Glycoconjugate J (1984) 1:155—169

The Carbohydrate Structure of Human Fibronectins: A Comparison between Normal Embryonic Lung Fibroblasts WI-38 and the SV40 Virus Transformed Cell Line VA13

KIMIE MURAYAMA[1], EVERETT J NICHOLS, STEVEN B LEVERY, WILLIAM G CARTER and SEN-ITIROH HAKOMORI*

Program of Biochemical Oncology/Membrane Research, Fred Hutchinson Cancer Research Center and Departments of Pathobiology, Microbiology and Immunology, University of Washington, 1124 Columbia Street, Seattle, Washington 98104, USA.
[1]*Present address: Central Laboratory of Medical Sciences, Juntendo University School of Medicine, Tokyo, Japan*

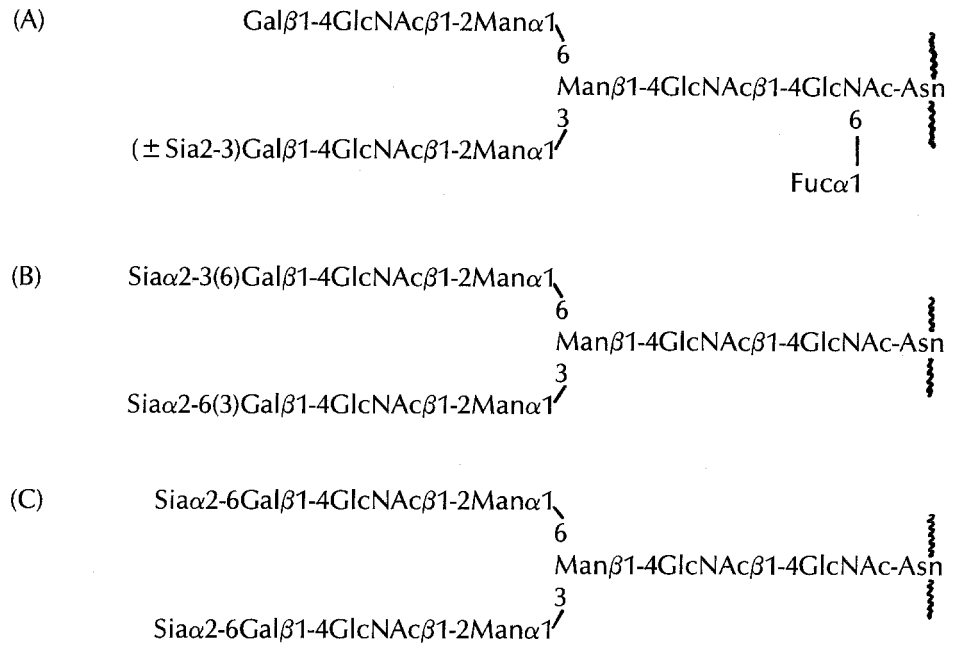
Received June 25, 1984.

Key words: fibronectin, bi-antennary carbohydrates

The carbohydrates of human fibronectin released from non-transformed human fibroblasts WI-38 have been compared with those of fibronectin released from SV40 virus transformed WI-38/VA13 cells and those of fibronectin prepared from human plasma. The majority of the bi-antennary glycopeptides of fibronectin released from WI-38 fibroblasts was not sialylated at the terminal galactosyl residues, but was fucosylated at the core *N*-acetylglucosaminyl residue directly linked to a peptide (structure A, below). Most of the minor sialylation detected was linked α2-3 to galactose. In contrast, the majority of the bi-antennary glycopeptides released from the transformed VA13 cells was highly sialylated at the terminal galactosyl residues with both α2-3 and α2-6 linkages, but was only partially fucosylated at the core *N*-acetylglucosaminyl residue (structure B, below). This structure was similar to that of the bi-antennary glycopeptide of human plasma fibronectin which was, however, predominantly sialylated with an α2-6 linkage (structure C, below). These human fibronectins, regardless of their source, lack a high molecular weight lactosaminoglycan structure.

In addition to the differences in bi-antennary structure described above, the quantity of tri- to tetra-antennary glycopeptides of fibronectin released from VA13 transformed cells was found to be much greater than the quantity of these glycopeptides of fibronectin released from normal WI-38 fibroblasts. Furthermore, there was a relatively small quantity of a glycopeptide fraction having a smaller molecular weight that did not bind to Con A-Sepharose and was separated on a Bio-Gel P-4 column. The presence of

* Author for correspondence



this fraction was characteristic for fibronectin released from transformed VA13 cells, and the fraction was absent in fibronectin from normal fibroblasts.

Fibronectin (FN) represents a class of multifunctional high molecular weight glycoproteins abundant in the pericellular matrix and in body fluids (for reviews, see refs. 1-5), and their expression at the cell surface is greatly reduced by oncogenic transformation [6, 7]. FN derived from plasma (plasma form, pFN) is distinctively different in its functional domains [8-11] and carbohydrate structure [12-14] from that present at the cell surface (cellular form, cFN). Hamster FN derived from fibroblasts (cFN) and from plasma (pFN) both contain carbohydrates with mainly bi-antennary structure, but cFN contains a fucosyl residue at the innermost *N*-acetylglucosamine residue of the core structure [12,13], in contrast to pFN which lacks this fucosyl residue [14]. The sialosyl residue in hamster pFN is exclusively NeuAcα2-6Gal [14]. In human FN, only the carbohydrate structure of pFN has been extensively studied [15,16]. It was found to be composed essentially of carbohydrates with a bi-antennary structure having NeuAcα2-6Gal substitution at the terminus [15]. However, there was a significant amount of Con A-unbound material which was assumed to contain tri- or tetra-antennary structures [16], although no chemical data for these structures were provided. In contrast to these common structures in hamster and human pFN, bovine pFN has a bi-antennary structure with an unusual side chain, NeuAcα2-6Galβ1-3[NeuAcα2-6]-GlcNAcβ1-2Man [17].

Carbohydrate changes in glycoproteins [18] or glycolipids [19] associated with oncogenic transformation have been well established, although the biological implication of these changes is still unclear. By analogy with a general change of cell surface carbohyd-

rates in glycoproteins [18], FN carbohydrate derived from transformed hamster fibroblasts shows a remarkable increase in the higher molecular weight Con A-unbound fraction as compared to that derived from non-transformed hamster fibroblasts NIL or BHK [19,20]. However, the chemical structure of carbohydrates in human FN released from transformed cells as compared with normal fibroblasts has not been elucidated. On the other hand, mouse and human teratocarcinoma FNs contain a carbohydrate with unusually high molecular weight, which is characteristic of lactosaminoglycan [21-23]. A similar lactosaminoglycan structure in human FN isolated from placenta has been described recently [24]. This paper describes a strict comparison of glycopeptides of cFN released from normal human fibroblasts WI-38 and SV40 virus transformants as well as those of pFN. The comparison is focused on a) the proportion of bi- and multi-antennary fractions, b) the structure of the bi-antennary fraction, and c) the presence or absence of lactosaminoglycan.

Materials and Methods

Cell Culture and Preparation of FN from Medium

Human embryonic lung fibroblast WI-38 and SV40 virus transformed cell line VA13 were obtained from the American Type Culture Collection, Rockville, MD, USA. Cells were cultured in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum. When cells were confluent, the medium was changed to, and cells were maintained in, the same DME medium without fetal calf serum for at least 24 h. The medium was harvested, and phenylmethylsulfonyl fluoride and EDTA were added at final concentrations of 1 mM and 5 mM, respectively. The harvested medium was filtered through Whatman No. 50 filter paper, and the medium was loaded on a gelatin-Sepharose column according to the procedure described by Engvall and Ruoslahti [25]. The column was washed with 0.5 M urea and FN was eluted with 4 M urea in 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). Eluted FN was dialyzed against 20 mM borate pH 7.8 containing 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA and stored at -20°C. Human pFN was purified from pooled human plasma, according to Engvall and Ruoslahti [25] and as described above. In some experiments cells were cultured in the presence of [^{3}H]labeled glucosamine (5 μCi/ml) or [^{14}C]galactose (0.5 μ Ci/ml) for 24 h. The yield of FN from media was as follows: 21.6 mg of FN was obtained from 55 l of media harvested from WI-38 normal fibroblasts (0.2 mg FN/l of medium/day); 61.4 mg of FN was released from 105 l of medium from WI-38 VA13 tumor cells (0.58 mg/l of medium/day). These FNs were analysed by sodium dodecylsulfate-polyacrylamide gel electrophoresis by the method of Laemmli [26] (see Fig. 1).

Preparation and Fractionation of Glycopeptides

FNs isolated from normal and transformed cell culture media as well as from human plasma were digested extensively by Pronase® (*Streptomyces griseus*, Calbiochem-Behring, San Diego, CA, USA) according to the procedure as described below. 20 mg of FN containing [^{3}H]glucosamine-labeled FN (1 × 10^5 cpm) and [^{14}C]galactose-labeled FN (1

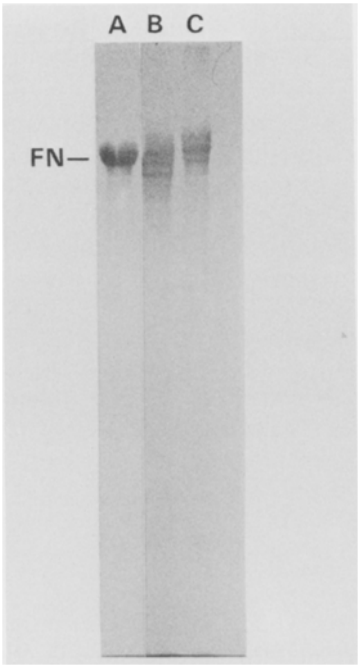


Figure 1. Comparative sodium dodecyl sulfate-5% polyacrylamide gel electrophoresis patterns of various FNs. Each line represents 10 μg of FN from human plasma (lane 1), WI-38 human fibroblasts (lane 2), and WI-38VA13 transformed human fibroblasts (lane 3).

$\times 10^4$ cpm) were lyophilized and dissolved in 5 ml of 100 mM borate buffer pH 7.8 containing 5 mM $CaCl_2$, and boiled for 5 min to effect denaturation. After cooling, 200 mg of Pronase (preincubated for 1 h at 37°C) was added and incubated at 37°C for 24 h. The Pronase addition followed by incubation was performed twice, and after three days the solution was centrifuged and the supernatant was loaded on a Bio-Gel P-6 column (1.2 $\times$ 85 cm) in 0.1 M pyridine acetate buffer pH 5.0. Elution was performed in this buffer solution and 50 drops of eluate were collected per fraction. Fractions from pFN were assayed for carbohydrates by orcinol-H_2SO_4. An aliquot of the glycopeptide-containing peak (co-eluting with P6II for normal and VA13 cFN) was labeled by reductive alkylation using [^{14}C]formaldehyde and sodium borohydride according to the method of Rice and Means [27], then combined with the unlabeled glycopeptides and fractionated on Con A-Sepharose as described below. The elution pattern is shown in Fig. 2. Two fractions separated by gel filtration on a Bio-Gel P-6 column were subjected to Con A-Sepharose column (1.0 $\times$ 36 cm: 30 ml) chromatography according to the method originally described by Ogata *et al.* [28]. The elution was performed first with 0.025 M Tris-HCl, pH 7.4, containing 0.25 M NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, followed by the same buffer solution containing 10 mM α-methyl mannoside. Finally the column was eluted with 100 mM α-methyl mannoside. Glycopeptides present in each fraction eluted from the Con A-Sepharose column were further purified on a Bio-Gel P-4 column (1 $\times$ 41 cm: 32 ml) eluted with distilled water; 50 drops were collected per fraction.

The glycopeptides from VA13 tumor cells contained a slow migrating fraction separated on Bio-Gel P-4 which was further purified on a Bio-Gel P-2 column (1 × 110 cm: 86 ml) eluted with distilled water, 50 drops per fraction. Fractionation on Bio-Gel P-2 resulted in a single peak (data not shown). The glycopeptide fractionation scheme is shown in Fig. 2.

Susceptibility of Glycopeptide to Endo-β-galactosidase Digestion

The high molecular weight range was collected and subjected to digestion with endo-β-galactosidase of *Escherichia freundii* [29]. The glycopeptide fraction (P6-I in Fig. 2) having 1.32×10^4 cpm was dissolved in 10 μl of endo-β-galactosidase (2.5 units) under "condition II" [29] and incubated at 37°C for 21 h. After incubation, the sample was passed through a Bio-Gel P-6 column (1.2 × 85 cm) equilibrated in 0.1 M pyridine-acetate buffer pH 5.0. Fifty drops of eluate were collected per fraction, and the activity of each fraction was determined in a scintillation counter.

Methylation Analysis

The glycopeptide fraction was analyzed by microadaptation with reduced volume of methylation reagent and hydrolysis solvent followed by preparation of partially methylated alditol acetates in reduced scale which will be described elsewhere (Levery SB, Hakomori S, manuscript in preparation). About 30-50 μg of sample was methylated in 300 μl of dimethyl sulfoxide and an equal volume of methylation reagent [30]. The reaction mixture was partitioned with chloroform and water, and the chloroform phase was evaporated to dryness and was subsequently hydrolyzed in 300 μl of 0.5 N sulfuric acid in 90% acetic acid for 6 h at 80°C under a nitrogen atmosphere with 0.5 N NaOH, reduced with 500 μl of NaB^2H_4 (10 mg/ml) in 0.025 N NaOH at 4°C. After reduction, excess NaB^2H_4 was destroyed with the addition of a few drops of acetic acid followed by addition of ethanol and evaporation with methanol. The residue was dried over P_2O_5 and finally acetylated in the presence of 500 μl of acetic anhydride at 100°C for 2 h. The product was evaporated with the addition of toluene and the dried residue was partitioned with chloroform and water. The chloroform layer was transferred to a small conical test tube and evaporated to dryness. Partially *O*-methylated alditol acetates were separated on a capillary DB-5 bonded phase fused silica column (30 m × 0.32 mm) (J & W Scientific, Rancho Cordova, CA, USA), and analyzed by chemical ionization mass spectrometry in a Finnigan 3300 Gas Chromatograph-Mass Spectrometer with a 6110 data system [31,32]. Chemical ionization was measured in methane (300μ), with electron energy 120 eV, emission current 500 μA, electron multiplier 2.4 kV and ion source temperature 100°C. The data were collected with a selected ion monitoring mode collecting intensities of 4 ions/scan. The program was set up in order to obtain the intensity of ions at m/z 234 and 262 for 2,3,4-tri-*O*-Me-Fuc, m/z 264 and 292 for 2,3,4,6-tetra-*O*-Me-Gal, m/z 292 and 320 for tri-*O*-Me-Hex, m/z 320 and 348 for di-*O*-Me-Hex, m/z 348 for mono-*O*-Me-Hex, m/z 305 for tri-*O*-HexNAc-Me, m/z 333 and 393 for di-*O*-Me-HexNAc-Me and m/z 361 and 421 for mono-*O*-Me-HexNAc.

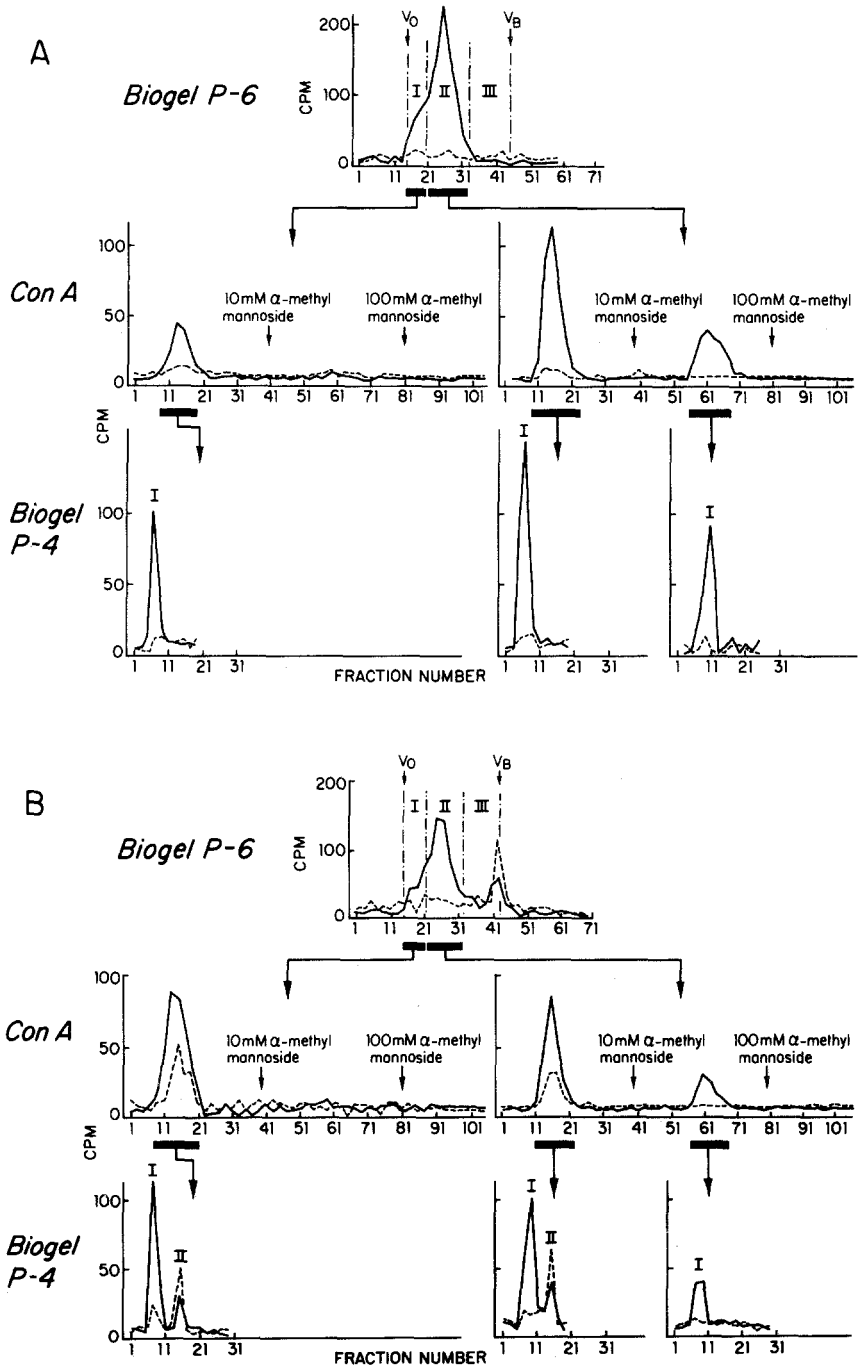


Figure 2. Comparative fractionation scheme for separation of FN glycopeptides by gel filtration and Con A-Sepharose chromatography. (A) FN from WI-38 human fibroblasts, (B) FN from VA13 transformed human fibroblasts. (—) [^{3}H]GlcN-labeled glycopeptides; (- - - -) [^{14}C]Gal-labeled glycopeptides.

Proton-NMR Spectroscopy

The 500 MHz ^{1}H-NMR spectra were recorded at 303°K on a Bruker (W. Germany) WM-500 spectrometer in the pulsed Fourier transform mode and using quadrature detection. The glycopeptide samples (100-150 μg) were deuterium-exchanged by lyophilization from $^{2}H_2O$, then dissolved in 0.4 ml $^{2}H_2O$ containing a trace of acetone, which was used as an internal standard, set at 2.225 ppm. 600-2000 free induction decays were accumulated; a Lorentzian to Gaussian function was applied before transformation [33]. A spectral width of 5000 Hz on 16K data points was used and the relaxation delay was 2 sec.

Binding Activities of FNs to Lentil Lectin and Anti-FN Antibodies

Lentil lectin (Vector Laboratories, Burlingame, CA, USA) and Protein A (Sigma, St. Louis, MO, USA) were labeled by chloramine-T oxidation [34]. FN solutions in PBS with a concentration of 50 μg/ml were diluted 1:3 in 96-well vinyl assay plates (Costar Scientific, Cambridge, MA, USA) and incubated overnight, followed by blocking with bovine serum albumin (BSA) and reaction with ^{125}I-labeled lentil lectin (7×10^4 cpm/well) or polyclonal rabbit antibodies to human FN (1:500) for 5 h. The plates were then washed 3 times with PBS with Ca^{2+}, Mg^{2+}, and 0.1% BSA. FN-coated wells reacted with anti-FN antibodies were then incubated with 1×10^5 cpm ^{125}I-labeled Protein A in PBS with Ca^{2+}, Mg^{2+}, and 0.1% BSA for 1 h and washed 3 times with the same buffer. Control wells containing either BSA or FN were incubated with ^{125}I-labeled Protein A alone.

Results

Purified intact FNs were homogeneous when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). FN released from VA13 transformed fibroblasts showed a slightly higher molecular weight than FN released from normal WI-38 fibroblasts or plasma.

The relative activity of glycopeptides, metabolically labeled with [^{3}H]glucosamine or [^{14}C]galactose, in various fractions (in Fig. 2) is shown in Table 1. As indicated, the glycopeptides from VA13 tumor cells had a significantly higher content of a Con A-unbound fraction, representing tri- and tetra-antennary structures, than glycopeptides from normal cFN and from pFN. The quantity of the glycopeptide fraction with a low molecular weight, "fraction III" separated on Bio-Gel P-6, was much higher in FN from VA13 cells than in FN from normal cells (Fig. 2 and Table 1). A glycopeptide with similar labeling characteristics to P6-III, i.e., higher incorporation of [^{14}C]galactose compared to [^{3}H]glucose was separated from the Con A-unbound fraction on Bio-Gel P-4 and was only detectable in cFN from transformed cells. In order to test the possible presence of the lactosaminoglycan structure in a higher molecular weight fraction, the fractions derived from cFN of WI-38 and VA13 cells were subjected to digestion with endo-β-galactosidase of *Escherichia freundii* [29]. There was no digestion of glycopeptides of FN from WI-38 and VA13 cells (Fig. 3).

Table 1. Relative activities of glycopeptide fractions from fibronectins from human embryonic lung fibroblast (WI-38), its SV40 transformant (VA13) and from human plasma FN.

Fractionation		WI-38 Normal FN[a]		WI-38VA13[a]		Human Plasma FN[b]	
		cpm	%	cpm	%	cpm	%
P6-I	Con A (-) P4I	0.71×10^4	16.9	0.92×10^4	28.0		
	Con A (-) P4II	absent		0.06×10^4	1.8	absent	
P6-II	Con A (-) P4I	1.96×10^4	46.7	1.50×10^4	45.7	0.59×10^6	35.3
	Con A (-) P4II	absent		0.26×10^4	7.9		
	Con A (+) P4I	1.53×10^4	36.4	0.54×10^4	16.5	1.06×10^6	64.7
Con A (-)/Con A (+) ratios		1.74		5.07		0.54	
P6-III			3.4[c]		15.0[c]	absent	

[a] [^{3}H]glucosamine metabolic labeled.
[b] [^{14}C]formaldehyde (reductive alkylation).
[c] Expressed as % of the total cpm in peaks I, II, III on Bio-Gel P-6.
Con A (-), unadsorbed on the Con A-Sepharose column; Con A(+), absorbed on the Con A-Sepharose column and eluted with 10mM α-methylmannoside under the conditions described in Materials and Methods.

The structures of the bi-antennary glycopeptides [28] derived from cFN of transformed VA13 fibroblasts were compared with those from cFN of normal WI-38 and those from pFN. As shown in Table 2, a much higher relative quantity of 2,4,6-tri-*O*-Me-Gal and 2,3,4-tri-*O*-Me-Gal was yielded from the permethylated cFN of transformed VA13 fibroblasts as compared with those sugar derivatives released from cFN of normal WI-38 fibroblasts. In contrast, the relative yield of 2,3,4,6-tetra-*O*-Me-Gal was much higher from cFN of normal WI-38 cells than from cFN of transformed VA13 cells. A weak acid hydrolysis (0.01 N HCl at 80°C in a water bath for 80 min) resulted in the absence of 2,4,6-tri-*O*-Me-Gal and 2,3,4-tri-*O*-Me-Gal and an increase in 2,3,4,6-tetra-*O*-Me-Gal, suggesting that the difference in the yield of these partially *O*-methylated galactose residues is due to a difference in sialylation. The results also suggest that the bi-antennary glycopeptide derived from transformed VA13 fibroblasts contains both sialosyl 2-6Gal and sialosyl 2-3Gal substitutions. In contrast to the results observed for cFN, sialylation of pFN was predominantly in a 2-6Gal linkage. Only minor quantities of terminal galactose (2,3,4,6-tetra-*O*-Me-Gal) were detected, indicating that most of the galactose was penultimate to terminal sialic acid. Desialylation of the pFN glycopeptide with mild acid removed approximately two sialosyl residues per glycopeptide with a corresponding increase in terminal galactose. Methylation data from human pFN are in agreement with those reported by Takasaki *et al.* [15]. Another outstanding difference between the bi-antennary glycopeptides derived from the cFN of transformed VA13 fibroblasts and normal WI-38 fibroblasts was found in the yield of 3-mono-*O*-Me-GlcNAcMe, which was derived from the innermost core *N*-acetylglucosamine residue linked to a peptide. A significant quantity of 3-mono-*O*-Me-GlcNAcMe was yielded from normal WI-38 fibroblasts, while a much lower yield of this sugar was obtained from transformed VA13 fibroblasts.

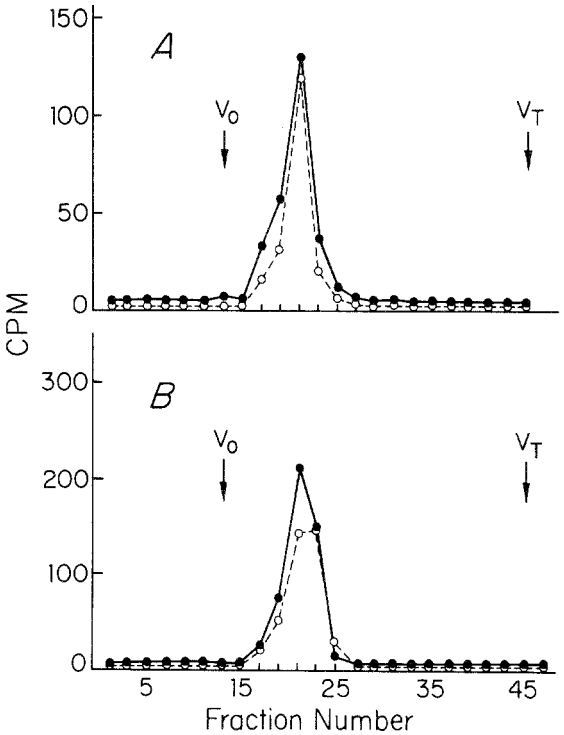


Figure 3. Endo-β-galactosidase digestion of fibronectin glycopeptides from transformed VA13 human fibroblasts followed by Bio-Gel P-6 column chromatography. Glycopeptide fraction P6-I (Fig. 2) from FN of VA13 cells was incubated with endo-β-galactosidase as described in Materials and Methods. (A) Before digestion. (B) After digestion. (—) [^{3}H]GlcN-labeled glycopeptides; (- - - -) [^{14}C]Gal-labeled glycopeptides.

Consistent with this, the yield of 2,3,4-tri-*O*-Me-Fuc from normal WI-38 cells was much higher than that of transformed VA13 fibroblasts. This sugar was not detected in the bi-antennary glycopeptide of pFN.

The structural differences between the glycopeptides derived from cFNs of transformed and normal fibroblasts and pFN were further analyzed by NMR spectroscopy (Fig. 4). Comparison of chemical shifts with published data showed the following features which have by now been well-characterized [35,36]. (i) All three glycopeptides have the distinctive bi-antennary pattern of Man H-2 resonances (Table 3, Fig. 4). (ii) The major resonances in the spectrum of the glycopeptide from normal WI-38 fibroblasts are all in agreement with those published for a completely non-sialylated structure [37], except for GlcNAc H-1 (see below). In agreement with the methylation analysis, smaller resonances can also be found which are characteristic of a NeuAcα2-3 terminal at 1.794 ppm and 2.758 ppm (NeuAc H-3ax and H-3eq) and at 4.548 ppm (Gal-**6** and/or -**6'** H-1). Resonances expected for the small amount of NeuAcα2-6 terminal are apparently below the detection limit. (iii) The spectrum of the glycopeptide from plasma FN, in agreement with its previously reported structure, shows resonances characteristic of a structure substituted primarily with NeuAcα2-6 residues on both branches (NeuAc H-3ax and H-3eq at

Table 2. Relative intensity of ions for partially methylated hexitol and hexosaminitol acetates yielded from permethylated bi-antennary fraction of glycopeptides. The partially methylated alditol acetates were separated by gas chromatography on capillary columns and determined by selected ion monitoring system in chemical ionization mass spectrometry.

	Selected ions m/z	Relative intensities[a] of ions from glycopeptides of:	
		Normal WI-38	SV40 transformed VA13
2,4-Me_2-Man	320;348	1.00	1.00
3,4,6-Me_3-Man	292;320	2.08	1.70
3,6-Me_2-GlcNAc-Me	333;393	3.52	4.24
3-Me-GlcNAc-Me	361;421	0.62	0.10
2,3,4,6-Me_4-Gal	264;292	0.94	0.54
2,3,4-Me_3-Gal	292;320	0.14	1.04
2,4,6-Me_3-Gal	292;320	0.19	0.97
2,3,4-Me_3-Fuc	234;262	0.44	0.28

[a] Normalized to 2,4-Me_2-Man as 1.00.

1.714 and 2.675 ppm, and Gal-**6/6'** H-1 at 4.447 ppm; the remaining resonances are in similar agreement with published data [38]). That a small amount of unsialylated material, probably on the (1-6)-branch, is present is indicated by smaller resonances at 4.474 and 4.930 ppm (Gal-**6'** and Man-**4'** H-1, respectively). (iv) In contrast to both of these, the glycopeptide of FN from transformed VA13 cells clearly shows H-3 signals from both α(2-3) and α(2-6)-linked NeuAc [36,38,39]. In addition, there are three apparent signals for Gal-**6/6'** H-1 at 4.449, 4.545 and 4.472 ppm, which are assigned to NeuAcα2-6 on the (1-6)-branch, NeuAcα2-3 on the (1-3)-branch, and NeuAc deletion, respectively. However, the confidence level for assigning sialylation types to any particular branch is low, particularly due to the low resolution of the spectrum. Assignments of other peaks by comparison with literature values [35] would seem to indicate an arrangement opposite to that deduced from Gal-**6/6'** H-1 shifts. The distribution of terminal types may in fact be random.

It is apparent that there is no correspondence between literature values for GlcNAc-**1** H-1 [35]. It may be broadened and appear at several shifts somewhat upfield of those usually found, due to incomplete digestion of the attached peptide with a subsequent heterogeneity in the peptide moiety. This interaction may also explain why no clear resonances were found for the Fucα1-6 residue, even though FN from normal WI-38 cells plainly contains a significant amount (methylation analysis, Table 2).

The reactivity of FN released from normal WI-38 fibroblasts, transformed VA13 fibroblasts, and normal plasma with lentil lectin was compared in solid phase binding assays (Fig. 5). The binding of lentil lectin to the cFN derived from normal fibroblasts was much higher than that to the cFN derived from transformed cells. Essentially no binding of lentil lectin to pFN was found.

Table 3. ^{1}H Chemical shifts of structural reporter groups of constituent monosaccharides for bi-antennary asparagine-linked glycopeptides of fibronectins.

Reporter Group	Residue	A plasma	B normal released	C VA13 released
H-1	**1**	5.029,5.059,5.080[a]	5.027,5.060,5.080[a]	5.025,5.058,5.077[a]
	2	4.622	4.618	4.625
	3	4.774	4.763	4.765
	4	5.140	5.125	5.123[g] (5.138[h])
	4'	4.948 (4.930[b])	4.924	4.924[i] (4.948[j])
	5	4.607	4.584	4.585[m]
				4.610[n]
	5'	4.607 (4.58[b])	4.584	4.576[o]
	6	4.447[c]	4.472[c]	4.545[k] (4.472[d])
	6'	4.447[c] (4.474[b,d])	4.472[c] (4.548[e,f])	4.449[j] (4.472[b])
H-2	**3**	4.253	4.246	4.247
	4	4.196	4.191	4.192
	4'	4.114[c]	4.107	4.107
H-3 ax	NeuAcα2-6	1.174	— (1.794[e,f])	1.712 (1.730)
	NeuAcα2-3	—	—	1.798 (1.796)
H-3 eq	NeuAcα2-6	2.675	— (2.758[e,f])	2.673
	NeuAcα2-3	—	—	2.761

[a] Signals arising from main components with respect to heterogeneity of peptide moiety. Signals are considerably broadened, indicating probably large peptide fragments.
[b] NeuAc deletion on (1-6) branch.
[c] Average values for two unresolved signals.
[d] NeuAc deletion possibly on (1-3) branch.
[e] NeuAcα2-3 on (1-6) [and possibly (1-3) branch].
[f] Insufficient resolution to assign strictly.
[g] NeuAc deletion and/or α2-3 on (1-3) branch.
[h] NeuAcα2-6 on (1-3) branch.
[i] NeuAcα2-3 and/or deletion on (1-6) branch.
[j] NeuAcα2-6 on (1-6) branch.
[k] NeuAcα2-3 on (1-3) branch.
[m] NeuAc deletion.
[n] NeuAcα2-6 on either branch.
[o] NeuAcα2-3 on either branch.

Discussion

In human fibroblasts, cell surface and matrix FN are greatly reduced on oncogenic transformation induced by tumor viruses [6,7]. In view of a possible role of carbohydrates in the determination of mobility, protease susceptibility, cellular localization, and turnover of proteins [4], it is important to determine the exact structural differences between the FNs of normal and transformed fibroblasts. On the other hand, species-specific differences in domain structure and carbohydrate structure of pFN have been increasingly clear. Hamster pFN lacks the fibrin-binding domain in one of its subunits (β

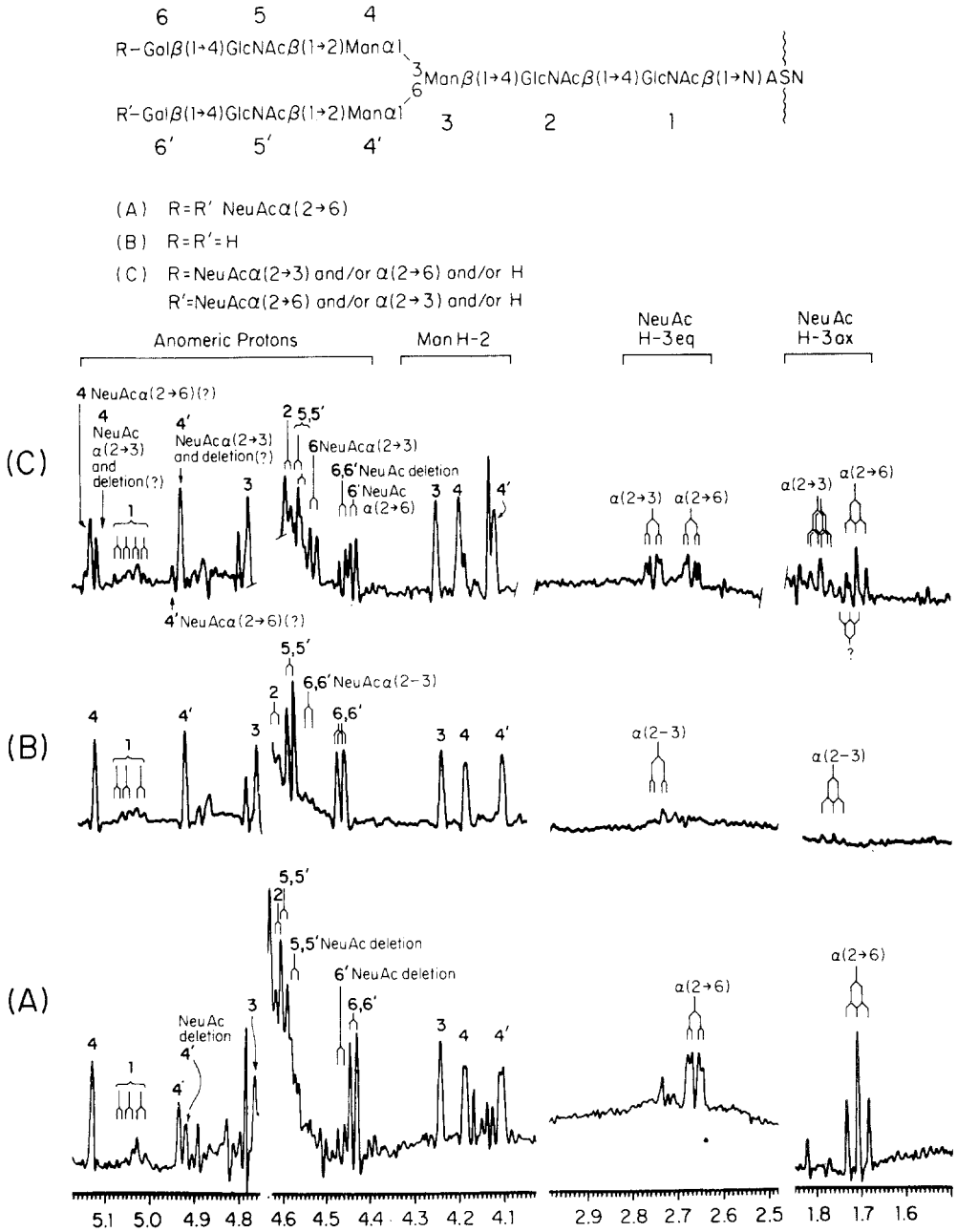


Figure 4. ^{1}H-NMR spectra of bi-antennary glycopeptides prepared from FNs of WI-38 fibroblasts, SV40 virus transformed VA13 cells, and plasma. (A) Glycopeptide from plasma FN. (B) Glycopeptide from FN of normal WI-38 fibroblasts. (C) Glycopeptide from FN of transformed VA13 cells.

Assignments of resonances due to anomeric protons of the sugar residues and mannose H-2 is indicated by arabic numbers **1-6** and **4' -6'**. Assignments of resonances of the H-3 proton of sialic acid residues are shown upfield.

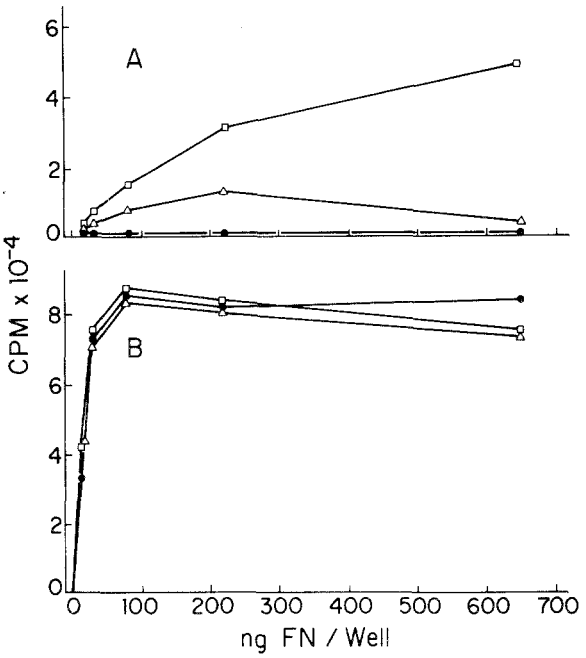


Figure 5. Quantitative reactivity of FN with lentil lectin by solid phase binding assay. (A) Reactivity of FNs from different sources with ^{125}I-labeled lentil lectin. (B) Reactivity of FNs from different sources with anti-FN antibody. ●, pFN; □, cFN from WI-38 fibroblasts; △, cFN from transformed VA13 cells.

subunit), while the fibrin-binding domain is present in both the α and β subunits of human pFN [8,9]. Bovine pFN has an unusual peripheral structure in the bi-antennary carbohydrate, NeuAcα2-4Galβ1-3[NeuAcα2-6]GlcNAc. However, hamster and human pFN have a common bi-antennary structure with a core of Galβ1-4GlcNAcβ1-2Manα1-6 (Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc [12-15]. Interestingly, both human and hamster pFN have a Siaα2-6Gal linkage and have no Fucα1-6GlcNAc substitution at the core structure [14,15]. In hamsters, FN derived from NIL fibroblasts contains a fucosyl substitution at the core *N*-acetylglucosamine residue [12,13], in striking contrast to pFN of this species, which lacks this structure [14]. Although these bi-antennary structures represent major components of glycopeptides in both hamster fibroblast FN and hamster pFN, a considerable amount of the fraction that does not bind to Con A-Sepharose was found in human pFN [16]. Such a fraction may represent a tri- or tetra-antennary glycopeptide, although its structure remains to be elucidated. Glycopeptides of FN from normal hamster NIL fibroblasts and NIL cells transformed by hamster sarcoma virus were recently studied by metabolic labeling [20]. The results showed a great increase in a Con A-unbound, higher molecular weight glycopeptide in transformed NIL cells as compared to non-transformed NIL cells. An enhanced label in the Con A-unbound glycopeptide derived from the FN of transformed BHK cells as compared to that of normal BHK cells was also reported by Delannoy *et al.* [21], in general agreement with the phenomenon previously described by Warren and his associates [18].

The results of this study using human WI-38 fibroblasts and their SV-40 virus transformants (VA13) support the enhanced concentration of the Con A-unbound high molecular weight glycopeptide in transformed VA13 cells as compared to normal WI-38 cells. In addition, the results of this study clearly indicate the following differences between the glycopeptides derived from FN of normal and transformed fibroblasts. i) There was a unique glycopeptide fraction detectable only in transformed cells as a slow-migrating fraction on a Bio-Gel P-4 column, although the chemical properties of this fraction remain to be elucidated. ii) The Con A-bound fraction representing the bi-antennary glycopeptide showed a remarkable difference in sialosyl and fucosyl substitutions between the FNs of normal and transformed cells.

The bi-antennary glycopeptide from normal fibroblasts was not sialylated at the galactosyl terminus, but it was fucosylated at the internal core *N*-acetylglucosamine through a Fucα1-6 linkage. The presence of a fucosyl residue in the core structure of cellular FN from fibroblasts was previously reported in hamster fibroblasts [12,13] and seems to be a general phenomenon for cellular FN. The bi-antennary glycopeptide derived from the FN of transformed VA13 cells contained both sialosyl 2-3 and 2-6 substitutions at the terminal galactose residue and showed very little evidence of a fucosyl residue at the internal *N*-acetylglucosamine core structure. Although these structural differences were found in the bi-antennary glycopeptide, the difference must also exist in the tri-antennary structure because intact FN derived from normal WI-38 cells showed much stronger binding activity with lentil lectin as compared to intact FN derived from transformed VA13 cells. It should be noted that plasma FN has no binding activity with lentil lectin. Kornfeld and his associates showed that binding activity with lentil lectin depends on the presence of a Fuc-GlcNAc residue at the core structure [40], and the absence of such a structure in plasma FN agrees well with the absence of binding activity with lentil lectin. In general, the glycosylation pattern of the bi-antennary structure in FN from transformed cells is similar to that of plasma FN in location of sialylation and fucosylation.

References

1 Pearlstein E, Gold LI, Garcia-Pardo A (1980) Mol Cell Biochem 29:103-28.
2 Mosesson MW, Amrani DL (1980) Blood 56:145-58.
3 Ruoslahti E, Engvall E, Hayman, EG (1981) Coll Relat Res 1:95-128.
4 Hakomori S, Fukuda M, Sekiguchi K, Carter WG (1984) in Extracellular Matrix Biochemistry, eds. Piez K, Reddi AH, Elsevier, New York, p 229-75.
5 Yamada KM (1983) Ann Rev Biochem 52:761-99.
6 Gahmberg CG, Hakomori S (1973) Proc Natl Acad Sci USA 70:3329-33.
7 Hynes RO (1973) Proc Natl Acad Sci USA 70:3170-74.
8 Sekiguchi K, Hakomori S (1980) Proc Natl Acad Sci USA 77:2661-65.
9 Sekiguchi K, Fukuda M, Hakomori S (1981) J Biol Chem 256:6452-62.
10 Sekiguchi K, Hakomori S (1983) J Biol Chem 258:3967-73.
11 Sekiguchi K, Siri A, Zardi L, Hakomori S (1983) Biochem Biophys Res Commun 116:534-40.
12 Carter WG, Hakomori S (1979) Biochemistry 18:730-38.
13 Fukuda M, Hakomori S (1979) J Biol Chem 254:5451-57.
14 Fukuda M, Levery SB, Hakomori S (1982) J Biol Chem 257:6856-60.

15 Takasaki S, Yamashita K, Suzuki K, Kobata A (1980) J Biochem (Tokyo) 88:1587-94.
16 Fisher SJ, Laine RA (1979) J Supramol Struct 11:391-99.
17 Takasaki S, Yamashita K, Suzuki K, Iwanaga S, Kobata A (1979) J Biol Chem 254:8548-53.
18 Warren L, Buck CA, Tuszynski GP (1978) Biochim Biophys Acta 516:97-127.
19 Hakomori S, Kannagi R (1983) J Natl Cancer Inst 71:231-51.
20 Wagner DD, Ivatt R, Destree AT, Hynes RO (1981) J Biol Chem 256:11708-15.
21 Delannoy P, DeBeire P, Montreuil J, Debray H (1983) in Proc 7th Int Symp Glycoconjugates, eds. Chester MA, Heinegård D, Lundblad A, Svensson S, Secretariat, Lund, p 173-74.
22 Cossu G, Andrews PW, Warren L (1983) Biochem Biophys Res Commun 111:952-57.
23 Cossu G, Warren L (1983) J Biol Chem 258:5603-7.
24 Zhu BC, Fisher SF, Pande H, Calaycay J, Shively JE, Laine RA (1984) J Biol Chem 259:3962-70.
25 Engvall E, Ruoslahti E (1977) Int J Cancer 20:1-5.
26 Laemmli UK (1970) Nature 227:680-85.
27 Rice RH, Means GE (1971) J Biol Chem 246:831-32.
28 Ogata S, Muramatsu T, Kobata A (1975) J Biochem 78:687-96.
29 Fukuda MN, Watanabe K, Hakomori S (1978) J Biol Chem 253:6814-19.
30 Hakomori S (1964) J Biochem 55:205-8.
31 Laine RA (1980) in Proc 27th Int Congr Pure and Applied Chemistry, ed. Varmavuori A, Pergamon Press, New York, p 193-98.
32 McNeil M, Albersheim P (1977) Carbohydr Res 56:239-48.
33 Ernst RR (1966) Adv Magn Reson 2:1-135.
34 Hunter WM, Greenwood FC (1962) Nature 194:495-96.
35 Vliegenthart JFG, Dorland L, van Halbeek H (1983) Adv Carbohydr Chem Biochem 41:209-374 and references cited therein.
36 Bhattacharyya SN, Lynn WS, Dabrowski J, Trauner K, Hull WE (1984) Arch Biochem Biophys 231:72-85.
37 Fournet B, Montreuil J, Strecker G, Dorland L, Haverkamp J, Vliegenthart JFG, Binette JP, Schmid K (1978) Biochemistry 17:5206-14.
38 Debray H, Fournet B, Montreuil J, Dorland L, Vliegenthart JFG (1981) Eur J Biochem 115:559-63.
39 Schut BL, Dorland L, Haverkamp J, Vliegenthart JFG, Fournet B (1978) Biochem Biophys Res Commun 82:1223-28.
40 Kornfeld K, Reitman ML, Kornfeld R (1981) J Biol Chem 256:6633-40.