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Additional fucosyl residues on membrane glycoproteins but not a secreted glycoprotein from cystic fibrosis fibroblasts

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Summary

Glycopeptides derived from peripheral membrane glycoproteins of skin fibroblasts of seven patients with cystic fibrosis (CF) had an increase in fucosyl residues when compared with those of seven age, race and sex matched controls (Pediatr Res 1985;19:368–374). To further define these results, the membrane glycopeptides which bound to immobilized lentil lectin and thereby enriched in fucosyl residues linked $\alpha 1 \rightarrow 6$ to *N*-acetylglucosamine attached to asparagine, were Pronase digested, partially purified and examined by 500-MHz ^{1}H-NMR spectroscopy. The CF derived glycopeptides had two different features when compared to those from Controls (1) an increased number of fucosyl residues linked $\alpha 1 \rightarrow 6$ to the *N*-acetylglucosamine attached to asparagine and (2) fucosyl residues linked $\alpha 1 \rightarrow 3$ to a branch *N*-acetylglucosamine. The glycopeptides from both sources were of the di and triantennary type containing sialic acid linked $\alpha 2 \rightarrow 3$ and $\alpha 2 \rightarrow 6$ to galactose in an approximate molar ratio of 3 : 2 and 2 : 1, from CF and Control, respectively. Glycopeptides derived from a glycoprotein, fibronectin, secreted from CF fibroblasts were also examined by ^{1}H-NMR spectroscopy and showed no evidence of fucosyl residues linked $\alpha 1 \rightarrow 3$ to branch *N*-acetylglucosamine and a lesser per-

Abbreviations: CF, cystic fibrosis; NMR, nuclear magnetic resonance; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GlcNAc-1, *N*-acetylglucosamine attached to asparagine; NeuNAc, *N*-acetyl neuraminic acid (sialic acid); α-meMan, α-methyl mannoside.

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centage of core fucose than found in the peripheral membrane glycopeptides. These results define further the altered fucosylation of the CF peripheral membrane glycoproteins.

Introduction

Increased fucosylation was first reported by Dische et al. [1] in the glycoproteins of duodenal secretions from patients with CF. This report laid dormant for a number of years and then a variety of studies were published indicating altered glycosylation of the mucins [2–4] from other CF secretions. Subsequently, the peripheral membrane glycoproteins obtained from skin fibroblasts of seven CF patients were shown by gas–liquid chromatography analysis to have an increased fucose content as well as an increased molar ratio of fucose to other monosaccharides when compared to those from seven matched controls [5]. It was also noted that a secreted glycoprotein [6] which was shown to be fibronectin [7] had minor differences in monosaccharide composition [6] and no differences detectable by affinity to a variety of immobilized plant lectins [7] selected to provide structural information [8,9].

Further analysis of the membrane glycoproteins, radiolabeled with L-[^{3}H]fucose, revealed that the glycopeptides from the three CF fibroblasts lines which were examined, bound more tightly to lentil lectin–Sepharose than similar fractions from three matched control fibroblast lines [5]. At that time a structure was proposed for the altered glycopeptides on the basis of affinity to immobilized lectins. In proposing the structure [5] we included two facts concerning fucosyl residues linked $\alpha 1 \rightarrow 3$ to an antennary GlcNAc: (1) Fucαl $\rightarrow$ 3GlcNAc did not interfere with binding to lentil lectin in the presence of Fucαl $\rightarrow$ 6GlcNAc-1 [8,10] which is among the requirements for binding [11] and (2) at least in one case, the presence of Fucαl $\rightarrow$ 3GlcNAc increased the affinity of the glycopeptides to lentil lectin [12]. To establish the structure of these fucosylated glycopeptides it was necessary to confirm it by more precise methodology. One such method is 500-MHz ^{1}H-NMR spectroscopy which has been developed to give the complete sequence of monosaccharides found in an oligosaccharide and in addition the anomeric linkage and branching [13,14]. We report here the results of these studies and show that the oligosaccharides from the membrane glycoproteins of the CF fibroblasts were fucosylated in two positions, whereas those from the Control fibroblasts were less fucosylated and fucose was present in only one position. The secreted CF glycoprotein, fibronectin, was partially fucosylated in only one position.

Materials and methods

Cell culture and harvest

Skin fibroblasts were obtained by biopsy of a CF patient and an age-, race- and sex-matched individual which served as a Control. The procedures and informed consent were approved by the Committee for the Protection of Human Subjects of

The Children's Hospital of Philadelphia. To obtain material for NMR spectroscopy the cells were grown as described [5,6] between passages 10–17. As previously reported [5] all growth conditions were rigidly controlled. When comparisons were made, the CF and Control cells were grown under the same conditions and were of the same passage numbers. The stock cultures were transferred every 7 days and the number of cells seeded per 75 cm^2 flask was always constant (1×10^6 cells). The cells were always refed two times and transferred on the same days. The same lot of serum and medium was used and every attempt was made to duplicate growth and processing conditions of both cell types. In order to follow the isolation of the glycopeptides some of the cells were made radioactive by metabolic labeling with D-[6-^{3}H]glucosamine (5 μC/75 cm^2 flask; 19 Ci/mmol New England Nuclear, Boston, MA) for 48 h prior to harvest on day 7. The cells were washed and removed from the monolayer by controlled trypsinization as described [15]. The characteristics of the cells which remained viable after this procedure were similar to those reported previously [5].

The culture medium from the fibroblasts was collected at Day 7, and was after refeeding the fibroblasts 48 h prior to harvest. In some cases, radioisotopes were added as described above.

Preparation of membrane glycopeptides for ^{1}H-NMR spectroscopy

The material which was removed from the surface of the cells by trypsin was centrifuged $12000 \times g$ for 30 min at 5 °C and the supernatant solution was lyophilized and designated 'peripheral membrane glycopeptides'. Peripheral membrane glycopeptides were collected from 5×10^9 CF fibroblasts and $1.5–3 \times 10^9$ Control fibroblasts. The lyophilized glycopeptides from CF or Control fibroblasts were desalted over a column (2.8×80 cm) of Sephadex G-50. After lyophilization and further desalting on Biogel P-2, a fraction, enriched in Fuc linked $\alpha 1 \rightarrow 6$ to GlcNAc-1, was selected by binding to a column (2.2×26 cm) of lentil lectin-Sepharose (Pharmacia LKB Biotechnology Inc, Piscataway, NJ). The glycopeptides were eluted with 0.2 mol/l α-meMan [5,11]. The CF glycopeptides, which contained non-radioactive glycopeptides were divided and purified on three such affinity columns whereas the Control glycopeptides were purified in two lots. The large glycopeptides which bound to lentil lectin–Sepharose were dialyzed against water for 18 h to remove the sugar, lyophilized and then digested exhaustively with Pronase [15]. The Pronase-digested material was separated on a column (1×110 cm) of Biogel P-10 in 50 mmol/l ammonium acetate and subsequently desalted in a column (1×50 cm) of Biogel P-2, repeating this latter procedure two or three times.

Preparation of a secreted glycoprotein for ^{1}H-NMR spectroscopy

Culture medium from approximately seventy (75 cm^2) flasks of CF fibroblasts was collected at day 7 and contained secreted glycoproteins from days 5 to 7 in culture. In some cases, the cells were metabolically labeled with D-[^{3}H]GlcN as described above. Immediately after collection the medium was centrifuged at $800 \times g$ for 15 min at 5 °C and the supernatant solution was stored at −40 °C until processed. The growth medium (200 ml) was combined after thawing, dialyzed

against water and lyophilized. Precipitation of the glycoproteins with heparin (sodium salt, ICN Biochemicals, Cleveland, OH) was as described [6,7] with the exception that proportionally larger amounts were used. After precipitation, the glycoproteins were suspended in 0.25 mmol/l Tris–HCl, pH 6.8, containing 0.1% sodium dodecyl sulfate, 2 mmol/l mercaptoethanol and 0.8% *p*-methylsulfonyl fluoride, boiled for 3 min and further purified on a column (1.5 × 50 cm) of Biogel P-200 in the same buffer, collecting fractions of 1.35 ml. An aliquot of the ^{3}H-material which was excluded from the column was examined by polyacrylamide gel electrophoresis under denaturing conditions [6,7]. These procedures, including the preparation of a fresh Biogel P-200 column, were repeated 5 times in order to process one liter of medium. The five fractions were combined and digested exhaustively with Pronase [15]. Further purification was on a column (1.0 × 130 cm) of Biogel P-10 in 50 mmol/l ammonium acetate buffer utilizing one third of the fraction each time followed by chromatography of the three combined fractions of included material and excluded material, each, over a column (1 × 35 cm) of Biogel P-2. A column (1.5 × 9 cm) of Con A–Sepharose (Pharmacia LKB Biotechnology Inc, Piscataway, NJ) [7,16] was used to separate further the Biogel P-10 included fractions. The bound glycopeptides were freed of α-meMan by filtration over Biogel P-10 in 10 mmol/l ammonium acetate followed by two times over Biogel P-2 in water. The Con A unbound glycopeptides were subjected to the same purification steps after rechromatography on Con A–Sepharose. In addition, the Con A unbound glycopeptides were purified further by binding to a column (0.8 × 18 cm) of serotonin-Sepharose. The glycopeptides were then desalted over a column (1.5 × 140 cm) of Biogel P-4 in water and twice over Biogel P-10 in water.

Affinity chromatography

Analytical amounts of the purified glycopeptides from the membrane and secreted glycoproteins were examined by affinity to immobilized plant lectins. The specificities of the lectins used have been described in addition to the procedural details and therefore only brief descriptions are given here. Con A–Sepharose will bind certain diantennary glycopeptides [7,16]. Lentil lectin-Sepharose binds certain di- or triantennary glycopeptides which are fucosylated α1 → 6 at GlcNAc-1 [5,11]. The presence of fucosyl residues in α1 → 3 linkage to branch GlcNAc will not interfere with binding in the presence of fucosylated GlcNAc-1 [8,10,12]. SNA (EY Labs, Inc, San Mateo, CA), a lectin from elderberry bark [17] binds glycopeptides which are sialylated in α2 → 6 linkage to Gal or GalNAc [18,19]. Datura stramonium lectin (EY Labs, Inc, San Mateo, CA) binds mainly polylactosamine-containing glycopeptides [20] although there are notable exceptions [21]. In addition to the lectins, an analytical column (0.8 × 5.5 cm) of serotonin-Sepharose was prepared and used as described [7,22]. Serotonin will bind glycopeptides containing NeuNAcα2 → 6 or α2 → 3. In all cases, glycopeptides with known binding characteristics [10,19,20] were used to standardize the affinity columns.

High resolution ^{1}H-NMR spectroscopy

A Bruker AM-500 spectrometer (SON hf. NMR facility, Department of Bio-

physical Chemistry, Nijmegen University or the Department of Organic Chemistry, Utrecht University, The Netherlands), equipped with an Aspect-3000 computer, was used. Further experimental details were described previously [13,14]. Prior to analysis, the glycopeptides were treated repeatedly with deuterium oxide at room temperature, with intermediate lyophilization. Finally, the samples were redissolved in 0.4 ml ^2H_2O (99.96 atom % ^2H; Aldrich Chemical Company, Inc, Milwaukee, WI). Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation [13,14]. The probe temperature was 300 K. The chemical shifts (δ) are expressed in ppm downfield from the methyl signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ2.225) with an accuracy of 0.002 ppm.

Results

Purification of peripheral membrane glycopeptides

It was reported previously that the peripheral membrane glycopeptides from CF fibroblasts bound more tightly to lentil lectin–Sepharose than those of control fibroblasts [5]. In order to show this effect, the glycopeptide fractions were enriched prior to Pronase digestion in glycopeptides which bound to lentil lectin [5]. Therefore, as part of the purification procedure the glycopeptides analyzed as described here were also enriched by binding to lentil lectin–Sepharose.

The lentil lectin-bound glycopeptides were subsequently digested with Pronase and separated on Biogel P-10. The CF glycopeptides (Fig. 1A) separated into two main groups; approximately 17% was excluded from Biogel P-10 whereas 81% was included when [^{3}H]GlcN was the metabolic label. On the other hand, although the Control glycopeptides (Fig. 1B) were separated into two main groups, the excluded group (31%) contained proportionately more radioactivity than those of the CF glycopeptides. The included material (63%) was heterogeneous in size and was found in a slightly larger size range than the CF glycopeptides.

Some characteristics of the Biogel P-10 excluded and included membrane glycopeptides obtained by affinity chromatography

The glycopeptides were from cells metabolically labeled with [^{3}H]GlcN and were a heterogeneous mixture therefore it was impossible to obtain structural information by affinity to various lectins. It was possible to show a few comparisons on the distribution of ^{3}H-glycopeptides and these points are noted from the data given in Table I. The Biogel P-10 excluded material showed some difference between CF and Control in total percentage of radioactivity bound to serotonin–Sepharose (77 vs. 97%, respectively) however 40% of the CF-derived radioactivity was tightly bound (Fig. 2A) whereas only 14% of the Control radioactivity was tightly bound (Fig. 2B). Serotonin–Sepharose binds sialylated glycopeptides therefore these results can be interpreted tentively to suggest that proportionately more of the CF glycopeptides excluded from Biogel P-10 were more highly sialylated than those of Control. On the other hand, the included glycopeptides derived from the CF and control [^{3}H]GlcN-labeled fibroblasts had similar percentages and distribution bound to

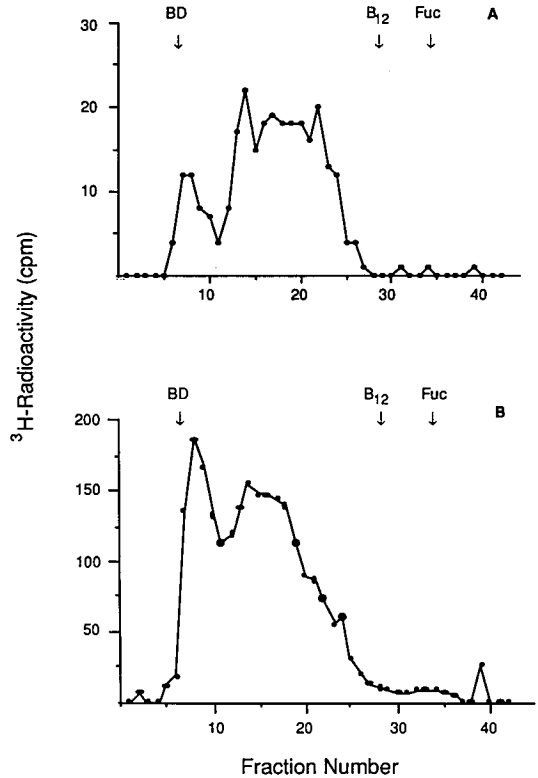


Fig. 1. Separation of peripheral membrane glycopeptides by chromatography on Biogel P-10. ^{3}H-Glycopeptides derived from A, CF and B, Control fibroblasts were each separated into two groups; fractions 5–10 were combined and designated *excluded* from the Biogel and fractions 11 to 25 were combined and designated *included.* BD, blue dextran 2000; B_{12}, cobalamine; and [^{3}H]Fuc marked the void volume, M_r 1350, and monosaccharides, respectively. See 'Materials and methods' for further details.

serotonin–Sepharose and the lectins (Table I). Approximately 10% of the radioactivity of the CF material which was included in Biogel P-10, bound to SNA whereas 23% of that of the Control glycopeptides bound to this immobilized lectin which binds glycopeptides containing NeuNAcα2 → 6 [17].

It was anticipated that a large percentage of the [^{3}H]GlcN-labeled glycopeptides which were excluded from Biogel P-10 would bind to Datura-agarose. Polylactosamine-containing glycopeptides are reported [20] to bind to this lectin since they have repeating sequences, Galβ1 → 4GlcNAcβ1 → 3, and are in a larger size class than the complex oligosaccharides. Therefore it was surprising that only 5 and 6% of the CF and Control ^{3}H-glycopeptides bound (Table I).

Approximately 25% of the radioactive material which was enriched in Fucα1 → 6GlcNAc-1 from both CF and Control sources bound to Con A–Sepharose (Table I) revealing the presence of diantennary glycopeptides with structural characteristics appropriate for binding [8,9].

Alkaline borohydride treatment of CF peripheral membrane glycopeptides

It was possible that the CF glycopeptides contained oligosaccharides *O*-linked to Ser or Thr as these oligosaccharides can be fucosylated and thus could provide the observed increase in fucosylation [5]. The excluded and included fractions from Biogel P-10 (Fig. 1A) were treated with alkaline borohydride under conditions which released *O*-linked oligosaccharides [23,24]. Essentially, no radioactivity was released from the excluded (Fig. 3A) or the included fractions (Fig. 3B). The fractions were from cells metabolically labeled with D-[^{3}H]GlcN and approximately 10% of *N*-linked oligosaccharides are released by the method utilized [24,25] and could thus account for the slight difference observed in the profiles (compare Fig. 1A and Fig. 3). Therefore it was concluded that at least the largest proportion of [^{3}H]GlcN-labeled oligosaccharides were not of the *O*-linked type, since they would have been readily released under the conditions used.

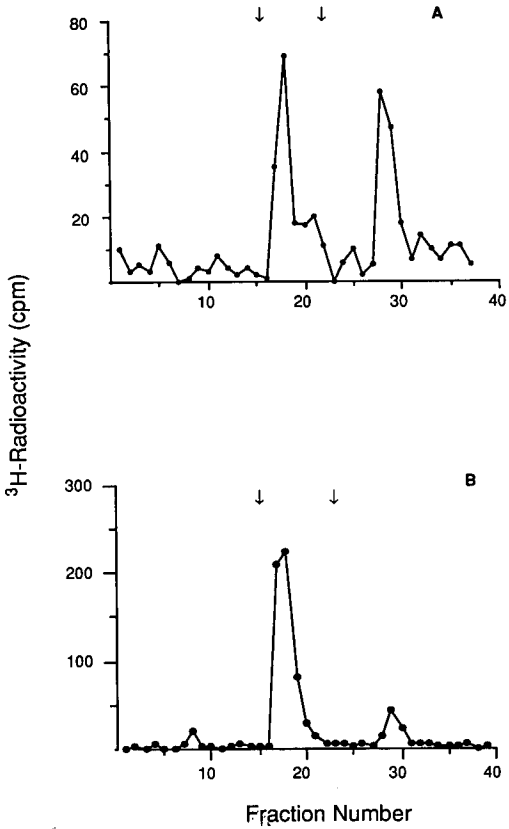


Fig. 2. Binding of peripheral membrane glycopeptides to serotonin–Sepharose. The ^{3}H-glycopeptides derived from A, CF and B, Control fibroblasts, metabolically labeled with D-[^{3}H]GlcN, were excluded from Biogel P-10 (Fig. 1) and were bound to serotonin–Sepharose and eluted with: first arrow, 0.1 mol/l Tris buffer, pH 7.2, and second arrow, the same buffer containing 0.5 mol/l NaCl. See 'Materials and Methods' for further details.

TABLE I

Binding of [^{3}H]GlcN-labeled membrane glycopeptides to immobilized lectins and serotonin

Affinity column [b]	Biogel P-10 fractions [a]			
	Excluded		Included	
	CF	C	CF	C
	Radioactivity (percentage bound):			
Lentil lectin [c]	31	35	44	40
Con A [d]	–	–	23	22
Datura [e]	5	6	14	11
Serotonin [f]	77	97	82	83
SNA [g]	–	–	11	23

[a] See Fig. 1. 17% and 31% of the radioactivity was excluded from the Biogel P-10 column for CF and Control (C) glycopeptides, respectively.

[b] Eluted with: [c] 10 mmol/l and 0.2 mol/l α-meMan [5]; [d] 0.1 mol/l α-meMan [16]; [e] partially degraded chitin [20]; [f] 0.1 mol/l Tris, pH 7.2 and the same buffer containing 0.5 mol/l NaCl [7]; [g] 50 mmol/l lactose [17].

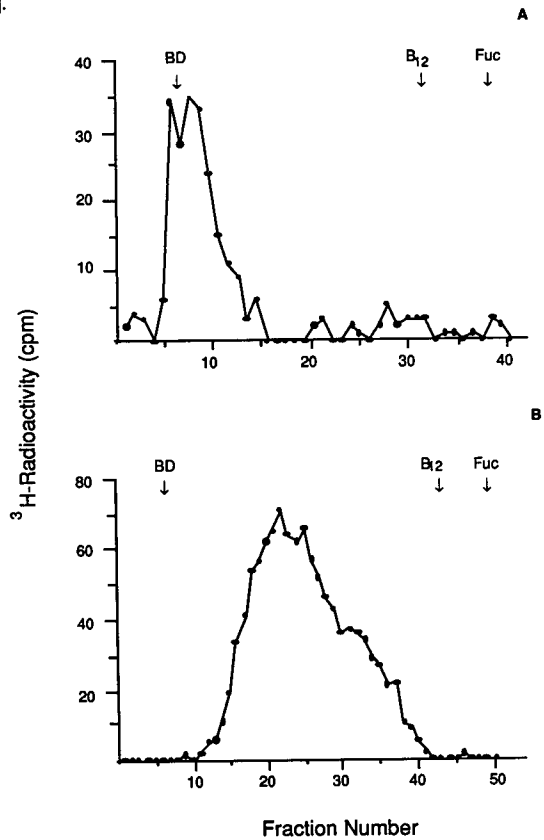
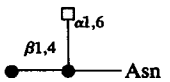
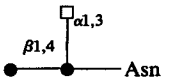
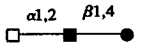
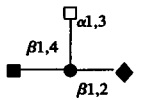
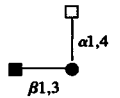


Fig. 3. Alkaline borohydride reduction of the CF peripheral membrane glycopeptides. The ^{3}H-glycopeptides which were A, excluded or B, included in Biogel P-10 were treated with 1 mol/l alkaline borohydride, 0.1 mol/l sodium hydroxide for 18 h at 37°C and rechromatographed on a column of Biogel P-10 in 50 mmol/l ammonium acetate [23]. Compare Fig. 1A, fractions 5–10 with A and fractions 11–25 with B. Note the position of the markers and size of the column (1×120) of Biogel P-10 in B.

TABLE II

^{1}H-Chemical shifts of pertinent structural-reporter group protons which discriminate the type of linkage of L-fucosyl residues

Fucose reporter group	Compound and schematic structure [a]				
					
	1 [b]	2 [c]	3 [b]	4 [b]	5 [d]
	Chemical shift of L-fucosyl residue:				
H-1	4.88	5.136	5.31	5.12–5.13	5.02
H-5	4.12–4.13	4.722	4.22	4.83	4.87
CH_3	1.20–1.21	1.285	1.24	1.17	1.18

[a] Chemical shifts are given at 300 K in ppm referenced to internal acetone at $\delta = 2.225$ ppm (in ^2H_2O). The types of linkage of the residues are given for the compounds represented by short-hand symbolic notation [10]: ●, GlcNAc; ■, Gal; □, Fuc; ◆, Man.

[b] Data from [13]; [c] data from [26]; [d] data from [27].

^{1}H-NMR spectroscopy of the Biogel P-10 included membrane glycopeptides

In order to determine more detailed oligosaccharide structures of the CF membrane glycopeptides which were more highly fucosylated than those from the controls [5], the Biogel P-10 included glycopeptides which were isolated by binding to lentil lectin were examined by 500-MHz ^{1}H-NMR spectroscopy. The main objective was to determine whether or not (1) GlcNAc-1 was fucosylated as suggested by lentil lectin binding and (2) branch GlcNAc's were fucosylated. The linkage would be $\alpha 1 \rightarrow 6$ and $\alpha 1 \rightarrow 3$ or $\alpha 1 \rightarrow 4$, respectively.

The chemical shifts of structural reporter group protons from five compounds which discriminate the types of fucosyl linkages are given in Table II. The ^{1}H-NMR spectrum for the CF glycopeptides which were included in Biogel P-10 (Fig. 1A) was complex, due to the presence of a mixture of glycopeptides. Therefore, only structural elements could be deduced from the spectrum. At least 50% of the GlcNAc-1 residues bear $\alpha 1 \rightarrow 6$ linked Fuc on the basis of the *N*-acetyl signal of GlcNAc-2 at δ 2.091 ppm and δ2.080 ppm. As typical for the structural element of Fuc$\alpha 1 \rightarrow$ 6GlcNAc-1 (Table II, Compound 1), a doublet was present at δ1.206. The presence of Fuc$\alpha 1 \rightarrow 3$ to peripheral GlcNAc is suggested by a methyl signal at δ1.172 (Table II, Compound 4). There were no detectable signals for the elements Fuc$\alpha 1 \rightarrow$ 4GlcNAc or Fuc$\alpha 1 \rightarrow$ 2Gal (Table II, Compounds 5 and 3, respectively). The intensity of the signals were compatible with Fuc$\alpha 1 \rightarrow 3$ to be present with NeuNAc in an approximate ratio of 1 : 10 whereas Fuc$\alpha 1 \rightarrow 6$ was in a ratio of approximately 1 : 5.

In marked contrast, the ^{1}H-NMR spectrum of the Control glycopeptides gave no indication of Fuc$\alpha 1 \rightarrow 3$ and the intensity of the signals for Fuc$\alpha 1 \rightarrow 6$ showed that it was present in a lesser proportion to NeuNAc, that is a ratio of 1 : 15. Based on the NAc signals of GlcNAc-2, only about 25% of the GlcNAc-1 residues were fucosylated

Signals were observed for both CF and Control glycopeptides indicating both NeuNAc$\alpha 2 \rightarrow 3$ and $\alpha 2 \rightarrow 6$ to be present. NeuNAc$\alpha 2 \rightarrow 3$ was predominant in both cases although present in an approximate molar ratio to NeuNAc$\alpha 2 \rightarrow 6$ of 3 : 2 and 2 : 1 for the CF and Control glycopeptides, respectively. Glycopeptides from the two sources were 60% or more of the triantennary type.

Based on the results of ^{1}H-NMR spectroscopy a tentative structure is proposed for the predominant glycopeptide from CF membrane glycoproteins, which were enriched in fucosyl residues (Fig. 4A). Substitution of Man-4 with an antennary GlcNAc rather than Man-4′ is proposed solely on the basis of known structures [28] and the pathways of biosynthesis [29] which suggest that there is a preference for Man-4 to be substituted before Man-4′. As indicated in Fig. 4A, the assignment of antennary Fuc to a particular antennae can not be made although its presence was defined from the spectral shifts (Table II).

Purification of a glycoprotein secreted from CF fibroblasts

In order to determine the generality of the increased fucosylation in the CF peripheral membrane glycoproteins, a glycoprotein, fibronectin, which was secreted from the CF fibroblasts was examined. It was previously reported that fibronectin

could be precipitated with heparin [6] from the fibroblast culture medium. Therefore, the heparin precipitated material was further purified on Biogel P-200 (Fig. 5A). The material which was excluded from the Biogel column was shown to have the same mobility on polyacrylamide gels as authentic fibronectin (M_r 220 000). The fibronectin-containing fraction was digested with Pronase and after filtration on Biogel P-10 (Fig. 5B) and desalting on Biogel P-2, the included material which was 95% of the ^{3}H-radioactivity was applied to Con A–Sepharose. 57% of the radioactivity bound to this column and was eluted with 10 mmol/l α-meMan (Fig. 5C).

The Con A-bound material was further purified over Biogel P-10 in 10 mmol/l ammonium acetate and desalted on Biogel P-2 twice. These glycopeptides were used for NMR spectroscopy. The Con A-bound fraction (100%) was unbound to SNA-Sepharose indicating that the ^{3}H-glycopeptides contained no NeuNAcα2 → 6. Moreover, the glycopeptides (87%) were unbound to serotonin–Sepharose indicating a lack of sialyl residues.

The Con A-unbound fractions, representing 37% of the [^{3}H]GlcN-labeled glycopeptides (Fig. 5C) were reapplied to Con A–Sepharose and approximately 100% of the ^{3}H-radioactivity remained unbound indicating that the radioactive fraction did not have the structural characteristics for binding. After subsequent filtration on Biogel P-10 and Biogel P-2 the fraction was further purified by binding to serotonin-Sepharose (Fig. 6A). Serotonin–Sepharose will bind sialic acid-containing glycopeptides and bound 75% of the Con A-unbound radioactivity. The serotonin-

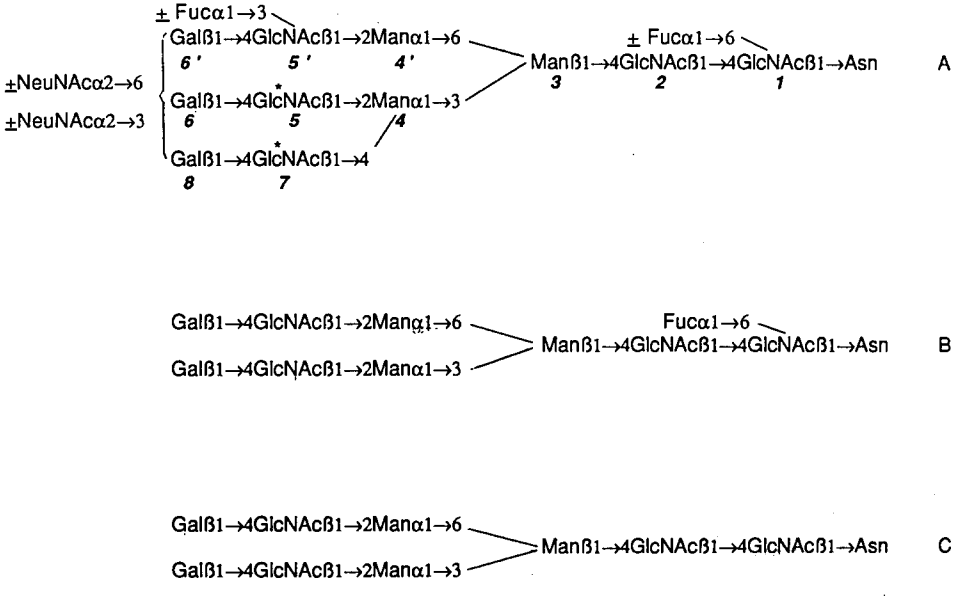


Fig. 4. Proposed structures for glycopeptides derived from CF fibroblasts. A, glycopeptide from peripheral membrane glycoproteins and B and C, glycopeptides from fibronectin isolated from the culture medium. A, numbers represent the position of the monosaccharides; * indicates alternate positions for Fucα1 → 3.

Sepharose bound material was eluted with 0.1 mol/l Tris-HCl, pH 7.2 and was desalted on Biogel P-4 and then on Biogel P-10 twice. These glycopeptides were used for NMR spectroscopy.

Although the Con A-unbound fraction bound to serotonin–Sepharose (Fig. 6A) indicating sialic acid, it was not bound to SNA–Sepharose (Fig. 6B) suggesting that it did not contain NeuNAcα2 → 6 or that there was an interfering substitution.

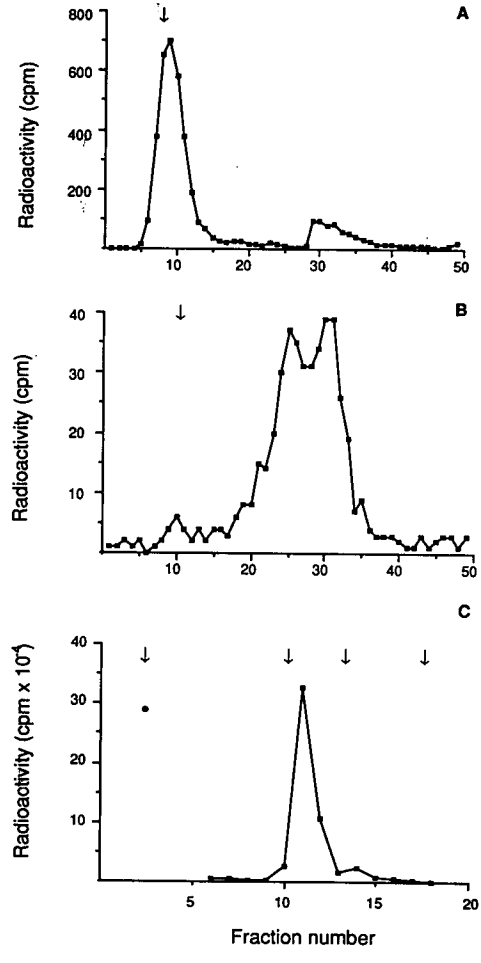


Fig. 5. Purification of ^{3}H-fibronectin and the glycopeptides thereof from CF fibroblast culture medium. ^{3}H-Fibronectin was precipitated by heparin from the CF fibroblast culture medium and further purified by filtration through A, Biogel P-200. Fractions 5 to 14 were combined and digested exhaustively with Pronase and the ^{3}H-glycopeptides were separated by filtration through B, Biogel P-10. Fractions 6 to 11 were combined and designated *excluded* and fractions 17 to 35 were designated *included*. C, The included glycopeptides were further purified on Con A-Sepharose, and separated into: first arrow, unbound and those which were eluted with: second arrow, 10 mmol/l α-meMan. Less than 10% of the radioactivity was eluted with: third and fourth arrows, 100 and 200 mmol/l α-meMan, respectively. All details are given in 'Materials and methods'.

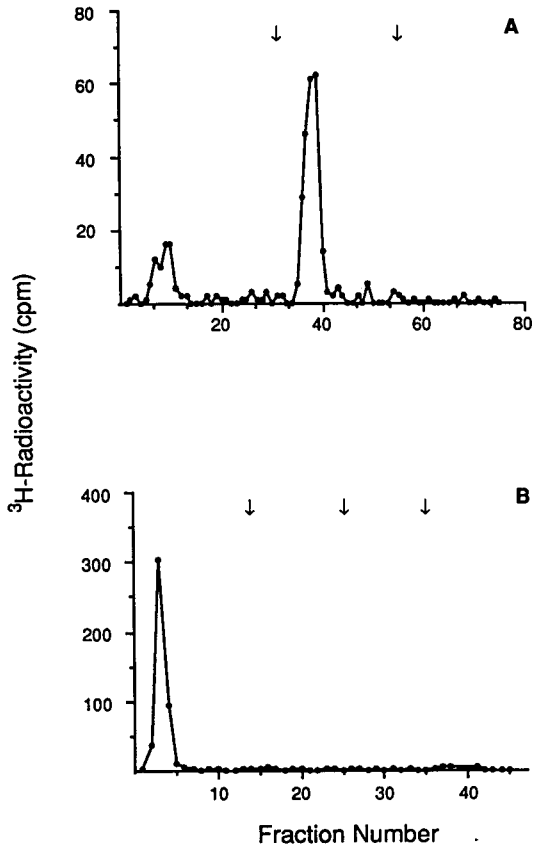


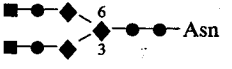
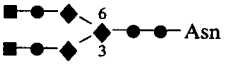
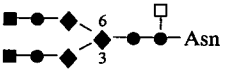
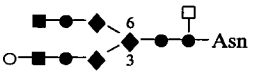
Fig. 6. Characterization of [^{3}H]GlcN-fibronectin glycopeptides derived from CF fibroblast culture medium. The presence of sialyl residues was examined by binding of the ^{3}H-fibronectin glycopeptides which were unbound to Con A–Sepharose to A, serotonin–Sepharose and B, SNA–Sepharose. Arrows left to right indicate the start of eluting buffers, A. 0.1 mol/l Tris buffer, pH 7.2, the same buffer containing 0.5 mol/l NaCl; B, 18 mmol/l phosphate buffer, pH 7.4, the same buffer containing 0.1 mol/l lactose and the same buffer containing 20 mmol/l ethylenediamine. See 'Materials and methods' for further details.

NMR spectroscopy of the glycopeptides from fibronectin

The ^{1}H-NMR spectrum of the Con A bound glycopeptides obtained from CF fibronectin showed a mixture of two diantennary glycopeptides. One of the glycopeptides (CF-2) had an $\alpha 1 \rightarrow 6$ linked fucosyl residue at GlcNAc-1 and the other glycopeptide (CF-1) was the afuco analogue of this. In Table III the ^{1}H chemical shift values of structural-reporter-group protons for the two glycopeptides are compiled, together with those for two reference compounds. The ratio of the fucose containing glycopeptide to the afuco glycopeptide was about 3 : 2. All structural-reporter-group protons except the ones for GlcNAc-1 of CF-1, matched those obtained for reference compound 8, bearing terminal galactose residues on both branches. The difference in the values for GlcNAc-1 was due to differences in the

TABLE III

^{1}H-Chemical shifts of structural-reporter group protons of the constituent monosaccharides for the glycopeptides CF-1 and CF-2 derived from fibronectin isolated from the culture medium of CF fibroblasts, together with those for reference compounds 8 and 42 [13]

Reporter group	Residue [a]	Compound and schematic structure [b]			
					
		8	CF-1	CF-2	42
H-1	GlcNAc-1	5.094	5.072	5.072	5.102 [c]
	GlcNAc-2	4.616	4.614	n.d.	4.682 [c]
	Man-3	4.765	n.d.	n.d.	4.771
	Man-4	5.121	5.117	5.117	5.134
	Man-4′	4.928	4.926	4.926	4.927
	GlcNAc-5	4.582	4.578	4.578	4.605
	GlcNAc-5′	4.582	4.578	4.578	4.579
	Gal-6	4.467	4.467	4.467	4.445
	Gal-6′	4.473	4.472	4.472	4.472
	Fuc	–	–	4.879	4.877
H-2	Man-3	4.246	4.251	4.251	4.258
	Man-4	4.190	4.192	4.192	4.191
	Man-4′	4.109	4.110	4.110	4.112
NAc	GlcNAc-1	2.004	2.014	2.014	2.006 [c]
	GlcNAc-2	2.079	2.078	2.093	2.094
	GlcNAc-5	2.050	2.051	2.051	2.069
	GlcNAc-5′	2.046	2.046	2.046	2.048
	NeuNAc	–	–	–	2.030
CH_3	Fuc	–	–	1.205	1.206 [c]

[a] For numbering of the monosaccharide residues, see Fig. 4.

[b] Chemical shifts are given at 300 K in ppm referenced to internal acetone at $\delta = 2.225$ ppm (in ^2H_2O). Compounds are represented by short-hand symbolic notation [13]: ●, GlcNAc; ◆, Man; ■, Gal; □, Fuc; ○, NeuNAcα2 → 6.

[c] Signals stemming from the main component(s) with respect to the heterogeneity of the peptide moiety. n.d.: not determined, because of overlap with the ^2H_2O-line.

peptide moiety. It has to be noted that in contrast to the CF membrane glycopeptides neither sialic acid signals nor signals stemming from branch fucose could be detected. The complete structures proposed for the oligosaccharide residues of the CF fibronectin glycopeptides which bound to Con A-Sepharose are shown in Fig. 4B and C. As defined by ^{1}H-NMR spectroscopy the oligosaccharides were asialo-diantennary with fucosyl residues in $\alpha 1 \rightarrow 6$ linkage to GlcNAc-1 and the afuco analogue.

The fibronectin glycopeptides which were unbound to Con A and bound to serotonin–Sepharose, representing approximately 30% of the [^{3}H]GlcN-fibronectin glycopeptides, proved to be a complex mixture. This fraction was not readily resolved by NMR spectroscopy. There were, however, no detectable signals for branch Fuc.

Discussion

The previous finding [5] that peripheral membrane glycoproteins from seven CF fibroblast lines were more highly fucosylated than those from seven matched Control fibroblast lines was supported in this study with the use of 500-MHz ^{1}H-NMR spectroscopy. Moreover, the analytical power of 500-MHz ^{1}H-NMR provided information on the anomeric linkage and position of these fucosyl residues. In addition, some information was obtained on the antennary structure of the oligosaccharides, the sequence and linkage of the other monosaccharides and the relative proportion of each. Two major differences were noted in the CF-derived glycopeptides when compared to the Control-derived glycopeptides. First, fucosyl residues were present in $\alpha 1 \rightarrow 3$ linkage to branch GlcNAc (Table II) and second, fucosyl residues in $\alpha 1 \rightarrow 6$ linkage to GlcNAc-1 were present in a ratio to NeuNAc of 1 : 5 whereas the ratio in Control-derived glycopeptides was only 1 : 15. Both of these results were supportive of our previous studies [5]. It was suprising, however, to find that the major oligosaccharides were of the triantennary type. It was suggested previously [5] that the fucosylation differences observed between CF and control resided on diantennary oligosaccharides since diantennary oligosaccharides are predominant on fibroblasts [8,15]. In this case, a selection of glycopeptides containing triantennary oligosaccharides has been made using lentil lectin and the resolving power of ^{1}H-NMR spectroscopy was necessary to define the branching of the oligosaccharides. Moreover, the presence of fucosyl residues in $\alpha 1 \rightarrow 3$ linkage to branch GlcNAc was observed for the first time on the CF fibroblast oligosaccharides. In this regard, serum from CF individuals has high reactivity with antibody CA-19/9 [30] indicating the presence of branch fucosyl residues in $\alpha 1 \rightarrow 4$ linkage on circulating glycoproteins to a much greater extent than those of controls. The presence of a surface fucosyltransferase as recently reported [31] may account for these differences.

There was approximately 50% more of the total (Biogel P-10 excluded and included) ^{3}H-glycopeptides from CF fibroblasts more tightly bound to lentil lectin than from Control fibroblasts (Table I). However, the dramatic difference observed previously [5] with [^{3}H]fucose-labeled glycopeptides was not apparent with [^{3}H]GlcN

as the metabolic label. In heterogeneous fractions of glycopeptides, factors such as branching and sialylation affect the amount of radioactivity present in each glycopeptide with [^{3}H]GlcN as metabolic label. Therefore, it is not possible to equate the percentages of radioactivity to the glycopeptides in a manner possible when the glycopeptides observed contain ^{3}H-labeled fucosyl residues.

In contrast to the peripheral membrane glycopeptides, the glycopeptides which were isolated from a purified secreted glycoprotein from CF fibroblast culture medium contained no branch fucosyl residues. There were no signals detected for Fucα1 → 3GlcNAc (Table III) among the fractions which were resolved by NMR spectroscopy. Since these glycopeptides were asialo-diantennary and contained approximately 60% of the [^{3}H]GlcN label they may represent > 60% of the glycopeptides derived from fibronectin. Although it is possible that other glycoproteins secreted by the fibroblasts are fucosylated on a branch GlcNAc, these results suggest a specificity of the α1 → 3Fucosyltransferase for the CF glycoproteins which are targeted to the surface membrane.

The exact antennae on which Fucα1 → 3 resides on the CF oligosaccharides and whether or not it occurs on the same antennae as NeuNAc is not known from the studies reported here (Fig. 4A). However, it is not likely to occur on the same antennae as NeuNAcα2 → 6. α1 → 3fucosyltransferase isolated from several different sources will not act when the oligosaccharide is sialylated α2 → 6 although the presence of NeuNAcα2 → 3 does not interfere with enzyme activity (summarized in ref. [32]). In this regard, the CF fibroblasts have α1 → 3fucosyltransferase activity (J.A. Voynow, T.F. Scanlin and M.C. Glick, unpubl.).

It has been proposed that the branch specificity of an oligosaccharide determines the sialylation [28]. As discussed above, the presence of fucosyl residues in α1 → 3 linkage to an antennary GlcNAc in some cases will determine the action of the sialyltransferases [33]. In addition, the presence of antennary fucose confers a marked conformational change in the oligosaccharide structure since it is a bulky residue preventing the folding of the oligosaccharide [34]. Thus this apparent subtle change in oligosaccharide structure could influence the sialylation or/and the conformation of the oligosaccharides and thereby alter functions such as adherence/clearance of bacteria and the uptake of growth factors. It will be interesting to determine if the putative membrane protein encoded by the CF gene [35] is also overfucosylated and, if it is, to define the role fucosylation plays in its function. It has already been suggested that post-translational processing such as glycosylation or sulfation [36] of the channel or a regulatory protein could be responsible for the defective Cl^- flux observed in CF [37]. The studies reported here suggest that this latter hypothesis [37] should be pursued, since the mechanism of the altered Cl^- flux remains elusive [38].

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