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Direct Evidence for Decreased Sialylation and Galactosylation of Human Serum IgA1 Fc O-Glycosylated Hinge Peptides in IgA Nephropathy by Mass Spectrometry

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Human serum immunoglobulin IgA1 is produced in bone marrow and interacts with specific cellular receptors that mediate biological events. In this study, we have analyzed the detailed glycoform structure of the human serum IgA1 Fc O-glycosylated hinge region by electrospray ionization liquid mass spectrometry. The IgA1 fragments containing the hinge glycopeptide were separated from 4 IgA nephropathy patient (IgAN) pooled sera, 10 non-IgAN pooled sera with other primary glomerulonephritides, and 5 healthy control subject pooled sera by trypsin treatment and Jacalin affinity chromatography. The molecular weights of IgA1 hinge glycopeptide were estimated using mass spectrometry, and 13 sialo and 8 asialo glycopeptide groups were identified. The results obtained clearly showed a decrease of GalNAc, Gal, and sialic acid in IgAN compared with non-IgAN and normal controls, and those strongly suggested the possibility that the decreased galactosylation and sialylation of the IgA1 hinge result in its glomerular deposition in IgAN.

Human serum IgA1 is one of the exceptional serum glycoproteins because it has O-linked oligosaccharides (O-glycans) in its hinge region (18-mer hinge peptide core (HP) + O-glycans) (1, 2). The majority of serum proteins are glycoproteins, and the sugar side chains linking to the proteins are usually N-linked oligosaccharides (N-glycans). O-glycans basically consists of N-acetyl-galactosamine (GalNAc), galactose (Gal), and sialic acid (neuraminic acid, NANA). The fundamental structure of the O-glycan is the linkage between the α-anomeric carbon atom in GalNAc and the hydroxy group of serine or threonine (2). The structural variety of the sugar side chains in glycoproteins widely observed under physiological conditions is called "microheterogeneity" of the carbohydrate. We have attempted to elucidate the microheterogeneity of O-glycans in the IgA1 hinge region (3–5) because the IgA1 molecule is the predominant subtype to deposit in the glomeruli in IgA nephropathy (IgAN) (6, 7). Mestecky *et al.* (8) and Allen *et al.* (9) have reported a decreased terminal galactose content.

There are many reports on the presence of an incompletely glycosylated O-linked oligosaccharide(s) on the IgA1 hinge region in some of IgAN patients (5, 8, 9, 11–21).

Previously, we succeeded in the specific release of galactosyl β1-3*N*-acetylgalactosamine (Galβ1-3GalNAc) side chains from the IgA1 hinge by residue, which had been found in monoclonal IgA1 by the alkaline degradation method of Baenziger and Kornfeld (1). We have also attempted to investigate the O-glycan side chains in the IgA1 hinge using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOFMS) and faced difficulty because of the presence of sialic acid residues located on the outside of O-glycans (4, 5).

In this study, we have analyzed the native sialo O-glycan glycoform structure in human serum IgA1 hinge region directly using electrospray ionization/liquid chromatography/mass spectrometry (ESI/LC/MS). The results showed decreased sialylation and galactosylation of serum IgA1 Fc hinge glycopeptides in IgAN compared with non-IgAN and normal controls, and strongly suggested that the reduction of sialic acid on the IgA1 hinge region resulted in its glomerular deposition in IgAN.

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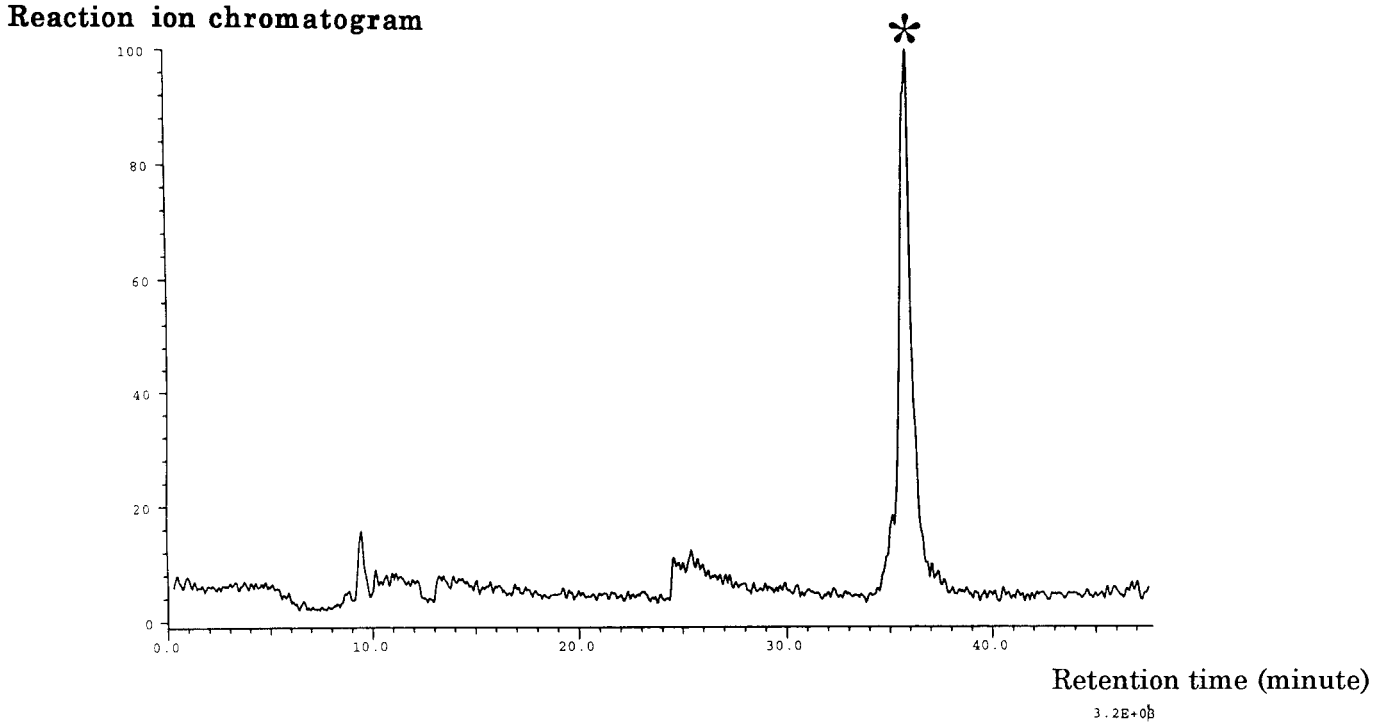

FIG. 1. The total ion chromatogram of purified normal serum IgA1 O-glycan hinge glycopeptide digested by trypsin. The * peak reveals the total ions of purified pooled normal control serum IgA1 Fc hinge peptides as described under Materials and Methods.

MATERIALS AND METHODS

Materials. The following compounds and materials were commercially obtained: Jacalin (a lectin specific for Gal-1,3-GalNAc)-agarose was purchased from Vector Laboratories Inc (Burlingame, CA) (10); PD-10 column from Pharmacia Biotech AB (Uppsala, Sweden); 4-vinyl pyridine from Nacalai Tesque. (Japan); Tripsin (TPCK-treated) from Sigma (St. Louis, MO).

Biological materials. Four patients with biopsy-proven IgAN (mean age, 46 ± 8 years), five healthy individuals (mean age, 45 ± 6 years), and ten patients with other primary glomerulonephritides (mean age, 49 ± 10 years) were the subjects of this study.

Preparation and subfractionation of IgA1 by Jacalin-agarose affinity chromatography. To obtain the subfraction of IgA1, the Jacalin-agarose affinity chromatography was carried out at room temperature (4).

At first, 1 ml of serum was precipitated with 50% ammonium sulfate twice, and the precipitated protein was dialyzed against 0.01 M phosphate buffer solution, pH 7.8 (PBS). Then, the lyophilized protein was applied to a Jacalin column (5 ml), and the column was washed with PBS, containing 0.15 M NaCl. The thoroughly washed column was first eluted with 0.8 M glucose and then with 0.1 M melibiose in the above buffer. The absorbance of the elute at 280 nm was read to detect the protein. The IgA1 subfractions obtained on stepwise elution with melibiose were desalted on a PD-10 column and then lyophilized.

Preparation of hinge glycopeptide from human serum IgA1. The desalted sample was dissolved in 500 ml 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 ml of a dithiothreitol solution (200 mg/ml) was added with stirring.

After heating at 50°C for 4 h, 1.6 ml 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 ml of 2.0 M formic acid. The reaction mixture was desalted on a PD-10 column and then lyophilized.

Preparation of hinge glycopeptide from a trypsin digest. About 0.5 mg of above S-pyridylethylated α1 chain was dissolved in 160 μl of 50 mM Tris–HCl buffer, pH 8.0, containing 2.0 M urea. Twenty microliters of a TPCK-treated 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 2 ml by adding 0.01 M PBS, pH 7.8. 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 of the column with 6 ml of 0.8 M glucose in 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 with 0.5 ml/min flow rate. Detection was performed by UV absorption monitoring at 220 nm. The material eluted at the peak position of the hinge glycopeptide around 20 min was collected and concentrated. The purified glycosylated peptide was analyzed by ESI/LC/MS.

ESI/LC/MS measurement. All of the glycosylated peptides were resolved by reversed-phase high performance liquid chromatography (RP-HPLC) and analyzed by ESI/MS using a TSQ 7000 triple stage quadruple mass spectrometer (Thermoquest, U.S.A.). RP-HPLC was conducted on a Monitor C-18 M column (5 mm, 150 × 4.6 mm ID, Column Engineering, U.S.A.) equilibrated with solvent A (0.03% TFA in H_2O), and eluted with a linear gradient to 50% solvent B (40% acetonitrile, 60% H_2O, 0.02% TFA) during the first 30 min; the column was then washed with 50% B for 20 min, with 100% B for 5 min, then re-equilibrated in 100% A for 20 min; flow rate was 0.3 ml/min. Elution time for above glycopeptide was approximately 35 min. For electrospray ionization, the ionizing energy, current of

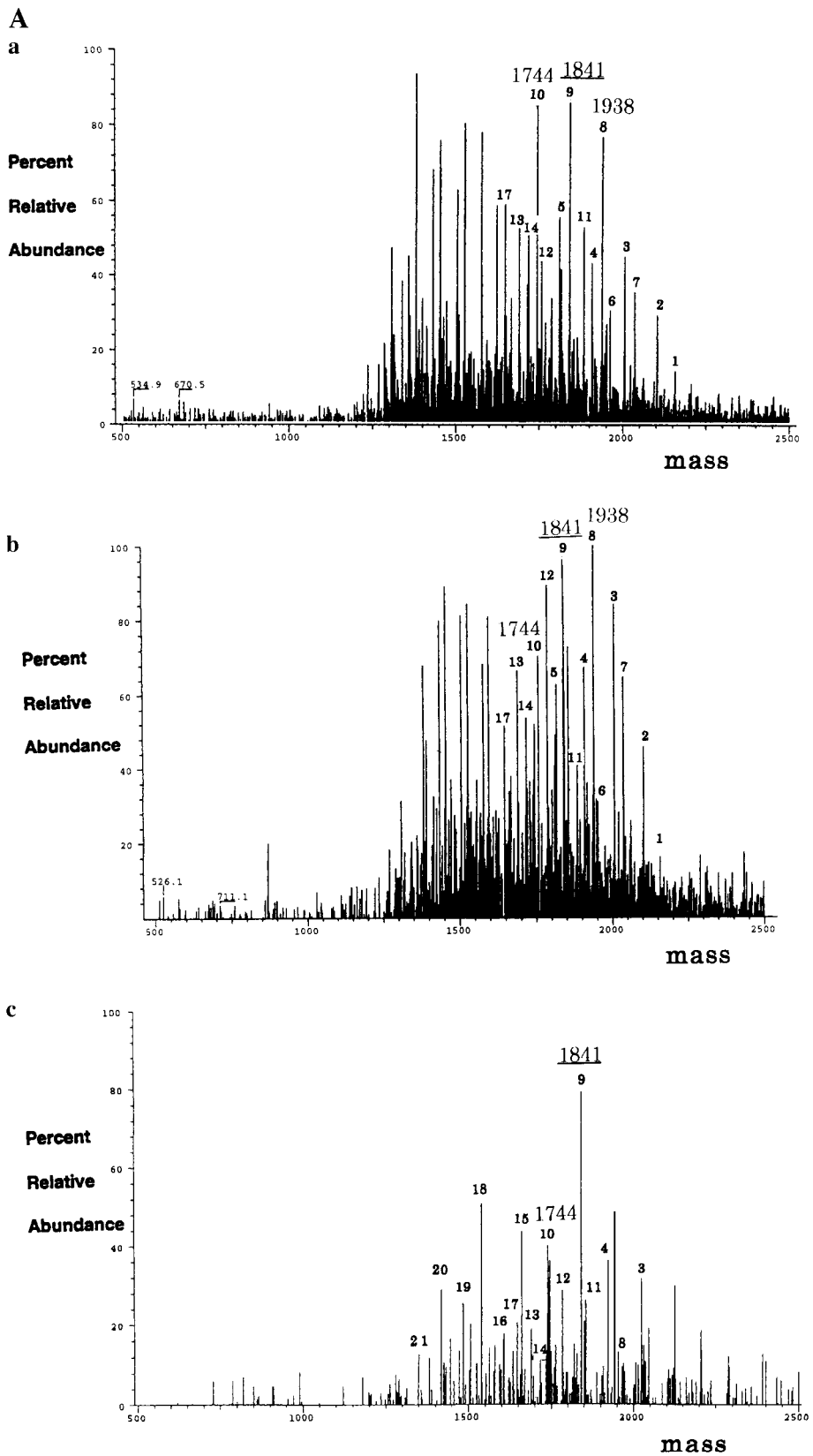

FIG. 2. (A) (a) The multiply charged protonated ion mass spectrum of the * peak in normal serum IgA1 in Fig. 1 by ESI/LC/MS; (b) the multiply charged mass spectrum of pooled non-IgAN serum IgA1; (c) the multiply charged mass spectrum of pooled IgAN serum IgA1. A(a)–A(c) show a *m/z* scan range from 500 to 2500 per 3.8-s scan rate in positive-ion mode. The number of peaks is consistent with the number of peaks shown in Table

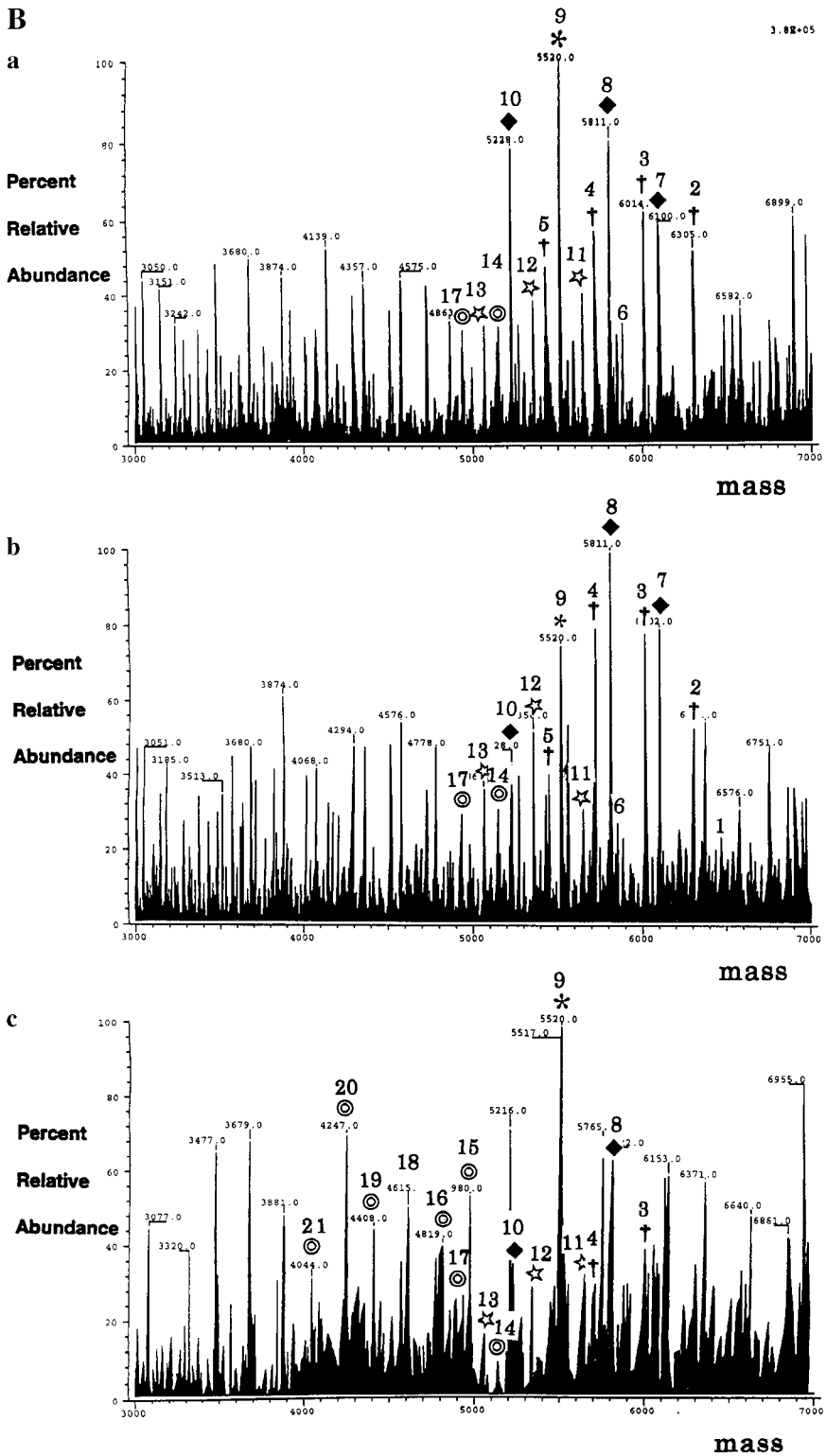

FIG. 2—*Continued*

1. (B) ESI mass spectra of the IgA1 hinge glycopeptide of (a) pooled normal human controls; (b) pooled non-IgAN (with 10 other primary glomerulo-nephritides); (c) pooled 4 primary IgAN after the deconvolution using the BIOMASS deconvolution program in A: † peak, HP + 5GalNAc + 4Gal + nNANA (n = 1 to 4); ◆ peak, HP + 4GalNAc + 4Gal + nNANA (n = 1 to 4) (* peak shows HP + 4GalNAc + 4Gal + 2NANA); ☆ peak, HP + 4GalNAc + 3Gal + n NANA (n = 1 to 3); ◎ peak, HP + nGalNAc (n = 2 to 5) + nGal (n = 1 to 4) (Table 1 shown). Each chromatograms in 2A(a) to A(c) are corresponding to each deconvolution ESI mass spectra in B(a) to (c), respectively.

TABLE 1

Molecular Weights of IgA1 Hinge Glycopeptides

Peak No.	Group	Theoretical MW	Measured MW	Positive ion (3+)	Peak area ratio to HP + 4 GalNAc + 4Gal + 2NANA		
					Control	Non-IgAN	IgAN
			Sialo groups				
1	HP + 5 GalNAc + 5Gal + 4NANA	6468	6465	2156	0.14	0.20	n.d.
2	HP + 5 GalNAc + 4Gal + 4NANA	6306	6307	2103	0.32	0.60	n.d.
3	HP + 5 GalNAc + 4Gal + 3NANA	6014	6014	2006	0.49	0.58	0.39
4	HP + 5 GalNAc + 4Gal + 2NANA	5723	5723	1909	0.65	0.50	0.43
5	HP + 5 GalNAc + 4Gal + 1NANA	5432	5430	1811	0.55	0.63	n.d.
6	HP + 5 GalNAc + 3Gal + 3NANA	5852	5852	1952	0.17	0.35	n.d.
7	HP + 4 GalNAc + 4Gal + 4NANA	6102	6102	2035	0.31	0.62	n.d.
8	HP + 4 GalNAc + 4Gal + 3NANA	5811	5811	1938	0.77	1.02	0.17
9	*HP + 4 GalNAc + 4Gal + 2NANA	5520	5520	1841	1.0	1.0	1.0
10	HP + 4 GalNAc + 4Gal + 1NANA	5228	5228	1744	0.97	0.54	0.31
11	HP + 4 GalNAc + 3Gal + 3NANA	5649	5648	1884	0.34	0.47	0.21
12	HP + 4 GalNAc + 3Gal + 2NANA	5358	5358	1787	0.54	0.80	0.18
13	HP + 4 GalNAc + 3Gal + 1NANA	5066	5066	1690	0.61	0.51	0.10
			Asialo groups				
14	HP + 5 GalNAc + 4Gal	5140	5141	1715	0.30	0.41	0.10
15	HP + 5 GalNAc + 3Gal	4978	4978	1661	n.d.	n.d.	0.60
16	HP + 5 GalNAc + 2Gal	4816	4819	1607	n.d.	n.d.	0.39
17	HP + 4 GalNAc + 4Gal	4937	4936	1646	0.64	0.48	0.31
18	HP + 4 GalNAc + 2Gal	4613	4615	1540	n.d.	n.d.	0.71
19	HP + 3 GalNAc + 2Gal	4410	4409	1471	n.d.	n.d.	0.24
20	HP + 3 GalNAc + 1Gal	4247	4247	1417	n.d.	n.d.	0.43
21	HP + 2 GalNAc + 1Gal	4044	4044	1349	n.d.	n.d.	0.27

Note. n.d., not determined.

spray, and voltage of spray were 72 eV, 1.5 mA, and 4.5 kV, respectively.

Data analyses. When we use ESI to ionize a peptide or protein, we observe a distribution of peaks in the ESI/MS spectrum relating to individual components. These peaks correspond to multiply-charged molecular ions (shown in Fig. 2A(a)–2A(c)). As we operated a TSQ 7000 system in the ESI positive-ion mode, we generated multiply-charged positive ions. In the presence of hydrogen ions, the resulting positive ions were defined as $(M + nH)^{+n}$, where M represented the intact molecule and n was the number of charges. To analyze and identify the multiply protonated ion mass spectrum, we used the BIOMASS Deconvolution program. This program was used to interpret the ESI/MS spectrum of a biopolymer to determine its molecular weight (MW). When we deconvolute the spectrum, the mass spectral plot of relative ion abundance versus mass-to-charge ratio (m/z) is mathematically transformed into a plot of relative abundance versus mass. The deconvoluted spectrum consists of sets of calculated peaks containing contributions from the real peaks in the original spectrum. Thus, through the deconvolution process by using the BIOMASS Deconvolution program, the ESI/MS spectrum of multiply-charged ions was transformed into a deconvoluted spectrum containing a single peak at the MW of the sample component (shown in Figs. 2B(a)–2B(c)).

RESULTS

Figure 1 showed the total ion chromatogram of the hinge glycopeptide prepared from a tryptic digest of normal serum IgA1, and Fig. 2A(a) presented the multiply charged protonated ion mass spectrum of the * peak in Fig. 1A. In other groups, non-IgAN and IgAN, they showed similar total ion chromatograms to normal control in Fig. 1.

To analyze and identify these multiply protonated ion mass spectra shown in Fig. 2A(a)–2A(c), we used the BIOMASS Deconvolution program as described above.

Figures 2B(a)–2B(c) showed the deconvoluted spectra containing a single peak at the MW of the hinge glycopeptides of pooled normal serum IgA1 (a), that of non-IgA pooled serum IgA1 (b), and that of pooled serum IgAN (c) mass spectra by using the above program. Many extremely sharp peaks appeared in Figs. 2B(a)–2B(c), perhaps because of their structural microheterogeneity. Fifteen kinds of IgA1 O-glycosylated hinge peptides were exactly identified by ESI/LC/MS in all subjects (Figs. 2B(a)–2B(c)), based on their MW of binding number of GalNAc, Gal, and sialic acid with Ser and Thr added HP on the hinge region peptides. Their calculated MW values and estimated MW values were quite consistent as shown in Table 1. The * peaks (MW: 5520) shown in Figs. 2B(a)–2B(c) meant HP + 4GalNAc + 4Gal + 2NANA, and this peak was constantly presented in serum IgA1 hinge peptides in this

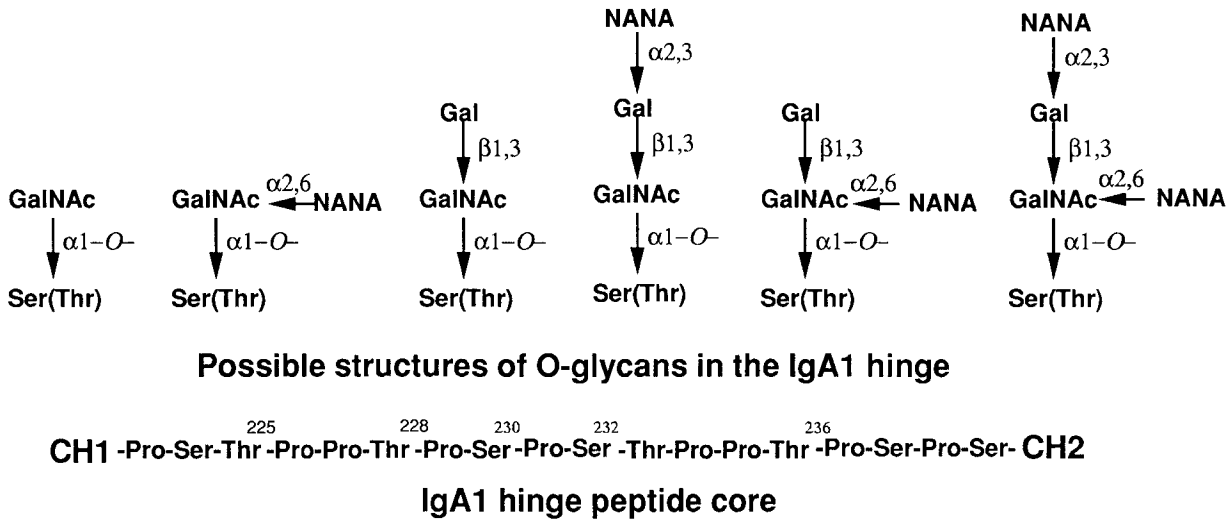

FIG. 3. Human serum IgA1 proline-rich O-glycan hinge peptide possible structure obtained from Jacalin lectin affinity-purified pooled serum IgA1 following digestion with trypsin. Thr (Ser)-n GalNAc (n = 2 *to* 5)- nGal (n = 1 to 5)- n NANA (0 to 4); NeuAc, NANA (sialic acid).

study. So we have estimated each glycopeptide population ratio to HP + 4GalNAc + 4Gal + 2NANA according to calculated each peak area based on each 3^+ ion chromatogram as shown in Table 1. But, in the case of IgAN in Fig. 2B(c), the number of sialylated glycopeptides was decreased and the number of the asialylated glycopeptides was increased compared to normal controls and non-IgAN in Fig. 2B(a) and (b) (Table 1 shown). This result clearly showed the decreased amount of sialic acid in IgAN compared with normal controls and non-IgAN.

Moreover, compared with each sugar chain distribution ratio to HP + 4GalNAc + 4Gal + 2NANA in three subjects, in 5GalNAc + 4Gal (A), 4GalNAc + 4Gal (B), and 4GalNAc + 3Gal (C) groups, the A: B: C ratios were 0.626: 1.0: 0.410 in normal controls, 0.743: 1.0: 0.486 in non-IgA, and 0.374: 1.0: 0.224 in IgAN. It was clarified that in IgAN the peak C/B (0.224) in 4GalNAc + 3Gal groups as well as the A/B (0.374) in 5GalNAc + 4Gal groups was significantly lower than those of normal controls (C/B: 0.410, A/B: 0.626) and non-IgAN (C/B: 0.486, A/B: 0.743).

DISCUSSION

The hinge region glycans of normal serum IgA1 consist mostly of mono-, di-, tri-, and tetrasaccharides linked to serine or threonine (2, 8, 22, 23). We have previously reported that the deposited IgA in the glomeruli of IgAN had reactivity to Jacalin (24). Although IgA1 is more resistant than IgG or IgM to cleavage by common proteolytic enzymes, the Jacalin affinity-purified pooled serum IgA1 was constantly digested with trypsin to generate Fc fragments containing the whole O-linked hinge region and the following secondary Jacalin affinity chromatography provided the whole O-linked hinge glycopeptide (Fig. 3 shown). IgA1 contains a prolin-rich hinge sequence between the Fab and Fc regions of the glycoprotein, and there are nine potential O-glycosylation sites in this sequence as shown in Fig. 3.

The first detailed structural analysis of the glycans of normal serum IgA1 was published by Field *et al.* (25), but as with other studies (26), the analysis was carried out on a desialylated glycan pool from intact IgA1 and sialylated glycans were not directly analyzed. Recently, Mattu *et al.* clarified the precise binding sites of O-glycans in the IgA1 hinge by N-terminal amino acid sequencing using an increased temperature sequencer cycle (22). They observed that O-glycans were located at Thr 228, Ser 230, and Ser 232, while the O-glycan sites at Thr 225 and Thr 236 were partially occupied.

In our previous reports, we found a phenomenon in which the enzymatic removal of NANA from normal human serum IgA1 induced the self-aggregation of part of the IgA1 (27). Aggregated human serum IgA1 induced by neuraminidase treatment had a lower number of O-linked sugar chains on the hinge region (4, 27, 28). The removal of N-glycan sugar chains from IgA1 by peptide N-glycanase treatment did not induce self-aggregation or binding to the asialo IgA1-Sepharose column (29). The N-glycan sugar chain is shown not to be involved in IgA1-IgA1 interactions (30). From our previous results, sialic acid containing sugar chain on IgA1 was found to play an important role inhibiting the aggregation of IgA1.

In this study, we have shown the direct detailed structures with sialylated and desialylated O-glycans from human serum IgA1 hinge region, and suggested

that under glycosylation of IgA1 hinge is directly involved in the pathogenesis of IgAN as we previously reported (3–5), and clarified the decreased sialylation of IgA1 hinge peptides in IgAN.

These results also showed that this chemical analysis method such as ESI/LC/MS would develop the detailed biochemical study about aberrant sugar chain diseases including IgA nephropathy.

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