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Structure Determination by 360-MHz ^{1}H-NMR Spectroscopy and Methylation Analysis of a Biantennary Glycan of the *N*-Acetyllactosaminic Type Isolated from Rat-Liver Plasma Membrane

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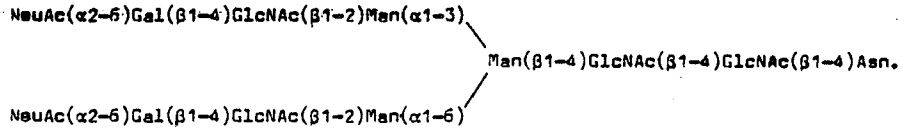
Glycopeptides obtained by exhaustive pronase digestion of delipidated rat liver plasmic membranes were purified by gel filtration on Sephadex G-25. These glycopeptides were further fractionated by affinity chromatography on a concanavalin-A–Sepharose 4B column into the following fractions: (a) glycopeptides which did not bind to the column (fraction 1); (b) glycopeptides with weak affinity for concanavalin-A–Sepharose, which could be eluted with buffer only (fraction 2); (c) glycopeptides retained on the column and which could be eluted specifically with buffer containing 0.2 M methyl α-glucoside (fraction 3).

On the basis of the carbohydrate composition, methylation analysis and 360-MHz ^{1}H-NMR spectroscopy, the following primary structure of a glycan in fraction 2 is proposed:



In recent years, evidence has accumulated indicating that carbohydrate moieties of surface glycoproteins and glycolipids play important roles in cellular functions such as cell-cell recognition, adhesion, cellular differentiation and that in malignant transformation these carbohydrate moieties may be altered, allowing the cell to escape control [1].

Changes in glycolipids associated with transformation have been well documented in a number of different systems and it has been demonstrated that transformed cells generally show a simplified pattern of their glycolipids [2]. On the other hand, surface glycoproteins of malignant cells seem to be characterized by the presence of glycans with a larger molecular weight than those of the control cells [3].

While structures of membrane glycolipids are now well documented, the analysis of the glycans of membrane glycoproteins is much more difficult due to their small amount. Therefore it is very important to develop a new methodology for the fractionation and the structure determination of the carbohydrate moieties of surface glycoproteins.

In the present paper, we describe a new approach to study the structure of the glycans of rat liver plasma membrane glycoproteins by combining affinity chromatography of glycopeptides derived from these glycoproteins on concanavalin-A–Sepharose and subsequent analysis of the fractionated glycopeptides by high-resolution ^{1}H-NMR spectroscopy at 360 MHz and methylation analysis [4].

A preliminary report on these results has been published [5].

Abbreviations. NMR, nuclear magnetic resonance; Fuc, L-fucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; NeuAc, *N*-acetyl-D-neuraminic acid; Asn, L-asparagine; IgM, immunoglobulins M.

Enzymes. 5′-Nucleotidase or 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5); glucose-6-phosphatase or D-glucose-6-phosphate phosphohydrolase (EC 3.1.3.9); succinate dehydrogenase (EC 1.3.99.1).

MATERIALS AND METHODS

Preparation of Delipidated Rat Liver Plasma Membrane

Hepatocyte plasma membranes were isolated from the liver of male Wistar Rats fed *ad libitum*, using a sucrose density gradient procedure according to Neville [6], as modified by Ray [7]. The purity of the plasma membrane fraction was controlled by electron microscopy and by the assay of marker enzymes: 5′-nucleotidase according to Emmelot et al. [8], glucose-6-phosphatase according to Ray [7] and succinate dehydrogenase according to Bonner [9].

Purified plasma membranes were washed extensively with cold distilled water to remove any residual sucrose and were lyophilized. 460 mg of lyophilized membranes were suspended in 12 ml 1.5 mM $MgCl_2$ solution and extracted at room temperature for 2 h by 19 vol. of a mixture of chloroform/methanol (2:1, v/v). The membrane suspension was centrifuged for 30 min at 13000 rev./min in a Beckman J-21 centrifuge (JA-20 rotor). The membrane pellet was resuspended in 15 ml of the chloroform/methanol mixture and extracted and centrifuged as before. This step was repeated twice and the delipidated membranes were dried under vacuum.

Pronase Digestion of Delipidated Membranes

The delipidated dry membranes (250 mg) were resuspended in 12 ml of distilled water and 2 ml of 1 M Tris/HCl pH 8.4, 0.1 M $CaCl_2$ was added. To prevent bacterial contamination, a few drops of toluene were added and the membranes were digested with 5 mg of pronase (B grade, Calbiochem) for 96 h at 37 °C, a further 2.5 mg of pronase being added at 24 h and 48 h. The digest was then adjusted to pH 7 and centrifuged to remove insoluble materials.

Fractionation of Glycopeptides

The pronase digest was first chromatographed on a column (2 × 50 cm) of Sephadex G-25 fine eluted with 10 mM pyridine/acetic acid buffer pH 5.0. Glycopeptides were detected using the phenol/H_2SO_4 reagent [10]. Purified glycopeptides were *N*-[^{14}C]acetylated on their peptide moiety with [^{14}C]-acetic anhydride (7 Ci/mol) obtained from the Commissariat à l'Energie Atomique (France), according to Koide et al. [11]. The acetylated glycopeptides were desalted on a column (2 × 50 cm) of Sephadex G-25 fine equilibrated with 10 mM pyridine/acetic acid buffer pH 5.0, and then fractionated by affinity chromatography on a column (15 × 2.5 cm) of concanavalin-A – Sepharose 4B [12].

Methods

Molar ratios of neutral monosaccharides and hexosamines were determined after methanolysis by 0.5 M HCl/methanol, at 80 °C during 24 h [13]. The amino acid composition of glycopeptides was determined with a Beckman Multichrom Analyser, hydrolysis of the glycopeptides being performed with 5.6 M HCl at 105 °C under vacuum during 24 h.

Permethylation of glycopeptides was carried out according to Hakomori [14]. Partially methylated monosaccharides were identified according to Fournet et al. [15].

For NMR analysis, glycopeptides (750 μg) were repeatedly exchanged in deuterium oxide. The 360-MHz ^{1}H-NMR spectra of the glycopeptide solutions in 0.5 ml ^2H_2O were recorded on a Bruker HX-360 spectrometer, operating in the Fourier transform mode, at probe temperatures of 20 °C and 40 °C. Chemical shifts are given relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate (indirectly to acetone in ^2H_2O : $\delta = 2.225$ ppm).

RESULTS

Fractionation of Glycopeptides by Affinity Chromatography on Concanavalin-A – Sepharose 4B

From [^{14}C]acetylated membrane glycopeptides chromatographed on a concanavalin-A – Sepharose 4B column, three fractions were obtained as shown in Fig. 1: (a) An unbound fraction (fraction 1) which might contain glycopeptides with *O*-glycosidic linkages and/or glycopeptides with the *N*-glycosylamine linkage, belonging to the *N*-acetyllactosamine type, with triantennary and/or tetraantennary structures according to the nomenclature of Montreuil [16]; (b) a retarded fraction (fraction 2) which presents a low affinity for concanavalin A and which is eluted with the buffer devoid of methyl α-D-glucoside; from previous experiments [12], it can be predicted that the glycopeptides present in this fraction are biantennary glycopeptides of the *N*-acetyllactosamine t
(c) the third fraction (fraction 3) possesses a higher affinity for concanavalin A and is eluted from the column by 200 mM methyl α-D-glucoside. Preliminary methanolysis results show that these latter glycopeptides are mainly of the oligomannosidic type, with structures similar to those of ovalbumin or thyroglobulin. type A glycopeptides [17, 18]. However, the presence of *N*-acetylneuraminic acid and galactose residues seems to indicate that glycopeptides with oligomannosidyl-*N*-acetyllactosamine type (mixed type) structures [19] are probably present in this fraction or that the oligomannosidyl structures are contaminated with glycans of the *N*-acetyllactosamine type.

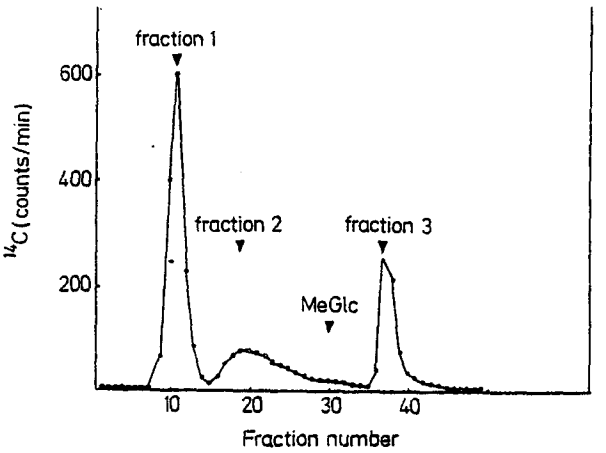


Fig. 1. *Fractionation of glycopeptides by affinity chromatography on concanavalin-A – Sepharose 4B.* MeGlc, position at which 0.2 M methyl α-D-glucoside was added to the elution buffer

The fractions were rechromatographed on the same concanavalin-A – Sepharose column after desalting; each of them gives only one peak. This group separation of *N*-glycosidic glycopeptides by concanavalin-A – Sepharose 4B chromatography seems to be in good agreement with the results of Ogata et al. [20] and those of Krusius et al. [21] as well as with our own previous results [12]. Starting from 250 mg of delipidated dry membranes, the amount of total sugar recovered in each fraction after affinity chromatography on concanavalin-Sepharose is as follows: fraction 1, 3.84 mg; fraction 2, 750 μg; fraction 3, 460 μg. From these results, we can estimate the sugar in the membrane material to be 2% of the total. In this study, only results obtained with the retarded fraction (fraction 2) are presented. The unbound (fraction 1) and eluted fraction (fraction 3) are still under investigation.

Composition of the Glycopeptides of Fraction 2

Carbohydrate and amino acid compositions of the glycopeptides recovered in fraction 2 are shown in Table 1.

Structure Determination of the Glycopeptides of the Retarded Fraction (Fraction 2)

NMR-Analysis. The 360-MHz ^{1}H-NMR spectrum of fraction 2 is given in Fig. 2 and relevant NMR data are summarized in Table 2. The interpretation of the spectrum was carried out as described by Dorland et al. who took ad-

vantage of the corresponding spectra of appropriate reference compounds [22–24]. In this spectrum, the resonance positions of the mannose C-1 and C-2 protons show that this glycopeptide has the biantennary structure. The chemical shifts of the neuraminic acid C-3 protons indicate that the sialic acid residues are bound to C-6 of the galactose residues 6 and 6′. The fact that the C-1 protons of mannose 4 and 4′ both shift downfield with respect to an asialo-biantennary glycopeptide [23] means that both branches are (α2–6) sialylated. The resonance signals at 4.87 ppm (spectrum recorded at 40 °C), 4.11 ppm and 1.20 ppm point to the presence of a fucose residue linked (α1–6) to the *N*-acetylglucosamine residue 1 [25]. The relative intensities of these signals indicate that a fucose analogue of the biantennary glycopeptide is present to about 10%. This agrees with the amount of fucose determined after methanolysis (Table 1). The ^{1}H-NMR spectral data of the major glycopeptide of fraction 2 are in

Table 1. *Molar composition in monosaccharides and amino acids of the glycopeptides of the retarded fraction (fraction 2)*

Amino acid	Amount
	mol/mol Asp
Aspartic acid	1
Threonine	0.5
Serine	0.54
Glutamic acid	0.36
Glycine	0.47
Alanine	0.21
Sugar	
	mol/3 mol Man
Galactose	1.8
Mannose	3
Fucose	0.12
N-Acetylglucosamine	4.2
N-Acetylgalactosamine	0
N-Acetylneuraminic acid	2.1

Table 2. *^{1}H-NMR chemical shifts of anomeric (C-1), mannose (C-2), N-acetylneuraminic acid (C-3) and N-acetyl protons for the major glycopeptide of the retarded fraction (fraction 2)*

Proton		Chemical shift
		ppm
Anomeric		
GlcNAc	1	5.049
GlcNAc	2	≈ 4.62
Man	3	4.768[a]
Man	4	5.135
Man	4′	4.936[a]
GlcNAc	5	4.607
GlcNAc	5′	4.607
Gal	6	4.444
Gal	6′	4.444
H-2		
Man	3	4.256
Man	4	4.200
Man	4′	4.118
H-3 equatorial		
NeuAc		2.670
H-3 axial		
NeuAc		1.724
N-acetyl		
GlcNAc	1	2.010
GlcNAc	2	2.080
GlcNAc	5	2.069
GlcNAc	5′	2.069
NeuAc		2.030

[a] Values determined at 40 °C.

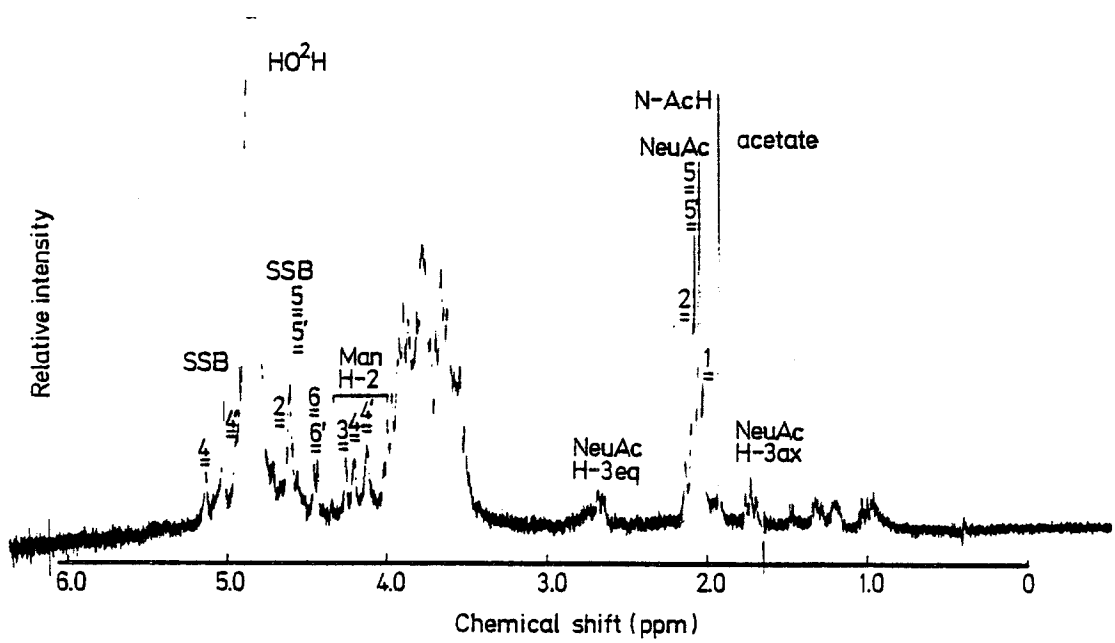


Fig. 2. *360-MHz ^{1}H-NMR spectrum of the fraction 2 glycopeptides in 2 H_2O at p^{2}H 7.* SSB = spinning side band; spectral width = 2500 Hz; data memory = 16 k; digital resolution = 0.3 Hz; acquisition time = 3.3 s; pulse angle = 90°; number of scans = 500

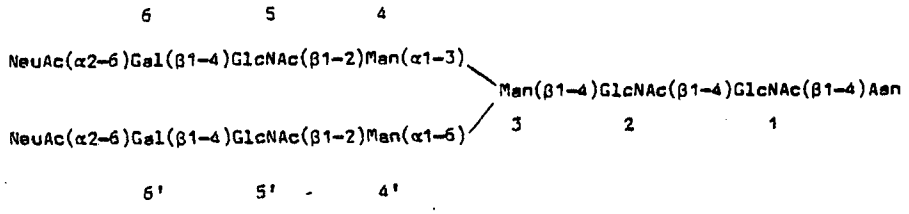


Fig. 3. *Primary structure of one of the glycans isolated from rat liver plasma membrane*

accordance with those of the sialoglycan structure of human [23] and rabbit [24] serotransferrin.

Permethylation Studies. Analysis of methyl derivatives from constituting monosaccharides of glycopeptides of fraction 2 are in a good accordance with the NMR data. On the other hand, the nature and the molar ratio of monosaccharide methyl ethers agree with those reported earlier for the corresponding carbohydrate unit derived from human serotransferrin [26]: 3,4,6-tri-*O*-methylmannose : 2,3,4-tri-*O*-methylmannose : 2,4-di-*O*-methylmannose : 3,6-di-*O*-methylmannose = 1.95 : 1.7 : 1 : 3.7.

DISCUSSION AND CONCLUSION

On the basis of the carbohydrate composition, methylation analysis and 360-MHz ^{1}H-NMR spectroscopy, the primary structure of a glycan of a glycopeptide fraction isolated from rat liver plasma membrane as given in Fig. 3 was deduced.

Considering that we had available only 750 μg of glycopeptide, such a structure determination could be carried out only by the combination of high-resolution ^{1}N-NMR spectroscopy and methylation analysis which were for the first time applied to the study of glycans of plasma membrane origin. Thus it is demonstrated that NMR spectroscopy can be successfully used for structural studies of the carbohydrate moiety of membrane glycoproteins and that it is a very promising method. One of the particular advantages of this method is the fact that it is not destructive and thus leaves open the possibility of subsequent chemical and enzymic investigations.

Work is in progress concerning fractions 1 and 3 of normal rat liver plasma membranes and corresponding glycopeptidic fractions of hepatoma cells.

This kind of biantennary glycan structure of the *N*-acetyllactosaminic type has already been found in several glycoproteins: human [23] and rabbit [24] serotransferrins, human α_1-acid glycoprotein [27], thyroxin-binding globulin [28], Waldenström IgM J chain [29], bovine prothrombin [30], human plasminogen 1 [31] and rat α-lactalbumin [32].

Moreover, this structure has been recently characterized in calf thymocyte plasma membrane [33]. On the other hand, this structure seems to be widely distributed in glycoproteins originating from various sources and playing very different biological roles. The problem is thus posed of the biological specificity of this kind of common structure.

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