Journal of Biomolecular NMR, **12:** 385–394, 1998.
KLUWER/ESCOM

Dynamics of the carbohydrate chains attached to the Fc portion of immunoglobulin G as studied by NMR spectroscopy assisted by selective ^{13}C labeling of the glycans

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Received 30 March 1998; Accepted 19 May 1998

Key words: carbohydrate chain, Fc, glycoprotein, IgG, stable isotope labeling

Abstract

A systematic method for ^{13}C labeling of the glycan of immunoglobulin G for NMR study has been developed. A mouse immunoglobulin of subclass IgG2b has been used for the experiment. On the basis of chemical shift and linewidth data, it has been concluded that (1) the mobility of the carbohydrate chain in IgG2b is comparable to that of the backbone polypeptide chain with the exception of the galactose residue at the nonreducing end of the Manα1–3 branch, which is extremely mobile and (2) agalactosylation does not induce any significant change in the mobility. The results obtained indicate that even in the agalactosyl form the glycans are buried in the protein. Biological significance of the NMR results obtained is also briefly discussed.

Abbreviations: C1q, component of the C1 complex of the classical complement cascade, responsible for binding to the Fc region of complexed IgG; CD2, cluster of differentiation 2; FID, free induction decay; HMQC, heteronuclear multiple-quantum correlation; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single-quantum correlation; MALDI-TOF MS, matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry; TFA, trifluoroacetic acid.

Introduction

Immunoglobulin G (IgG) consists of two distinct regions, Fab and Fc. The Fab portion carries the recognition site for antigenic determinants, whereas the Fc region promotes effector functions through interactions with complements or cellular receptors. The Fc portion of IgG, which is composed of two C_H2 and two C_H3 domains, possesses one conserved glycosylation site at Asn-297 in each of the C_H2 domains, where complex-type oligosaccharides are expressed (Figure 1). It has been reported that aglycosyl IgG molecules, which have been prepared by treating hybridoma cells with tunicamycin or by site-directed mutagenesis at position 297, retain little ability in activating complements and in binding to Fc receptors. These results indicate that the oligosaccharide linked to Asn-297 is of vital importance for the expression of the proper effector functions of IgG (Nose and Wigzell, 1983; Leatherbarrow et al., 1985; Tao and Morrison, 1989).

It is known that the oligosaccharides exhibit various microheterogeneities depending upon species, age, and pathological states (Mizuochi et al., 1987,

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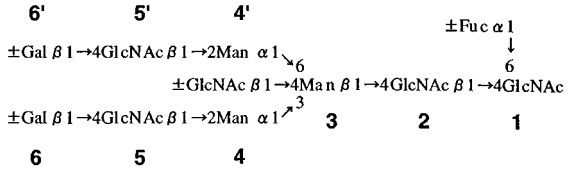

Figure 1. The major structures of the oligosaccharides linked to Asn-297 of IgG Fc.

1990; Rothman et al., 1989; Parekh et al., 1985; Youings et al., 1996; Rademacher et al., 1994).

This includes the presence or absence of core Fuc, bisecting GlcNAc and nonreducing terminal Gal residues. In the case of mouse IgG antibodies, the *N*-linked oligosaccharides, which lack bisecting GlcNAc, are almost fully fucosylated (Mizuochi et al., 1987; Rothman et al., 1989). It has been reported that serum IgG from patients suffering from rheumatoid arthritis contains a significantly reduced amount of Gal (Parekh et al., 1985; Youings et al., 1996). The decrease in the galactosylation of IgG oligosaccharides has also been found in autoimmune MRL/Mp-lpr/lpr mice (Mizuochi et al., 1990). It has been demonstrated that agalactosyl IgG glycoforms are directly associated with pathogenicity in murine collagen-induced arthritis and exhibits a significant degree of reduction of the ability to bind to C1q and Fc receptor (Rademacher et al., 1994; Tsuchiya et al., 1989).

In order to understand the biological and pathological roles of the Fc carbohydrate moieties, the glycan has to be investigated from a structural point of view. X-ray crystallographic studies that have been done by using IgG and Fc fragments isolated from IgGs of various species and subclasses have indicated that the two oligosaccharides are packed between the two C_H2 domains. It has also been demonstrated that significant differences in conformation of the glycans exist among the crystal structures (Deisenhofer, 1981; Sutton et al., 1983; Harris et al., 1997). However, we cannot rule out the possibility that structural differences result from microheterogeneities of the carbohydrate chains.

NMR spectroscopy has also been in use for gaining structural information concerning the carbohydrate chains of glycoproteins in solution. One of the important features of NMR is that it can potentially provide us with information on the dynamics of the carbohydrate chains (de Beer et al., 1994, 1996; Wyss et al., 1995a; Weller et al., 1996). One-dimensional ^1H and ^{13}C NMR had been used to gain information on dynamical properties of the carbohydrate chains attached to Fc (Rosen et al., 1979; Malhotra et al., 1995; Wormald et al., 1997). Dwek and his co-workers have suggested that the carbohydrate chains which lack Gal-6′ do not interact with the peptide chain, resulting in exposure of previously covered region of the inner surface of the C_H2 domain and making the glycan more accessible to other molecules (Malhotra et al., 1995; Wormald et al., 1997). In these studies, however, severe overlap of carbohydrate signals hampered the collection of the spectral data at atomic resolution. In order to overcome this difficulty of signal overlap, a successful attempt has been made by Gilhespy-Muskett et al. (1994), who observed the ^1H-^{13}C HMQC peaks originating from the terminal Gal residues of human IgG4 Fc by use of D-[U-^{13}C]Gal, which is enzymatically attached onto the nonreducing end of the carbohydrate chains. This method is undoubtedly quite promising, but can only be applied to the NMR analyses of terminal residues of glycans in glycoproteins.

In view of the current situation described above, we clearly have to establish a systematic stable-isotope labeling method for introducing NMR probes to desired selective positions of the glycans. It is also necessary to develop a general method of trimming the carbohydrate chains in order that all glycoforms are of uniform structure. Establishment of these procedures would make it possible to discuss the structural and biological role of the glycans in IgG in solution by using NMR.

In the present paper, we describe a systematic method for ^{13}C labeling of the carbohydrate chains of a mouse immunoglobulin of subclass IgG2b. The Fc fragment with the trimmed carbohydrate chains that are labeled with ^{13}C at selected positions have been used for the NMR spectral analyses. On the basis of the chemical shift and relaxation data obtained, dynamics of the carbohydrate chains will be discussed. We will also discuss briefly the biological significance of the results obtained.

Materials and methods

Materials

D-[1-^{13}C]Glc, D-[1-^{13}C]Gal, and [^{13}C]NaCN were purchased from ICON. Isotope enrichment is 99% for each product. UDP-Glc, UDP-Gal, galactosyltransferase from bovine milk, galactokinase from Gal-adapted yeast, UDP-Glc pyrophosphorylase from

Bakers yeast, Gal-1-phosphate uridyltransferase from Gal-adapted yeast, potassium phosphoenolpyruvate, pyruvate kinase from rabbit muscle, inorganic pyrophosphatase from Bakers yeast and papain were purchased from Sigma. V8 protease, acetonitrile, dithiothreitol, *N*-ethylmaleimide and iodoacetic acid were from Wako Pure Chemical Industry. β-Galactosidase from *Streptococcus* strain 6646K and glycoamidase A from almond (obtained as glycopeptidase A) were purchased from Seikagaku Kogyo.

Synthesis of [1-^{13}C]GlcN·HCl

D-[1-^{13}C]GlcN·HCl was prepared from D-arabinose and [^{13}C]NaCN according to the literature (Walker and Barker, 1978).

Cell lines and stable-isotope labeling

Cell line 7D7, which produces mouse anti-progesterone IgG2b(κ) (Sawada et al., 1987), was generously provided by Dr. T. Terao and Dr. J. Sawada. Hybridoma cells adapted to a modified Nissui NYSF 404 serum-free medium (Yabe et al., 1986), where dihydroxyethylglycine had been replaced by 15 mM HEPES, were grown in tissue culture flasks (Corning) at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

Metabolic ^{13}C-labeling of IgG was performed by making use of three types of serum-free media (media A, B, and C) prepared from a modified NYSF 404 serum-free medium with additional modifications. In medium A, 2 g/L of unlabeled D-Glc was replaced by the equal amount of D-[1-^{13}C] Glc and 100 mg/L of L-Ala was added. In medium B, 0.2 g/L of D-[1-^{13}C]GlcN·HCl and 200 mg/L of L-Ala were added and D-glucose content was reduced by half whereas contents of sodium succinate, succinic acid and sodium pyruvate were increased twice. The composition of medium C was identical with medium B except that unlabeled D-Glc and D-[1-^{13}C]GlcN·HCl were replaced by D-[1-^{13}C]Glc and unlabeled D-GlcN·HCl, respectively.

After cell growth, the supernatant was concentrated with a Millipore Minitan ultrafiltration system and then applied to an Affi-Gel protein A column (Biorad). A typical yield was 70 mg, 40 mg and 40 mg of purified IgG2b per liter of cell culture in media A, B and C, respectively.

Preparation of the Fc fragment

The Fc fragment of IgG2b was prepared by papain digestion at 37 °C for 3 h in 75 mM sodium phosphate buffer pH 7.0, which contains 75 mM NaCl, 2 mM EDTA, and 5 mM NaN_3. The protein concentration was 5 mg/ml and the ratio of papain/IgG2b was 1:500 (w/w). The digestion products were loaded onto a Mono Q column (Pharmacia Biotech) equilibrated with 20 mM Tris-HCl buffer pH 8.0, and eluted at varying NaCl concentrations in the range 0–400 mM. Fraction containing the Fc was further separated on a Superose 12 column (Pharmacia Biotech) equilibrated with 10 mM sodium phosphate buffer pH 7.3, containing 150 mM NaCl. Purity of the isolated Fc fragment was checked by SDS-polyacrylamide gel electrophoresis.

Galactosidase treatment

Galactosidase treatment of Fc was carried out according to the literatures (Tsuchiya et al., 1989; Kiyohara et al., 1976) with some modifications. Fc was dissolved in 50 mM sodium acetate buffer pH 5.5, at a concentration of 2 mg/ml, and incubated at 37 °C for 48 h in the presence of 0.02 units/ml of β-galactosidase with 10 mM $MnCl_2$. After addition of 20 mM EDTA, the reaction mixture was dialyzed against 20 mM Tris-HCl pH 8.0, at 4 °C, and then Fc was purified by use of an Affi-Gel protein A column. Homogeneity of the Fc carbohydrate chains after the β-galactosidase treatment was checked by *N*-linked oligosaccharide analyses as described below.

Galactosylation of Fc

Fc was galactosylated by exposure to galactosyltransferase and UDP-Gal. While commercially available UDP-Gal could be used for galactosylation with unlabeled D-Gal, we need to generate UDP-[1-^{13}C]Gal *in situ* in the reaction mixture for galactosylation with D-[1-^{13}C]Gal.

Galactosylation with use of D-[1-^{13}C]Gal was performed according to the protocols described in the literature (Ichikawa et al., 1992; Gilhespy-Muskett et al., 1994) with some modifications. Fc was dissolved at a concentration of 2 mg/ml in 100 mM HEPES buffer, pH 7.4, which contains 20 mM KCl, 5 mM $MgCl_2$, 10 mM ATP, 4 units/ml of galactokinase and 10 mM D-[1-^{13}C]Gal, and incubated at 37 °C for 30 min. After addition of 0.4 units/ml of Gal-1-phosphate uridyl transferase and 10 mM UDP-Glc, the reaction mixture was incubated at 37 °C for 4 h. Then, 5 mM $MnCl_2$, 2 units/ml of galactosyltransferase, 20 mM potassium phosphoenolpyruvate, 40 units/ml of pyruvate kinase, 2 units/ml of inorganic pyrophosphatase and 2 units/ml of UDP-Glc pyrophosphorylase were added to the reaction mixture, which was fur-

ther incubated at 37 °C for three days. After addition of 20 mM EDTA, the reaction mixture was dialyzed against 20 mM Tris-HCl pH 8.0, and then the Fc fragment was purified by use of an Affi-Gel protein A column.

Galactosylation with unlabeled D-Gal was carried out according to the protocol by Fujii et al. (1990) with slight modifications. Fc (2.5 mg/ml) was dissolved in 50 mM Tris-HCl buffer pH 7.4, that contains 10 mM $MnCl_2$, 0.6 units/ml of galactosyltransferase, and incubated at 37 °C in the presence of 0.1 mM UDP-Gal for 24 h (protocol A) or in the presence of 20 mM UDP-Gal for 48 h (protocol B). The Fc fragment was purified as described above. *N*-linked oligosaccharide analyses of the Fc after the reactions revealed that 1) in protocol A, the Manα1–6 branch was fully galactosylated but the Manα1–3 branch was partially galactosylated, and 2) in protocol B, both of the Manα1–3 and Manα1–6 branches were fully galactosylated.

Oligosaccharide analyses

Fc protein (0.5 to 1.0 mg) was used for the structure analyses of *N*-linked oligosaccharides. Fc glycopeptides obtained by trypsin plus chymotrypsin digestion were treated with glycoamidase A (Takahashi et al., 1987). The oligosaccharide fraction collected by gel filtration on a Bio-Gel P-4 column was reductively aminated with 2-aminopyridine by use of sodium cyanoborohydride (Yamamoto et al., 1989). The pyridylamino derivatives of oligosaccharides purified by gel filtration on a Sephadex G-15 column were first applied on a DEAE-5PW anion-exchange HPLC column (Tosoh) for the analysis of the sialylated oligosaccharides. Neutral pyridylamino oligosaccharides were then separated and identified on a Nakanopak ODS-A reverse-phase HPLC column (Nakano) as described previously (Takahashi et al., 1987).

Isolation of glycopeptides

Fc was dissolved at a concentration of 1 mg/ml in 0.5 M Tris-HCl buffer pH 8.5, containing 6 M guanidinium chloride, 16 mM dithiothreitol and 2 mM EDTA, which was kept under N_2, at 50 °C for 3 h, and then 37 °C overnight. After addition of 32 mM iodoacetic acid and 32 mM NaOH, the reaction mixture was incubated at room temperature for 30 min, dialyzed against 50 mM NH_4HCO_3 buffer pH 7.9 at 4 °C, and then lyophilized. The lyophilized sample was re-dissolved in 50 mM NH_4HCO_3 buffer pH 7.9, at an Fc concentration of 1 mg/ml and then incubated in the presence of V8 protease at an enzyme/substrate molar ratio of 1:30 at 37 °C for 12–24 h. The reaction mixture was lyophilized, re-dissolved in 0.1% (v/v) TFA at a total protein concentration of 1 mg/ml and loaded onto a YMC ODS A-312 reverse-phase column (Yamamura Chemical Laboratories) connected to a Shimadzu LC-6A pump system equilibrated with an aqueous solution containing 5% acetonitrile and 0.1% (v/v) TFA. The digestion products were eluted at a flow rate of 1.0 ml/min with a linear gradient of 5–50% (v/v) acetonitrile in 0.1% (v/v) TFA for 80 min at 40 °C. Absorbance was detected at 210 nm with a Shimadzu CTO-6A detector. Fractionated peptides were lyophilized and then subjected to an amino acid sequence analysis on an Applied Biosystems protein sequencer model 477A/120A and a MALDI-TOFMS analysis on a Shimadzu Kompact MALDI IV mass spectrometer. The isolated glycopeptide originating from a segment spanning residues Asp-295 to Glu-318 was subjected to an NMR analysis.

NMR measurements

The Fc glycoprotein or the glycopeptide was dissolved in 0.4 mL of 5 mM sodium phosphate buffer pH 7.3 (uncorrected meter reading made with an electrode standardized with H_2O buffer), containing 200 mM NaCl and 3 mM NaN_3 in ^2H_2O (99.96%). An NMR sample tube of 5 mm diameter was used with a final protein or peptide concentration of 0.1–1.5 mM.

^1H-^{13}C HSQC experiments were carried out on a Bruker DRX-400 spectrometer at a ^1H frequency of 400 MHz The spectral widths were 10 kHz in the F1 (^{13}C) dimension and 6 kHz in the F2 (^1H) dimension. The data points were 256–512 in the F1 (^{13}C) dimension and 1,024 in the F2 (^1H) dimension. The probe temperature was set to 52 °C. For each F1 (^{13}C) increment with the time-proportional phase increment mode, 64–600 transients of FID were recorded. ^{13}C enrichment was estimated on the basis of the relative intensity of the center peak to its satellite peaks in a ^{13}C-coupled ^1H NMR spectrum of the glycopeptide.

The linewidth of each anomeric ^{13}C resonance was measured in one-dimensional ^{13}C NMR spectra of the Fc fragments in which the anomeric carbons of selected sugar residues are labeled with ^{13}C. One-dimensional ^{13}C NMR measurements were made at 100 MHz on a JEOL GSX-400 spectrometer with ^1H decoupling in a composite pulse mode. The probe temperature was set to 37 °C unless otherwise stated. 130 000–350 000 transients of FID after a 30° pulse

were recorded with a repetition period of 2 s, with 32 768 data points and a spectral width of 24 000 Hz. FID was multiplied by an exponential window function with a broadening factor of 5 Hz prior to Fourier transformation. All chemical shifts were given in ppm from external 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt.

Results

Identification of the carbohydrate chains

For the NMR analyses of glycoproteins, it is essential to obtain information concerning their glycoforms. Takahashi et al. (1987) have established a method to identify *N*-linked oligosaccharides rapidly on the basis of HPLC elution profiles of their pyridylamino derivatives. Figure 2A shows a typical HPLC elution profile of the *N*-linked oligosaccharides of the IgG2b Fc used in the present study. As reported for mouse IgGs previously (Mizuochi et al., 1987; Rothman et al., 1989), the great majority of the carbohydrate chains is fucosylated and lack bisecting GlcNAc (oligosaccharides *E*, *F*, *G*, and *H* in Figure 2D). Especially, two components were outstanding: One has no Gal residue (oligosaccharide *E*), the other has a single Gal residue at the nonreducing terminal of the Manα1–6 branch (oligosaccharide *F*).

Trimming of the carbohydrate chains

In order to avoid any spectral complexities resulting from the heterogeneity of the Fc glycoforms, the carbohydrate chains were trimmed to become uniform structure by β-galactosidase or galactosyltransferase treatment. In the present NMR study, Fc fragments that exclusively possess the agalactosylated oligosaccharides (designated as Fc(G0)) and the di-galactosylated oligosaccharides (designated as Fc(G2)) were prepared. The Fc(G0) and Fc(G2) preparations gave HPLC profiles as indicated in Figures 2B and 2C, respectively. These Fc preparations fully retained a protein A-binding activity, indicating that no significant denaturation occurred during the papain digestion and the trimming procedures.

Metabolic labeling of the carbohydrate chains

Figure 3A compares the anomeric region of the ^{1}H-^{13}C HSQC spectrum of the Fc(G0) metabolically labeled with D-[1-^{13}C]Glc with that of the glycopeptide derived from it by a V8 protease digestion. The ^{1}H and ^{13}C chemical shifts of the anomeric peaks of the

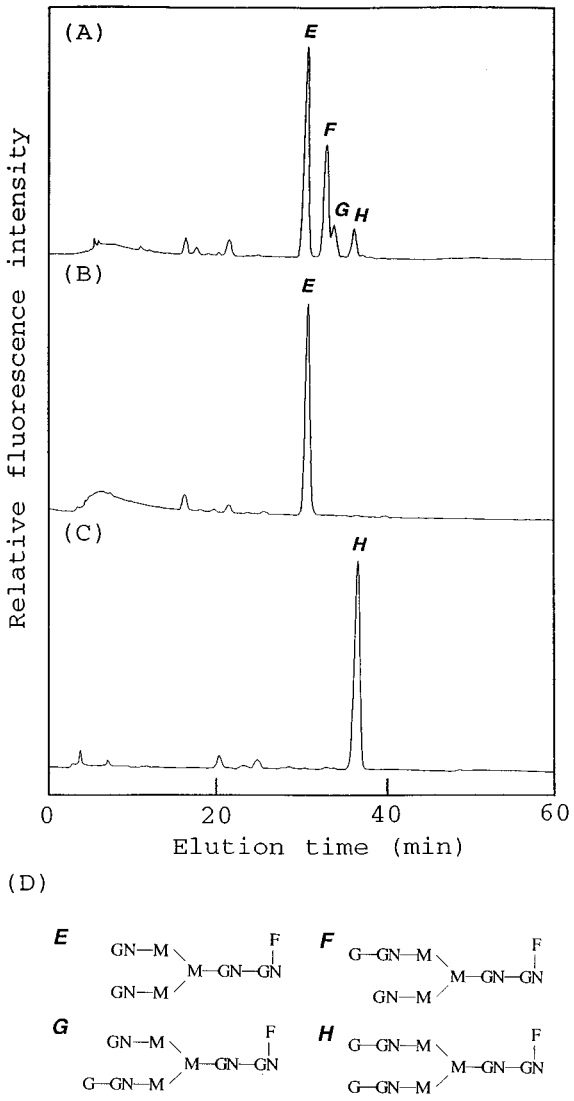

Figure 2. Elution profiles on an ODS-silica column of pyridylamino derivatives of the *N*-linked oligosaccharides obtained from the IgG2b Fc fragments used in the present study (A–C) and the oligosaccharide structures corresponding to each of the peaks (D). A, native Fc; B, Fc after treatment by β-galactosidase from *Streptococcus* strain 6646K; C, Fc after exposure to UDP-Gal and galactosyltransferase from bovine milk. The alphabetical codes *E*, *F*, *G*, and *H* are used for the identification of the oligosaccharide structure according to the literature (Takahashi et al., 1987). No sialylated *N*-linked oligosaccharide was contained in the native Fc sample (data not shown). In D, one-letter codes are used to indicate the sugar residues as follows: G, D-galactose; GN, N-acetyl-D-glucosamine; M, D-mannose; F, L-fucose.

glycopeptide are in agreement with those of isolated oligosaccharides and their derivatives (Vliegenthart et al., 1983; Damm et al., 1987; Lu and Halbeek, 1996) and therefore can unequivocally be assigned. Significant differences were observed in the chemical shifts of most of the cross-peaks between the Fc(G0) and the isolated glycopeptide. Therefore, it was not possible to assign most of the individual anomeric peaks of Fc(G0) solely on the basis of a chemical shift comparison. The only exception is peak a, which was assigned to GlcNAc-1 judging from its characteristic ^{13}C chemical shift (∼80 ppm) of the C-N linkage.

In order to classify the anomeric peaks for the Fc(G0) on the basis of carbohydrate residue-types, residue-selective ^{13}C labeling of the carbohydrate chains was performed. Figure 3B shows a ^{1}H-^{13}C HSQC spectrum of an Fc(G0) that was prepared by cultivating the hybridoma cells in the medium containing D-[1-^{13}C]GlcN. Only four peaks (a, e, f and g) were observed in the spectrum. As expected, the glycopeptide isolated from this Fc sample gave anomeric resonances, all of which originated from the four GlcNAc residues (Figure 3B). Therefore we concluded that peaks a, e, f and g originate from the anomeric groups of the GlcNAc residues of the Fc carbohydrate chains. On the basis of the relative intensities of the center peaks to their satellite peaks in a ^{13}C-coupled ^{1}H NMR spectrum of the glycopeptide, it was concluded that ^{13}C enrichments at the anomeric carbons of the GlcNAc residues were 94% (data not shown).

As shown in Figure 3C, the Fc(G0) prepared by making use of a medium with a combination of D-[1-^{13}C]Glc and unlabeled D-GlcN gave a different set of four peaks, b, c, d and h, which were assigned to the three Man residues and the single Fuc residue. An Fc(G0) prepared by making use of a medium containing 100 mg/L of D-[1-^{13}C]Man also gave four peaks b, c, d and h, indicating that Man had been converted into Fuc during the cell growth (data not shown). Thus differentiation of the Man resonances from the Fuc resonance by selective isotope labeling *via* biosynthesis was not possible at the present stage.

In vitro ^{13}*C labeling of the Gal residues at the non-reducing ends*

We followed a method employed by Gilhespy-Muskett et al. in order to observe selectively the anomeric resonance originating from the terminal Gal residues of the Fc(G2) (Gilhespy-Muskett et al., 1994). D-[1-^{13}C]Gal was attached onto the nonreducing ends of the carbohydrate chains of an unlabeled Fc(G0). Figure 4A shows a ^{1}H-^{13}C HSQC spectrum of the Fc(G2), in which the anomeric carbons of Gal-6 and Gal-6′ have been selectively labeled with ^{13}C. Two peaks, i and j, are clearly observed in the anomeric region. As shown in Figure 4B, peak i is much narrower in linewidth than peak j.

In order to assign peaks i and j to each of the Gal residues, we prepared another Fc(G2) sample in which Gal-6 and Gal-6′ were labeled with ^{13}C in different degrees, taking advantage of a branch specificity of the galactosyltransferase. In the case of IgG Fc, galactosylation of non-bisected, biantennary complexes at the Manα1–6 branch predominates over those at the Manα1–3 branch (Fujii et al., 1990). It was confirmed that exposure of Fc(G0) to the galactosyltransferase and unlabeled UDP-Gal according to protocol A (see Materials and methods) results in full and partial (47%) galactosylations of Manα1–6 and Manα1–3 branches, respectively (data not shown). This Fc fragment was isolated and then fully galactosylated with D-[1-^{13}C]Gal, giving rise to Fc(G2) enriched with ^{13}C only at the anomeric carbon of Gal-6. The Fc(G2) thus prepared gave only peak i in the ^{13}C spectrum (Figure 4C). The result led us to assign peaks i and j to Gal-6 and Gal-6′, respectively.

NMR analyses of the dynamics of the carbohydrate chain

In order to examine the effect of the presence of the nonreducing terminal Gal residues upon the structure of the Fc carbohydrate chains, Fc(G2) was prepared using unlabeled D-Gal by *in vitro* galactosylation of Fc(G0) that had been labeled with D-[1-^{13}C]Glc. Figure 5 compares the anomeric region of the ^{1}H-^{13}C HSQC spectrum for Fc(G2) with that for Fc(G0). It is indicated that all peaks except for peaks c, g and h have same chemical shifts.

Table 1 summarizes the assignments and ^{13}C linewidths of the peaks originating from the anomeric carbons of the carbohydrate chains in Fc(G0) and Fc(G2).

Discussion

NMR spectral features of the carbohydrate chain

As shown in Figure 3A, we have successfully observed the ^{1}H-^{13}C HSQC peaks originating from the individual anomeric groups of carbohydrate chains attached to the Fc of IgG by using the metabolic labeling established in the present study, in spite of the fact that

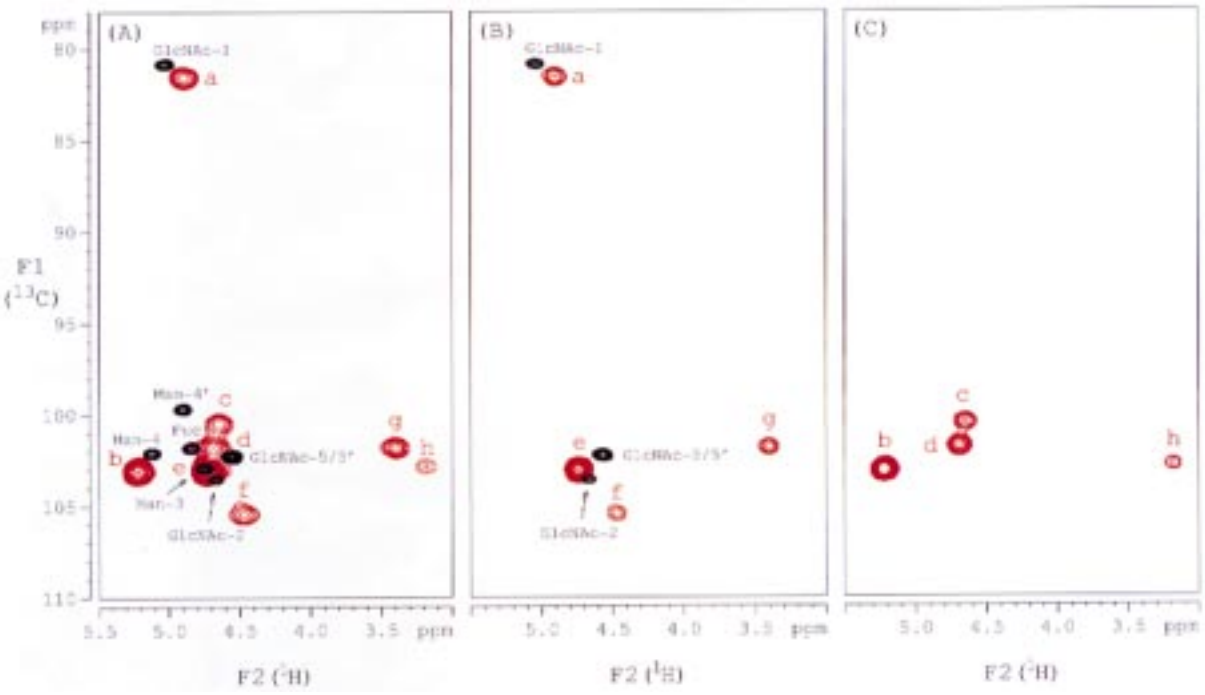

Figure 3. The anomeric regions of the ^{1}H-^{13}C HSQC spectra observed for the Fc(G0) labeled with D-[1-^{13}C]Glc (red) and the glycopeptide derived from it (black) (A), Fc(G0) labeled with D-[1-^{13}C]GlcN (B), Fc(G0) labeled with D-[1-^{13}C]Glc in the presence of unlabeled D-GlcN (C). The Fc(G0) samples for A, B, and C were prepared by use of media A, B, and C, respectively, as described in the text. Assignments of the resonances originating from the glycopeptide were made on the basis of the literature data of free oligosaccharides (Vliegenthart et al., 1983; Damm et al., 1987; Lu and Halbeek, 1996) and shown in A. It should be noted that the peaks originating from GlcNAc-5 and GlcNAc-5′ overlaps with each other in the spectra for the glycopeptide. ^{13}C enrichments at the anomeric carbons were 60% and 94% for A and B, respectively.

Table 1. Linewidth of ^{13}C resonances originating from the anomeric carbons of the carbohydrate chains linked to the Fc fragments[a]

Peak	Assignment	Fc(G0)	Fc(G2)
a	GlcNAc-1	55	52
b	Fuc/Man	–[b]	–
c	Fuc/Man	45	51
d	Fuc/Man	37	43
e	GlcNAc	30	32
f	GlcNAc	50	49
g	GlcNAc	49	52
h	Fuc/Man	–	–
i	Gal-6		13
j	Gal-6′		39

[a] The linewidths at half heights of the resonances observed in one-dimensional ^{13}C NMR spectra are indicated in Hertz.

[b] Linewidth was not available because of degeneracy of resonances b and h.

the Fc has a molecular mass of 50 kDa. The labeling technique has also enabled us to assign the resonances to each of GlcNAc and Man/Fuc residues (see Figures 3B and 3C). To our knowledge, these are the first NMR spectra of glycoproteins in which selected sugar residues are metabolically labeled.

It has been shown that most of the anomeric peaks originating from the carbohydrate chains attached to Fc give different chemical shifts from those in the spectrum of the glycopeptide isolated from Fc (Figure 3A). This result is in marked contrast to those previously reported for human CD2 (Wyss et al., 1995b) and human chorionic gonadotropin α subunit (de Beer et al., 1996). In the spectra of these glycoproteins, most of the resonances originating from the carbohydrate chains gave chemical shifts which are similar to those for the isolated oligosaccharides. This indicates that the carbohydrate moieties of these glycoproteins are located on the protein surface and exposed to solvent. In contrast, the present NMR data strongly suggest that the carbohydrate chains linked

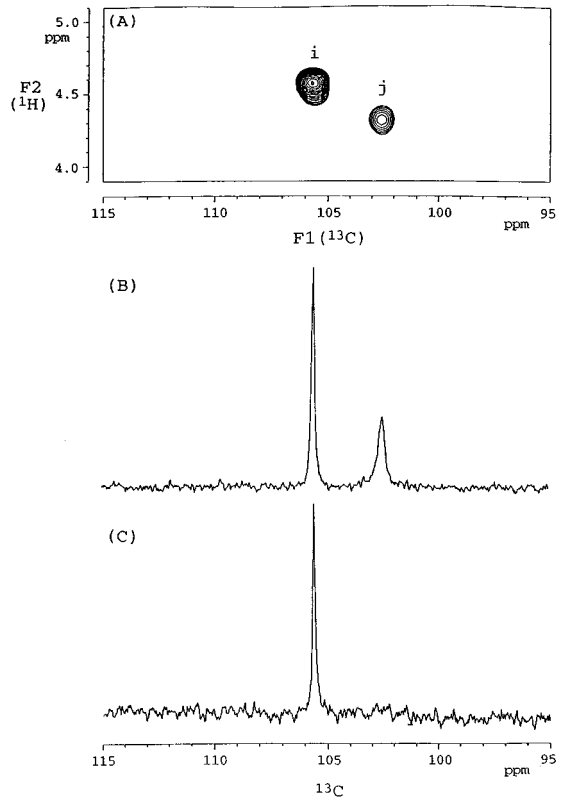

Figure 4. The ^{1}H-^{13}C HSQC (A) and one-dimensional ^{13}C NMR (B, C) spectra of the Fc(G2) fragments in which the anomeric carbons of the terminal Gal residues were fully (A, B) or partially (C) labeled with ^{13}C. In A and B, the Fc(G2) prepared from unlabeled Fc(G0) by a full galactosylation with D-[1-^{13}C]Gal was used. In C, the Fc(G2) prepared from unlabeled Fc(G0) by a two-step galactosylation was used: In the first step, with unlabeled D-Gal, the Manα1–6 branch was fully galactosylated and the Manα1–3 branch was partially (47%) galactosylated according to protocol A for galactosylation. In the second step, the Manα1-3 branch that had not been galactosylated in the first step was fully galactosylated with D-[1-^{13}C]Gal. The probe temperature was 52 °C.

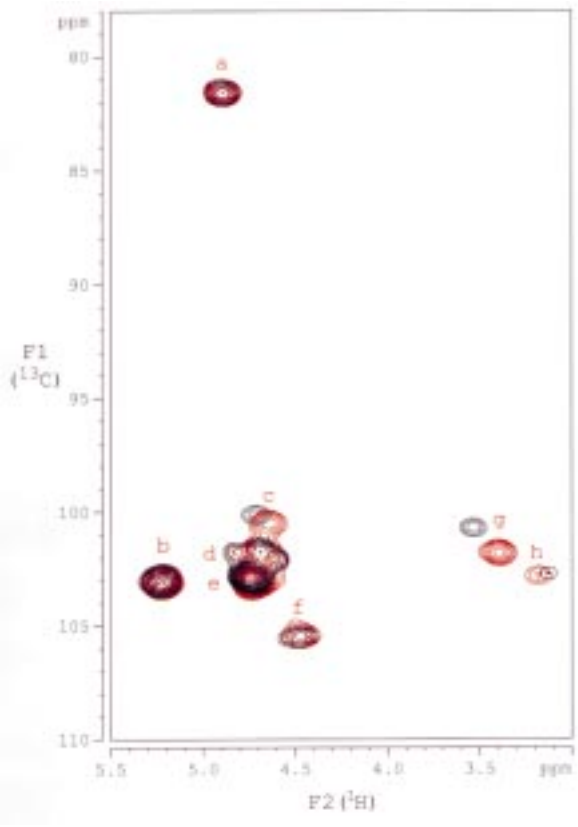

Figure 5. Comparison of the anomeric regions of the ^{1}H-^{13}C HSQC spectra observed for the Fc(G0) metabolically labeled with D-[1-^{13}C]Glc (red) and the Fc(G2) prepared from the same Fc(G0) by galactosylation with unlabeled D-Gal according to protocol B (black).

to Fc are located in an environment which is different from that of the isolated oligosaccharide. This is quite consistent with the previous X-ray crystallographic data of the IgG and the Fc fragments. In the crystal structures, the carbohydrate chains are buried inside the molecule, exhibiting conformations which are different from that predicted by NMR and theoretical calculations performed for free oligosaccharides in solution (Deisenhofer, 1981; Sutton et al., 1983; Harris et al., 1997; Homans, 1994).

The crystal structures show that the core and the Manα1–6 branch of the carbohydrate chain make contacts with an inner surface of the C_H2 domain (Deisenhofer, 1981; Sutton et al., 1983; Harris et al., 1997). On the other hand, the conformation of the Manα1–3 branch, which protrudes to the space between the C_H2 domains, is significantly diverse among the crystal structures. In human IgG Fc, the Manα1–3 branches of the pair of the carbohydrate chains form a weak bridge between the C_H2 domains (Deisenhofer, 1981). In rabbit IgG Fc, the pair of the carbohydrate chains exhibits an asymmetric structure, in which the Manα1–3 branch of one carbohydrate chain makes extensive contacts with the core of the other carbohydrate chain (Sutton et al., 1983). No contact exists between the pair of the carbohydrate chains in intact mouse IgG2a (Harris et al., 1997).

In the ^{1}H-^{13}C HSQC spectrum of either Fc(G0) or Fc(G2), each of the resonances originating from the two carbohydrate chains is observed as a single peak (Figure 5). The results obtained from the present study indicate that asymmetric arrangement of the Fc carbohydrate chains as shown in the rabbit Fc crys-

tal does not exist on NMR time-scale. An intriguing possibility is that different Fc crystal structures reflect different snapshots of the Manα1–3 branches of the carbohydrate chains in solution. We also cannot rule out the possibility that the diversity of the observed sugar conformations depends on the glycoforms of the Fc used for the crystallographic studies.

Dynamics of the Fc carbohydrate chain

The present study has shown that the Fc carbohydrate chain gives the anomeric ^{13}C resonances with linewidths of 30 to 55 Hz except for Gal-6 of Fc(G2), which gives an extremely narrow ^{13}C resonance with a linewidth of 13 Hz (Table 1). It should be noted that information on the dynamics of the Gal-6 and 6′ part can be selectively extracted on the basis of the assignment established in the present study. The linewidth of the ^{13}C resonances of the Cα carbon of the Fc polypeptide is of the order of 40 Hz (Yamaguchi et al., unpublished data). Therefore, we conclude that mobility of the carbohydrate chain is comparable to that of the polypeptide backbone except Gal-6, which is much more mobile than the rest of the carbohydrate chain. Thus, the mobilities of the terminal Gal residues are significantly different for the Manα1–3 and the Manα1–6 branches.

An early ^{13}C NMR observation at natural abundance for human IgG Fc (Rosen et al., 1979) along with an EPR analysis (Nezlin, 1990) have indicated that mobility of the carbohydrate chains in Fc is comparable to that of the Fc polypeptide chains. These results are consistent with those obtained in the present NMR analyses except for Gal-6. In the ^{13}C NMR spectrum obtained at natural abundance, it was difficult to identify the resonances of Gal-6 and Gal-6′ individually. The EPR data lacked information on the localization of an introduced spin label. In addition, glycoforms of the Fc fragments were not described in these studies. It is therefore virtually impossible to discuss the mobility of Gal-6 on the basis of the previous ^{13}C NMR and EPR data.

As mentioned above, the crystal structures indicate that the core and the Manα1–6 branch of the carbohydrate chain make contacts with the inner surface of the C_H2 domain (Deisenhofer, 1981; Sutton et al., 1983; Harris et al., 1997). It is quite conceivable that these contacts restrict the motion of the core and the Manα1–6 branch of the carbohydrate chains of Fc in solution. The present NMR data also indicate that the Manα1–3 branches except Gal-6 are restricted in mobility in solution. It is possible that the interactions between the two carbohydrate chains in one Fc molecule, which have been observed in the crystal structures of human IgG Fc and rabbit IgG Fc, are responsible for the restriction of the movement of the Manα1–3 branches.

Dwek and co-workers have reported that agalactosylation induces a significant change in conformation of the glycans in Fc making the glycans more accessible to the effector molecules (Malhotra et al., 1995; Wormald et al., 1997). It was suggested that the exposed glycans can activate complement through binding to the collageneous lectin mannose-binding protein, resulting in inflammation in rheumatoid arthritis. Their model was based on the comparative NMR relaxation data, which showed that rheumatoid IgG Fc (at a G0 fraction of 55%) gives a much more slowly relaxing subset of proton resonances observed between 3.50 ppm and 4.25 ppm as compared with the control sample (at a G0 fraction of 26%). On the basis of these data, they have concluded that 1) the carbohydrate chains with Gal-6′ have relaxation properties quite similar to those of the peptide backbone and thus do not show independent motion, 2) the carbohydrate chains that lack the Gal residue give the relaxation time which is 30 times longer than that of the peptide and therefore 3) a higher degree of mobility becomes acquired for the carbohydrate chains. Their conclusion apparently contradicts to the present NMR data, which indicate that galactosylation induces no significant change in mobility of carbohydrate chains (Table 1). It is unlikely that the difference in species of IgG between mouse and human is responsible to the above discrepancy, because the residues in contact with the carbohydrate chain are completely conserved between human IgG1 and mouse IgG2b with an only exception that Glu-258 of human IgG1, which makes contact with Gal-6′ in the crystal structures, is replaced by Lys in mouse IgG2b. It is virtually impossible to distinguish the resonances originating from the carbohydrate chains from those from the peptide chains and therefore all resonances in the spectral range 3.50–4.25 ppm had to be used as a whole. Thus the ^{1}H NMR relaxation data should be interpreted with great care.

On the basis of the present ^{13}C relaxation data along with the chemical shift data, we conclude that 1) agalactosylation induces no significant change in the dynamics of the Fc carbohydrate chains and 2) the carbohydrate chains are buried into the Fc molecule even in the agalactosyl form. The present NMR data also indicate that removal of the terminal Gal residues induces a structural change in the limited part

(one GlcNAc and two Man/Fuc residues) of the carbohydrate chains in Fc. As shown in Figure 5, three resonances c (Fuc or Man), g (GlcNAc) and h (Fuc or Man) showed small but significant chemical shift changes upon galactosylation, while the rest of the anomeric resonances were not perturbed at all. Therefore, it is possible that the local structural change on the carbohydrate chain, which is accompanied by the structural change of the surrounding polypeptide chain, is responsible for the reduced reactivity of agalactosyl IgG to C1q and Fc receptors and also for the pathogenicity in rheumatoid arthritis.

For a better understanding of the structural correlate of the glycans to the effector functions of immunoglobulins, an attempt is now underway in our laboratory for the site-specific spectral assignments of all observed carbohydrate resonances. It is hoped that the method developed would eventually become a general method for the discussion of the structural basis of glycoproteins.

Acknowledgements

We thank Drs T. Terao and J. Sawada (National Institute of Health Sciences) for generously providing us with the cell line 7D7. We also thank H. Araki for HPLC analyses of the oligosaccharides used in the present study. This research was supported in part by a grant from the Japan Society for the Promotion of Science (JSPS-RFTF96L00505) and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Sciences, Culture and Sports of Japan (06276102).

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