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CHARACTERIZATION OF THE NEUTRAL GLYCOPEPTIDES CONTAINING THE STRUCTURE α-L-FUCOPYRANOSYL-(1→3)-2-ACETAMIDO-2-DEOXY-D-GLUCOSE FROM HUMAN NEUROBLASTOMA CELLS*,†

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ABSTRACT

Human tumor cells of neurectoderm origin contain a high proportion of α-L-fucosyl linkages were determined by high-resolution, 500-MHz, ^{1}H-n.m.r. spectroscopy which gave signals characteristic for α-L-Fuc*p*-(1→3)-D-GlcNAc residues these L-fucosyl residues. This was shown by use of a specific α-L-fucosidase from almond emulsin and a broad-spectrum α-L-fucosidase from rat testes. The exact α-L-fucosyl linkages were determined by high-resolution, 500-MHz, ^{1}H-n.m.r. spectroscopy which gave signals characteristic for α-L-Fuc*p*-(1→3)-D-GlcNAc residues linked to branches and for α-L-Fuc*p*-(1→6)-D-GlcNAc residues linked to the core. More than 95% of the asparagine-linked GlcNAc residues were substituted with (1→6)-α-L-fucosyl groups. Further definition of the range of neutral glycopeptides was obtained with immobilized lectins. Binding to E-PHA–agarose suggested the presence of a β-D-mannopyranosyl residue substituted at O-4 by a 2-acetamido-2-deoxy-D-glucopyranosyl group. α-L-Fuc*p*-(1→3)-GlcNAc interfered with this binding since removal of α-L-fucosyl groups by almond emulsin α-L-fucosidase increased the binding by 100%. These studies demonstrate the ability of a combination of high-resolution ^{1}H-n.m.r., enzyme degradation, and lectin-binding affinities to delineate structural elements of small amounts of oligosaccharide residues.

*Dedicated to Professor Elvin A. Kabat.

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INTRODUCTION

One of the major problems in characterizing oligosaccharide structures derived directly from surface membrane glycoproteins of mammalian cells other than erythrocytes has been the lack of sufficient material for complete structural analysis. Because of this, very few of such glycoproteins have been characterized in detail[1]. One attempt to overcome this difficulty has been with the use of monoclonal antibodies to isolate membrane glycoproteins that have a particular biological characteristic defined by immunological properties[2]. However, even in these cases, the glycoproteins may be only minor components of the membrane. With the advent of high-resolution 500-MHz ^{1}H-n.m.r. spectroscopy, it is now possible to delineate the detailed structures of nanomoles of heterogeneous glycopeptides and oligosaccharides[3]. Moreover, possible structures can be predicted from even smaller quantities of glycopeptides by the use of exo- and endo-glycosidases, and immobilized lectins with defined specificities[4]. On this guidance, the more interesting structures can be isolated in such amounts that they can subsequently be confirmed by n.m.r. analysis.

The glycopeptides of human tumor cells of neurectoderm origin are of particular interest since they contain[5,6] an unusually high proportion of α-L-Fuc-(1→3 or 4)-D-GlcNAc groups. For example, the human neuroblastoma cell line, CHP-134, contains 33% of the α-L-fucosyl groups in this linkage[6]. In contrast, other cell types such as human, mouse or hamster fibroblasts, and mouse neuroblastoma cells contain none[7]. The α-L-fucosyl groups were detected with an α-L-fucosidase from almond emulsin which is specific[8,9] for the α-L-Fuc*p*-(1→3 or 4)-D-GlcNAc linkage. The remaining α-L-fucosyl groups were cleaved by a general α-L-fucosidase from rat testes[10] and were predicted to be linked (α1→6) to the asparagine-linked D-GlcNAc residue. The exact linkages could not be determined with the enzymes and, therefore, high-resolution ^{1}H-n.m.r. analysis was used to provide this detailed information.

The α-L-Fuc*p*-(1→3)-D-GlcNAc group is of particular interest since, in addition to its presence on human tumors, it affects distal moieties of glycopeptides as substrates for glycosyltransferases, and is also a recognition marker for a hepatic lectin[11]. We report herein the partial characterization of neutral glycopeptides from CHP-134 cells that are enriched in α-L-Fuc*p*-(1→3)-D-GlcNAc groups by applying 500-MHz ^{1}H-n.m.r. spectroscopy in conjunction with enzymic degradation and binding affinity to immobilized lectins.

EXPERIMENTAL

Materials. — L-[5,6-^{3}H]Fucose (56 Ci/mmol) and L-[1-^{14}C]fucose (50.8 mCi/mmol) were obtained from New England Nuclear Corporation; D_2O was from Aldrich. DEAE–Sephacel, concanavalin A–Sepharose, and lentil–Sepharose were from Pharmacia. The immobilized erythroagglutinating and leukoagglutinating lec-

tins from *Phaseolus vulgaris*, E-PHA- and L-PHA-agarose (special high density), Ulex-I-agarose (2 mg/mL), and pea lectin-agarose (10 mg/mL) were from E.Y. Laboratories. Trypsin (3 times crystallized) and soybean trypsin inhibitor were from Worthington Biochemicals and Pronase was from Calbiochem Laboratories. Almond emulsin, the starting material for the specific α-L-fucosidase[12], was obtained as "β-D-glucosidase" from Sigma Chemical Co. The broad spectrum α-L-fucosidase was isolated from rat testes[10]. 2-Acetamido-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine was from Vega-Fox Biochemicals and Man_5-[1-^{3}H]GlcNAcol was a gift from Dr. M. Fukuda, La Jolla, CA.

Cell culture and harvest. — Human neuroblastoma-cell line, CHP-134, was grown for 7 days as described[13]. The cells were labeled with L-[^{3}H]- or [^{14}C]-fucose (5 μCi per 75 cm^2 of flask) for 48 h before harvest. For harvesting, cells were washed 3 times with 0.02M Tris · HCl, pH 7.5, in 0.15M sodium chloride (TBS) and removed from the monolayer with trypsin (1 mg/1 mL of TBS) for 5 min at room temperature, followed by soybean trypsin inhibitor (1 mg/0.5 mL of TBS). The cells were centrifuged at 250*g* for 5 min, the supernatant solution was removed, and this material was considered to contain the membrane glycoproteins. The cells containing the remaining glycoproteins not cleaved by trypsin were washed 3 times

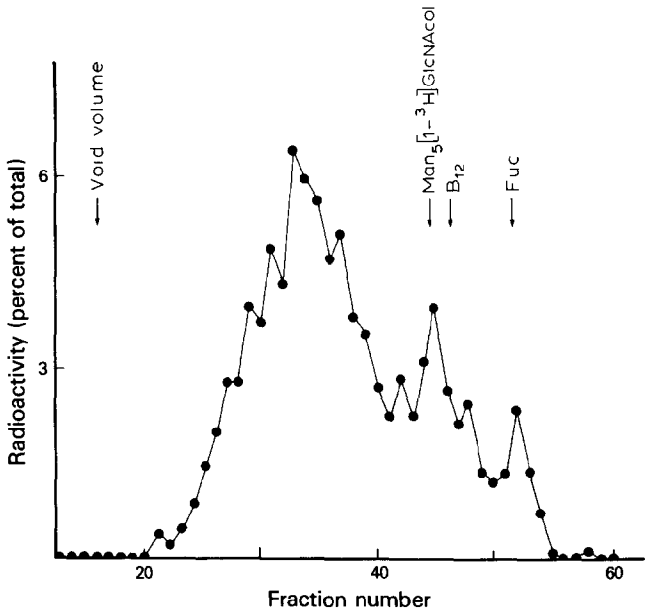


Fig. 1. Profile of elution on Bio-Gel P-10 of total Pronase-digested material from CHP-134 cells. The total L-[^{3}H]fucose-labeled Pronase digests, diluted 1:2 with water, were chromatographed on a Bio-Gel P-10 column (0.9 ×90 cm) in 50mM ammonium acetate containing 0.02% of sodium azide. Fractions (1 mL) were collected and aliquots examined for radioactivity. Fractions 29–39 were pooled for further separation on DEAE-Sephacel and fractions 40–51 pooled and designated low-molecular-weight glycopeptides. The void volume contained all material measurable at $Abs._{280}$. The column was precalibrated with bovine serum albumin (void volume), Man_5-[1-^{3}H]GlcNAcol, vitamin B_{12}, and L-[^{14}C]fucose.

with 0.16M sodium chloride, and aliquots were removed for cell count, radioactivity, and protein determination[14]. The cells were 91% viable after these procedures, as measured by the uptake of Trypan Blue, and contained 85% of the total membrane and cell radioactivity. The specific activity was 1.2×10^4 c.p.m./mg of protein.

Purification of the cell glycopeptides. — The harvested cell pellet was suspended in 0.1M Tris · HCl, pH 7.8, containing 20mM calcium chloride, and digested exhaustively with Pronase[15]. Separation of the resulting glycopeptides into molecular-weight groups was on Bio-Gel P-10. The glycopeptides from fractions 29–39 (Fig. 1) were combined, lyophilized, and desalted on Bio-Gel P-2. Further separation on the basis of charge was on DEAE-Sephacel as described[12]. The glycopeptides which were not retained on DEAE–Sephacel in 0.5mM ammonium acetate were defined as neutral glycopeptides. The neutral glycopeptides were lyophilized, rechromatographed over DEAE-Sephacel, and subsequently passed through immobilized concanavalin A. The unbound material was dialyzed, lyophilized, fractionated on Bio-Gel P-10, and desalted. This fraction was used for ^{1}H-n.m.r. analysis.

The lower-molecular-weight, Pronase-digested glycopeptides, representing 20% of the total cell-radioactivity, were eluted from Bio-Gel P-10, fractions 40–51 (Fig. 1), and were combined, lyophilized, and chromatographed over Bio-Gel P-4 in 50mM ammonium acetate. Separation on Dowex 50-X8 (H^+) was with a gradient of hydrochloric acid[13].

For some experiments as indicated, the Pronase-digested glycopeptides were filtered through Bio-Gel P-2 in water. The material that was in the void volume of the column was further separated on DEAE–Sephacel.

Lectin-affinity chromatography. — Glycopeptides were applied in 0.5 mL of the appropriate buffer in all cases. The void volume was marked with a dye (B_{12}) or radioactive L-fucose. All columns were brought to room temperature and all buffers contained 0.02% of sodium azide. A column (1 × 13 cm) of Con A–Sepharose was washed with 10mM Tris–0.1M sodium chloride[16] (pH 7.5) containing mM Ca^{2+}, Mg^{2+}, and Mn^{2+} to a total of 40 mL after the void volume, and then eluted sequentially with 40 mL of 10mM and 100mM methyl α-D-mannopyranoside in the same buffer. Chromatography on pea and lentil lectins was as described[17], and the glycopeptides were eluted with 0.2M methyl α-D-mannopyranoside. A column (0.3 × 60 cm) of E-PHA–agarose was washed with buffer as described[18,19] except that fractions (0.5 mL) were collected. A column (0.7 × 26 cm) of L-PHA–agarose was washed with buffer as described[18,19]. Ulex-I–agarose was washed with 25mM sodium phosphate buffer–0.15M sodium chloride[20], pH 7.2, 15 min after application of the glycopeptides, and fractions (0.12 mL) were collected. The glycopeptide-binding specificities of these lectins have been described[16–21].

Almond-emulsin α-L-fucosidase. — α-L-Fucosidase was prepared from almond-emulsin[8,9] with further purification[12] on Sephacryl S–200. The enzyme, which does not hydrolyze synthetic α-L-fucosides, was assayed with total [^{3}H]glycopeptides from human neuroblastoma cells, and the released L-[^{3}H]fucose was de-

tected[12] by t.l.c. or filtration through a Bio-Gel P-2 column, thus reducing the detection time by 2 days over the previously-described assay[8,9]. With saturating amounts of enzyme, 30% of the L-fucosyl residues from CHP-134 total glycopeptides were released. This enzyme preparation cleaved α-L-Fuc*p*-(1→3 or 4)-D-GlcNAc but not α-L-Fuc*p*-(1→2)-D-Gal or α-L-Fuc*p*-(1→6)-D-GlcNAc[9,12]. Each glycopeptide fraction (500 c.p.m.) to be examined was desalted on Bio-Gel P-2 and incubated[12] with α-L-fucosidase (2 units) in 0.2M sodium monohydrogenphosphate–0.1M citric acid, pH 5.5, in a total volume of 25 μL for 16 h at 37°. The free L-fucose was separated from the incubation mixture on a Bio-Gel P-2 column or on a silica gel plate in the presence of L-[^{14}C]fucose-containing CHP-134 glycopeptide as internal standard. The thin-layer plates were scraped in the area of L-fucose, and the content of radioactivity released by the enzyme determined by scintillation counting[12]. In all cases, a control without enzyme was similarly treated.

Rat testis α-L-fucosidase. — The general α-L-fucosidase from rat testis was purified and utilized as described[10]. The neuroblastoma neutral glycopeptides were treated with the enzyme (2 I.U.) by incubation for 72 h at 37° in 0.1M citrate buffer, pH 5.5.

^{1}H-N.m.r. 500-MHz spectroscopy. — A Bruker WM-500 spectrometer (SON hf.-n.m.r. facility, Department of Biophysics, Nijmegen University, The Netherlands), operating in the Fourier-transform mode, and equipped with an Aspect-2000 computer, was used. Further experimental details were described previously[3,25]. For solvent-peak suppression, a water-elimination, Fourier-transform pulse sequence (180°–τ–90°-acquisition, with composite, nonselective, 180° pulse) was applied[26]. Prior to analysis, the glycopeptides were treated repeatedly with deuterium oxide at room temperature, with intermediate lyophilization, and finally with 99.96% deuterium oxide. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation[3,25]. The probe temperature was 27°. The chemical shifts (δ) are expressed in p.p.m. downfield from the methyl signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ 2.225) with an accuracy of 0.002 p.p.m.

RESULTS

α-L-Fucosyl groups in neuroblastoma glycopeptides. — The neutral glycopeptides from the human neuroblastoma cell-line, CHP-134, represented 24% of the total cellular glycopeptides. Approximately 90% of the neutral glycopeptides were recovered in Bio-Gel P-10, fractions 29–39 (Fig. 1 and Table I). The neutral glycopeptides from Bio-Gel P-10, fractions 29–39 (Fig. 1), were separated from the charged glycopeptides by two chromatographies on DEAE-Sephacel, and from Con A-bound glycopeptides by passage through Con A–Sepharose (Table II). The Con A-nonbound neutral glycopeptides (N-1) were 73% of the total neutral glycopeptides and provided the fraction for the subsequent analyses.

Fraction N-1 was treated with the specific α-L-fucosidase from almond emul-

TABLE I

FRACTIONATION BY CHARGE OF CHP-134 GLYCOPEPTIDES

Elution buffer[b] (mM)	*High-molecular-weight, radioactive glycopeptides*[a]					
	Bio-Gel P-2 (total)	*Bio-Gel P-10*		*Lentil-lectin bound, Con A nonbound*[c]		
		Proportion of total (%)	*Proportion released by α-L-fucosidase*[d] *(%)*	*Proportion of total (%)*	*Proportion of fraction (%)*	*Proportion released by α-L-fucosidase*[d] *(%)*
0.5	24	21	57	4	16	42
30	13	10	29	5	38	33
50	11	11	23	4	38	24
100	25	10	17	4	17	23
300	23	4	7	5	22	7

[a]The high-molecular-weight glycopeptides were obtained in the void volume of Bio-Gel P-2 (total) or separated by Bio-Gel P-10 (Fractions 29–39, Fig. 1), lyophilized, desalted on Bio-Gel P-2, and subsequently separated on DEAE–Sephacel with a linear gradient of ammonium acetate[13]. [b]Molarity of ammonium acetate at the radioactive peak. [c]The total, high-molecular-weight glycopeptides from Bio-Gel P-2 were passed through Con A–Sepharose and lentil–Sepharose in series. The latter was eluted with 0.1M methyl α-D-mannopyranoside. The lentil-bound glycopeptides were treated with α-L-fucosidase from almond emulsin, and the radioactivity (%) released was determined as described in the Experimental section. [d]From almond emulsin.

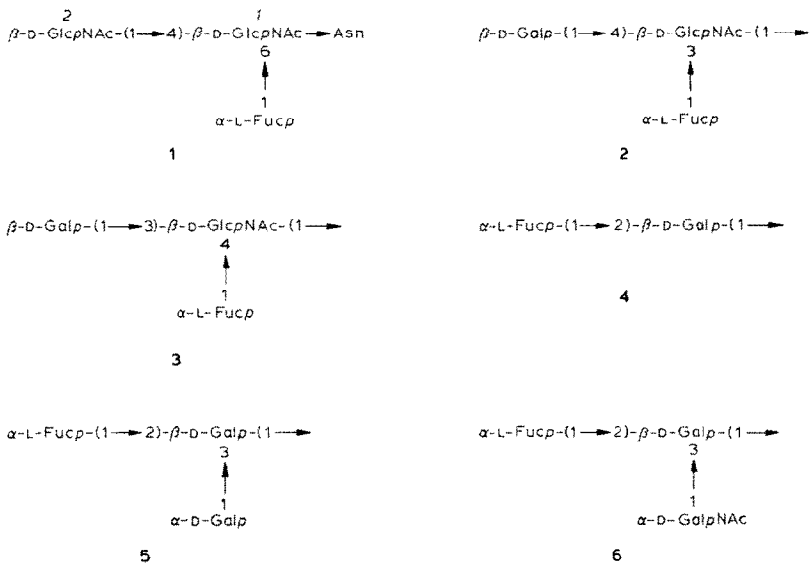
TABLE II

NEUTRAL GLYCOPEPTIDES SEPARATED BY IMMOBILIZED CON A[a]

Con A fraction	*Radioactivity*	
	Proportion of total (%)	*Proportion of α-L-Fucp-(1→3)-D-GlcNAc in fraction (%)*
Unbound[b]	73	61
Slightly retarded	9	49
Retained and eluted by		
10mM methyl α-D-mannopyranoside	15	56
100mM methyl α-D-mannopyranoside	2	ND

[a]The neutral glycopeptides purified on Bio-Gel P-10 and DEAE–Sephacel (Table I) were applied to a column (1 × 13 cm) of Con A–Sepharose. The column was washed with 3.5 times the void volume, and successively eluted with 10 and 100mM methyl α-D-mannopyranoside[16]. [b]Fraction analyzed by 500-MHz, ^{1}H-n.m.r. spectroscopy.

sin and ~60% of the α-L-fucosyl groups were released (Table II), demonstrating the presence of α-L-Fuc*p*-(1→3 or 4)-D-GlcNAc groups. Analysis of Fraction N-1 by 500-MHz ^{1}H-n.m.r. spectroscopy (Table III) showed the presence of an α-L-Fuc*p*-(1→3)-D-GlcNAc, as L-fucosyl structural-reporter-group signals were found at δ 5.128 (H-1) and 1.177 (methyl). In addition, a galactose H-1 doublet was ob-



Scheme 1

TABLE III

^{1}H-CHEMICAL SHIFTS (δ) OF PERTINENT STRUCTURAL REPORTERS FOR NEUTRAL GLYCOPEPTIDES FROM HUMAN NEUROBLASTOMA, CHP-134, COMPARED TO ^{1}H-CHEMICAL SHIFT DATA KNOWN TO BE DISCRIMINATIVE FOR TYPE OF LINKAGE AND ENVIRONMENT OF L-FUCOPYRANOSYL RESIDUES

Structure[a]	*Chemical shift of* L-*fucosyl residue*			*Other typical features*		*References*
	H-1	*H-5*	*CH$_3$*	*Reporter*	*Chemical shift*	
Previously reported:						
1	4.876	4.125	1.21	H-1 of GlcNAc-2	4.69	3,25,27
				NAc of GlcNAc-2	2.095	
2	5.12	4.83	1.17	H-1 of Gal	4.45	3,25,28,29
				NAc of GlcNAc	$\Delta\delta$ −0.01[b]	
3	5.02	4.87	1.18	H-1 of Gal	4.43	30
4	5.2–5.3	4.2–4.3	1.24	H-1 of Gal	4.5–4.6	25,29,31,32
5	5.35	4.32	1.24	H-1 of α-Gal	5.28	33[c]
				H-1 of β-Gal	4.55	
6	5.19[d]	4.34	1.24	H-1 of GalNAc	5.39[d]	31
				H-1 of Gal	4.72	
Observed for neutral glycopeptides from human neuroblastoma CHP-134:						
1	4.87	[e]	1.206	NAc of GlcNAc-2	2.096	
2	5.128	[e]	1.177	H-1 of Gal	4.445	

[a]See scheme. [b]As compared to a fucosyl analog. [c]For blood-group B determinant (and for related structures), ^{1}H-chemical shifts have been reported also in ref. 34; however, those data were obtained under conditions completely different from ours; we failed to coordinate them with the data listed here. [d]Assignments may have to be interchanged. [e]The value could not be determined.

served at δ 4.445. From ^{1}H-n.m.r.-spectral integration of the appropriate structural-reporter-group signals (namely, the methyl doublets at δ 1.206 and 1.177), it appeared that 50% of the α-L-fucosyl groups of Fraction N-1 were present in this linkage, confirming the proportion cleaved with the almond emulsin α-L-fucosidase (Table II). These conclusions are based upon comparison of these chemical shift values with ^{1}H-n.m.r. literature data useful for recognition of a fucosyl-linkage type and its structural environment; some of the latter have been included in Table III.

After removal of the α-1-fucosyl groups from Fraction N-1 with almond emulsin α-L-fucosidase, the fraction was further incubated with the general α-L-fucosidase from rat testes. This enzyme cleaved the remaining α-1-fucosyl groups suggesting that 2-acetamido-2-deoxy-D-glucosyl residues were substituted at O-6 with α-L-fucosyl groups or D-galactosyl residues were substituted at O-2 with α-L-fucosyl groups. ^{1}H-N.m.r. analysis indicated the substitution at O-6 of the asparagine-linked GlcNAc-*1* residue with an α-L-fucosyl group since the anomeric signal of this latter group was found at $\delta \simeq 4.87$ and its methyl doublet at δ 1.206. In addition, the *N*-acetyl singlet of the GlcNAc-*2* residue was observed at δ 2.096, exclusively (Table III). On the basis of the absence of a signal at δ 2.078 for the *N*-acetyl group of the GlcNAc-*2* residue, it may be concluded that at least 95% of the GlcNAc-*1* residues are substituted with (1→6)-linked α-1-fucosyl residues.

Although the presence of other α-L-fucosyl groups cannot be ruled out completely by ^{1}H-n.m.r. analysis, no ^{1}H-n.m.r. detectable (<5%) glycopeptides contained an α-L-Fuc*p*-(1→4)-D-GlcNAc group since the characteristic signals were not seen. In addition, there was no evidence for an α-L-Fuc*p*-(1→2)-D-Gal group (Table III).

Presence of O-α-L-*fucopyranosyl*-(*1*→*3*)-*2-acetamido-2-deoxy*-D-*glucosyl groups on glycopeptide.* — Previously, it was shown[12] that the neuroblastoma membrane glycopeptides contained α-L-Fuc*p*-(1→3)-D-GlcNAc groups on the oligosaccharide branches by sequential enzyme degradation with exoglycosidases and endoglycosidase D. Only those glycopeptides that were treated with almond-emulsin enzyme prior to neuraminidase, β-D-galactosidase, and *N*-acetyl-β-D-hexosaminidase provided the substrate for endoglycosidase D, suggesting the presence of α-L-Fuc*p*-(1→3)-D-GlcNAc groups on branches.

Analysis of Fraction N-1 by ^{1}H-n.m.r. spectroscopy confirmed the presence of α-1-Fuc*p*-(1→3)-D-GlcNAc groups on double- and triple-branched (bi- and triantennary) carbohydrate chains containing a β-D-Gal*p*-(1→4)-D-GlcNAc residue (Table III). In addition, no signals were observed for β-D-Gal*p*-(1→3)-D-GlcNAc or α-D-Gal*p*-(1→3)-D-Gal residues in the ^{1}H-n.m.r. spectrum. Owing to the rather poor quality of the spectra obtained with the very low amount of material available, the location of the 2-acetamido-2-deoxy-D-glucosyl residues could not be assigned.

Properties of radioactive neutral glycopeptides on immobilized concanavalin A and influence of O-α-L-*fucopyranosyl*-(*1*→*3*)-*2-acetamido-2-deoxy*-D-*gluco-*

pyranosyl groups on binding affinity. — When the L-fucose-labeled neutral glycopeptides were applied to a column of Con A–Sepharose, three fractions were observed: nonbound (N-1), slightly retarded, and retained (Table II). The latter glycopeptides were eluted sequentially with 10mM and 100mM methyl α-D-mannopyranoside. Most (73%) of the L-fucose-labeled glycopeptides were not bound to Con A–Sepharose (N-1), and 2.0% were tightly bound, being eluted with 100mM methyl α-D-mannopyranoside. All of the oligomannoside-type glycopeptides were removed by Con A–Sepharose, and this was corroborated by ^{1}H-n.m.r. analysis of the glycopeptides before and after passage through a Con A–Sepharose column.

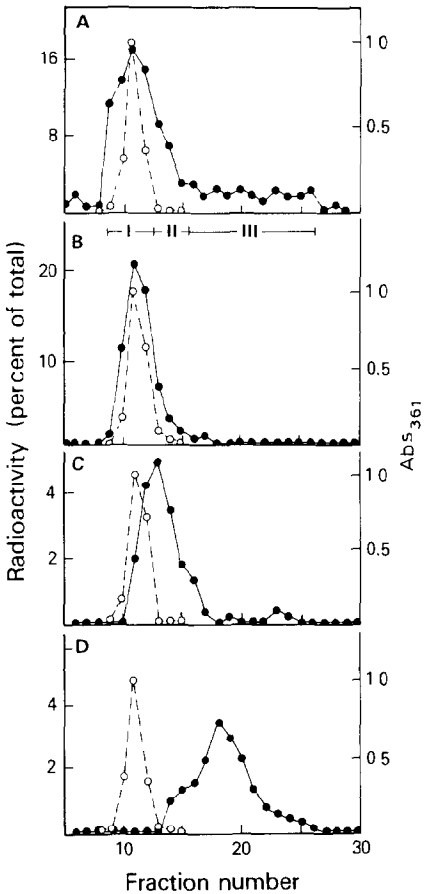


Fig. 2. Profiles of elution of Fraction N-1 on E-PHA–agarose. A: Fraction N-1 was applied to E-PHA–agarose in 0.5 mL of buffer as described in the Experimental section, and fractions (0.5 mL) were collected and tested for radioactivity (●——●). The fractions were pooled as indicated (I–III), desalted, and reapplied to the same column. B, C, and D: Rechromatography of I, II, and III, respectively. The void volume is marked by vitamine B_{12} (○---○), Abs $_{361}$; the peak fraction was designated 1.0.

A small portion (9%) of the L-fucose-labeled glycopeptides were slightly retarded (Table II), being eluted slowly with 14–30 mL of buffer, after the void volume. When this fraction was rechromatographed on Con A–Sepharose, it was eluted as a symmetric peak with the same amount of buffer. Treatment of this slightly retarded fraction with almond-emulsin α-L-fucosidase released 49% of the α-L-fucosyl groups. Moreover, when the treated glycopeptides were rechromatographed on Con A–Sepharose, 50% were now retained and eluted with 10mM methyl α-D-mannopyranoside. The rest no longer was eluted as a symmetric peak, but still was eluted with buffer, although more slowly. On the other hand, when Fraction N-1 and the glycopeptides that were eluted originally from Con A–Sepharose with 10mM methyl α-D-mannopyranoside were treated with α-L-fucosidase, both fractions retained the original elution-position. In this case, 61 and 56%, respectively, of the α-L-fucosyl groups were removed (Table II). Thus, the binding affinity of only those glycopeptides that were slightly retarded on Con A–Sepharose was increased by removal of α-L-Fuc*p*-(1→3)-D-GlcNAc groups.

Properties of Fraction N-1 on immobilized E-PHA and influence of α-L-Fucp-(1→3)-D-GlcNAc groups on binding affinity. — Immobilized E-PHA has been shown to retard D-galactose-containing double-branched (bi-antennary) glycopeptides having a 2-acetamido-2-deoxy-β-D-glycopyranosyl group linked (1→4) to the β-D-mannopyranosyl residue of the asparagine-linked core[18]. The glycopeptides are, however, only retarded on the immobilized lectin and are eluted with continued application of buffer. When Fraction N-1 was applied to a column of E-PHA–agarose, similar to that described earlier[18], three fractions were obtained (Fig. 2). One fraction in the void volume (Fig. 2B), one slightly retarded (Fig. 2C), and one more highly retarded (Fig. 2D) contained 62, 19, and 18%, respectively, of the radioactivity of Fraction N-1 and, as shown in Fig. 2, retained their original position after rechromatography on E-PHA–agarose. Thus, 37% of Fraction N-1 had some affinity for immobilized E-PHA, but only 18% had the affinity characteristic of double-branched glycopeptides with a 2-acetamido-2-deoxy-D-glucosyl group linked (1→4) to the β-D-mannosyl residue[18]. None of these glycopeptides was detected by ^{1}H-n.m.r. analysis; however, the amounts that were present may have precluded the detection of the characteristic H-1 signal at $\delta \simeq 4.47$ of the 2-acetamido-2-deoxy-D-glucose unit, because of interference with the H-1 doublets at this position of the D-galactose units.

Properties of Fraction N-1 on other immobilized lectins and influence of α-L-Fucp-(1→3)-D-GlcNAc groups on binding affinity. — Pea and lentil lectins have similar binding specificities[17,23], except that binding to pea lectin requires the presence of an α-L-Fuc*p*-(1→6)-β-D-Glc*p*NAc-(1→4)-Asn structure, whereas binding to lentil lectin requires only an α-L-Fuc*p*-(1→6)-D-GlcNAc structure and no asparagine residue[21,24]. When N-1 glycopeptides were passed through immobilized pea and lentil lectins in series, only 2% of the radioactivity bound to the pea lectin, whereas 16% bound to the lentil lectin. The removal of L-fucose by α-L-fucosidase

TABLE IV

BINDING OF FRACTION N-1 TO IMMOBILIZED LECTINS

Lectin	*Radioactivity (% of total fraction)*	
	Bound	*Additionally bound after α-L-fucosidase treatment*[a]
Sequence pea lectin, lentil lectin, E-PHA, and L-PHA[b]:		
Pea lectin	2	0
Lentil lectin	16	0
E-PHA	17	24
L-PHA	0	15
Sequence E-PHA and L-PHA:		
E-PHA	18	
L-PHA	0	

[a]A portion of each nonbound fraction was treated with almond emulsin α-L-fucosidase and rechromatographed through the respective lectin column. [b]The nonbound fraction of Fraction N-1 from each immobilized lectin was passed through the subsequent lectin column.

from almond emulsin did not influence the binding affinity to the immobilized pea or lentil lectin (Table IV).

When the lentil lectin-bound fraction was dialyzed, lyophilized, and passed through E-PHA–agarose, 12% of the radioactivity was retarded, similar to Fraction N-1 (Fig. 2D). Treatment of the E-PHA-nonbound fraction with α-L-fucosidase did not increase the amount bound. In contrast, when the lentil lectin-nonbound fraction was passed through E-PHA–agarose, and then treated with α-L-fucosidase and returned to E-PHA–agarose, the amount retarded increased by more than 100% (Table IV).

Fraction N-1 did not bind to immobilized L-PHA either before or after affinity chromatography on a series of immobilized lectins (Table IV). However, when the E-PHA-nonbound glycopeptides were treated with almond-emulsin α-L-fucosidase, 15% of the radioactivity was slightly retarded on L-PHA–agarose. This retardation was not to the extent observed for other fractions, such as that which was eluted with 0.3M ammonium acetate from DEAE–Sephacel[35] or as reported by Cummings and Kornfeld[18,19].

Comparison of the neutral and charged glycopeptides. — The neutral glycopeptides were enriched in α-L-Fuc*p*-(1→3)-D-GlcNAc groups. This was shown by separating the cellular glycopeptides into various fractions on DEAE–Sephacel with a salt gradient. When the charged fractions, representing 13, 11, 25, and 23%, respectively, of the radioactivity incorporated into the glycopeptides (Table I) were treated with the almond emulsin enzyme, α-L-fucosyl groups were released from each charged fraction. However, the proportion of L-fucose released

was inversely proportional to the charge, with the neutral glycopeptides containing the highest proportion of α-L-Fuc*p*-(1→3)-D-GlcNAc groups (Table I).

The proportion of glycopeptides that bound to lentil lectin–Sepharose and were not bound to Con A–Sepharose was similar for the most highly-charged and neutral classes of glycopeptides (22 and 16%, respectively). However, among these lentil lectin-bound glycopeptides, the proportion that contained α-L-Fuc*p*-(1→3)-D-GlcNAc groups were markedly different. Only a small percentage (7%) of α-L-fucosyl groups were released by almond-emulsin α-L-fucosidase from the most highly-charged glycopeptides, whereas 42% were released from the neutral glycopeptides (Table I).

Retardation of a small glycopeptide on immobilized Ulex-I lectin — The lower-molecular-weight L-[^{3}H]fucose glycopeptides (fractions 40–51, Fig. 1) con-

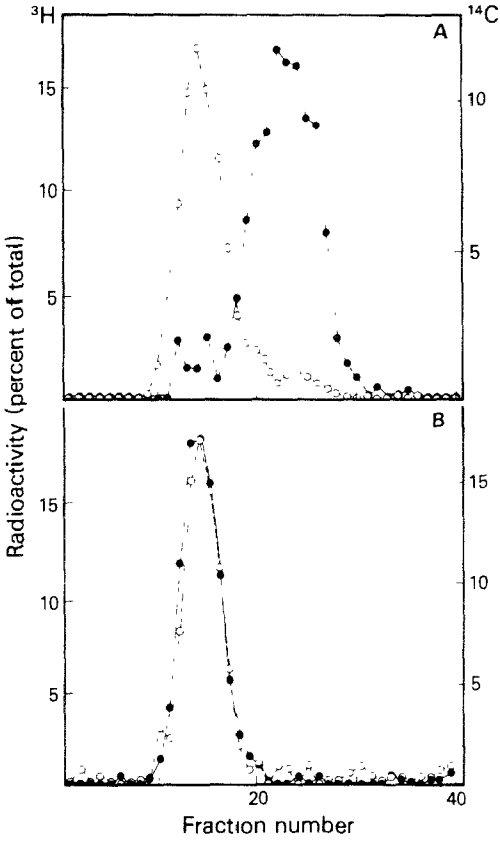


Fig. 3. Profiles of elution on Ulex I–agarose of glycopeptides from CHP-134 cells L-[^{3}H]Fucose-labeled glycopeptides, fractions 29–39 (Fig. 1) (○---○), were mixed with the lowest-molecular-weight glycopeptide (U-1) labeled with L-[^{14}C]fucose (●——●) and applied to a column of Ulex I–agarose as described in the Experimental section. (A) The column was then washed with the same buffer and fractions (0.12 mL) were collected. No further radioactivity was eluted with 0.2M L-fucose in the aforementioned buffer or with 0.8% sodium dodecyl sulfate in 0.1M Tris buffer, pH 9.5. (B) The experiment was repeated with 0.2M L-fucose added to the initial and the wash buffer.

tained 20% of the radioactivity of the cells; they were neutral on DEAE–Sephacel and not retained by Con A or lentil lectin. Some of the lower-molecular-weight glycopeptides were retained on Ulex I–agarose, however, in contrast to Fraction N-1, which showed no retention. When the lower-molecular-weight glycopeptides were further separated on Bio-Gel P-4, the Ulex I-retained material was completely found in one fraction (U-1), being eluted from Bio-Gel P-4 with R_{Fuc} 0.76. Fraction U-1 represented the lowest-molecular-weight, conjugated, ^{3}H-labeled molecule found in the cells. No L-fucose was released when it was treated with almond emulsin α-L-fucosidase. However, subsequent treatment with α-L-fucosidase from rat testis released 92% of the radioactive α-L-fucosyl groups from this fraction.

Fraction U-1 was the only neuroblastoma glycopeptide that was bound or retarded on Ulex I–agarose. It was retarded but not retained (Fig. 3A). The retardation was specific since it was abolished by the inclusion of 0.2M L-fucose in the eluting buffer (Fig. 3B). In addition, Fraction U-1 was bound to Dowex 50 (H^+) and eluted with a gradient of hydrochloric acid just prior to β-D-Glc*p*NAc-(1→4)-Asn. On paper chromatography (12:5:4, v/v, ethyl acetate–pyridine–water), Fraction U-1 showed $R_{\beta\text{-D-Glc}p\text{NAc-(1}\rightarrow\text{4)-Asn}}$ 0.9. In double-label experiments, Fraction U-1 comigrated in all of these systems with α-L-Fuc→D-GlcNAc→Asn derived from degradation[12] of membrane glycopeptides with endoglycosidase D. All of these properties were consistent with the characterization of the structure of this fraction as α-L-Fuc*p*-(1→6)-β-D-Glc*p*NAc-(1→4)-Asn. Insufficient material was available for confirmation by ^{1}H-n.m.r. analysis.

DISCUSSION

Analysis of the neutral glycopeptides from human neuroblastoma cells, CHP-134, has provided evidence that a large proportion of the α-L-fucosyl groups occur as α-L-Fuc*p*-(1→3)-D-GlcNAc groups on the branches. Most of the glycopeptides containing α-L-fucopyranosyl groups linked (1→3) to peripheral 2-acetamido-2-deoxy-D-glucose units also contain α-L-fucopyranosyl groups linked (1→6) to the asparagine-bound 2-acetamido-2-deoxy-D-glucose units. These results were predicted on L-[^{3}H]fucose-containing glycopeptides by use of a specific α-L-fucosidase, and by the binding affinity to immobilized lectins of known specificity (Tables I and IV). Subsequent confirmation by 500-MHz ^{1}H-n.m.r. spectroscopy (Table III) demonstrates the resolving power of a combination of these techniques. Homogeneous glycopeptides were not necessary in order to obtain the desired information, which was the exact location of the linkage of the α-L-fucosyl groups susceptible to cleavage with almond emulsin α-L-fucosidase.

α-L-Fucosyl groups having this linkage have been shown previously by Lloyd *et al.*[36] to be found on blood-group-active oligosaccharides. Since then, they have been shown to be present on minor oligosaccharide residues of circulating glycoproteins[37]. The interesting presence of the α-L-Fuc*p*-(1→3)-D-GlcNAc group in

large proportions on glycoproteins from human tumor cells of neurectoderm origin but not on mouse neuroblastoma or human fibroblasts[7] has led to the speculation that the presence of the unusual proportions of this group on glycoproteins is a vestige of early differentiation of these cells with subsequent re-expression during oncogenesis[4].

The content of α-L-Fuc*p*-(1→3)-D-GlcNAc groups was enriched in the neutral glycopeptide fraction (Table I), since >50% of the glycopeptides contained α-L-fucosyl groups in branches, whereas, in the most highly-charged class of total glycopeptides, only 7% of the glycopeptides contained these α-L-fucosyl groups. There was, in fact, an inverse correlation between the elution position from DEAE–Sephacel and the proportion of α-L-Fuc*p*-(1→3)-D-GlcNAc groups in each fraction. This was also true for the glycopeptides retained by lentil lectin–Sepharose and not Con A–Sepharose (Table I). A reciprocal relationship between the presence of α-NeuAc-(2→6)-D-Gal and α-L-Fuc*p*-(1→3)-D-GlcNAc structures on the same branch was observed, since α-L-Fuc*p*-(1→3)-[β-D-Gal-(1→4)]-D-GlcNAc could not serve as a substrate for β-D-galactoside α-(2→6)-sialyltransferase[11]. The multi-sialylated nature of some of the membrane glycopeptides which contained α-L-Fuc*p*-(1→3)-D-GlcNAc groups led us to believe that at least some of the branches contain both sialic acid and L-fucose[12]. In addition, the finding[38] that the major glycopeptide of rat brain was α-NeuAc-(2→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-GlcNAc suggested that the (2→6)- and the (2→3)-sialyltransferases have different substrate requirements.

The presence of α-L-Fuc*p*-(1→3)-D-GlcNAc groups decreased the affinity of the neutral glycopeptides to immobilized Con A, E-PHA, and L-PHA (Tables II and IV). Although the influence of α-L-Fuc*p*-(1→3)-D-GlcNAc on binding to Con A has been reported previously[39], the influence on the lectins from *P. vulgaris* has heretofore not been observed. Most striking was the increased amount retarded on immobilized E-PHA after removal of α-L-fucosyl groups by α-L-fucosidase from almond emulsin (Table IV). The few glycopeptides retarded on immobilized L-PHA only after removal of L-fucosyl groups located in branches were not those previously[18,19] defined by L-PHA since the affinity for the lectin was much lower. No glycopeptides from Fraction N-1 showed an increased affinity for Con A after removal of branch L-fucosyl groups, although a fraction that was retarded on the high-affinity lectin was more tightly bound (Table II). In the total membrane glycopeptides, branch L-fucosyl groups increased the affinity for lentil lectin[12]. However, in Fraction N-1, this affinity was not demonstrably changed by removal of these L-fucosyl groups. This was also true for the affinity to pea lectin- and Ulex-I–agarose. The latter lectin bound only one species of glycopeptide, Fuc→GlcNAc→Asn[12,40].

The observation that 40% of Fraction N-1 was not retarded or retained on any of the immobilized lectins that were used, even with the removal of the branch α-L-fucosyl groups, may be explained by the presence of a large portion of glycopeptides containing α-D-mannosyl residues substituted at O-2 and -4, rather

than at O-2 and -6. The latter substitutions are a requirement for binding to immobilized lentil, pea[17], and L-PHA[18] lectins. Fraction N-1 was chromatographed on immobilized lectins in series that removed double-branched glycopeptides (Con A), triple-branched glycopeptides with an asparagine core (pea lectin), triple-branched oligosaccharides, and double-branched glycopeptides containing β-D-Glc*p*NAc-(1→4)-β-D-Man*p* residue (lentil lectin). The latter group was also removed by E-PHA. Therefore, it was anticipated that some of the remaining glycopeptides were quadruple-branched and would be retained[18] on L-PHA. Since this was not the case, we favor the explanation that human neuroblastoma cells contain a high proportion of triple- and quadruple-branched glycopeptides having α-D-mannosyl residues substituted at O-4.

Several other points can be made concerning the N-1 glycopeptide structures. There was no ^{1}H-n.m.r. indication for α-L-Fuc*p*-(1→4)-D-GlcNAc or α-L-Fuc*p*-(1→2 or 6)-D-Gal structures. The latter was found previously[41], by mass-spectrometry, for the secretory immunoglobulins A. The persistence of potential double-branched glycopeptides after Fraction N-1 was passed through immobilized Con A allowed the prediction of a 2-acetamido-2-deoxy-β-D-glucopyranosyl group (1→4)-linked to the β-D-mannosyl residue, since glycopeptides with this structure are not bound to Con A–Sepharose[22]. The observation that 17% of these glycopeptides bound to E-PHA–agarose (Table IV) suggested the β-D-Glc*p*NAc-(1→4)-β-D-Man*p* structure. E-PHA–agarose has been shown to bind such structures[18]. On the other hand, no clear ^{1}H-n.m.r. signal for this 2-acetamido-2-deoxy-D-glucopyranosyl group was observed, even though it was shown previously in other neutral glycopeptides of the *N*-acetyllactosamine type[42]. Therefore, binding to E-PHA may be influenced by oligosaccharide residues other than those described[18]. Additional material will be necessary to confirm this suggestion by ^{1}H-n.m.r. analysis.

Similar to the structural analyses of the blood-group substances so elegantly done by immunochemical techniques[43], lectins provide the tools for structural analysis when the binding affinities have been defined[16–24]. With the advances in high-resolution, 500-MHz ^{1}H-n.m.r. spectroscopy, structural analyses of glycopeptides are possible on nmol quantities[3,25]. By use of a combination of these techniques, it is now feasible to delineate the oligosaccharide structures of biologically relevant molecules, such as the purified glycoproteins of the voltage-sensitive sodium channel[44].

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