), and recombinant wild-type IgA2 (lanes 3 and 6) after being either untreated (lanes 1 to 3) or incubated with N-glycosidase F (lanes 4 to 6) and probed with anti-human IgA-peroxidase conjugate. Positions of molecular mass markers in kilodaltons are shown on the left. Treatment of the two-banded hybrid IgA2/A1 half hinge glycoprotein with N-glycosidase F revealed the presence in lane 6 of trace amounts of non-IgA glycoproteins.

FIG. 3. Western blot analysis of proteins separated under nonreducing conditions of recombinant IgA1 (lanes 1 and 5), recombinant hybrid IgA2/A1 half hinge (lanes 2 and 6), and recombinant IgA2 (lanes 3 and 7) after probing either with anti-human IgA-peroxidase conjugate (lanes 1 to 3) or, after reaction with biotinylated jacalin, probing with streptavidin-peroxidase conjugate (lanes 5 to 7). Positions of molecular mass markers (lanes 4 and 8) in kilodaltons are shown at the right. The amounts of IgA in lanes 1 to 3 were the same as their equivalents in lanes 5 to 7. The lectin jacalin bound to recombinant wild-type IgA1 and recombinant hybrid IgA2/A1 half hinge but not to recombinant IgA2, indicating the presence in IgA2/A1 half hinge of O-glycosylated jacalin binding amino acids in the half hinge. The highly sensitive jacalin binding analysis revealed the presence in lane 6 of trace amounts of non-IgA glycoproteins.

It is now appreciated that disulfide bonds can be formed between H and L chains in IgA2m(1) but with low frequency (8), and this obviously also applies to the IgA2/A1 half hinge molecule.

The hybrid IgA2/A1 half hinge molecule was examined for the presence of O-glycosylated amino acids in the half-hinge region by determining its ability to bind the biotinylated lectin jacalin (from Artocarpus integrifolia). The results showed that the wild-type IgA1, the hybrid IgA2/A1 half hinge molecule bound jacalin and thus contained O-glycosylated amino acids, whereas, as expected, the wild-type IgA2 molecule did not (Fig. 4).

FIG. 4. Western blot analysis of proteins separated under reducing conditions of recombinant IgA1 (lanes 1 and 5), recombinant hybrid IgA2/A1 half hinge (lanes 2 and 6), and recombinant IgA2 (lanes 3 and 7) after probing either with anti-human IgA-peroxidase conjugate (lanes 1 to 3) or, after reaction with biotinylated jacalin, probing with streptavidin-peroxidase conjugate (lanes 5 to 7). Positions of molecular mass markers (lanes 4 and 8) in kilodaltons are shown at the right. The amounts of IgA in lanes 1 to 3 were the same as their equivalents in lanes 5 to 7. The lectin jacalin bound to recombinant wild-type IgA1 and recombinant hybrid IgA2/A1 half hinge but not to recombinant IgA2, indicating the presence in IgA2/A1 half hinge of O-glycosylated jacalin binding amino acids in the half hinge. The highly sensitive jacalin binding analysis revealed the presence in lane 6 of trace amounts of non-IgA glycoproteins.

TABLE 1. Functional activity of recombinant IgA molecules by rosetting assay

<table>
<thead>
<tr>
<th>NIP-erythrocytes coated with:</th>
<th>% Rosettes</th>
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<tr>
<td></td>
<td>Test 1</td>
</tr>
<tr>
<td>IgA1</td>
<td>55</td>
</tr>
<tr>
<td>IgA2m(1)</td>
<td>65</td>
</tr>
<tr>
<td>IgA2/A1 half hinge</td>
<td>50</td>
</tr>
<tr>
<td>No antibody</td>
<td>0</td>
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* A rosette was defined as ≥3 erythrocytes bound to a neutrophil. In each test, 100 neutrophils were counted three times and the mean is shown. Tests 1 and 2 were performed with two different neutrophil preparations.
zyme preparations were shown to have IgA1 protease activity, for they all cleaved wild-type IgA1 in the hinge region to generate Fab and Fc fragments but were unable to cleave wild-type IgA2 (data not shown).

Examination of the sensitivity of wild-type IgA1 and IgA2/A1 half hinge to the different streptococcal IgA1 proteases showed that although all of the enzymes were active and cleaved IgA1 to Fab and Fc fragments, the IgA2/A1 half hinge hybrid was sensitive only to those of *S. pneumoniae* and *S. sanguis* and resistant to that of *S. oralis* (Fig. 6). Interpretation of the relative sizes of the cleavage products requires careful consideration of both the N-linked sugar moieties in the Fc of IgA1 (two per Fc H chain) and of IgA2/A1 (three per Fc H chain) and also the differing presence of contaminating glycosidase activity in the protease preparations. Although the proteases from *S. pneumoniae* and *S. oralis* cleaved the wild-type IgA1 to produce IgA1 fragments of the same sizes of ca. 28 and 27 kDa (Fig. 6, lanes 2 and 4), the fragments generated by cleavage with *S. sanguis* protease (Fig. 6, lane 3) were marginally larger. Moreover, the size of the fragments generated by cleavage of the IgA2/A1 half hinge by the protease from *S. pneumoniae* (Fig. 6, lane 6) differed in size from those resulting from cleavage with *S. sanguis* protease (Fig. 6, lane 7). The reason for this is thought to be a consequence of the *S. pneumoniae* (and *S. oralis*) protease preparations, but not that of *S. sanguis*, also having glycosidase activity as has been observed previously (33). Thus, when the *S. pneumoniae* cleavage of IgA2/A1 half hinge was repeated in the presence of 25 mM EDTA to inhibit the *S. pneumoniae* IgA1 metalloproteinase activity, although there was no proteolytic cleavage of IgA2/A1, the hybrid antibody was nevertheless deglycosylated to a protein of ca. 56 kDa (Fig. 7). The difference in the sizes of the IgA fragments produced by the different enzymes is more marked for the IgA2/A1 half hinge than for the wild-type IgA1, presumably because of the loss of three sugar moieties in the former, compared with just two in the latter.

The action on wild-type IgA1 and the IgA2/A1 half hinge of the IgA1 proteases of *S. pneumoniae*, *N. meningitidis* type 2, and *N. gonorrhoeae* type 1, which cleave at different sites in the hinge in IgA1 (Fig. 1), is shown in Fig. 8. All of these enzymes which cleaved IgA1 were also found to be able to cleave the hybrid IgA2/A1. The lower molecular weight of the fragments produced by the *S. pneumoniae* protease than of the fragments produced by the different *Neisseria* proteases is presumably due to the additional activity of glycosidases in the *S. pneumoniae* preparation (see above). The hybrid IgA2/A1 was also found to be sensitive to the type 1 protease of *N. meningitidis* and the type 2 protease of *N. gonorrhoeae* (results not shown), which cleave the same peptide bonds in IgA1 as *N. gonorrhoeae* type 1 protease and *N. meningitidis* type 2 protease, respectively. Moreover, the hybrid IgA2/A1 was also sensitive to *P. melaninogenica* protease, which cleaves IgA1 at the opposite end of the full hinge in IgA1 (Fig. 1) to that cleaved by the type 1 protease of *N. meningitidis* (Fig. 9).

Investigations into the sensitivity of IgA2/A1 half hinge to
type 1 and type 2 IgA proteases of *H. influenzae* showed that although the hybrid immunoglobulin was cleaved by the type 2 enzyme, it was much more resistant to the type 1 enzyme of strain HK368. Further investigation with type 1 enzymes from seven different *H. influenzae* strains of biotypes I (R27), II (R12, R14, and R16), III (R20), IV (R11), and VII (R25) showed that the IgA2/A1 hybrid was sensitive to four of these type 1 proteases but resistant to three of them (Fig. 10).

**DISCUSSION**

Microbial IgA proteases are extremely specific. Excepting peptide bonds present in precursors of the enzymes which are cleaved in their processing, LAMP1 (the major integral membrane protein of lysosomes) (19), and some outer membrane proteins of *N. gonorrhoeae* (36), the only known substrate of IgA1 protease is IgA1 of humans, gorillas, chimpanzees, and orangutans (32). Little is known about what determines the specificity of IgA1 proteases. The fact that they cleave human IgA1 at specific sites in only one of the two available duplicated sites in the hinge suggests that the two duplicated half hinges have different conformations or that the enzymes recognize additional elements distant from the cleavage site.

In an attempt to gain further information about the requirements of IgA for sensitivity to IgA1 proteases and the determinants of specificity of IgA1 proteases, seven amino acids representing a half hinge region of human IgA1 were introduced into protease-resistant human IgA2 to create an artificial half hinge. The hybrid IgA2/A1 molecule was found to have an arrangement similar to that of IgA2m(1) with regard to the bonding of its H and L chains and was deemed to be functionally active in that it could form rosettes and bind efficiently to FcεR on neutrophils and trigger a respiratory burst. However, it now possessed the O-glycosylated amino acids of half the hinge of IgA1 and showed sensitivity to several diverse IgA1 proteases.

Determination of the exact site of cleavage by the enzymes in the IgA2/A1 hybrid was felt beyond the scope of this study because of the work involved in analyzing the cleavage products generated by so many different enzymes. However, because of the known extreme specificity of bacterial IgA1 proteases, it is not unreasonable to assume that the cleavage site for each enzyme in the IgA2/A1 half hinge hybrid is identical to its natural cleavage site in one of the duplicated half hinge regions of the wild-type IgA1 molecule, although conclusive proof requires amino acid sequence analysis of the cleavage products.

The IgA1 proteases active on the IgA2/A1 half hinge hybrid included representatives of those which cleave at specific sites in both of the duplicated half hinges of IgA1, namely, the IgA1 proteases of *S. pneumoniae*, *S. sanguis*, *H. influenzae*, *N. meningitidis* 1 and 2, and *P. melanogenumica* (Fig. 1). Thus, for these enzymes it would appear that although they cleave IgA1 naturally at a specific peptide bond in only one of the duplicated half hinge areas (the preferred cleavage site), if the specific peptide is represented only once as in the IgA2/A1 half hinge hybrid, these enzymes will still cleave IgA, thereby overriding the determinants of site selectivity. The results also indicate that for these enzymes the recognition site for IgA cleavage is contained in a half hinge region or that if additional more distal elements are required, the framework of IgA2 substitutes reasonably adequately for that of IgA1. These results support the work of Pohler et al. (29) and that of others (1) who have suggested that the consensus target sequence for serine-type IgA1 proteases of *Neisseria* and *Haemophilus* is either P-P | S/T-P or P-X-P | S/T-ST-P, where X is any amino acid, S/T is serine or threonine, and S/T-ST is serine or threonine or both. These sequences are provided in the IgA2/A1 half hinge molecule. A proline as the amino acid N-terminal to the cleaved peptide bond is a requirement for *Haemophilus* and *Neisseria* IgA1 proteases (1). Proline residues introduce bends into polypeptide chains, and these may expose sites for essential protein-protein interactions.

It was not very surprising, therefore, to find that the IgA2/A1 hybrid was sensitive to most of the IgA1 proteases of *Haemophilus* and *Neisseria* spp., for these are serine-type proteases that can be inhibited by short peptides. Bachovchin et al. (1) showed that tri- and tetrapeptide prolylboronic acid analogues could block the active site of the serine-type IgA1 proteases of *Haemophilus* and *Neisseria* (but not that of the metalloproteinase IgA1 protease of *S. sanguis* and that synthetic short peptides were cleaved more slowly than the IgA1 hinge. This suggests that maximum efficiency in cleavage occurs only when the substrate has the correct length and conformation. In support of this conclusion, it was repeatedly found that the cleavage of the IgA2/A1 hybrid by IgA1 proteases from some organisms was less complete than that of wild-type IgA1 after incubation for similar periods. This suggests not only that cleavage of IgA requires the presence of a cleavable peptide bond at the correct location but also that other parts of the
molecule influence its sensitivity to IgA protease cleavage. Although in this study substrate-enzyme reactions were usually incubated for 72 h, this was done in order not to miss substrate cleavage by any slow-acting IgA1 protease on the IgA2/A1 hybrid. In fact, all IgA1 proteases that hydrolyzed the hybrid demonstrated cleavage within 16 h. A more detailed comparison of the kinetics of IgA1 protease cleavage of the IgA2/A1 half hinge with that of wild-type IgA1 is to be the subject of a separate investigation.

The reason for the resistance of the IgA2/A1 hybrid to some *H. influenzae* type 1 proteases is not clear. It is known for organisms like *H. influenzae* (and *N. meningitidis*), which produce type 1 and type 2 proteases which cleave at different sites within one of the duplicated half hinge sites, that the site of cleavage is determined by a region near the amino-terminal end of each protease known as the cleavage site determinant (CSD) (12). Comparisons between the CSDs of different organisms have shown that the CSD length varies with the enzyme and is proportional to the distance between the interchain disulfide bridge at the top of the CH2 domains prior to the hinge, i.e., Cys 241, and the specific peptide bond cleaved by the enzyme (21). It has been suggested that the CSD acts as a spacer between the catalytic site and the substrate recognition site. Consistent with this is the finding that cleavage appears to be dependent on hinge structural features C-terminal to the susceptible peptide bond because sequential incubation with different IgA1 proteases resulted in cleavage of Fc but not Fab fragments (21). As the CSD of *H. influenzae* type 1 protease is bigger than that of all of the other enzymes (21) and there is known to be much variation in the CSDs of type 1 proteases of *H. influenzae*, it is possible that for some *H. influenzae* type 1 proteases a single half hinge site is too small to accommodate such a large CSD spacer and the enzyme is directed to act at a site outside the half hinge where a Pro-Ser bond is not present for cleavage. Alternatively, because the IgA1 proteases of *H. influenzae* are the most antigenically diverse, more than 30 antigenic types having been described on the basis of antibody neutralization tests (17, 22), it may be that some are unable to cleave the IgA2/A1 hybrid because of steric hindrance due to their increased bulk and the closer approach of the Fab arms.

The inability of the protease of *S. oralis* alone among the streptococcal IgA1 proteases to cleave the IgA2/A1 hybrid is difficult to understand and explain, for all are metalloproteinases, the cleavage site on IgA1 is the same for all, and their amino acid sequences are highly homogeneous (31). Moreover, unlike the situation with *H. influenzae* type 1 proteases, the proteases of *S. oralis* are all of one antigenic type (33). However, the iga protease gene in *S. oralis* contains elements displaying subtle differences from those of *S. sanguis* and *S. pneumoniae* which may contribute to a particular cleavage site specificity. It is also possible that the *S. oralis* protease requires structures outside the half hinge in the hybrid antibody for which IgA2 elements are not an acceptable alternative to IgA1 elements for substrate recognition.

The susceptibility of IgA1 to IgA1 proteases can be influenced by its state of glycosylation (33), and it is possible that the carbohydrates in the hinge region contribute to the specificity of IgA1 proteases. The IgA1 hinge region contains several potential sites for O-linked glycosylation (2, 33). Classically, in studies of IgA1 myelomas, these have been considered to be the five serine residues, four of which have galactosyl-β1-3-N-acetylgalactosamine groups (and possibly sialic acid 33) whereas Ser 224 has N-acetylgalactosamine (10). More recently, however, analysis of IgA1 from serum indicates that the sugars are O-linked via both serine and threonine residues asymmetrically distributed between the two duplicated halves of the hinge (22). Thus, it could be argued that the two duplicated half hinges in wild-type IgA1 are distinguishable on the basis of differences in glycosylation.

The half hinge region of the IgA2/A1 hybrid is believed to be O-glycosylated, for it was found to bind the biotinylated lectin jacalin, which is specific for the O-linked sugars restricted to the hinge of IgA1. Although the exact state of glycosylation of the half hinge region is not known, it can at best presumably resemble only one of the half hinges of IgA. Thus, it is unlikely that the O-linked sugars act as determinants of specificity and direct the protease specifically to one of the duplicated half hinge regions in IgA1 because representatives of proteases acting in each of these different regions in IgA1 were all able to cleave IgA2/A1 half hinge. If the half hinge in the IgA2/A1 hybrid is underglycosylated or glycosylated incorrectly in other ways, it is unlikely that this is the reason for the resistance of the molecule to the *S. oralis* and some *H. influenzae* type 1 proteases because we have observed (M. R. Batten, B. W. Senior, M. Kilian, and J. M. Woof, unpublished data) that when O-linked glycosylation of the IgA1 hinge region was perturbed through substitution of its serine residues with alanine and that of threonine 225 with valine, the modified IgA1 molecules nevertheless remained sensitive to virtually all types of IgA1 proteases, including those of *S. oralis* and *H. influenzae* type 1.

A molecular model for human IgA1 based on small-angle X-ray and neutron-scattering analysis has recently been generated (4). The average conformation of the antibody is predicted to be T shaped, with the Fab arms widely separated. The hinge peptides are suggested to adopt extended, exposed structures, presumably readily accessible to IgA1 proteases. In the IgA2/A1 hybrid, a similar structural arrangement may be present but with the Fab arms held much closer to the Fc portion. A maintained exposure of the shortened hinge might then explain the continued access, recognition, and thus sensitivity of the molecule to most of the IgA1 proteases, whereas the closer proximity of the Fab and Fc regions may for some IgA1 proteases present an unfavorable arrangement for access or substrate recognition or both.

In summary, this study has shown that through the insertion into protease-resistant IgA2 of seven amino acids representing half the hinge region of IgA1, a hybrid IgA2/A1 molecule that was functionally active and sensitive to many different bacterial IgA1 proteases was formed.

**ACKNOWLEDGMENTS**

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