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Estimation of the Number of O-Linked Oligosaccharides per Heavy Chain of Human Serum IgA1 by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOFMS) Analysis of the Hinge Glycopeptide[1]

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In our previous study, gas-phase hydrazinolysis was used to analyze the glycoform of the O-linked oligosaccharide of human serum IgA1. All O-linked oligosaccharide chains are known to be present in the hinge portion. However, the number of O-linked oligosaccharide chains on IgA1 remained unclear. In order to determine the number of linked sugar chains, we applied matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) to the hinge glycopeptide prepared from human serum IgA1. MALDI-TOFMS did not show clear peaks, probably due to the microheterogeneity of the structure of each sugar chain. However, elimination of peripheral sialic acid and galactose residues by sequential treatment with neuraminidase and β-galactosidase gave clear mass spectra with several sharp peaks. On the basis of these spectra, we conclude that IgA1 prepared from normal human serum carries different numbers of sugar chains. There are two major populations, one contains five GalNAc residues and the other four GalNAc residues. On the other hand, the hinge glycopeptide prepared from myeloma IgA1 was composed mainly of one population containing four GalNAc residues. Earlier, we reported incomplete glycosylation of IgA1 isolated from the serum of an IgA1 myeloma patient. In this experiment, the presence of four O-linked oligosaccharides per heavy chain of IgA1 from a myeloma patient was found. The reason why only four out of five sites on the hinge glycopeptide were fully glycosylated in the IgA1 from the IgA1 myeloma patient is not clear.

Key words: IgA1, IgA nephropathy, IgA1 myeloma, MALDI-TOFMS, O-linked oligosaccharide.

Human serum IgA1 is one of the most exceptional glycoproteins among human serum glycoproteins because it has five O-linked oligosaccharides in its hinge portion in addition to two N-linked carbohydrate chains in its structure (*1-3*). In our previous study, the glycoforms of the O-linked oligosaccharides of IgA1s from a healthy control and IgA1 myeloma patients was analyzed using gas-phase hydrazinolysis. Three glycoforms of IgA1 in myeloma patients and only one glycoform in healthy individuals were found, although it was later found out that the IgA1 from the healthy controls was composed of heterogeneous components having mutually different glycoforms (*4, 5*). All the results were expressed qualitatively as the relative content of each oligosaccharide. As another example, analysis of the O-linked oligosaccharide released from the IgA1 of an IgA nephropathy patient indicated an increase in the proportion of asialo-Galβ1,3GalNAc in the IgA1 hinge region (*6*). Our results and other reports suggested the presence of a molecule having incompletely glycosylated O-linked oligosaccharide(s) in the IgA1 hinge region in some IgA nephropathy patients (*7-10*). IgA1 purified from the serum of a healthy control was also subfractionated depending upon different affinities toward jacalin and different heat stabilities. The glycoforms of the O-linked oligosaccharides in the hinge region of these IgA1 subfractions also differed from each other (*5*).

Meanwhile, Baenziger and Kornfeld reported that one of five possible glycosylation sites on the α1 chain showed only a GalNAc residue difference from other sites (*2*). They suggested that there might be one specific site which is difficult to glycosylate.

In this experiment, the number of O-linked sugar chains per heavy chain of IgA1 was estimated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) using a hinge glycopeptide. Mass spectra of the hinge glycopeptide first showed the presence of prominent heterogeneous components. However, sequential removal of peripheral sialic acid and galactose

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residues resulted in simple spectra which allowed the number of attached sugar chains to be determined by counting of the remaining *N*-acetylgalactosamine residues.

EXPERIMENTAL PROCEDURE

Materials—The following materials were purchased from the sources indicated: IgA1 was from Chemicon International (Ei Segundo, CA, USA); PD-10 from Pharmacia Biotech AB (Uppsala, Sweden); 4-vinyl pyridine from Ardrich Chem. (Milwaukee, WI, USA); jacalin-agarose from Vector Laboratories (Burlingame, USA); neuraminidase from *Arthrobacter ureafaciens* from Boehringer Mannheim (Germany); β-galactosidase from bovine testes and trypsin from Sigma Chem. (St. Louis, USA); and α-*N*-acetylgalactosaminidase from *Acremonium* sp. from Seikagaku (Tokyo).

Preparation of IgA1 by Jacalin-Agarose Affinity Chromatography—If not otherwise stated, the jacalin-agarose affinity chromatography was carried out at room temperature. Ten milliliters of serum was applied to a jacalin column (10 ml) and then the column was thoroughly washed with 0.1 M Tris-HCl buffer, pH 7.6, containing 0.02% sodium azide. The thoroughly washed column was first eluted with 0.8 M glucose (50 ml) and then with 0.8 M galactose (50 ml) in the above buffer. The absorbance of the eluate at 280 nm was read to detect the protein. Nonspecifically bound materials were eluted with glucose. The IgA1 subfractions obtained on stepwise elution with galactose were precipitated with 50% ammonium sulfate, and the precipitated IgA1 was dialyzed against distilled water and then lyophilized.

Preparation of the Hinge Glycopeptide from Human Serum IgA1—About 1 mg of IgA1 was first desalted on a PD-10 column. The desalted sample was dissolved in 500 μl of 0.4 M Tris-HCl buffer, pH 8.6, containing 6 M guanidine-HCl and 0.2 M EDTA. To dissociate the disulfide linkage, 5.4 μl of a dithiothreitol solution (200 mg/ml) was added with stirring. After heating at 50°C for 4 h, 1.6 μl of 4-vinyl pyridine was added and the reaction mixture was allowed to stand for 90 min at room temperature. The reaction was terminated by the addition of 50 μl of 2.0 M formic acid.

Preparation of the S-Pyridylethylated α1 Chain from IgA1—The α-chain dissociated as described above was fractionated by HPLC on a Cosmosil 5C4-300 column (Nacalai Tesque; 4.6×150 mm) in a Shimadzu LC-4A. Elution was carried out with a linear gradient, 60 min, of from 10 to 90% acetonitrile in 0.1% trifluoroacetic acid (TFA). The material eluted at the peak position around 30 min was collected and concentrated.

Preparation of the Hinge Glycopeptide from a Trypsin Digest of the Heavy Chain—About 0.5 mg of heavy chain was dissolved in 160 μl of 50 mM Tris-HCl buffer, pH 8.0, containing 2.0 M urea. Twenty microliters of a trypsin solution (10 μg trypsin/20 μl of the above buffer) and 20 μl of 0.1 M $CaCl_2$ were added, and then the reaction mixture was incubated overnight at 37°C. The trypsin digest was made up to 1 ml by adding 0.8 ml of 0.175 M Tris-HCl buffer, pH 7.6. The sample was applied to a jacalin-agarose column (2 ml) and the passed fraction was eluted with 6 ml of the above buffer. After further washing the column with 6 ml of the buffer, the hinge glycopeptide fraction was eluted with 6 ml of 0.1 M melibiose in the buffer. Purification of the glycopeptide by HPLC was carried out on a Cosmosil 5C18-300 column (Nacalai Tesque; 4.6×150 mm). Elution was carried out with a linear gradient, 60 min, of from 0 to 90% acetonitrile in 0.1% TFA. Detection was performed by UV absorption monitoring at 220 nm. The material eluted at the peak position of the hinge glycopeptide around 23 min was collected and concentrated.

Treatment of the Glycopeptide with Exo-Glycosidases—The purified glycopeptide was dissolved in 95 μl of 0.2 M sodium acetate buffer, pH 5.0. Five microliters of neuraminidase from *Arthrobacter ureafaciens* (1 U/100 μl) was added to the solution followed by incubation at 37°C overnight. The de-sialylated glycopeptide was purified by HPLC under the gradient conditions of 90% acetonitrile to 0% in 0.1% TFA. The purified de-sialylated glycopeptide was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS). The asialo-glycopeptide was then treated with β-galactosidase as follows. The sample was dissolved in 30 μl of 0.2 M acetate buffer, pH 5.0, and then 20 μl of β-galactosidase from bovine testes (1.0 U/ml) was added to the sample. The reaction mixture was incubated overnight at room temperature. HPLC purification of the asialo-, agalacto-glycopeptide was carried out as described above. A part of the product was analyzed by MALDI-TOFMS.

For final treatment of the glycopeptide with α-*N*-acetylgalactosaminidase, the asialo-, agalacto-glycopeptide was dissolved in 83 μl of 50 mM sodium citrate buffer, pH 4.5, and then to the solution was added 17 μl of α-*N*-acetylgalactosaminidase from *Acromonium* sp. (13 U/ml). Incubation of the reaction mixture was performed overnight at room temperature. Purification of the deglycosylated peptide was carried out by HPLC on a Cosmosil 5C18-300 column (4.6×150 mm) under the same conditions as described above. The purified deglycosylated peptide was analyzed by MALDI-TOFMS.

MALDI-TOFMS Analysis of the Hinge Glycopeptide and Glycosidase-Treated Glycopeptide—The hinge glycopeptide was analyzed by MALDI-TOFMS in the negative ion mode to suppress the production of the adduct ion. The mass spectrometer used in this work was a Finnigan Lasermat (Finnigan MAT, Hemel Hempstead, UK). The matrix used was α-cyano-4-hydroxy cinnamic acid (10 mg/ml) in a 70% acetonitrile solution. The mass spectra were obtained with almost the same laser power and 10 shots were summed. The accuracy of the mass spectra was less than 0.3% error, except in the case of the deglycosylated glycopeptide (<0.6% error). TOFMS analysis of the glycopeptide sequentially treated with neuraminidase, β-galactosidase, and α-*N*-acetylgalactosaminidase was also carried out under the above conditions.

RESULTS AND DISCUSSION

Figure 1 shows the HPLC profiles of the hinge glycopeptide prepared from a trypsin digest of human serum IgA1 and exo-glycosidase-treated hinge glycopeptides. The detailed conditions were given under "EXPERIMENTAL PROCEDURE." Figure 2A shows the results of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) analysis of the purified hinge glycopep-

tide. A very broad peak corresponding to a molecular mass of around 5,500 was obtained. The results obtained are due to the attached heterogeneous sugar chains, as reported previously (*5*). Figure 2, B, C, and D, shows the mass spectra of the asialo-glycopeptide, asialo-, agalacto-glycopeptide, and deglycosylated peptide, respectively. Neuraminidase treatment of the glycopeptide reduced the heterogeneity but still resulted in a slightly broad peak. Next, the removal of galactose residues from the asialoglycopeptide produced two major peaks, which corresponded to a component containing five GalNAc residues (Observed: $m/z=4,499$; Calculated: 4,491) and a partially glycosylated component having four GalNAc residues (Observed: $m/z=4,294$; Calculated: 4,288). A minor peak corresponded to a component containing three GalNAc residues (Observed: $m/z=4,088$; Calculated: 4,085). Removal of the GalNAc residues by treatment with α-*N*-acetylgalactosaminidase produced a single core peptide (Observed: $m/z=3,486$; Calculated: 3,476). The peptide portion consisted of 33 amino acids. The presumed amino acid sequence of the hinge glycopeptide of IgA1 and the typical structural change resulting from sequential glycosidase treatment of the glycopeptide are summarized in Fig. 3. The cleavage of the seryl cysteine bond at the C-terminal position in the hinge glycopeptide by trypsin was unusual, and it might be due to the presence of a neighboring unusual structure, two sequential pyridylethylated cysteine residues.

A similar experiment was carried out on myeloma IgA1, as shown in Fig. 4. Based on the established quantitative significance of the relative intensities of peaks in the mass spectra of *N*-glycan glycopeptides, a comparative study of the mass spectra of glycosidase-treated glycopeptides derived from IgA1s of a healthy control and a myeloma patient was carried out (*11*). Differing from that of the IgA1 from the healthy control, the myeloma IgA1 was composed of a major component having four GalNAc residues (Observed: $m/z=4,291$; Calculated: 4,288) (see Figs. 2C and 4C). The molecular weight of the obtained free peptide (Observed: $m/z=3,518$; Calculated: 3,476) was the same as that from normal IgA1 (see Figs. 2D and 4D). Figure 4B shows that the asialo-glycopeptide corresponds to a component having four galactose and four *N*-acetylgalactosamine residues (Observed: $m/z=4,944$; Calculated: 4,936). Thus, most of the sugar chains was fully galactosylated in the myeloma IgA1. On the other hand, Fig. 2B shows two slightly broad peaks corresponding to components bearing four GalNAc and four Gal (Observed: $m/z=4,966$; Calculated: 4,936), and five GalNAc and four Gal (Observed: $m/z=5,172$; Calculated: 5,139), respectively. The major and largest asialo-glycopeptide was not the fully glycosylated

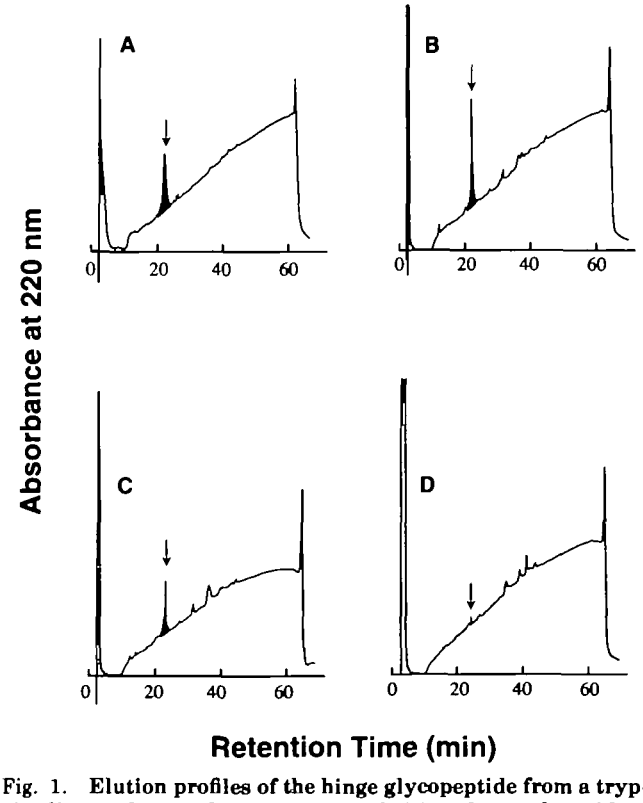

Fig. 1. **Elution profiles of the hinge glycopeptide from a trypsin digest of normal human serum IgA1 and exo-glycosidase treated glycopeptides.** A, glycopeptide prepared from a trypsin digest of human serum IgA1 from a healthy control; B, asialo-glycopeptide; C, asialo-, agalacto-glycopeptide; D, deglycosylated peptide. Preparation of the glycopeptide and the elution conditions on HPLC were described under "EXPERIMENTAL PROCEDURE." Arrows in the figure indicate the elution positions of the glycopeptide and deglycosylated peptide.

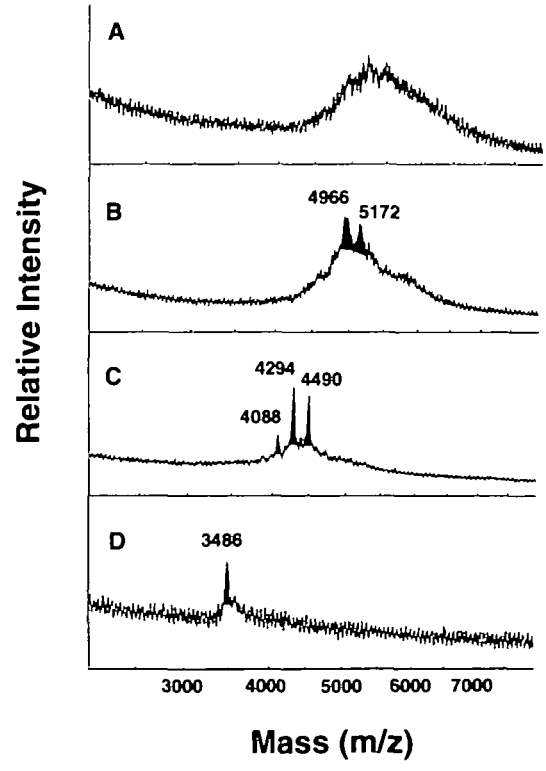

Fig. 2. **MALDI-TOFMS analysis of the hinge glycopeptide from a trypsin digests of normal human serum IgA1 and exo-glycosidase-treated glycopeptides.** A, glycopeptide prepared from a trypsin digest of human serum IgA1 from a healthy control; B, asialo-glycopeptide; C, asialo-, agalacto-glycopeptide; D, deglycosylated peptide. MALDI-TOFMS analysis was carried out under the conditions given under "EXPERIMENTAL PROCEDURE." The m/z value for the major ion is indicated in the figure.

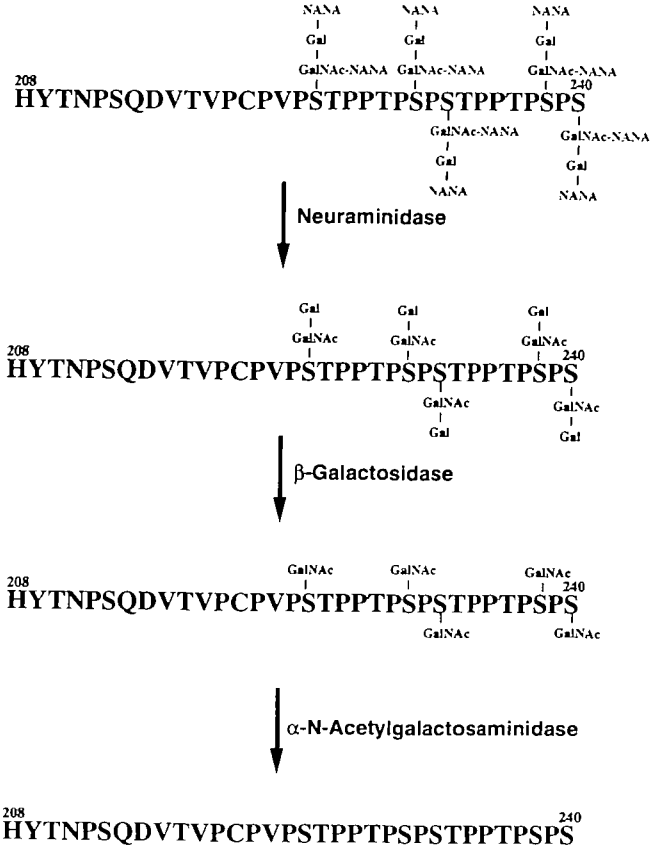

Fig. 3. **Presumed amino acid sequence of the hinge glycopeptide of IgA1 and the typical structural change due to sequential exo-glycosidase treatment of the glycopeptide.** The amino acid sequence of the peptide was deduced from the results of amino acid sequencing. The structure of the carbohydrate chain of the glycopeptide is presented as a presumed fully glycosylated form.

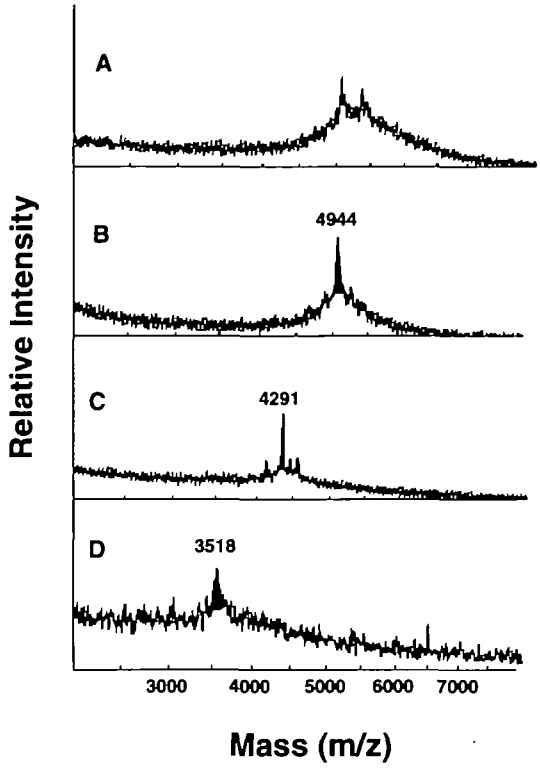

Fig. 4. **MALDI-TOFMS analysis of the hinge glycopeptide from a trypsin digests of IgA1 from an IgA1 myeloma patient and exo-glycosidase-treated glycopeptides.** A, glycopeptide prepared from a trypsin digest of human serum IgA1 from an IgA1 myeloma patient; B, asialo-glycopeptide; C, asialo-, agalacto-glycopeptide; D, deglycosylated peptide. MALDI-TOFMS analysis was carried out under the conditions given under "EXPERIMENTAL PROCEDURE." The m/z value for the major ion is indicated in the figure.

component having five GalNAc and five Gal. This result was consistent with the report by Baenziger and Kornfeld that one of five possible glycosylation sites in the hinge portion of the $\alpha 1$ chain shows only a GalNAc residue difference from the others, Galβ1,3GalNAc, in the *O*-linked sugar chains of IgA1. There might be one specific site which is difficult to *N*-acetylgalactosaminylate and galactosylate.

As an alternate explanation for the above results, two kinds of polypeptide *N*-acetylgalactosaminyltransferase were recently cloned (*12*). Because the specificities for peptide sequences of these transferases were different, a disorder of the balanced expression of these transferases might have occurred in a IgA1 myeloma patient.

O-Linked oligosaccharides in the hinge portion of IgA1 will play a very important role in the stability of human serum IgA1. Jentoft reported that the presence of a GalNAc residue attached to the peptide portion in a mucin-type glycoprotein was most important for stabilization of the peptide conformation (*13*). Therefore, the number of sugar chains on IgA1 will be meaningful in relation to some kinds of disease. We reported recently that heat-labile IgA1 from a healthy control and IgA1 from an IgA nephropathy patient exhibited a relative abundance of incomplete *O*-linked oligosaccharides with respect to their glycoforms. Comparison of the number of *O*-linked oligosaccharides per heavy chain of IgA1 between that from a healthy control and that from an IgA nephropathy patient will be the next step.

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