Determination of Aberrant O-Glycosylation in the IgA1 Hinge Region by Electron Capture Dissociation Fourier Transform-Ion Cyclotron Resonance Mass Spectrometry*

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In a number of human diseases of chronic inflammatory or autoimmune character, immunoglobulin molecules display aberrant glycosylation patterns of N- or O-linked glycans. In IgA nephropathy, IgA1 molecules with incompletely galactosylated O-linked glycans in the hinge region (HR) are present in mesangial immunodeposits and in circulating immune complexes. It is not known whether the Gal deficiency in IgA1 proteins occurs randomly or preferentially at specific sites. To develop experimental approaches to address this question, the synthetic IgA1 hinge region and hinge region from a naturally Gal-deficient IgA1 myeloma protein have been analyzed by 9.4 tesla Fourier transform-ion cyclotron resonance mass spectrometry. Fourier transform-ion cyclotron resonance mass spectrometry offers two complementary fragmentation techniques for analysis of protein glycosylation by tandem mass spectrometry. Infrared multiphoton dissociation of isolated myeloma IgA1 hinge region peptides confirms the amino acid sequence of the de-glycosylated peptide and positively identifies a series of fragments differing in O-glycosylation. To localize sites of O-glycan attachment, synthetic IgA1 HR glycopeptides and HR from a naturally Gal-deficient polymeric IgA1 myeloma protein were analyzed by electron capture dissociation and activated ion-electron capture dissociation. Multiple sites of O-glycan attachment (including sites of Gal deficiency) in myeloma IgA1 HR glycoforms were identified (in all but one case uniquely). These results represent the first direct identification of multiple sites of O-glycan attachment in IgA1 hinge region by mass spectrometry, thereby enabling future characterization at the molecular level of aberrant glycosylation of IgA1 in diseases such as IgA nephropathy.

Several human diseases of autoimmune or chronic inflammatory character exhibit abnormal glycosylation of serum proteins, including immunoglobulins (1–7). The distinctive carbohydrate side chains of IgA1 molecules play a pivotal role in the pathogenesis of IgA nephropathy (IgAN)1 (8–10). IgA1 contains a hinge region (HR) between the first and second heavy chain constant domain regions with a high content of proline (Pro), serine (Ser), and threonine (Thr) (Fig. 1). Several groups have identified three to five O-linked glycan chains within the IgA1 HR (11–15). In normal human serum IgA1, glycosylated sites have been localized to Ser/Thr residues 225, 228, 230, 232, and 236 by N-terminal sequencing methods (14) showing that Ser/Thr residues 228, 230, and 232 were occupied in most IgA1 molecules (14). IgA1 O-linked glycans consist of GalNAc with a β1,3-linked Gal (11, 14, 16). Sialic acid (NeuAc) may be attached to GalNAc by an α2,6-linkage or to Gal by an α2,3-linkage (11, 14, 16). Carbohydrate composition of O-linked glycans in the HR of normal human serum IgA1 is variable, and the prevailing forms include Gal-GalNAc disaccharide and its mono- and disialylated forms (11, 12, 14). A variant with terminal GalNAc or sialylated GalNAc is rare for normal serum IgA1 (12, 14) but is more common in IgAN patients (8, 10, 17–20).

Recent reports from several laboratories support the earlier findings that O-linked glycans in the HR of some IgA1 molecules in the circulation of IgAN patients are deficiently galactosylated (8, 17–22). The Gal-deficient IgA1 in the circulation is exclusively present in circulating immune complexes and is mostly a J-chain-containing polymer (20). In the absence of Gal, the terminal sugar is GalNAc (19, 20). Subsequently, these aberrant O-glycans or HR glycopeptides (23, 24) are recognized by naturally occurring antibodies with anti-glycan or anti-HR peptide specificities (20, 24), and thus circulating immune complexes are formed (25). It is hypothesized that these Gal-deficient IgA1-containing circulating immune complexes are not efficiently cleared in IgAN patients and thus deposit in the mesangium where they bind to and activate the resident mesangial cells, inducing cellular proliferation and matrix overproduction (9, 26). In fact, these Gal-deficient IgA1-containing

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1 The abbreviations used are: IgAN, IgA nephropathy; HR, hinge region; FT-ICR, Fourier transform-ion cyclotron resonance; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RRMPD, infrared multiphoton dissociation; ECD, electron capture dissociation; A-FECD, activated ion-ECD; ESI, electrospray ionization; SWIFT, stored waveform inverse Fourier transform; W, watt; CID, collision-induced dissociation.
complexes bind to mesangial cells more efficiently than uncomplexed IgA1 or similarly sized IgA1-containing complexes from healthy controls (25). Furthermore, Gal-deficient IgA1 was detected in glomerular immune deposits (27, 28). These observations support the hypothesis that aberrantly glycosylated IgA1-containing immune complexes participate in the pathogenesis of IgAN (4, 9, 22, 26).

Available techniques for analysis of IgA1 O-glycosylation, such as lectin binding assays, can identify the presence of Gal-deficient O-glycan chains (27–31). However, it is not known whether the Gal deficiency in IgAN patients occurs randomly or preferentially at specific sites. Mass spectrometric analysis of IgA1 HR O-glycosylation has characterized the heterogeneity of glycoforms (15, 28, 30) but has not localized specific sites of O-glycosylation. Tandem mass spectrometry (MS/MS) has become a standard tool for the structural analysis of carbohydrates (for a review of complex carbohydrate mass spectrometry (MALDI-TOF MS). The following HR peptide and glycopeptides used: HP, VPSTPPTPSPSTPPTPSPS, 4-HP (GalNAc attached to Thr and so on), VPS-(GalNAc)TPPTPSTPPTPSPS, 7-HP, VPSTPPTP-(GalNAc)SPS; 11-HP, VPSTPPTP-(GalNAc)SPS; 15-HP, VPSTPPTP-(GalNAc)SPS; 9-HP, VPSTPPTP-(GalNAc)SPS; 19-mer hinge region peptide and glycopeptides, with an N-glycan sequence and linkages of the sugars in complex carbohydrate structures (32). Although localization of sites of glycan attachment in O-linked glycopeptides (32–35) and N-linked glycopeptides (36–38) is possible by CID fragmentation, the task is complicated by the dominance of fragments resulting from cleavage of glycosidic linkages rather than peptide backbone cleavages in the tandem (CID) MS/MS spectra. Also, as the size and number of glycan chains increase, so does the dominance of glycosidic fragments (34).

Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR MS) offers two complementary fragmentation techniques for analysis of protein glycosylation by MS/MS (39). Infrared multiphoton dissociation (IRMPD) (40) induces selective dissociation of the glycosidic bonds on N-linked glycans of glycopeptides. Electron capture dissociation (ECD) fragmentation, first demonstrated by Zubarev et al. (41) and reviewed recently in Refs. 42 and 43, results in complementary cleavage of the backbone N–Cα bond with minimal loss of post-translational modifications (44, 45). Haselmann et al. (46) unambiguously localized four sites of O-glycosylation in a 6.8-kDa glycopeptide by use of ECD fragmentation. Håkansson et al. (40, 47) implemented both of these fragmentation techniques in a single FT-ICR mass spectrometer configuration and successfully demonstrated their use in analysis of glycoproteins. These studies suggest ECD FT-ICR MS could be a valuable tool for the analysis of Gal-deficient IgA1 HR.

Here we analyze O-glycans of synthetic IgA1 HR and a naturally Gal-deficient polymeric (p) IgA1 myeloma protein. We have shown previously (20) that this IgA1 can inhibit re-association of IgA1-containing immune complexes, suggesting that this IgA1 protein has properties similar to the aberrantly glycosylated IgA1 present in the circulation of IgA nephropathy patients. We observed a heterogeneous population of glycosylated IgA1 HR as in prior studies (15, 28, 30). FT-ICR MS/MS experiments localized sites of O-glycosylation in the IgA1 HR. Four and five glycan chains composed of either a GalNAc-Gal disaccharide or a GalNAc monosaccharide were localized to 5 of the 10 possible sites of O-glycosylation in the myeloma IgA1 HR. These analytical approaches will be used in the future for characterization of O-glycans in IgA1 from IgAN patients.

MATERIALS AND METHODS

Synthesis of IgA1 HR Peptide and Glycopeptide Variants—A panel of 19-mer hinge region peptide and glycopeptides, with an O-Linked GalNAc residue, corresponding to the amino acid sequence of the human glucosyltransferase, were synthesized by and purchased from the Peptide Institute Inc. (Osaka, Japan). Purity and molecular weight of the preparations were confirmed by high pressure liquid chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The following HR peptide and glycopeptides used: HP, VPSTPPTPSPSTPPTPSPS; 4-HP (GalNAc attached to Thr and so on), VPS-(GalNAc)TPPTPSTPPTPSPS; 7-HP, VPSTPPTP-(GalNAc)SPS; 11-HP, VPSTPPTP-(GalNAc)SPS; 15-HP, VPSTPPTP-(GalNAc)SPS; 9-HP, VPSTPPTP-(GalNAc)SPS; 19-mer hinge region peptide and glycopeptides, with an N-glycan sequence and linkages of the sugars in complex carbohydrate structures (32). Although localization of sites of glycan attachment in O-linked glycopeptides (32–35) and N-linked glycopeptides (36–38) is possible by CID fragmentation, the task is complicated by the dominance of fragments resulting from cleavage of glycosidic linkages rather than peptide backbone cleavages in the tandem (CID) MS/MS spectra. Also, as the size and number of glycan chains increase, so does the dominance of glycosidic fragments (34).

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ions and C-terminal unambiguously localizes four of the glycosylations (to Thr7, Ser9, Ser11, and Thr15) and the final glycosylation to Ser3/Thr4. N-terminal irradiation event, the trap electrodes were set to 4–10 V; the cathode were irradiated with electrons for 10 or 20 ms. During the electron following photon irradiation, the isolated ion precursor populations for 300 ms at 16% (6.4 W) or 20% (8 W) laser power. Immediately SWIFT isolated ion precursor populations were photon-irradiated time-domain data). Displayed spectra represent the sum of 50 time-domain transients. For ECD of synthetic IgA1 HR glycopeptides, quadrupole and SWIFT isolated ion precursor populations were irradiated with electrons for 10–100 ms. During the ECD fragmentation event, the trap electrodes were set at 10 V; the cathode was biased at −10 V, and the extraction grid was pulsed to −200 V. Immediately following the ECD event, the trap electrodes were reset to 2 V, and potentials of 5 V for the grid and 10 V for the cathode were applied for 1 ms to remove the remaining electrons. At all other times the cathode bias voltage was −0.1 V and the grid potential was −200 V. The cathode heating power was 11 W, corresponding to an electron emission current of ~300 nA. Ions were frequency-sweep (“chirpy”) excited (48–640 kHz, at 150-Hz/μs) and detected in direct mode (512 kWord time-domain data). Displayed spectra represent the sum of 100–200 time-domain transients. The AI-ECD FT-ICR MS/MS spectra were internally frequency to mz calibrated with respect to the precursor and the charge-reduced species. An indirectly heated 10-mm diameter dispenser cathode (1109; Heat Wave, Watsonville, CA) mounted on the central axis of the system provided the electrons for ECD; IRMPD was performed with a 40-W, 10.6-μm, CO2 laser (Synrad, Mukilteo, WA), fitted with a 2.5× beam expander. The laser beam is directed to the center of the cell through an off-axis BaF2 window (47). Activated ion (AI)-ECD was performed with the dispenser cathode and CO2 laser in tandem as described below.

For ECD of synthetic IgA1 HR glycopeptides, quadrupole and SWIFT isolated ion precursor populations were irradiated with electrons for 10–100 ms. During the ECD fragmentation event, the trap electrodes were set at 10 V; the cathode was biased at −10 V, and the extraction grid was pulsed to −200 V. Immediately following the ECD event, the trap electrodes were reset to 2 V, and potentials of 5 V for the grid and 10 V for the cathode were applied for 1 ms to remove the remaining electrons. At all other times the cathode bias voltage was −0.1 V, and the grid potential was −200 V. The cathode heating power was 11 W, corresponding to an electron emission current of ~300 nA. Ions were frequency-sweep excited (48–480 or 45–480 kHz, at 150-Hz/μs) and detected in direct mode (1 MWord time-domain data). Displayed spectra represent the sum of 60–100 time-domain transients. The AI-ECD FT-ICR MS/MS spectra were internally frequency to mz calibrated with respect to the precursor, the charge-reduced species, and z+ ions. All FT-ICR MS and MS/MS spectra were analyzed by use of the modular ICR data acquisition system analysis software package (55).

RESULTS

FT-ICR MS/MS of Synthetic HR Peptide and Glycopeptides—ECD fragmentation typically cleaves the peptide backbone N–C–α bond with minimal loss of post-translational modifications. The exception to that rule is proline, which is cyclic around the N–C–α bond and does not cleave into the characteristic N-terminal c and C-terminal z fragments (68) seen by ECD (69). The IgA1 HR is a proline-rich segment with 9–10 Ser/Thr residues interspersed among 12 prolines. For that reason, pilot experiments to determine the feasibility of characterizing IgA1 HR glycosylation sites by ECD FT-ICR MS/MS were performed with synthetic HR peptides. Fig. 2 shows the FT-ICR mass spectra obtained following ECD of the peptides HP, 9-HP, 4-HP, and 4-15-HP. Table I lists the identified fragments. For peptide HP (i.e. the unglycosylated peptide), c and/or z+ fragments were detected for each amino acid except proline. Taken together, the identified N-terminal c and C-terminal z+ fragment ions provide amino acid sequence identification for the unglycosylated peptide HP (Fig. 2A). With the addition of a single GalNAc (9-HP and 4-HP, Fig. 2, B and C) or multiple GalNAc residues (9–15-HP, Fig. 2D), the observed c and z+ ions allow localization of the site of glycosylation based on the additional mass of a GalNAc (203.08) in a given fragment ion. For peptide 9-HP, the observation of c8 without associated GalNAc and [c10 + GalNAc]+ unambiguously localizes the glycan to Ser9. The remaining fragments (listed in Table I) elim-
iniate the remaining serines and threonines as the site(s) of glycan attachment. Similarly, the significant fragments detected for peptide 4-HP were \([z_{16} + \text{GalNAc}]^+\), \(z_{16}\), and \([c_6 + \text{GalNAc}]^+\). In contrast to MS/MS of the unglycosylated peptide, no \(z_{16}\) fragment was detected. The results allow location of the glycan to Ser\(^3\) or Thr\(^4\). Singly glycosylated peptides 7-HP and 11-HP were also subjected to ECD analysis (data not shown). In both cases the ECD fragmentation pattern allowed for unambiguous localization of the glycan (Thr\(^7\) in 7-HP; and Ser\(^{11}\) in 11-HP). ECD analysis of the quintuply glycosylated peptide 4-15-HP (Fig. 2D) enabled unambiguous localization of four of the glycans to Thr\(^7\), Ser\(^9\), Ser\(^{11}\), and Thr\(^{15}\) and the final glycan to Ser\(^3\) or Thr\(^4\). These results demonstrate that direct assignment of native IgA1 HR O-glycosylation sites is possible by ECD FT-ICR MS.

**Analysis of Heterogeneity in Synthetic HR Glycopeptides**—To assess the ability of ECD fragmentation to distinguish between homogeneous and heterogeneous glycosylation, mixtures of the synthetic peptides were subjected to ECD and their fragmentation patterns examined. Fig. 3 shows the ECD FT-ICR mass
IgA1 O-Glycosylation Sites Identified by FT-ICR MS

**Fig. 3.** ECD FT-ICR positive ion mass spectrum obtained from a 1:1 mixture of the peptides, 7-HP and 9-HP (VPSTPP-[GalNAc]-TPSPSTPPTPSPS-NH₂ and VPSTPPTP-[GalNAc]-SPSTPPTPSPS-NH₂). The two species have identical masses; however, the ECD fragmentation pattern reveals different sites of glycosylation. Absence of C₆GalNAc and C₁₀ serves to locate the sites of glycan attachment at Thr⁴ and Ser⁶ within the mixture.

**Fig. 4.** ESI FT-ICR positive ion mass spectra of isolated IgA1 HR peptide. Top, peptides released from IgA1 by trypsin-pepsin digestion. The dominant series of triply charged ions are separated by 1/3 of the masses of GalNAc and Gal units. Middle, one of the triply charged ions seen in the top spectrum isolated externally by a quadrupole mass filter and internally by SWIFT excitation. Bottom, IRMPD MS/MS product ion spectrum of the isolated triply charged species. The spectrum shows series of triply and doubly charged product ions corresponding to the loss of multiple GalNAc (open squares) and Gal (filled circles) residues, indicating the attachment of several O-glycan chains in the peptide.

A 1:1 mixture of 7-HP, 11-HP, and 15-HP was analyzed by ECD FT-ICR MS (not shown). The results confirmed heterogeneity in the glycosylation pattern. Glycosylation at positions 11 and 15 was confirmed, as was the absence of glycosylation at position 9. However, evidence for glycosylation at positions 9 and 11 was ambiguous. Similarly, ECD analysis of a mixture of all five peptides confirmed heterogeneity and the presence of glycosylation at positions 4 and 15. The presence of glycosylation at positions 7, 9, and 11 was ambiguous. The ambiguity associated with heterogeneity can be attributed to the absence of z fragments bracketing the residues of interest in these mixtures. The results for 7-HP, 9-HP, and 11-HP were unambiguous because the glycans are at adjacent glycan attachment sites. Thus the lack of z fragments did not interfere with the interpretation of the results. Similarly, unambiguous results would also be expected for the following mixtures: 4-HP and 7-HP; 7-HP and 11-HP; and 11-HP and 15-HP.

**FT-ICR MS/MS Analysis of Isolated Myeloma IgA1 HR Glycopeptides**—HR glycopeptides were isolated from IgA1 myeloma protein by trypsin-pepsin digestion of IgA1 as described previously (49). Monosaccharide compositional analysis by gas-liquid chromatography determined the presence of GalNAc, Gal, and NeuAc (results not shown), indicating that HR was free of contaminating glycopeptides containing N-linked glycans. Isolated IgA1 HR was desalted (C₁₈, ZipTip, Millipore) and analyzed by ESI FT-ICR MS. Fig. 4 (top) shows the mass spectrum (10-s ion accumulation, sum of 25 time-domain transients) of the desialylated IgA1 HR peptide separated from a trypsin-pepsin digest by size exclusion and affinity chromatography. The mass spectrum exhibits a series of triply charged ions (m/z 1479.011, 1533.028, 1600.033, 1654.737) separated by 1/3 of the mass of hexose (162.05 Da) and N-acetylhexosamine (203.08 Da). Although no species corresponding to an unglycosylated IgA1 HR were detected, the series of ions suggests that the isolated IgA1 HR peptide is a heterogeneous mixture of differentially glycosylated species, as seen in other myeloma IgA1 HR by mass spectrometry and lectin-binding assays (15, 28, 30). The single species, m/z 1600.033⁺, was isolated (Fig. 4 (middle)) and fragmented by infrared multiphoton dissociation (IRMPD, Fig. 4 (bottom)). The IRMPD product ion spectrum shows a series of triply and doubly charged ions corresponding to the loss of multiple Gal (162.05) and GalNAc (203.08) residues from the isolated parent ion.

To establish the sequence of the isolated IgA1 myeloma HR glycopeptide, an equivalent sample was treated with neuraminidase and O-glycanase to remove all glycans. The predicted peptide from a trypsin-pepsin digestion is [216VTVPCPVPSTPPSSTPSCCHPRL]. However, two mass ions corresponding to IgA1 residues [Val₂₁₆–Leu₂₄₆]³⁺ and [Val₂₁₆–Val₂₁₇–Leu₂₄₆ + GalNAc]⁵⁺ were observed. Fig. 5 shows the IRMPD FT-ICR MS/MS product ion spectrum of the triply charged species of m/z 1046.166, [Val₂₁₆–Leu₂₄₆]³⁺.

Eighteen (of 30) peptide backbone bonds are broken, confirming the sequence of the isolated IgA1 HR as [317AI-ECD of a single IgA1 HR glycoform ([216VTVPCPVPSTPPSSTPSCCHPRL]⁺). From the known glycan composition, the accurate mass of the glycosylated ion series in the desialylated IgA1 HR sample (Fig. 4), and the confirmed amino acid sequence of the de-glycosylated IgA1 HR peptide (Fig. 5), glycan structures for the mixture may be inferred for the dominant series of ions seen in Fig. 4 and are listed in Table II. ESI FT-ICR MS/MS analysis of sialylated IgA1 HR indicated a similar heterogeneous mixture of differentially glycosylated species including multiple NeuAc residues (data not shown). For localization of sites deficient in Gal by ECD FT-ICR MS/MS, IgA1 HR trypsin-pepsin preparations were desialylated to reduce the IgA1 HR glycopeptide heterogeneity for each sample.

**Localizing Sites of O-Glycosylation by ECD FT-ICR MS/MS**—Based on pilot studies with synthetic IgA1 HR and the identification of multiple glycosylated variants in the isolated myeloma IgA1 HR, ECD for three IgA1 HR glycopeptides serves to localize specific sites of O-glycan attachment. Unlike ECD fragmentation of the synthetic HR peptide, ECD of [IgA1 HR + 4 GalNAc + 4 Gal]⁵⁺ resulted in just a few detectable ionic fragments, including two y-type ions ([y₂₆ + 4 GalNAc + 4 Gal]³⁺ and [y₂₆ + 4 GalNAc + 4 Gal]⁴⁺). Typically, y ions represent a minor fragmentation channel in ECD (69).

Much more extensive e and z ECD fragmentation may be achieved by prior or simultaneous activation by infrared radiation (at lower IR power than would be used for direct IRMPD) (45, 47, 70). Fig. 6 shows the mass spectrum obtained following AI-ECD of a single IgA1 HR glycoform ([216VTVPCCVPST-
Fig. 5. IRMPD FT-ICR MS/MS of deglycosylated IgA1 HR [1046.166]+ precursor ions. Eighteen (of 30) peptide backbone bonds are broken, confirming the sequence of the isolated IgA1 HR as 216TVPCPVTSPTPSTPPSPSCCHPRL246. N-terminal b fragment ions and C-terminal y fragment ions are indicated in the IgA1 HR sequence.

**TABLE II**

Dominant glycopeptide series (monoisotopic neutral masses) observed in the tryptic-peptidic preparation of IgA1 HR (Fig. 4A)

| Measured mass (Da) | Calculated mass (Da) | Error (ppm) | Predicted no. GalNAc residues | Predicted no. Gal residues |
|--------------------|----------------------|-------------|-------------------------------|---------------------------|
| 4434.011           | 4433.995             | 3.6         | 4                             | 3                         |
| 4596.062           | 4596.048             | 3.0         | 4                             | 4                         |
| 4799.094           | 4799.127             | -6.9        | 5                             | 4                         |
| 4961.187           | 4961.180             | 1.4         | 5                             | 5                         |
| 5164.294           | 5164.259             | 6.7         | 6                             | 5                         |

Three additional fragments (c, z, and b) are seen. Taken together, these fragments enable unambiguous assignment of 4 O-glycan chains (each composed of a GalNAc-Gal disaccharide) to 4 peptides, 18/246 out of 30 possible sites in the IgA1 HR peptide. These results are consistent with previous reports localizing sites of O-glycosylation by other techniques (11, 14). Localization of O-glycosylation sites proceeds in a stepwise fashion by identification of fragments that come from the N terminal (b and c) followed by fragments that come from the C terminus (y and z) to identify (and eliminate) specific serines and threonines with (or without) attached glycans. From the N terminus, detection of b3, c8, and c9 ions eliminates Thr217 and Ser224 as sites of O-glycan attachment. Fragments (b11 + GalNAc + Gal)† [c12 + GalNAc + Gal]† localize one glycan chain to Thr225. The detection of [c16 + 3 GalNAc + 3 Gal]† indicates 2 GalNAc and 2 Gal residues are distributed between Thr224 and Ser230. The only remaining N-terminal fragment [c28 + 4 GalNAc + 4 Gal]† indicates that the remaining two glycans are attached C-terminal to Thr233 (five possible sites). From the C terminus, the detection of c14 eliminates Ser230, c23, Thr236, and Thr233, respectively, as sites of O-glycan attachment. The second and third glycan chains are localized to Ser232 and Ser230 by the detection of [b15 + GalNAc + Gal]† followed by [c17 + 2 GalNAc + 2 Gal]†, respectively. With the assignment of disaccharide chains at Thr225 and Ser230, the remaining GalNAc and Gal residues observed in the [c16 + 3 GalNAc + 3 Gal]† fragment can be assigned only to Thr225. That assignment is consistent with the two remaining C-terminal fragments, [y26 + 4 GalNAc + 4 Gal]2+ and [z28 + 4 GalNAc + 4 Gal]2+, which indicate that the remaining 2 glycan chains are attached N-terminal to Ser230 (4 possible sites), but because Thr217 and Ser224 are eliminated by the presence of c8 and c9, and because the first glycan chain is similarly assigned to Thr225, the only remaining site of glycan attachment is Thr228.

With the successful localization of four glycan chains in a single ion species, we chose two other IgA1 HR glycoforms seen in the original series (see Fig. 4 and Table II) to fragment by AI-ECD. Based on the known structure of IgA1 O-glycans and the fragment ions detected in the [Val216–Leu246 + 4 GalNAc + 4 Gal]3+ glycoform, we hypothesized that the predicted [Val216–Leu246 + 4 GalNAc + 4 Gal]3+ and [Val216–Leu246 + 5 GalNAc + 4 Gal]3+ glycoforms would have O-glycan chains deficient in Gal. Fig. 7 shows the mass spectrum obtained following AI-ECD of the [IgA1 HR + 4 GalNAc + 3 Gal]3+ glycoform ([C16VTVPVPVTSPSTPPSPSCCHPRL246 + 4 GalNAc + 3 Gal + 3H]3+ ions after AI-ECD fragmentations). The fragmentation pattern (17 fragment ions) is similar to that of the previous hinge region glycoform. Three GalNAc-Gal disaccharide chains are localized to Thr225, Thr228, and Ser230 as before. The difference between z14 and z14 corresponds to the mass of a serine residue + GalNAc, indicating a glycan chain composed of a GalNAc monosaccharide at Ser232, thus localizing a site of Gal deficiency.

Fig. 8 shows the mass spectrum obtained following AI-ECD of the [IgA1 HR + 5 GalNAc + 4 Gal]3+ glycoform ([C16VTVPVPVTSPSTPPSPSCCHPRL246 + 5 GalNAc + 4 Gal + 3H]3+ ions after 300-ms photon irradiation, followed by 10-ms electron irradiation). Although fewer fragment ions were detected (11 fragments) compared with the other glycoforms, [c12 + GalNAc + Gal]† and [c14 + 2 GalNAc + 2 Gal]† fragment ions localize two glycan chains to Thr225 and Thr228. From the
C terminus, the detection of $z_9$ (without attached glycans) combined with the detection of $z_{14}$/Gal14/H11001, GalNAc/H11001 localizes a third glycan chain to either Thr233 or Thr236. Subsequently the detection of $z_{17}$/Gal17/H11001, 3 GalNAc/H11001, 2 Gal suggests that two additional glycan chains are attached at the only two possible sites, Ser230 and Ser232, as seen in the [Val216–Leu246/4 GalNAc/4 Gal/3H]3/H11545 glycoform (Fig. 4). Although the difference between $z_{17}$ and $z_{14}$ corresponds to the mass of SPS + 2 GalNAc + 2 Gal, the absence of $z_{15}$/Gal15/H11001, 2 GalNAc/H11001, 1 Gal prevents direct assignment of a glycan chain at each serine residue.

**DISCUSSION**

The present results represent the first direct identification of multiple sites of O-glycan attachment in the IgA1 HR by mass spectrometry. A series of FT-ICR MS and MS/MS experiments provides a comprehensive characterization of IgA1 HR O-glycosylation.

**FT-ICR MS Characterization of IgA1 HR**—IgA1 HR-containing trypsin-pepsin fragment is a proline-rich sequence with a cluster of 10 serines and threonines that are potential sites of O-glycan attachment (Val216–Leu246) (71, 72). Because c and z fragments rarely arise within proline (69), initial ECD FT-ICR MS/MS experiments were performed with a synthetic version of IgA1 HR. ECD FT-ICR MS/MS of synthetic IgA1 HR showed that ECD fragmentation results in enough products to localize each possible site of O-glycan attachment (Fig. 2). ECD of several versions of synthetic IgA1 HR with a single GalNAc monosaccharide attached at various sites can distinguish between homogeneous and heterogeneous populations of glycosylated IgA1 HR (e.g., Fig. 3).

For HR isolated from IgA1 myeloma protein, IRMPD successfully confirms the sequence of the de-glycosylated hinge region. However, IRMPD of the glycosylated IgA1 HR demonstrates the complexity of identifying multiple sites of O-glycan attachment within a single glycoform. Although the loss of multiple GalNAc and Gal residues is easily recognized (Fig. 4, bottom), identifying sites of O-glycan chain attachment and which chain is Gal-deficient by IRMPD or other slow heating methods (i.e., CID) is impossible for several reasons. 1) The IgA1 HR glycan chains are all similar or identical in composition. 2) The 10 serines and threonines are clustered. 3) The HR amino acid sequence is a tandem repeat (49). All of these factors prevent the selective removal of a single glycan chain by slow heating methods and subsequent determination of its site of attachment by neutral loss. Only with the glycan chains intact could the site deficient in Gal be identified. Based on our pilot experiments, ECD fragmen-
tation of the HR thus offered the greatest promise for localization of sites of O-glycan attachment.

Identification of IgA1 HR O-Glycosylation Sites—The pathogenesis of IgAN is characterized by immunodeposits in the renal mesangium. These immunodeposits contain IgA1 with incompletely galactosylated O-linked glycans in the HR (27, 28). It is not known whether the Gal deficiency in IgAN occurs randomly or at specific sites. In analysis of IgA1 HR O-glycosylation, previous studies have used a combination of techniques to assess sites of O-glycan attachment (N-terminal sequencing and IgA specific proteases (14)), sites deficient in Gal (glycan specific lectins + specific and nonspecific proteases (27–31)), and O-glycan heterogeneity (MALDI-TOF MS (28, 30)). The present results show that FT-ICR MS and MS/MS can achieve the equivalent of all of these analyses from a single preparation of IgA1 HR.

The HR glycopeptide, Val216–Leu246, was isolated from IgA1 by a trypsin-pepsin digest. The initial FT-ICR MS spectrum showed a series of species corresponding to the predicted mass of Val216–Leu246 with the addition of varying numbers of GalNAc and Gal residues (Fig. 4, top). This series is consistent with previous mass analyses of the heterogeneity of O-glycosylation in the HR (15, 28, 30). Three IgA1 HR glycopeptides were chosen for further characterization by AI-ECD FT-ICR MS/MS. The AI-ECD product ion spectrum for the predicted [Val216–Leu246 + 4 GalNAc + 4 Gal + 3H]3+ (Fig. 6) reveals four GalNAc + Gal disaccharide chains unambiguously localized to Thr225, Thr228, Ser230, and Ser232. For a second glycoform (Fig. 7), four O-glycan chains could again be localized to the same sites, with a monosaccharide (GalNAc) instead of a disaccharide attached at Ser232. The AI-ECD product ion spectrum of a third glycoform (Fig. 8) localizes the same sites of attachment for four GalNAc-Gal disaccharide chains plus a fifth GalNAc monosaccharide at either Thr233 or Thr236.

Baenziger and Kornfeld (11) reported that IgA1 myeloma protein (Oso, different from the IgA1 myeloma protein reported here) contained O-glycans at Ser residues 224, 230, 232, 238, and 240 with a GalNAc monosaccharide at Ser224. Mattu et al. (14) localized sites of attachment to HR residues Thr225, Thr228, Ser230, Ser232, and Thr236 from pooled human serum IgA1, with a fraction of the population not glycosylated at residues Thr225 and Thr236. In our analysis, Thr225 in the three HR glycoforms always contained an attached GalNAc-Gal disaccharide. These differences could be attributed to the difference in source of the IgA1 HR. One distinct difference is that N-terminal sequencing methods sequence the entire population of IgA1 HR isolated from IgA1, whereas we isolate individual HR glycoforms and sequence by AI-ECD fragmentation. It is also worth noting that methods established previously rely on the depression or absence of expected signal at particular cycles in the amino acid sequencing (14), whereas an AI-ECD product ion spectrum directly identifies sites with and without an attached glycan. Recently, it was discovered that a single enzyme, GalNAc-transferase 2, is responsible for addition of GalNAc to IgA1 HR.
sites (73). Although there is a clear site preference, the enzyme is capable of adding GalNAc to all potential sites in IgA1 HR. Thus, the determination of which of the potential sites in IgA1 HR are glycosylated is likely related to the properties and regulation of this single enzyme. Differential regulation of this enzyme in different IgA1-producing cells may therefore result in the heterogeneity of sites with attached glycans. We believe that the FT-ICR MS/MS techniques described here will be applicable to the analysis of O-linked glycans of IgA1 isolated from patients with diseases such as IgAN.

**Conclusion**—The present approach represents a new level of analysis of aberrant O-glycosylation found in IgA-associated diseases. Previous mass spectrometric studies of IgA1 heterogeneity concluded that the three glycoforms characterized here are found in normal human serum IgA1 (15). However, assignment of glycan structures and attachment sites in previous studies was based on existing knowledge of IgA1 O-glycan structure and not direct analysis of individual IgA1 glycoforms as reported here. This study demonstrates the wealth of structural information that FT-ICR MS and MS/MS analysis can provide from a single preparation, instead of multiple techniques and preparations required to assess IgA1 O-glycan heterogeneity and localize sites of attachment. Furthermore, our FT-ICR MS/MS analysis of individual IgA1 HR glycoforms represents the first direct identification of sites deficient in Gal. The ability to analyze comprehensively individual IgA1 HR glycoforms from a single source should lead to an accurate and complete profile of IgA1 HR glycoforms from various sources, including IgA1 myeloma protein as well as IgA1 isolated from normal human serum, IgAN patients, and IgA1 immunodeposits in the mesangium. The successful identification of sites of attachment of O-glycans in the HR of a IgA1 myeloma protein enables characterization at the molecular level of the aberrant glycosylation of IgA1 from IgAN patients, and gives rise to new opportunities to understand the pathogenesis of diseases with altered glycosylation pattern of immunoglobulins or other glycoproteins.

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