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Heterogeneity of *O*-glycosylation in the hinge region of human IgA1

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Abstract

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was applied to studies of the molecular heterogeneity of desialylated human IgA1 hinge region glycopeptides released with two IgA1 proteases. Typically, the hinge region of an α1 chain contains three to five *O*-linked glycan chains. Variants of the hinge region peptides released from IgA1(Kni) myeloma protein carrying 0, 1, 2, or 3 GalNAc residues were observed in the mass spectra as well as the nonglycosylated peptide. Variable numbers of Gal residues indicated additional heterogeneity in *O*-glycosylation of IgA1. In the hinge region preparation from normal human serum IgA1, glycopeptides carrying 2, 3, 4, or 5 GalNAc residues with variable numbers of Gal residues were detected. In conclusion, our new approach using the site-specific cleavage with two IgA1 proteases allowed precise and sensitive MALDI-TOF mass spectrometric analysis of *O*-glycosylation heterogeneity in IgA1 hinge region.
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Keywords: IgA1; Hinge region; *O*-linked glycans; IgA1 protease; Mass spectrometry

1. Introduction

Human IgA is represented by two structurally and functionally distinct subclasses, IgA1 and IgA2 (Mestecky and Russell, 1986). IgA1 differs from IgA2 molecules in the total content of glycans, number and type of glycan side-chains (two or more *N*-linked and three to five *O*-linked side-chains per heavy chain in IgA1, and four or more *N*-linked side-chains per heavy chain in IgA2 molecules), and carbohydrate composition (the presence of mannose [Man], galactose [Gal], *N*-acetylglucosamine [GlcNAc], fucose [Fuc], and sialic acid [NeuAc] in both subclasses, and *N*-acetylgalactosamine [GalNAc] in IgA1 but not IgA2) (Baenziger and Kornfeld, 1974; Endo et al., 1994; Field et al., 1989; Tomana et al., 1972, 1976; Torano et al., 1977). A high degree of heterogeneity with respect to *N*-linked glycans has been observed in IgA from healthy individuals as well as in IgA myeloma proteins (Endo et al., 1994; Field et al., 1989; Tomana et al., 1972, 1976). The *N*-glycosylation site in the Cα2 domain of IgA1 contains mostly biantennary glycans, while the tailpiece site contains either triantennary structures (Mattu et al., 1998) or fully galactosylated and fucosylated biantennary glycans (Tanaka et al., 1998). Over 90% of the *N*-linked glycans in IgA1 are sialylated.

The human α1 heavy chain contains also *O*-linked chains attached to the Ser and Thr residues in the hinge region (Baenziger and Kornfeld, 1974; Mattu et al., 1998). This segment of the α1 chain displays a primary structure (repeated sequences of Pro, Thr, and Ser

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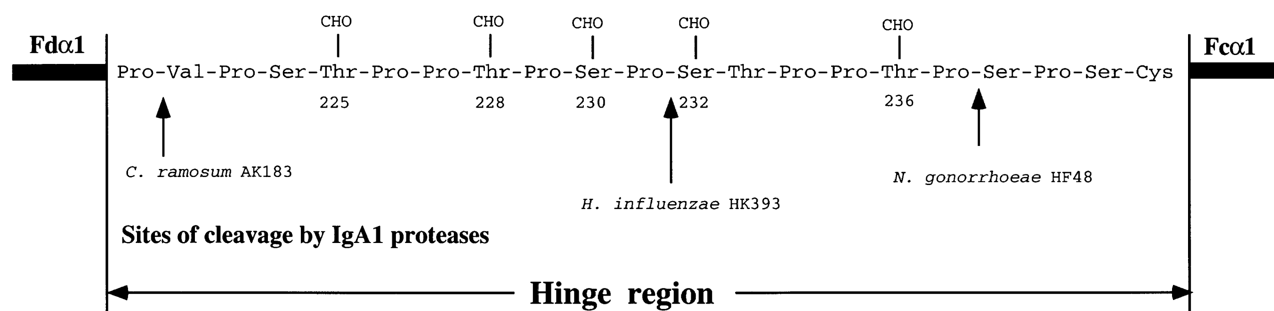

Fig. 1. Sites of attachment of *O*-linked glycans (CHO) and the IgA1 protease cleavage sites (arrows) in the hinge region of human IgA1. In an IgA1 protein, usually three to five sites per molecule of α chain are glycosylated (Baenziger and Kornfeld, 1974; Field et al., 1989; Mattu et al., 1998).

residues) unique among all immunoglobulin isotypes (Baenziger and Kornfeld, 1974; Frangione and Wolfenstein-Todel, 1972). Furthermore, IgA1 hinge region is susceptible to site-specific cleavage by IgA1 proteases from bacterial pathogens that frequently colonize mucosal surfaces (Kilian and Russell, 1999; Reinholdt et al., 1990). *O*-linked glycan chains in the hinge region of IgA1 consist of GalNAc with a β1,3 linked Gal, which may be sialylated (Baenziger and Kornfeld, 1974; Field et al., 1989). NeuAc can also be attached to GalNAc by an α2,6 linkage (Field et al., 1989). In normal serum IgA1, *O*-glycosylation sites include residues Thr(225), Thr(228), Ser(230), Ser(232), and Thr(236) (Mattu et al., 1998) (Fig. 1), however, the Thr(225) and Thr(236) are not always occupied (Mattu et al., 1998). Monosaccharide composition of *O*-linked glycans in the hinge region of normal human IgA1 is variable and the prevailing forms include Gal-GalNAc disaccharide, and its mono- and di-sialylated forms (Field et al., 1989; Mattu et al., 1998) (Fig. 2). A variant represented by terminal GalNAc appears to be absent from *O*-glycans of normal serum IgA1 (Mattu et al., 1998).

Contrary to the earlier opinion, a growing body of evidence indicates that glycan moieties of glycoproteins, including immunoglobulins play an essential role in many biological processes and functions (Brockhausen, 1999; Schachter and Jaeken, 1999; Varki, 1993). Glycans profoundly influence the cell–cell interactions as well as interactions of soluble glycoproteins with corresponding receptors expressed on various cell populations (Imai et al., 1991; Pahlsson et al., 1995; Priatel et al., 2000). Glycans on immunoglobulin molecules (specifically *O*-linked glycans in IgA1) are involved in the binding, internalization, and subsequent catabolism by hepatocytes (Baenziger and Fiete, 1980; Baenziger and Maynard, 1980; Stockert et al., 1980, 1982; Tomana et al., 1988a, 1985), *N*-linked glycans in various IgA are involved in binding to Fc receptors on phagocytic cells (Abadeh et al., 1997; Chuang and Morrison, 1997; Monteiro et al., 1990, 1995; Nose and Wigzell, 1983; Tao and Morrison, 1989; Tao et al., 1993), in activation of complement (Nikolova and Russell, 1995; Nikolova et al., 1994; Nose and Wigzell, 1983; Tao and Morrison, 1989; Tao et al., 1993; Zhang and Lachmann, 1994) and opsonization of antigens for phagocytosis (Tao and Morrison, 1989).

Polymorphism in glycosylation of immunoglobulin molecules has functional and pathologic implications. For example, differences in IgG galactosylation could account for the variable interactions with Fcγ RIII in vitro (Hadley et al., 1995). These findings reflect a general phenomenon, glycosylation of IgG may diversify antibody function; antibodies with high levels of terminal galactose on the *N*-linked glycans are tailored for opsonization (Hadley et al., 1995). The possible physiological function of differential glycosylation is further supported by observation that glycosylation pattern of IgG changes during immune response (Axford, 1997; Murray and Brown, 1988). Polymorphism of *O*- and *N*-linked glycosylation of IgA1 affects clearance from the circulation, removal of immune com-

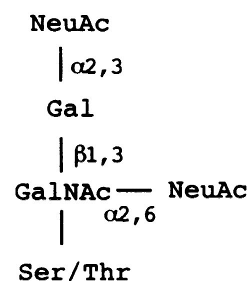

Fig. 2. Structure of the largest oligosaccharide chain found in the hinge region of normal serum IgA1 (Baenziger and Kornfeld, 1974; Field et al., 1989; Mattu et al., 1998).

plexes, complement activation, and interactions with bacteria (Baenziger and Kornfeld, 1974; Baenziger and Fiete, 1980; Basset et al., 1999; Chuang and Morrison, 1997; Grossetete et al., 1998; Mestecky et al., 1995; Nikolova et al., 1994; Rifai et al., 2000; Wold et al., 1990, 1994; Zhang and Lachmann, 1994).

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry is a technique of choice for studies of glycoproteins and glycopeptides (Rahbek-Nielsen et al., 1997; Sutton et al., 1994). MALDI-TOF mass spectrometry was used for estimating the number of *O*-linked oligosaccharides in isolated tryptic fragments containing serum IgA1 hinge region (Iwase et al., 1996). The same isolation procedure combined with either MALDI-TOF or electrospray ionization mass spectrometry detection was recently applied to the analyses of IgA1 hinge glycopeptides in IgA nephropathy (Hiki et al., 1998; Odani et al., 2000). The approach required a relatively large amount of IgA1 protein (about 1 mg) for the cleavage and following purification of the tryptic fragment containing the hinge region. In this study, the hinge region of IgA1 myeloma proteins was analyzed by MALDI-TOF mass spectrometry after site-specific cleavage of a small amount of desialylated IgA1 protein (1–4 μg) with two bacterial IgA1 proteases, without need for purification of IgA1 fragments. In addition to the low consumption of the sample, this approach provided mass spectra that readily allowed interpretation of the heterogeneity of glycosylation of the hinge region of IgA1 proteins.

2. Materials and methods

2.1. IgA samples

Monoclonal IgA1 myeloma proteins were isolated from serum or plasma of patients with multiple myeloma as described earlier (Mestecky and Kilian, 1985). Briefly, serum or plasma samples were precipitated with ammonium sulfate (50% saturation); dissolved and dialyzed precipitates were fractionated by ion-exchange chromatography on DEAE-cellulose, affinity chromatography on Jacalin-agarose (Sigma Chemical Company, St. Louis, MO, USA) and size-exclusion chromatography on columns of Sephadex G-200 or Ultrogel AcA22 (Mestecky and Kilian, 1985). Staphylococcal protein G immobilized on agarose (Sigma) was used to remove traces of IgG. Serum IgA1 was isolated from a normal healthy donor using Jacalin-agarose. Purity of the preparations was determined by Western blots and immunoelectrophoresis using polyvalent reagents against IgA, IgG, and human serum (Biosource Int., Inc., Camarillo, CA). The molecular forms of the IgA1 proteins (polymers and monomers) were determined by size-exclusion chromatography, SDS-polyacrylamide gel electrophoresis, and by the presence of the J-chain (Mestecky and Kilian, 1985). To confirm the presence of GalNAc that is characteristic for *O*-glycans in IgA1 hinge region, the monosaccharides from purified IgA1 or its Fab fragment were assayed as trifluoroacetates of methyl-glycosides by gas chromatography (Tomana et al., 1984). The analyses were performed with a Hewlett-Packard model 5890 gas chromatograph equipped with a 25 m fused silica (0.22 mm i.d.) OV-1701 WCOT column (Chrompack, Bridgewater, NJ), electron capture detector and a HP model 3396 integrator.

2.2. IgA1 proteases

IgA1 proteases from *Clostridium ramosum* AK183, *Haemophilus influenzae* HK393, and *Neisseria gonorrhoeae* HF48 (abbreviated by the strain names AK183, HK393, and HF48, respectively) were purified from media after growth of the particular microorganism as described before (Kilian et al., 1996; Reinholdt et al., 1990). The cleavage sites of individual IgA1 proteases in the IgA1 heavy chain are shown in Fig. 1. Enzymatically desialylated (Tomana et al., 1997) IgA1 protein (1–4 μg) was incubated overnight in 10 μl phosphate buffer saline (PBS) buffer (pH 7.4) with 1 μl of the respective IgA1 protease preparation at 37°C (Kilian et al., 1980). After cleavage, the sample was reduced with DTT (5 μl of 2 mg/ml solution, 3 h at room temperature) and immediately analyzed by MALDI-TOF mass spectrometry. To release the hinge region, the IgA1 proteins were incubated with two IgA1 proteases, AK183 and HF48; the sample was then reduced with DTT and desalted using ZipTips C-18 (Millipore, Bedford, MA, US) as recommended by the manufacturer. The eluted sample was then analyzed by MALDI-TOF mass spectrometry.

2.3. MALDI-TOF mass spectrometry and sample preparation

Samples were analyzed in the positive mode on a Voyager Elite mass spectrometer with delayed extraction technology (PerSeptive Biosystems, Framingham, MA). The acceleration voltage was set at 25 kV, and 50–100 laser shots were summed. Sinapinic acid (Aldrich, Milwaukee, WI, US) dissolved in acetonitrile:0.1% trifluoroacetic acid (1:1, v/v) was used as a matrix for protein samples. The mass spectrometer was calibrated with bovine serum albumin (BSA) or horse skeletal apomyoglobin (Sigma), depending on the expected sample mass range. Samples were either diluted 1:10 or 1:1 with matrix, and 1 μl was pipetted onto a smooth plate.

The samples of the released hinge region glycopeptides were desalted using ZipTips, then mixed (1:1, v/v)

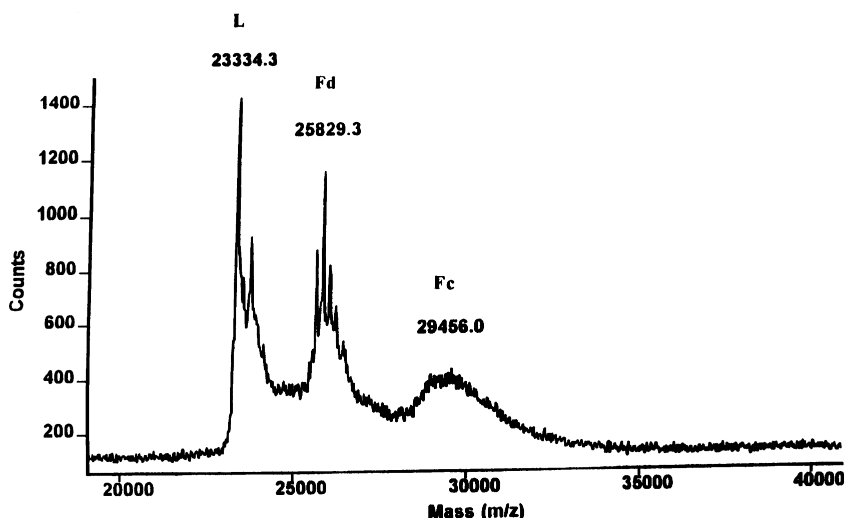

Fig. 3. MALDI-TOF MS spectrum of IgA1 (Kni) cleaved with the HK393 IgA1 protease. The sample was reduced with DTT. L, Fd, and Fc in the spectrum mark ions originating from light chain, Fd fragment and Fc fragment of α chain, respectively.

with a saturated solution of 2,5-dihydrobenzoic acid in acetonitrile. Samples were analyzed as stated earlier, except the acceleration voltage was set at 20 kV, and the instrument was calibrated using neurotensin and angiotensin for peptide samples.

3. Results and discussion

A monomeric IgA1 myeloma protein (Kni) was digested with three IgA1 proteases cleaving at the N-terminal, middle, and C-terminal parts of the hinge region (AK183, HK393, and HF48) to generate the respective Fab fragments for mass spectrometric analyses. MALDI-TOF mass spectra of the nonreduced samples contained singly- and doubly-charged molecular ions (for example, m/z values of 48 590 and 24 346, respectively, for Fab fragment of IgA1 generated with HK393 protease). Site specificity of cleavage by the three IgA1 proteases earlier determined by amino acid sequencing of the N-termini of the respective Fc fragments was confirmed by calculating the molecular masses of the released Fab fragments.

In addition, reduction of the Fab fragments under the acidic conditions of the matrix solution resulted in separation of L chains and Fd fragments of α1 chains normally linked by disulfide bonds, thus allowing more precise determination of molecular masses by MALDI-TOF mass spectrometry. The IgA1 myeloma protein (Kni) was cleaved with HK393 IgA1 protease and reduced before analysis. The three major singly charged ions observed in the spectra of the reduced samples were interpreted as L chain, Fd and Fc fragments of the α1 chain (Fig. 3). L chain was detected as an ion with a mean m/z value of 23 334. The width of the peak with the average m/z value 29 456 corresponding to Fc fragment of the α1 chain indicated considerable heterogeneity. This is consistent with the observed heterogeneity of *N*-linked oligosaccharide chains present on IgA proteins (Endo et al., 1994). The Fd fragment displayed several distinct peaks that corresponded to fragments differing in *O*-linked oligosaccharides; mean m/z value of the major ion was 25 829. Masses of both Fc and Fd fragments indicated average molecular mass of the α1 chain to be 55 283 Da, which was consistent with that determined by SDS polyacrylamide electrophoresis (56–59 kDa).

Major glycosylation variants of the Fd fragment of this monoclonal IgA1 protein (not treated with neuraminidase) could be interpreted by using the theoretical molecular masses for the sugar residues found in *O*-linked oligosaccharides of human myeloma proteins (GalNAc 203, Gal 162, NeuAc 291). Four major glycosylation variants of the Fd fragment were observed (major ion with m/z value 25 829; ions with addition of GalNAc (theoretical 203 mass units; measured 207); GalNAc-Gal (theoretical 365 mass units; measured 381); and GalNAc-Gal-NeuAc (theoretical 656 mass units; measured 652), respectively). These data also indicated that hinge region glycans in the Fd fragment of this IgA1 myeloma protein were not extensively sialylated, in agreement with results of other analyses. When the Fd fragment was further cleaved with AK183 IgA1 protease, the enzyme that releases the residual hinge region, only one major ion for Fd fragment was observed. This finding confirmed that the heterogeneity described above originated from a diversity in the glycosylation of the hinge region.

The mass range of the observed ions should allow determination of the actual mass with an error of about 13–15 mass units considering the average instrument error of 0.05%. Thus, the assignment of the differences

in masses to a different glycan composition should not be theoretically problematic, considering the differences in masses of Gal and GalNAc (162 and 204 mass units, respectively). However, the assignment of glycan variants was complicated due to a heterogeneity of the samples that could not be readily resolved at the range of m/z values around 20 000–30 000. It was, therefore, important to reduce the size of the analyzed ions. We have made an effort to release hinge region glycopeptide from a desialylated IgA1 by using a combination of two proteases (e.g. IgA1 protease and trypsin or two IgA1 proteases). While trypsin generated many fragments and thus increased background and generated non-hinge region ions in the spectrum, the use of two IgA1 proteases was more productive. Although the cleavage of the IgA1 protein with two different IgA1 proteases (AK183 and HF48) was verified by both SDS-PAGE and MALDI-TOF mass spectrometry, we were not able to detect the hinge glycopeptides in the mass spectra. However, after desalting the digested samples on reverse-phase microcolumns (ZipTip, Millipore), we observed signals of the hinge region glycopeptides. In the samples eluted from the microcolumns, we detected a series of ions with m/z values of 1562, 1766, 1927, 2131, 2293 and 2497 that were absent in controls and whose molecular masses matched those expected for hinge region glycopeptides (Fig. 4). Additional ions, apparently Na^+ and K^+ adducts, respectively, of the hinge region glycopeptides were also observed in the spectrum. The nonglycosylated hinge region peptide (calculated average molecular mass of $[M+H]^+$ ion 1561 Da; observed mass 1562 Da) was present, as well as a mixture of glycopeptides carrying 1, 2, or 3 O-linked glycans. This heterogeneity was further increased by the presence of variable numbers of Gal residues (Table 1). As evident from the Fig. 4 and Table 1, the interpretation of these mass spectra was more straightforward as compared with the spectra of Fd fragments.

An open question is the precise localization of the glycosylated sites in various glycosylation variants. For example, is the GalNAc residue on the hinge peptide with single GalNAc attached to a specific Ser/Thr residue or is the site variable and thus the glycopeptide is present as a population of peptides with the same amino acid and monosaccharide composition but differing in the site of GalNAc attachment? Although the analyses of Fd fragments cleaved by various IgA1 proteases may be useful in this respect, the relatively wide width of the signals seemed to limit the extent of data interpretation. We attempted to use HK393 protease that cleaves the hinge region between IgA1 protease AK183 and HF48 cleavage sites, in combination with one of the other two proteases at a time. This approach would provide additional information on localization of O-glycans. Mass spectra, however, did not show presence of any ions derived from the hinge region. It thus appears that further studies and development of alternative approaches will be required to resolve the complex issue of localization of the O-glycans.

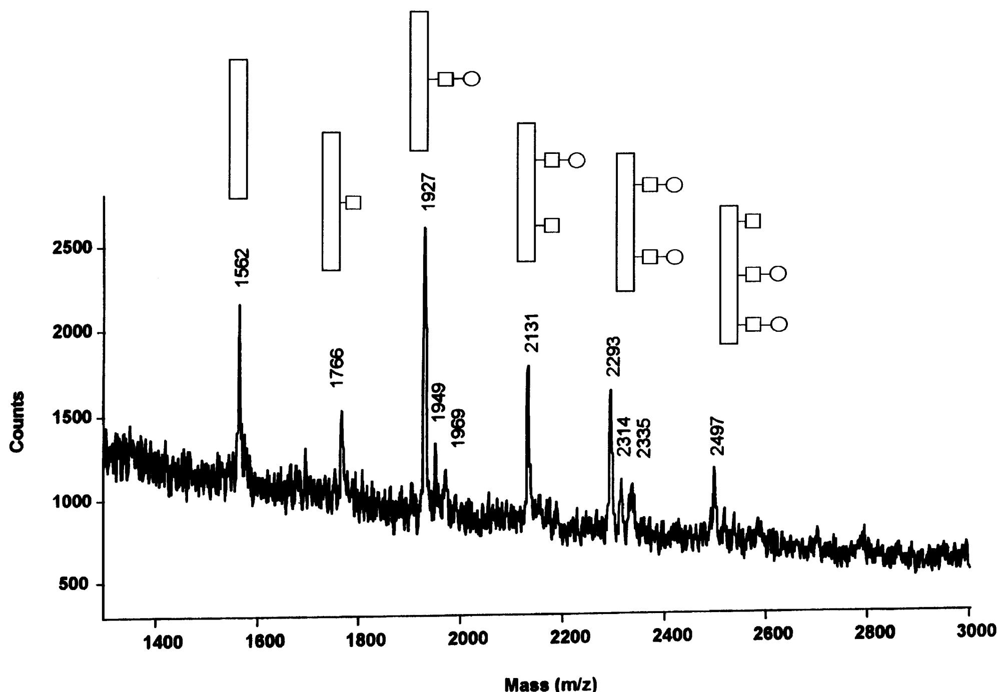

Fig. 4. Hinge region of desialylated IgA1 (Kni) released by two IgA1 proteases (AK183 and HF48). The schemas above the MALDI-TOF MS spectrum depict the observed glycosylation variants (rectangle, hinge peptide; square, GalNAc; circle, Gal).

Table 1
Glycosylation variants of the hinge region glycopeptides released from desialylated IgA1 (Kni) myeloma protein using AK183 and HF48 IgA1 proteases[a]

Theoretical average mass of $[M+H]^+$ (Da)	Measured mass of $[M+H]^+$ (Da)	Glycosylation variant of the hinge peptide (Val222-Pro237)	
		Number of GalNAc residues	Number of Gal residues
1561	1562	0	0
1764	1766	1	0
1926	1927	1	1
2129	2131	2	1
2291	2293	2	2
2495	2497	3	2

[a] Theoretical mass of $[M+H]^+$ was calculated using average values.

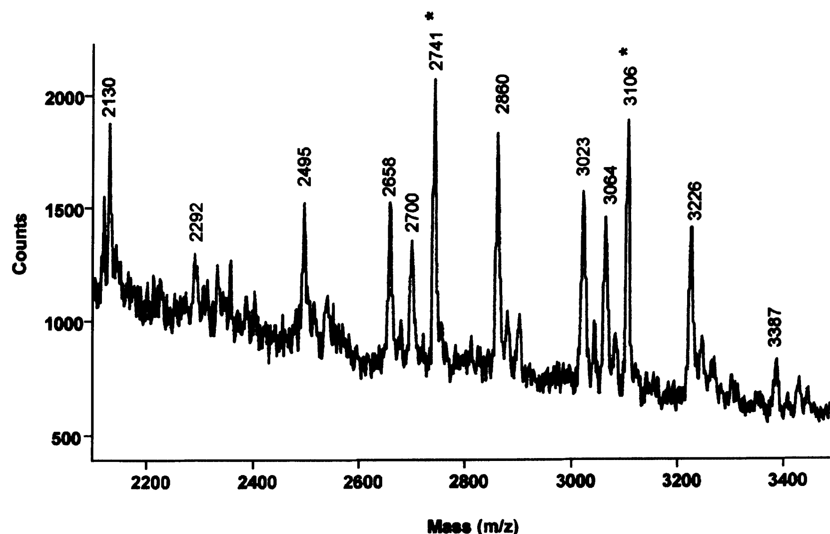

Fig. 5. Hinge region of desialylated normal serum IgA1 released by two IgA1 proteases (AK183 and HF48). IgA1 was desialylated before the proteolytic digest. Two ions (*m/z* 2741 and 3106) were also detected in a control sample not cleaved with IgA1 proteases and do not belong to the hinge region glycopeptide series (*).

After analysis of the hinge region from the monoclonal myeloma IgA1 protein, we applied the same procedure to normal polyclonal serum IgA1. Hinge region from this IgA1 preparation was released by the same procedures as described above (e.g. using IgA1 proteases AK183 and HF48). A series of ions ranging from *m/z* of 2130–3387 was observed in the spectrum (Fig. 5). These ions corresponded to hinge region glycopeptides with 2, 3, 4, and 5 GalNAc residues and variable numbers of Gal residues (Table 2). Two ions (*m/z* 2741 and 3106), detected also in a control sample not cleaved with IgA1 proteases, did not belong to the hinge region glycopeptide series (Fig. 5). In this sample, nonglycosylated hinge region peptide was not observed, unlike in a hinge region preparation from IgA1 myeloma protein (Fig. 4, Table 1). Furthermore, the spectrum of glycosylation variants in the hinge region preparation from the myeloma IgA1 protein was different compared with that from normal serum IgA1. The latter showed variants with more *O*-linked glycans attached and no nonglycosylated peptide. It is, therefore, apparent that biosynthesis of *O*-linked glycans in the hinge region of IgA1 is regulated, both in terms of number of glycans attached and their composition, and that the glycosylation pattern can change in a disease such as multiple myeloma. It is also unclear whether the IgA1 proteins with relatively lower heterogeneity of *O*-glycans exhibit correspondingly lower heterogeneity of *N*-linked glycans, and vice versa. If so, one would expect a slower clearance from the circulation (via the asialoglycoprotein receptor in the liver) of IgA1 proteins with lower number of glycans. It would be of interest to study this topic of glycosylation polymorphism and heterogeneity using antigen-specific IgA1 antibodies.

Deficiencies of some monosaccharides, especially Gal, have been described in several human diseases such as rheumatoid arthritis, inflammatory bowel dis-

ease, Tn syndrome, Sjögren's syndrome, and IgA nephropathy (Allen et al., 1999, 1995, 1998; Andre et al., 1990; Couser, 1999; Dueymes et al., 1995; Hiki et al., 1995, 1999; Julian et al., 1999; Malhotra et al., 1995; Mestecky and Tomana, 1997; Mestecky et al., 1993; Rademacher et al., 1988, 1994; Thurnher et al., 1992, 1993; Tomana, 1996; Tomana et al., 1997, 1999, 1994, 1988b, 1992; Tsuchiya et al., 1993). Role of glycans in autoimmune disease was demonstrated using experimental genetic remodeling of protein glycosylation. Mice with a mutation inactivating the gene encoding α-mannosidase II (enzyme regulating the hybrid to complex branching pattern of *N*-linked glycans) developed a systemic autoimmune disease similar to human systemic lupus erythematosus (Chui et al., 2001).

Aberrantly glycosylated glycoproteins display altered functions, distribution in various body fluids, and decreased or increased reactivity with cellular receptors and other proteins (Baenziger and Fiete, 1980; Mestecky et al., 1995; Monteiro et al., 1990, 1995; Nikolova et al., 1994; Nose and Wigzell, 1983; Stockert, 1995; Stockert et al., 1982; Tao and Morrison, 1989; Tomana et al., 1988a, 1985; Wold et al., 1990, 1994). Furthermore, enzymatic removal of the IgA glycans resulted in activation of the alternative complement pathway (Nikolova et al., 1994; Russell and Mansa, 1989; Zhang and Lachmann, 1994). The essential role of glycans in complement activation was most dramatically demonstrated on Gal-deficient IgG (Abadeh et al., 1997; Malhotra et al., 1995), GlcNAc as a terminal carbohydrate in Gal-deficient IgG binds mannose-binding lectin, because of the structural homology of mannose and GlcNAc. Thus, Gal-deficiency of IgG may lead to complement activation with subsequent local inflammatory consequences as seen in the afflicted joints of rheumatoid arthritis patients.

Glycosylation of glycoproteins is mediated by a large family of enzymes — glycosyltransferases — specific for individual carbohydrates (Brockhausen, 1999; Furukawa and Sato, 1999; Schachter and Roseman, 1980). These enzymes catalyze the transfer of intracellular monosaccharides (e.g. UDP-Gal for galactosyltransferase) to a suitable acceptor (e.g. GalNAc or GlcNAc) on specific glycoproteins. The decreased levels, absence or structural modification of glycosyltransferases with aberrant kinetic properties result in reduced or absent glycosylation by the corresponding monosaccharide. Functional deficiency of such enzymes is more frequent than the deletion of an encoding gene. For example, the functional deficiency of β1,4 galactosyltransferase in IgG-producing cells appears to be responsible for deficiency of Gal in *N*-linked side chains of IgG molecules isolated from sera of patients with rheumatoid arthritis or inflammatory bowel disease (Axford et al., 1987; Dube et al., 1990; Furukawa et al., 1990; Furukawa and Sato, 1999; Malhotra et al., 1995; Tomana, 1996; Tomana et al., 1988b). In a disease called Tn syndrome, *O*-glycans are deficient in Gal, resulting in a permanent mixed-field polyagglutinability (Thurnher et al., 1992). This defect is caused by permanent repression of a functional allele for β1,3 galactosyltransferase (Thurnher et al., 1993). In IgA nephropathy, a reduced activity of this enzyme (Allen et al., 1997) is likely responsible for Gal deficiency of IgA1 (Allen et al., 1999, 1995; Andre et al., 1990; Coppo and Emancipator, 1994; Hiki et al., 1995, 1999; Mestecky et al., 1993; Tomana et al., 1997). Gal-deficient IgA1 is recognized by antiglycan antibodies (IgG, IgA1, IgM) and circulating immune complexes are formed (Tomana et al., 1997, 1999, 2000). These immune complexes are thought to be pathogenic due to their reduced clearance and increased affinity for the kidney (Julian et al., 2000, 1999; Mestecky et al., 1995; Mestecky and Tomana, 1997; Mestecky et al., 1993; Novak et al., 2000).

Table 2
Glycosylation variants of the hinge region glycopeptides released from desialylated normal human serum IgA1 using AK183 and HF48 IgA1 proteases[a]

Theoretical average mass of $[M+H]^+$ (Da)	Measured mass of $[M+H]^+$ (Da)	Glycosylation variant of the hinge peptide (Val222-Pro237)	
		Number of GalNAc residues	Number of Gal residues
2129	2130	2	1
2291	2292	2	2
2495	2495	3	2
2657	2658	3	3
2698	2700	4	2
2860	2860	4	3
3022	3023	4	4
3063	3064	5	3
3225	3226	5	4
3387	3387	5	5

[a] Theoretical mass of $[M+H]^+$ was calculated using average values.

Glycosylation of immunoglobulins and polymorphisms and changes in the glycosylation fundamentally affect the physiological functions of immunoglobulins or may be part of pathologic processes in several human diseases. Aberrancies and heterogeneity of *O*-glycans in the hinge region of IgA1 are an example. Other studies convincingly demonstrated that *N*-linked glycan chains of IgA1 display a high degree of heterogeneity (Endo et al., 1994) which in turn influence their reactivities with complement (Nikolova et al., 1994), Fcα receptors expressed on monocytic and myeloid cells (Monteiro et al., 1990), the hepatic asialoglycoprotein receptor (Baenziger and Maynard, 1980; Tomana et al., 1988a, 1985), and lectins (Wold et al., 1994). However, the lack of convenient and highly informative techniques limited progress of the studies on IgA1 *O*-glycosylation. We have, therefore, tested various approaches to overcome the obstacle and developed a procedure using IgA1 proteases and mass spectrometry. This technique demonstrated that *O*-linked glycan chains of IgA1 also display a high degree of heterogeneity even in a single myeloma protein as well as in normal serum IgA1. Further studies will be needed, however, to develop a reliable and sensitive procedure for localization of the attachment sites of the *O*-glycans in the hinge region of various hinge region glycoforms. The high sensitivity and specificity of this approach might facilitate studies of aberrancies of IgA1 *O*-glycosylation in diseases such as IgA nephropathy.

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