

# Analysis of the Specificity of Bacterial Immunoglobulin A (IgA) Proteases by a Comparative Study of Ape Serum IgAs as Substrates†

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**Immunoglobulin A (IgA) proteases are bacterial enzymes with substrate specificity for human serum and secretory IgAs. To further define the basis of this specificity, we examined the ability of IgA proteases of *Clostridium ramosum*, *Streptococcus pneumoniae* (EC 3.4.24.13), *Neisseria meningitidis* (EC 3.4.21.72), and *Haemophilus influenzae* (EC 3.4.21.72) to cleave serum IgAs of gorillas, chimpanzees, and orangutans. All enzymes cleaved the IgAs of the three apes despite differences in ape IgA1 hinge sequence relative to the human prototype. To directly compare the ape and human hinge cleavage sites, the sites were identified in eight ape IgA digests. This analysis confirmed that ape proteins were all cleaved in the IgA hinge region, in all but one case after proline residues. The exception, *C. ramosum* protease, cleaved gorilla and chimpanzee IgAs at peptide bonds having no proline, but the scissile bonds were in the same hinge location as the Pro-221–Val-222 cleaved in human IgA1. These data indicate that proline is not an invariant substrate requirement for all IgA proteases and that the location of the scissile bond, in addition to its composition, is a critical determinant of cleavage specificity.**

Immunoglobulin A (IgA) proteases are among the putative virulence factors for the human bacterial pathogens *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* (13–16, 21, 22, 24–27). These enzymes cleave serum and secretory IgA1 in the hinge region of the heavy (alpha) polypeptide chain to yield Fab and Fc fragments, and this may enable the organisms to circumvent IgA1-mediated host mucosal defense mechanisms. A remarkable characteristic of IgA proteases is their high substrate specificity for human IgA1. Other proteins, including human IgA2, are not cleaved, with the exception that the A2m(1) allotype of human IgA2 was shown to be susceptible to *Clostridium ramosum* IgA protease (3, 4). IgAs from several other animal species, including monkeys, are also not substrates (28), but serum IgAs from gorillas and chimpanzees and secretory IgA of chimpanzees (4) are cleaved by *H. influenzae*. Recent comparative studies on the ape IgA constant-region gene structure and deduced amino acid sequence show many similarities in the hinge composition among chimpanzee, gorilla, orangutan, and human IgAs (11, 12). In human and gorilla IgA1s, the gene has a 15-bp tandem repeat sequence, while the C gene encoding the alpha chain of Old World monkeys lacks such a reiterated sequence in the hinge region (11, 12). However, the IgA1 hinge region is not identical among apes and humans, as shown in Fig. 1, and the observed differences include several of the peptide bonds that IgA proteases cleave in human IgA1. Human and gorilla

IgA1 hinge regions differ in only two amino acid residues, while the orangutan hinge is substantially different and has no repeated segment.

In this study, we evaluated serum IgA1s from gorillas, chimpanzees, and orangutans as substrates for five IgA proteases: *C. ramosum* and *S. pneumoniae* proteases, *N. meningitidis* protease types 1 and 2, and *H. influenzae* protease type 1. Protease types are based on the peptide bond cleaved in human IgA, as detailed elsewhere (25, 26). The main purpose of this study was to determine if differences in primary structure of the ape hinge region relative to the human hinge region would more clearly define the basis for IgA protease specificity. However, since apes are occasionally used to study pathogenic mechanisms and immune evasion strategies of these bacteria, information on the relative susceptibility of various ape IgA proteins to the enzymes may be of value.

## MATERIALS AND METHODS

**Bacterial strains and preparation of IgA proteases.** IgA proteases were obtained from ammonium sulfate precipitates of liquid medium culture supernatants of pathogens (23). *C. ramosum* (strain 269; kindly provided by Y. Fujiyama, Shiga University, Shiga, Japan) was cultured in Trypticase-glucose-yeast medium for 48 h with agitation at 30 rpm in an atmosphere of 95% nitrogen–5% CO<sub>2</sub>. *S. pneumoniae* (ATCC 27336) was cultured in Todd-Hewitt broth for 16 h without agitation in an atmosphere of 5% CO<sub>2</sub> in air. *N. meningitidis* strains yielding enzymes type 1 and 2 were isolated from cerebrospinal fluid of patients with meningitis, and enzyme type was determined as previously described (5). These cells were cultured in brain heart infusion broth containing IsoVitalX (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Md.) for 16 h at 100 rpm in air. *H. influenzae* strain Rd–(2), which produces type 1 IgA protease, was cultured in brain heart infusion broth supplemented with 10 µg each of NAD and hemin (both from Sigma Corp., St. Louis, Mo.) per ml for 16 h at 100 rpm in air.

The enzyme preparations were free of other proteases capable of cleaving IgA, as determined by their inability to cleave human serum IgA2 substrate after 7 days of incubation and the absence of secondary degradation of IgA fragments once formed. Also, as indicated in Results, certain ape IgA proteins were di-

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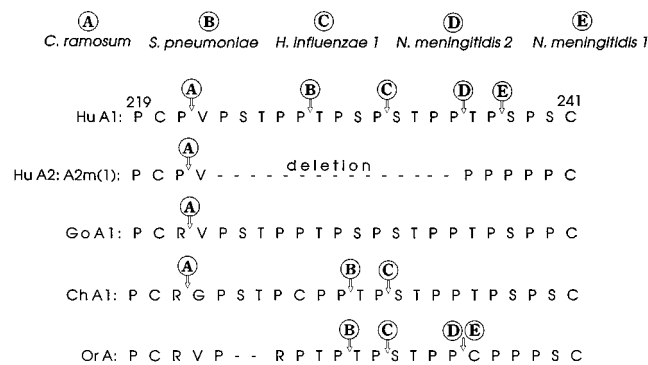


FIG. 1. Linear amino acid sequences of ape IgA hinge region segments and cleavage sites of various IgA proteases: human IgA1 (Hu A1), human IgA2 [Hu A2:A2m(1)], gorilla (Go A1), chimpanzee (Ch A1), and orangutan (Or A). Human IgA cleavage data are included for comparison, and the human IgA2-A2m(1) allotype, insusceptible to all but the *C. ramosum* enzyme, is also shown. Amino acid numbering of the human sequence is according to reference 32. A gap (---) is introduced in the shortened orangutan hinge to maintain alignment of cysteine residues.

gested for up to 10 days with these enzyme preparations, without evidence of any proteolytic products.

**Preparation of ape serum proteins.** Ape IgA proteins were purified from serum pools from six gorillas (*Gorilla gorilla*), eight chimpanzees (two *Pan troglodytes* and six *P. paniscus*), and seven orangutans (*Pongo pygmaeus*). Proteins were precipitated from these pools by gradual addition of 50% saturated ammonium sulfate and then centrifuged at  $9,000 \times g$  for 30 min at 8°C. The precipitate was dissolved in 20 mM phosphate-buffered saline-0.85% NaCl-0.1% thimerosal (pH 7.2) (PBS), dialyzed in the same buffer with stirring at 8°C for 18 h (Spectropor 1; molecular weight, 6,000 to 8,000; Spectrum, Los Angeles, Calif.), diluted with an equal volume of PBS, and filtered through a 0.45- $\mu$ m-pore-size filter (Acrodisc; Gelman, Ann Arbor, Mich.).

**Purification of IgA from ape serum proteins.** IgA was purified from serum proteins by using jacalin affinity chromatography. Two milliliters of 4% beaded agarose bearing jacalin lectin (from *Artocarpus integrifolia*; Sigma) was washed three times with PBS. One milliliter of the packed beads was placed in a 15-ml polyethylene tube and mixed with 2 ml of ape protein solution at 20 mg/ml. After gentle rotation for 16 h at 4°C, the beads were settled by low-speed centrifugation and then washed 10 times with 10-ml aliquots of PBS. Bound IgA was eluted from the beads by addition of 50 ml of 1 M galactose in PBS containing 0.1% NaN<sub>3</sub>. Eluates were concentrated on PM10 membranes (Amicon Corp., Lexington, Mass.), washed with multiple additions of PBS, and finally concentrated to a 2-ml solution enriched in ape IgA.

Further purification of IgA was by affinity chromatography using goat-anti-human IgA linked to agarose beads (Sigma). Two milliliters of IgA protein solution was mixed with 2 ml of the absorbant in PBS and shaken gently for 16 h at 4°C. The agarose beads were washed 10 times with PBS, after which IgA was eluted with 0.2 M glycine buffer (pH 2.8). The eluate was immediately brought to pH 7 by adding 0.5 M Na<sub>2</sub>HPO<sub>4</sub> and then concentrated to 0.5 ml on PM10 membranes. To displace glycine and phosphate ions, approximately 60 ml (in 20-ml aliquots) of 50 mM Tris HCl buffer (pH 7.5) containing 0.1% NaN<sub>3</sub> was added to the solution, and the material was repeatedly reconcentrated by filtration. The final IgA concentration was adjusted to 0.6 mg/ml, as measured by Bio-Rad protein assay, with bovine IgG as the standard.

**Protease assay and ape IgA digestion.** Quantitation of the enzyme units in each preparation, provided in Table 1, was based on rate of cleavage of human serum IgA1 substrate (23).

For cleavage of ape IgA proteins, each tube received 60  $\mu$ g of IgA in 100  $\mu$ l of 50 mM Tris HCl buffer (pH 7.5) containing 0.1% NaN<sub>3</sub>. IgA proteases were then added in either 10- or 20- $\mu$ l volumes, the amount depending on enzyme activity (Table 1). Tubes were incubated at 37°C for 2 to 10 days, depending on the rate of substrate hydrolysis as determined during incubation by periodically testing for IgA cleavage products by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with Coomassie blue staining (10). Additional enzyme was added to some digests, as detailed in Table 1.

Human serum IgA1 myeloma paraprotein (from patient Vie) in the same buffers as the ape IgA protein served as a control. Vie protein was treated with all enzyme preparations and was also incubated without enzyme as a buffer control.

**SDS-PAGE.** SDS-PAGE (17) was performed with vertical slab gels (5% stacking, 10% separating), using 75 V for the stacking gel and 150 V for the separating gel. Samples contained 3 to 7  $\mu$ g of IgA. For Western blots (immunoblots), proteins were transferred to nitrocellulose membranes in Tris-glycine buffer containing 20% methanol at 0.5 A for 2 h at 4°C (8, 31). Goat anti-human IgA

TABLE 1. Enzyme preparations used for digestion of ape IgA

Enzyme from:	Enzyme concn (U/ml) <sup>a</sup>	$\mu$ l of enzyme added to begin digest	Supplemental enzyme <sup>b</sup> (days added) to:
<i>C. ramosum</i>	108	10	Chimpanzee (4, 7)
<i>S. pneumoniae</i>	45	20	Chimpanzee (3, 5, 7, 9) Orangutan (4, 7)
<i>H. influenzae</i> type 1	67	20	Orangutan (4, 6)
<i>N. meningitidis</i>			
Type 2	763	10	None
Type 1	804	10	None

<sup>a</sup> One unit = activity cleaving 1  $\mu$ g of human IgA1 per min at 37°C.

<sup>b</sup> All supplemental enzyme aliquots had same number of units as used to begin the digest.

(alpha chain specific) conjugated with alkaline phosphatase (Sigma) was used to localize the intact alpha chain and its Fc and Fd fragments on stained gels.

**Amino acid sequencing.** IgA fragments were transferred from SDS-polyacrylamide gels to Immobilon P membranes (Millipore Corp., Bedford, Mass.) for sequence analysis. Transfer was at 0.5 A for 1 h at 4°C in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer (Sigma) containing 10% methanol and adjusted to pH 11.0 with 5 M NaOH. Transferred proteins were stained with 0.1% Coomassie blue in 50% methanol for 5 min, destained several times with 50% methanol-10% acetic acid, rinsed in deionized water, and dried in air. The Fc peptide fragment was cut from the stained membrane and subjected to limited amino-terminal sequence analysis (18) by automated Edman degradation on a model 477A pulsed liquid protein sequencer (Applied Biosystems, Foster City, Calif.) in the Protein Analysis Facility of Tufts University.

## RESULTS

The primary amino acid sequences of gorilla, chimpanzee, and orangutan IgA1 hinge regions are based on published information (11, 12) and are aligned with the human IgA1 hinge region sequence (19) in Fig. 1. All five IgA proteases used in this study readily cleaved human IgA1 protein Vie (Fig. 2A); the bonds cleaved, which had earlier been published for each enzyme, are shown in Fig. 1.

Gorilla, chimpanzee, and orangutan serum IgAs were also cleaved by each of the five enzymes. Western blot analysis was

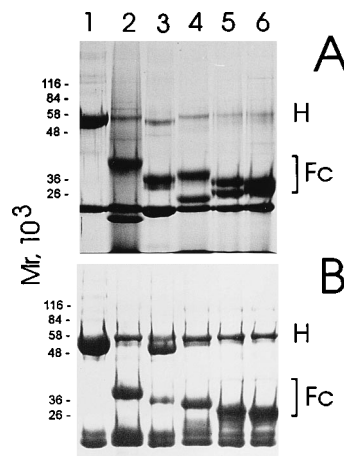


FIG. 2. Stained SDS-polyacrylamide gels of human IgA (A) and gorilla IgA (B) digested by five bacterial IgA proteases. Lanes: 1, control IgA, no enzyme; 2 to 6, samples digested by proteases of *C. ramosum*, *S. pneumoniae*, *H. influenzae* (enzyme type 1), *N. meningitidis* (enzyme type 2), and *N. meningitidis* (enzyme type 1), respectively. H, uncleaved heavy chain. Brackets enclose Fc fragments. Bands below the Fc fragment include the IgA light chain and the Fd remnant of the heavy chain. The gorilla Fd fragments are polyclonal and thus not as sharp as the monoclonal Fd fragments from human IgA in panel A. The five proteases cleave different peptide bonds along the hinge, and so Fc fragment sizes vary.

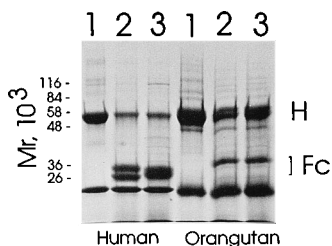


FIG. 3. Coomassie blue-stained SDS-polyacrylamide gels of human and orangutan serum IgAs digested by *N. meningitidis* IgA protease. Lanes: 1, control IgA, no enzyme; 2 and 3, samples digested with meningococcal type 2 and type 1 proteases, respectively. H, uncleaved heavy chain; Fc, fragments blotted for sequencing. In human digests, the paired fragment bands are Fc on the top and the Fd remnant of the heavy chain on the bottom. Orangutan Fc bands are identical, because both enzyme types cleaved the same bond (see Fig. 1). The Fd fragments from orangutan IgA are difficult to distinguish from light chains, with which they comigrate.

used to localize the heavy-chain segment of each Fc region on SDS-polyacrylamide gels. Representative stained gels showing the cleavage products are shown in Fig. 2B, 3, and 4. In Fig. 2B, the gorilla IgA Fc fragment sizes varied, depending on which peptide bond was cleaved. In these digests, the Fd fragments were less distinct because they originated from polyclonal gorilla IgA1 and thus have variable amino acid compositions at their amino termini. Also, they are almost the same size as light chains, with which they comigrated in SDS-polyacrylamide gels. Digests of polyclonal orangutan IgA also yielded heterogeneous Fd fragments that comigrated with light polypeptide chains in SDS-polyacrylamide gels (Fig. 3).

There was much variation in the time required for IgA proteases to cleave ape substrates. Although rates were not formally measured, fast and slow (but no intermediate) patterns of hydrolysis were noted. Fast hydrolysis occurred at the same rate as for human IgA with a given enzyme, required only a single addition of enzyme, and left little uncut substrate after overnight incubation. Slow hydrolysis required supplemental enzyme at periodic intervals (Table 1), and there was residual substrate in 8- to 10-day digests. Rapid cleavage was found for all five enzymes on gorilla IgA and for both *N. meningitidis* protease types on all ape substrates. Slow cleavage was noted for *S. pneumoniae* and *C. ramosum* enzymes with chimpanzee IgA and for *S. pneumoniae* and *H. influenzae* against orangutan IgA. In one case, chimpanzee IgA treated with *S. pneumoniae* protease, no detectable cleavage occurred after 10 days of incubation. However, limited proteolysis did occur once the substrate was reduced with dithiothreitol (1.0 mM) and held under reducing conditions during the incubation (Fig. 4).

Eight ape digests were selected for analysis of the specific hinge peptide bond cleaved. Fc fragment analyses were done mainly on chimpanzee and orangutan digests, as their hinge peptides differ more from the human prototype than do gorilla hinge peptides. However, the cleavage site for *C. ramosum* in the gorilla enzyme was of special interest since the human and gorilla Fc fragment products had the same size, suggesting hydrolysis of the gorilla enzyme at a bond not involving proline.

Amino-terminal sequences of the Fc fragments in the eight digests are presented in Table 2. In each case, the Edman-type analysis yielded a sequence that unambiguously aligned with the published hinge region sequence of that species, identifying the site cleaved (Fig. 1). With the exception of the *C. ramosum* enzyme, all enzymes cleaved after proline. In the gorilla and chimpanzee samples, *C. ramosum* did not cleave after proline, but cleaved Arg-221-Val-222 and Arg-221-Gly-222, respec-

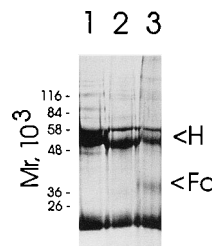


FIG. 4. Coomassie blue-stained SDS-polyacrylamide gels of chimpanzee serum IgA digested by *S. pneumoniae* IgA protease. Lanes: 1, control incubation with no enzyme; 2, native IgA incubated for 7 days with protease, showing no proteolysis; 3, dithiothreitol-treated IgA incubated for 7 days with protease. The Fc product is indicated. The shift in size of uncleaved heavy chain (H) is due to deglycosylation by other pneumococcal enzymes. The unshifted, uppermost band is unidentified.

tively, at hinge locations corresponding exactly to the Pro-221-Val-222 cleaved in human IgA1.

## DISCUSSION

The five IgA proteases used in this study cleave human IgA1 after proline residues in the hinge region of the heavy chain (13, 25, 26) (Fig. 1). Because this segment has a tandemly duplicated octapeptide rich in proline, it contains multiple Pro-X bonds (where X is either Pro, Thr, Ser, or Val). The experiments reported here confirm that all five enzymes also cleaved hinge peptide bonds in IgAs of gorillas, chimpanzees, and orangutans, and with the exception of the *C. ramosum* enzyme discussed below, proline contributed the carboxyl group to all of the ape peptide bonds cleaved. When the human and ape sequences are aligned as in Fig. 1, the ape cleavage sites all lie within two residues of the site cleaved in human IgA. However, *C. ramosum* IgA protease cleaved gorilla and chimpanzee IgAs at bonds not involving proline. The cleaved gorilla sequence (Arg-Val) and the corresponding chimpanzee sequence (Arg-Gly) both align with the Pro-221-Val-222 that *C. ramosum* cleaves in human IgA1. Further defining the similarity among these *C. ramosum* cleavage sites is the finding that all immediately follow an invariant Cys-220. The *C. ramosum* enzyme had already been recognized as unique among IgA proteases by its ability to cleave not only human IgA1 but also human IgA2 proteins having the A2m(1) allotype (6, 7, 32), and both were hydrolyzed at the same site, Pro-221-Val-222. In contrast, IgA2 proteins having the A2m(2) allotype are

TABLE 2. Amino-terminal sequence analysis of Fc fragments of ape IgAs

Enzyme from:	IgA	
	Source	Amino terminus of Fc fragment <sup>a</sup>
<i>C. ramosum</i>	Gorilla	V-P-S-T-P-P-?-P-?-P-?-T
	Chimpanzee	G-P-S-T/G-P/G-?-P-P-T-?
<i>S. pneumoniae</i>	Chimpanzee	?-P-S/Q-T-P-P-T-P-S-P-?
	Orangutan	?-P-S-T-P-P-?-P-P-P-S-?
<i>H. influenzae</i> type 1	Chimpanzee	?-T-P-P-T-P-S-P-S-?-?-H
	Orangutan	S-T-P-P-?-P-P-P-S-?
<i>N. meningitidis</i>	Orangutan	?-P-P-P-S-?-G-Q-P-Q-L
	Type 1	?-P-P-P-S-?-G-Q-P-Q-L

<sup>a</sup> Amino acids are shown as single letters. T/G, P/G, and S/Q indicate ambiguity in the experimental result, with the most likely residues underlined; ? indicates a residue not identified.

not cleaved by *C. ramosum*, which was attributed to the presence of Arg-221–Val-222 at this position (6, 7, 30). However, the data reported here show that absence of proline in ape IgA proteins does not confer resistance to *C. ramosum*, and they also show that Arg–Val in the gorilla substrate is rapidly cleaved. We conclude that the peptide bond cleaved by *C. ramosum* in human, gorilla, and chimpanzee IgAs is determined by its location in the alpha chain and not strictly its amino acid composition. These data also lead to the conclusion that in order to accommodate the *C. ramosum* enzyme, the definition of IgA proteases as post-proline-cleaving enzymes must be amended.

Analysis of other ape cleavage sites provides additional evidence that bond location is decisive for specificity. In orangutan IgA1, Pro-233–Cys-234 cleaved by *N. meningitidis* type 2 and Pro-229–Ser-230 cleaved by *H. influenzae* type 1 enzymes align exactly with the bonds cleaved in human IgA1. Since the orangutan hinge is two residues shorter than the human and other ape hinges (11, 12), the observed similarity of cleavage sites depends on how one arranges the hinge amino acid sequences. In Fig. 1, we have placed a two-amino-acid gap in the orangutan hinge following Pro-223, which keeps the invariant Cys-241 and Cys-220 aligned for this analysis and maximizes homology of cleavage sites relative to human IgA1. Regardless of how sequences are drawn, both orangutan IgA1 and human IgA1 are cleaved by *N. meningitidis* type 2 enzyme five residues from the invariant Cys-241 and by *H. influenzae* type 1 enzyme nine residues from this cysteine, although there is no indication that hinge cysteine residues in themselves provide information guiding specificity. Also, an STPP tetrapeptide stretch is between these cleaved sites in both proteins, further emphasizing the similarity of the scissile bonds in orangutan and human IgAs. The role which distal segments of the heavy chain have in specificity is yet to be defined, but Lomholt et al. (20), who analyzed the cleavage specificity determining region (CSD) (9) of the gram-negative *iga* genes, noted that the location of the susceptible peptide bond in human IgA1 is proportional to the length of the CSD. These investigators suggest that the CSD may be a spacer between a substrate recognition site in the Fc region and the bond cleaved in the hinge. Other bacterial proteolytic enzymes are also known to require long stretches of protein substrates for activity. For example, botulinum type B neurotoxin (29) and the light chain of tetanus toxin (33), both recently recognized as zinc-metalloendoproteases, require extended regions in substrates (at least 10 residues to either side of the scissile bond) for full expression of proteolytic activity. An additional link between these particular bacterial enzymes and the neisserial IgA proteases is a shared ability to cleave the PPAP motif in synaptobrevin II, a protein essential for exocytosis in neurons and chromaffin cells (1).

*S. pneumoniae* IgA protease did not cleave the native form of chimpanzee IgA, perhaps because of Cys-227 near the predicted cleavage position. We have no direct evidence that Cys-227 participates in a disulfide bond, but addition of a disulfide reducing agent in part overcame the resistance of this IgA to hydrolysis. The resulting very slow hydrolysis of this and certain other ape IgA proteins indicate that good substrates must have properties in addition to multiple proline residues in the hinge. We also cannot explain why both *N. meningitidis* proteases cut the same site in orangutan IgA, but there are three consecutive prolines (237 to 239) near the predicted cleavage site of meningococcal type 1, and no known protease is capable of cleaving a Pro-Pro peptide bond. The sluggish cleavage of orangutan IgA1 by *S. pneumoniae* and *H. influenzae* type 1 enzymes occurred despite the similarity of the surrounding sequence to human IgA1, suggesting that the charged Arg-224,

unique to the orangutan hinge, could influence the hydrolysis rate. It should be noted that since all of the purified ape IgA proteins that we used in this study were polyclonal, they may contain a subpopulation of protease-inhibiting antibody (3, 5). However, we have found that neither the chimpanzee nor the orangutan IgA protein that we used was able to inhibit *S. pneumoniae* and *H. influenzae* hydrolysis of human IgA1 (data not shown).

In summary, these studies have exploited the sequence differences among the hinge peptides of ape and human IgAs to examine the basis of IgA protease specificity. The data show that activity is not solely defined by enzyme recognition of specific hinge peptide bonds and their immediate surrounding residues but also depends on the location of the cleaved bond within the context of a much larger segment of alpha chain.

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