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PRODUCT-IDENTIFICATION AND SUBSTRATE-SPECIFICITY STUDIES OF THE GDP-L-FUCOSE: 2-ACETAMIDO-2-DEOXY-β-D-GLUCOSIDE (FUC→ ASN-LINKED GlcNAc) 6-α-L-FUCOSYLTRANSFERASE IN A GOLGI-RICH FRACTION FROM PORCINE LIVER*

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ABSTRACT

Golgi-rich membranes from porcine liver have been shown to contain an enzyme that transfers L-fucose in α-(1→6) linkage from GDP-L-fucose to the asparagine-linked 2-acetamido-2-deoxy-D-glucose residue of a glycopeptide derived from human α_1-acid glycoprotein. Product identification was performed by high-resolution, ^{1}H-n.m.r. spectroscopy at 360 MHz and by permethylation analysis. The enzyme has been named GDP-L-fucose: 2-acetamido-2-deoxy-β-D-glucoside (Fuc→Asn-linked GlcNAc) 6-α-L-fucosyltransferase, because the substrate requires a terminal β-(1→2)-linked GlcNAc residue on the α-Man (1→3) arm of the core. Glycopeptides with this residue were shown to be acceptors whether they contained 3 or 5 Man residues. Substrate-specificity studies have shown that diantennary glycopeptides with two terminal β-(1→2)-linked GlcNAc residues and glycopeptides with more than two terminal GlcNAc residues are also excellent acceptors for the fucosyltransferase. An examination of four pairs of glycopeptides differing only by the absence or presence of a bisecting GlcNAc residue in β-(1→4) linkage to the β-linked Man residue of the core showed that the bisecting GlcNAc prevented 6-α-L-fucosyltransferase action. These findings probably explain why the oligosaccharides with a high content of mannose and the hybrid oligosaccharides with a bisecting GlcNAc residue that have been isolated to date do not contain a core L-fucosyl residue.

INTRODUCTION

L-Fucose (6-deoxy-L-galactose) occurs in mammalian glycosphingolipids and protein-bound oligosaccharides of both the *N*- and *O*-glycosyl types[1,2]. It is invariably in a terminal, non-reducing position, as follows: (*a*) in the core of *N*-glycosyl oligosaccharides (1→6)-α-linked to the asparagine-linked GlcNAc residue; (*b*) as the human blood-group H determinant, Fuc(α1-2)Gal, in both *N*- and *O*-glycosyl oligo-

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saccharides and in glycosphingolipids; (*c*) as the human blood-group Lewis determinant (1→4)-α-linked to GlcNAc in the Gal(β1-3)GlcNAc sequence of *O*-glycosyl oligosaccharides and of glycosphingolipids; (*d*) in (1→3)-α linkage to GlcNAc in the Gal(β1-4)GlcNAc sequence of both *N*- and *O*-glycosyl oligosaccharides and of glycosphingolipids; (*e*) in (1→3)-α linkage to Glc in the Gal(β1-4)Glc sequence of milk oligosaccharides. Fucosyltransferases catalysing the incorporation of L-fucose from GDP-β-L-fucose into all of the above linkages have been described[2,3] and some of these have been purified to homogeneity[4–6]. We now report on the enzyme catalyzing the first linkage in the above list, namely, GDP-L-fucose: 2-acetamido-2-deoxy-β-D-glucoside (Fuc→Asn-linked GlcNAc) 6-α-L-fucosyltransferase; this enzyme will be termed the 6-α-fucosyltransferase in subsequent discussion.

Bosmann *et al.*[7] first described in HeLa cell extracts a fucosyltransferase acting on fetuin or α_1-acid glycoprotein from which sialic acid and galactose had been removed to expose terminal GlcNAc residues. Subsequently, we reported a similar fucosyltransferase activity in porcine liver[8] and human serum[9], and later showed that the enzyme activity was enriched 35-, 40-, 15-, and 24-fold in Golgi-rich fractions from porcine liver, rat liver, rat testis, and mouse testis, respectively[10,11]. However, none of these early studies reported a proper product-identification and the linkage synthesized by the transferase was not determined. In 1976, Wilson *et al.*[12] digested [^{14}C]-L-fucose-labelled glycopeptide product with endo-β-*N*-acetylglucosaminidase C_1 and showed that the radioactive label remained with the GlcNAc-Asn fragment, indicating that fucose was incorporated into the asparagine-linked GlcNAc residue rather than into a more peripheral GlcNAc residue as had previously been postulated. We have confirmed these findings by carrying out a more definitive characterization of the Fuc-GlcNAc-Asn-X fragment formed by endo-glycosidase cleavage of the product. We now report on this study, which also establishes the linkage to be (1→6)-α and presents some substrate-specificity studies of the porcine liver 6-α-fucosyltransferase. A preliminary report of this work has appeared[13].

EXPERIMENTAL

Materials. — [1-^{14}C]Acetic anhydride (27.2 mCi/mmol) and [^{3}H]acetic anhydride (50 mCi/mmol) were used undiluted. Non-radioactive UDP-GlcNAc was used to dilute UDP-*N*-acetyl-D-[6-^{3}H]glucosamine (6.6 Ci/mmol; from New England Nuclear) to a specific activity of either 10^5 or 2000 d.p.m. per nmol. GDP-[^{14}C]-L-fucose (112 mCi/nmol) was obtained from Amersham, and was diluted to lower specific activities (usually 9070 d.p.m. per nmol) by GDP-L-fucose synthesized[8] from L-fucose by the successive use of L-fucose kinase and GDP-L-fucose pyrophosphorylase from porcine liver. AG 50W-X2 (200–400 mesh), Bio-Gel P-2 (200–400 mesh), P-6 (100–200 mesh), and P-10, Chelex 100 (100–200 mesh, Na^+ form), and Bio-Beads SM-2 were from Bio-Rad Laboratories. Sephadex G-25 and G-75 and Concanavalin A/Sepharose 4B were from Pharmacia. Ovalbumin (grade V, crystalline) and D-galactose dehydrogenase from *P. fluorescens* were purchased from

Sigma, and pronase grade B was from Calbiochem. L-Fucose dehydrogenase was prepared[14] from porcine liver. Endo-β-*N*-acetylglucosaminidases C_I and C_{II} were purified[15] from the culture supernatant of *Cl. perfringens*. Endo-β-*N*-acetylglucosaminidase H was kindly donated by Dr. P. W. Robbins (Massachusetts Institute of Technology). Glycopeptides were prepared, as described below, from human myeloma IgG (Tem), ovalbumin, and human α_1-acid glycoprotein. IgG (Tem) was purified from serum kindly supplied by Dr. W. Pruzanski (Wellesley Hospital, Toronto). Human α_1-acid glycoprotein was prepared from Cohn-fraction supernatant V supplied by Connaught Laboratories (Toronto), or was donated by the American Red Cross National Fractionation Center. *Cl. perfringens* culture supernatant was used to prepare[16] β-D-galactosidase and β-*N*-acetylglucosaminidase. Bovine kidney α-L-fucosidase was purchased from Boehringer.

Glycopeptides from human IgG. — IgG (Tem) was subjected to pronase digestion and subsequent fractionation on Sephadex G-25, Sephadex G-75, and AG 50W-X2 (200–400 mesh) as described by Narasimhan *et al.*[17]. Chromatography of the pronase digest on AG 50W-X2 yielded an acidic glycopeptide GS which did not adhere to the column, and several neutral glycopeptides which were eluted from the column after prolonged washing with sodium acetate (pH 2.6, mM in Na^+). Glycopeptide GS was subjected to sequential glycosidase digestion, methylation analysis, and ^{1}H-n.m.r. spectroscopy at 360 MHz; it was found to be a biantennary structure with a terminal (2→6)-α-linked sialic acid on the (1→3)-α-linked Man arm and a terminal Gal residue on the (1→6)-α-linked Man arm. IgG glycopeptides are named according to the sugars present at each of the non-reducing ends of the various branches or antennae; the structures and names of the various IgG glycopeptides that have been prepared in this laboratory are shown in Fig. 2 of the paper by Narasimhan *et al.*[18]. The structures of IgG glycopeptides MM, MGn, and MGn(Gn) used in the present report were determined by ^{1}H-n.m.r. spectroscopy at 360 MHz (Table I) and are shown in Table II. Glycopeptides MM and MGn were prepared from glycopeptide GS by sequential glycosidase digestion[17] followed by preparative, high-voltage paper electrophoresis in 1% aqueous sodium tetraborate[18]; glycopeptide MGn(Gn) was prepared from the neutral IgG glycopeptide fraction by a similar approach[18].

Since the IgG glycopeptides all have a fucose residue attached to the asparagine-linked GlcNAc, this fucose had to be removed prior to testing the glycopeptides as substrates for the 6-α-fucosyltransferase. Fucose was removed from glycopeptides MM and MGn by treatment with 0.8M HCl at 80° for 1 h. The digest was passed through a column of Bio-Gel P-2 and the decrease in bound fucose was determined by analysis of the glycopeptide for fucose by g.l.c.[19]. Free fucose released by mild hydrolysis with acid was also monitored by analysis with L-fucose dehydrogenase[20]. Fucose loss was 57% and 37% for glycopeptides MM and MGn, respectively, with no detectable changes in Man or GlcNAc contents of these glycopeptides.

However, mild hydrolysis of IgG glycopeptide MGn(Gn) with acid resulted in appreciable losses of the GlcNAc residue (1→4)-β-linked to the β-linked Man residue (the intersecting GlcNAc residue). For this reason, fucose was removed from

TABLE I

CHEMICAL SHIFTS (δ) OF GLYCOPEPTIDE PROTONS[a] DERIVED BY 360-MHz N.M.R. SPECTROSCOPY

Protons[b]	*IgG glycopeptides*								*Ovalbumin glycopeptides*			
	MM (+Fuc)		*MGn (+Fuc)*		*MGn(Gn) (+Fuc)*		*MGn(Gn) (−Fuc)*		*IV-1 GnGnGn(Gn)*		*V-1 GnGn(Gn)*	
	Temp. (deg.)	δ	*Temp. (deg.)*	δ	*Temp. (deg.)*	δ	*Temp. (deg.)*	δ	*Temp. (deg.)*	δ	*Temp. (deg.)*	δ
H-1 of Man	70		70		70		70		23		23	
1		4.773		4.773		4.741		4.742		4.683		4.696
2		5.128		5.138		5.057		5.059		5.067		5.059
3		4.923		4.922		4.960		4.960		5.000		5.006
H-2 of Man	24		24		23		23		23		23	
1		4.261		4.255		4.188		4.190		4.138		4.176
2		4.068		4.188		~4.25		~4.25		4.272		4.249
3		3.972		3.969		~4.00		~4.00		4.138		4.151
H-1 of GlcNAc	70		70		70		70		23		23	
I		—		4.581		4.543		4.543		4.54[c]		4.557[d]
II		—		—		—		—		4.51[c]		4.547[d]
III		—		—		4.455		4.460		4.452		4.467
IV		4.693		4.690		4.682		4.630		4.612		4.615
V		5.074		5.071		5.081		5.088		5.07		5.07
VI		—		—		—		—		4.51		—

N-*Ac of GlcNAc*	70	[redacted] 70	70	70	23	[redacted] 23	
I		—	2.054	2.066	2.068	2.06	2.058[e]
II		—	—	—	—	2.06	2.049[e]
III		—	—	2.066	2.068	2.06	2.066
IV		2.089	2.088	2.083	2.068	2.079	2.081
V		2.021	2.020	2.016	2.015	2.045	2.010
VI		—	—	—	—	2.06	—
H-1 of Fuc		4.878	4.876	4.872	—	—	—
CH_3 of Fuc		1.214	1.212	1.216	—	—	—

[a]Molar ratios were determined by integration of peak areas; every signal in the chart represents one residue. Minus sign indicates absence of a peak. Glycopeptide structures are given in Table II. [b]Residues are identified by the numbers in parentheses in the formula. [c–e]Signals may be interchanged.

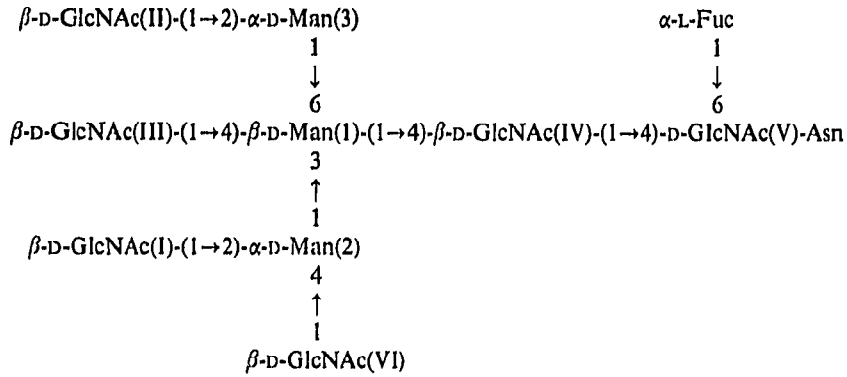
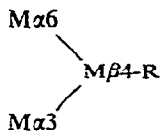
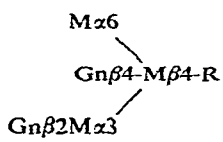
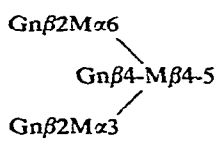
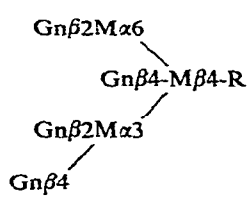
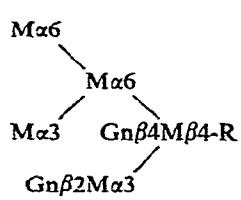
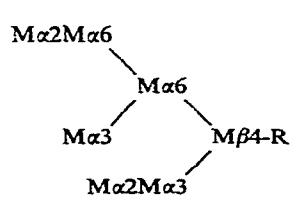


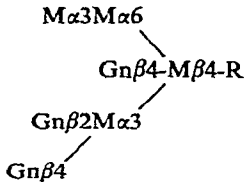
TABLE II

SUBSTRATE SPECIFICITY[a] OF 6-α-FUCOSYLTRANSFERASE FROM GOLGI-RICH MEMBRANES OF PORCINE LIVER

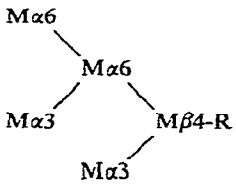
Inactive glycopeptides

Source and name[b]	*Structure*
IgG–MM	
IgG–MGn(Gn)	
Ovalbumin–GnGn(Gn)	
Ovalbumin–GnGnGn(Gn)	
Ovalbumin–III-A	
Ovalbumin–III-B	

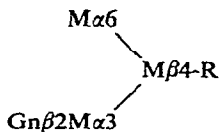
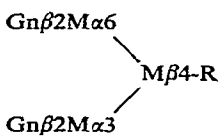
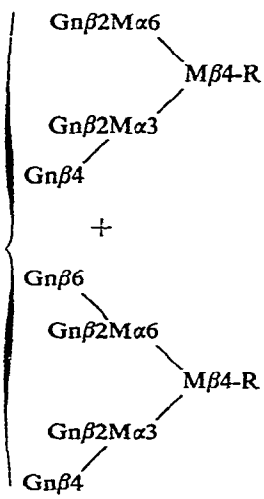
Ovalbumin–III-C



Ovalbumin–V

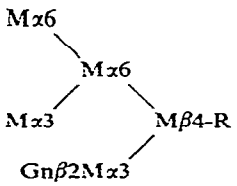


Active glycopeptides

Source and name	*Structure*	K_m *(mM)*	V_{max} *(nmol/μg/h)*
IgG–MGn		0.6	0.19
α_1-acid glycoprotein–GnGn		0.08	0.11
α_1-acid glycoprotein–GnGnGn + GnGnGnGn[c]		0.3	0.18

(continued on p. 372)

TABLE II (*Continued*)

Active glycopeptides			
Source and name	*Structure*	K_m *(mM)*	V_{max} *(nmol)/(μg/h)*
Gn-transferase I product-GnM_5Gn_2-Asn		—	0.09 at 0.6mM

[a]Inactive glycopeptides gave a velocity of <0.007 nmol/μg of protein/h at 0.5mM; active glycopeptides usually gave a velocity of ~0.1 nmol/μg/h at comparable concentrations. [b]Abbreviations: M, D-mannose; Gn, *N*-acetyl-D-glucosamine; R, Gnβ4Gn-Asn (fucose, when present, was removed as described in the text). [c]An ~1:1 mixture of GnGnGn and GnGnGnGn with ~1 in 10 glycopeptide molecules carrying a fucosylated antenna, as described in the text.

this glycopeptide by incubating 200 nmol of MGn(Gn) with 0.4 unit of α-L-fucosidase in 0.13M sodium acetate buffer (pH 4.5) in a total volume of 0.3 ml at 32°. After 24 h, a further 0.4 unit of α-L-fucosidase was added. The reaction was terminated after a total of 66 h and the digest was subjected to gel filtration on a column (1.5 × 30 cm) of Bio-Gel P-4 followed by passage through a column (1.5 × 30 cm) of Bio-Gel P-6, both equilibrated in water. The low molecular weight region of the P-4 column chromatogram was analyzed for free L-fucose by the L-fucose dehydrogenase method[20]; ~80% of the bound fucose had been released. ^{1}H-N.m.r. spectra at 360 MHz of MGn(Gn) before and after treatment with α-L-fucosidase confirmed almost complete removal of L-fucose and showed no detectable losses in either Man or GlcNAc residues (Table I).

Glycopeptides from ovalbumin. — Tai *et al.*[21–23] studied the structures of the ovalbumin oligosaccharides by fractionating a pronase digest on AG 50W-X2 in sodium acetate (pH 2.6, mM in Na^+), to obtain 6 fractions (I–VI) of asparaginyl oligosaccharides. Oligosaccharides were removed from I–VI by endo-β-*N*-acetylglucosaminidase H or C_{II} and the oligosaccharides were further fractionated by paper chromatography; the structures obtained for oligosaccharides III-A, III-B, III-C, IV, V, and VI are shown in the papers by Tai *et al.*[21–23]. Narasimhan *et al.*[18] subjected glycopeptide preparations III–VI from an AG 50W-X2 column to preparative, high-voltage paper electrophoresis in 1% aqueous sodium tetraborate. Fraction III separated into 3 fractions (III-1, III-2, and III-3, with increasing mobility towards the

anode). Ovalbumin glycopeptides III-1, III-2, and III-3 were shown to have oligosaccharide structures equivalent to III-C, III-A, and III-B, respectively[18]; see Table II for structures.

Fraction IV from the AG 50W-X2 column also separated into three bands in high-voltage electrophoresis. The band with the fastest mobility towards the anode (IV-3) has been identified as having an oligosaccharide structure equivalent to ovalbumin oligosaccharide IV reported by Tai *et al.*[21-23]. The middle band has not been identified. The slowest band (IV-1) has been prepared in relatively large amounts and has been identified, primarily by ^{1}H-n.m.r. spectroscopy at 360 MHz, as glycopeptide GnGnGn(Gn), the structure of which is shown in Table II; Table I gives the chemical shifts for this preparation.

Fraction V from the AG 50W-X2 column can be resolved by high-voltage paper electrophoresis into a rapidly moving, major fraction (V-3) and two minor components (V-1 and V-2). Glycopeptide V-3 has an oligosaccharide structure identical to that reported by Tai *et al.*[21] for oligosaccharide V; see Table II for this structure. The middle band has not been identified. Glycopeptide V-1 has been purified and shown by ^{1}H-n.m.r. spectroscopy to be glycopeptide GnGn(Gn), the structure of which is shown in Table II; Table I shows the chemical shifts for this preparation.

Glycopeptides GnGn(Gn) and GnGnGn(Gn) were not reported in the detailed studies on ovalbumin by Tai *et al.*[21-23]. A commercial preparation of ovalbumin was used in our studies and it is possible that the two novel structures derive from contamination of our ovalbumin preparation with ovotransferrin[24].

Glycopeptides from α_1-acid glycoprotein. — Human α_1-acid glycoprotein has been shown to contain 5 classes of *N*-glycosyl oligosaccharides, *i.e.*, bi-antennary, tri-antennary, tetra-antennary, and tri- and tetra-antennary with a fucose on one of the antennae[25-27]. From the yields obtained, it can be estimated that ~12–18% (w/w) of the oligosaccharides are bi-antennary and ~21% of the total are fucosylated[25].

Human α_1-acid glycoprotein was treated with 0.1M H_2SO_4 at 80° for 1 h, dialyzed against water, and lyophilized, to yield asialo-α_1-acid glycoprotein. This glycoprotein (0.5 g) was digested in 6.0 ml of 0.1M sodium phosphate buffer (pH 6.0; containing 0.01% of sodium azide and 2mM aldono-1,4-lactone derivative[28,29] of GlcNAc) with 3.6 units of *Cl. perfringens* β-D-galactosidase. After 16 h at 37°, another 0.9 unit of β-D-galactosidase was added and incubation was continued for a further 6 h. D-Galactose release was determined by use of D-galactose dehydrogenase[20], and GlcNAc release was measured by the method of Reissig *et al.*[30]; 87% of the protein-bound galactose and 0.95% of protein-bound GlcNAc were released. The 13% of resistant galactose is believed to be present on antennae carrying fucose residues, whereas the small amount of GlcNAc released is due to contamination of the β-D-galactosidase preparation with β-*N*-acetylglucosaminidase. The enzyme digestion mixture was heated at 60° for 30 min and then dialyzed extensively against 0.01M Tris-HCl (pH 7.8) containing 0.01% of sodium azide. The dialyzed asialo, agalacto-α_1-acid glycoprotein solution was adjusted to 12 mL with 0.01M Tris-HCl

(pH 7.8, containing 2mM $CaCl_2$), and 25 mg of pronase was added. Incubation was carried out under toluene at 37° for 24 h, a further 25 mg of pronase was added, and the incubation was terminated after a total of 48 h. The pH was maintained at 7–8 during digestion.

The pronase digest was passed through a column (2.5 × 75 cm) of Sephadex G-25 equilibrated in 25mM ammonium hydroxide; the glycopeptide fraction was detected by the phenol–sulfuric acid method[31] and was re-treated with pronase exactly as described above. The second pronase digest was also passed through a similar column of Sephadex G-25.

To remove residual peptide material, the pronase glycopeptides were purified by descending paper chromatography on Whatman 3MM paper with 1-butanol–acetic acid–water (12:3:5) at room temperature for 3 days. Guide strips were stained with fluorescamine[18] to detect free amino-groups and with periodate–benzidine[32] to detect carbohydrate. The glycopeptide region of the paper was eluted[33] with water. The yield of glycopeptide (by amino acid analysis) was 50.4 μmol or 81%. Amino acid analysis showed one residue of Asp, 0.3 of Thr, and traces of Ser and Glu. Sugar analysis by g.l.c. and amino acid analyzer (for hexosamine) showed the following composition (in residues relative to Asp): sialic acid, 0; Gal, 0.13; Fuc, 0.11; GlcNAc, 5.1; and Man, 3.0. On analysis of the glycopeptide preparation on Concanavalin A/Sepharose[17], ~20% of the material adhered to the column. The mixture was therefore fractionated preparatively on a column (1.5 × 32 cm) of Concanavalin A/Sepharose equilibrated in 10mM Tris-HCl (pH 7.4, containing 0.1M NaCl). The column was washed with 400 mL of this buffer and the adherent fraction was then eluted with 400 mL of buffer containing 0.1M methyl α-D-glucopyranoside. The two glycopeptide fractions were concentrated and purified by gel filtration on columns of Bio-Gel P-2 (1.5 × 100 cm) and P-6 (1.5 × 37 cm) equilibrated with 25mM ammonium hydroxide. The glycopeptide fraction which adhered to Concanavalin A/Sepharose required final purification by preparative, high-voltage paper electrophoresis in 1% aqueous sodium tetraborate[18].

The oligosaccharides known to be present on human α_1-acid glycoprotein[25–27] and the properties previously established[17] for adherence to Concanavalin A/Sepharose suggest that the Concanavalin A-negative glycopeptide fraction prepared above is a mixture primarily of structures GnGnGn and GnGnGnGn (see Table II for these structures), but should also contain some fucosylated tri- and tetra-antennary material with Gal(β1-4)[Fuc(α1-3)]GlcNAc antennae. Our analyses for Fuc and Gal (see above) indicate that the original glycopeptide mixture contained ~0.1 mol of fucosylated antennae per mol of asparagine. Characterization of the non-adherent fraction was carried out by analytical, high-voltage paper electrophoresis in borate[18], by analytical gel filtration on a column (2.0 × 200 cm) of Bio-Gel P-6 calibrated with glycopeptide standards[34], and by ^{1}H-n.m.r. spectroscopy at 360 MHz. The electrophoretic mobility and size of the glycopeptide fraction is consistent with the structures GnGnGn and GnGnGnGn. Further, the n.m.r. spectrum of this fraction at 75° showed a chemical shift for H-1 of the (1→6)-α-linked Man residue at 4.871

p.p.m. rather than at 4.92–4.94 p.p.m., consistent with a doubly substituted Man residue[26]. Integration of the n.m.r. signals indicates roughly equivalent amounts of tri- and tetra-antennary glycopeptides in the preparation.

The 20% yield of Concanavalin A/Sepharose-adherent glycopeptide is consistent with the amount of bi-antennary oligosaccharide in α_1-acid glycoprotein[25]. The adherent glycopeptide fraction behaved in analytical, high-voltage paper electrophoresis in borate[18] and in Concanavalin A/Sepharose chromatography[17] as expected for the bi-antennary glycopeptide GnGn (see Table II for structure); it should be noted that this GnGn preparation from α_1-acid glycoprotein differs from the previously described[17] GnGn preparation from IgG in lacking a fucosyl residue. Fucose-free glycopeptide GnGn has also been prepared by mild, acid hydrolysis of the IgG preparation; either preparation of GnGn serves as an excellent acceptor for the 6-α-fucosyltransferase (see later).

Based on the release of free GlcNAc during the preparation of asialo,agalacto-α_1-acid glycoprotein (see above), the maximum contamination of the above preparations with Man-terminated antennae or with GlcNAc(β1-4)Man(α1-3) or GlcNAc-(β1-6)Man(α1-6) termini should be <2% (an average of 3 out of every 5 GlcNAc residues are involved in antennae).

Preparation of glycopeptide GlcNAc(Man)$_5$(GlcNAc)$_2$Asn. — This glycopeptide was prepared by incubating ovalbumin glycopeptide V (see Table II for structure) with UDP-GlcNAc and highly purified UDP-GlcNAc: α-D-mannoside 2-β-acetamidodeoxyglucosyltransferase I from bovine colostrum, as previously described by Harpaz and Schachter[35,36]; it was purified by passage through a column of Bio-Gel P-2, a column of Blue Sepharose to remove albumin present as a stabilizing agent in the transferase preparation, and preparative, high-voltage paper electrophoresis in borate[18] to remove unreacted substrate. This glycopeptide preparation has previously been characterized[36] by analytical, borate high-voltage electrophoresis, gel filtration through calibrated columns of Bio-Gel P-6, permethylation analysis, adherence to Concanavalin A/Sepharose, susceptibility to cleavage by endo-β-*N*-acetylglucosaminidases H and C$_{II}$, resistance to cleavage by endo-β-*N*-acetylglucosaminidases D and C$_I$, and, most importantly, by high-resolution ^{1}H-n.m.r. spectroscopy. The glycopeptide was labelled either by acetylation with radioactive acetic anhydride, or by using radioactive UDP-GlcNAc as a glycosyl donor, or both.

N-*Acetylation of glycopeptides.* — Glycopeptides were acetylated with either non-radioactive or radioactive (^{14}C- or ^{3}H-labelled) acetic anhydride, as previously described[17,18]. Glycopeptide (5–100 nmol) was incubated in 0.1 mL of 2.5mM acetic anhydride in 0.08M sodium hydrogencarbonate at room temperature for 1 h. The reaction was terminated by the addition of 1 μL of 0.7M ammonium hydroxide. After a further 1 h at room temperature, the mixture was dried by repeated flash-evaporation from water and subjected to descending paper chromatography with 1-butanol–acetic acid–water (12:3:5); the acetylated glycopeptide was then eluted from the paper. In some cases, non-radioactive *N*-acetylation was performed by adding glycopeptide (5 nmol in 50 μL of water) to 5 μL of pyridine–acetic anhydride

(5:1) and incubating at room temperature overnight; the sample was then dried in a nitrogen stream, washing with water three times.

Preparation and characterization of Golgi-rich membranes from porcine liver. — Porcine liver (10 g) was subjected to the procedure described by Sturgess *et al.*[37] for the preparation of Golgi-rich membranes from rat liver. The Golgi-rich membrane pellets were stored at −20° and resuspended in water prior to use, to give a protein concentration of 7.4 mg/mL. Electron microscopy[37] showed a predominance of smooth membranes and cisternal stacks typical of the Golgi apparatus. The Golgi membranes were enriched 30-fold when the 6-α-fucosyltransferase acted on asialo, agalacto-α_1-acid glycoprotein[8], 18.3-fold for UDP-Gal: GlcNAc 4-β-D-galactosyltransferase[38], and 18.0-fold for CMP-sialic acid: asialo-α_1-acid glycoprotein sialyltransferase[39]; these values are in excellent agreement with the previous report[10] on Golgi-rich membranes from porcine liver.

Large-scale preparation of enzyme product. — In order to characterize the linkage synthesized by the Golgi fucosyltransferase from porcine liver, a large-scale incubation was carried out using as acceptor the glycopeptides purified from asialo,agalacto-α_1-acid glycoprotein (see above): the preparation used for this work was not fractionated by Concanavalin A/Sepharose and contained a mixture of bi-, tri-, and tetra-antennary glycopeptides with terminal GlcNAc residues (all known to be substrates for the enzyme, see later) and ~10% of tri- and tetra-antennary glycopeptides with a single fucosylated antenna, Gal(β1-4)[Fuc(α1-3)]GlcNAc. The incubation (final volume of 3.75 mL) contained: GDP-[^{14}C]-L-fucose (2500 d.p.m./nmol), 0.38mM; GTP, 2.5mM; $MgCl_2$, 40mM; Tris-HCl (pH 8.0), 50mM; Triton X-100, 1% (v/v); glycopeptide acceptor, 4mM; and 4.4 mg of Golgi-rich, membrane protein from porcine liver. The enzyme was added in 2 stages: 3.7 mg at zero time and 0.7 mg after 2.5 h; more GDP-[^{14}C]-L-fucose (0.2 μmol) was also added at 2.5 h. The incubation was kept at 37° for a total of 8 h. The reaction was terminated by the addition of 0.80 mL of 0.5M EDTA in 2% aqueous sodium tetraborate. An aliquot (15 μL) of the reaction mixture was spotted on Whatman 3MM paper, and high-voltage electrophoresis was performed in 1% aqueous sodium tetraborate (pH 9.0) at 0.95 V/cm^2, for 2.0 h. Glycopeptide product (9880 d.p.m.) was detected at the origin, indicating that ~1 μmol (6.5%) of added substrate had been fucosylated.

The reaction mixture was concentrated under nitrogen to 2.5 mL and applied to a column (2.0 × 28 cm) of Bio-Gel P-10. The column was developed with 25mM NH_4OH, and fractions were monitored for radioactivity and absorbance at 280 nm. Radioactive fractions were combined, concentrated to 3.0 mL, and applied to a column (2 × 34 cm) of Bio-Gel P-2 developed with 25mM NH_4OH. Fractions were monitored as described above and also for absorbance at 252 nm to detect guanosine-containing compounds. Two radioactive peaks were detected, and an aliquot of each was analyzed by borate high-voltage electrophoresis against standards. The first peak (0.8 μmol of radioactive fucose) was identified as fucosylated glycopeptide; the second, minor peak was discarded as it appeared to be free fucose.

To make the product more amenable to analysis, it was digested with glycosi-

dases from *Cl. perfringens*. Since the presence of an unsubstituted α-mannose residue (1→3)-linked to the β-mannose of the core is essential for the action of endo-β-*N*-acetylglucosaminidase C_I, the fucosylated glycopeptide was first treated with β-*N*-acetylglucosaminidase to expose this terminal Man residue. The presence of fucose linked to the GlcNAc attached to Asn does not interfere[15] with the action of endo-β-*N*-acetylglucosaminidase C_I. The fucosylated glycopeptide preparation also contains ~14 μmol of unreacted, substrate glycopeptide; thus, sufficient glycosidases were added to ensure cleavage of ~15 μmol of glycopeptide. Glycopeptide, containing both non-radioactive substrate and ^{14}C-fucosylated product, was resuspended in 2.0 mL of 0.1M sodium phosphate buffer (pH 6.3; containing 0.01% of sodium azide and 0.25 unit of β-*N*-acetylglucosaminidase), and the mixture was incubated at 37° for 5 h. Endo-β-*N*-acetylglucosaminidase C_I (0.2 mL) was added and the incubation continued overnight at 37°. After 20 h, a further 0.1 unit of β-*N*-acetylglucosaminidase and 0.1 mL endo-β-*N*-acetylglucosaminidase C_I were added and the incubation was continued at 37° for 8.0 h. The mixture was then heated at 70° for 30 min and applied to a column (1.5 × 30 cm) of Bio-Gel P-4 which was developed with 25mM NH_4OH. Fractions were monitored for radioactivity, and all radioactive fractions were combined and concentrated to 3.0 mL.

Bio-Beads SM-2 have been reported[40] to decrease the concentration of Triton X-100 in solution. Bio-Beads SM-2 were swollen in 10 volumes of methanol by stirring for 15 min, filtered off, washed with methanol and water, and resuspended in water. The sample eluted from the P-4 column (3.0 mL) was added to the Bio-Beads (10.0 mL), and the mixture was stirred for 1 h at room temperature, poured into a small column, and washed with water. The fractions eluted from the column were monitored for radioactivity and absorbance at 275 nm (Triton X-100, $E^{1\%}_{1\,cm}$ = 21.0 at 275 nm and pH 7.2) before and after treatment with Bio-Beads SM-2. The Triton X-100 concentration decreased from 1% to 0.01%, and the recovery of product (radioactive fucose) was 0.72 μmol. The sample was next applied to a column (2.0 × 200 cm) of Bio-Gel P-6, which was developed with 0.1M NaCl containing 0.01% of sodium azide during 2 days. Fractions (0.90 mL) were monitored for radioactivity. The void volume of the column (V_o) was determined with hemoglobin, the included volume (V_i) was determined with [^3H]-L-fucose, and the retention constant K_R for a fraction with elution volume V_e was calculated from $K_R = (V_e - V_o)/(V_i - V_o)$.

Three radioactive peaks were obtained from this fractionation: I, K_R = 0.58, yield = 14%; II, K_R = 0.66, yield = 28%; III, K_R = 0.76, yield = 58%. None of these peaks bound to Concanavalin A/Sepharose, suggesting that they were either cleavage products of endo-β-*N*-acetylglucosaminidase C_I or were tri- or tetra-antennary glycopeptides which had resisted cleavage by endo-glycosidase. The K_R values from the P-6 column show that only peak I is large enough to be uncleaved glycopeptide, as determined by comparison with K_R values of known glycopeptide standards. Peak II is probably the cleavage product [^{14}C]-L-Fuc–GlcNAc–Asn-X; there was too little material for definitive structural work. The major product, peak III, was

desalted on a column (2 × 100 cm) of Bio-Gel P-2 equilibrated with 25mM ammonium hydroxide. Amino acid analysis revealed Asp with only traces of other amino acids; the molar ratio of Asp to [^{14}C]-L-fucose was 4:1. Thus, peak III is probably a 3:1 mixture of GlcNAc–Asn and [^{14}C]-L-Fuc–GlcNAc–Asn. This mixture was subjected to preparative, descending chromatography on acid-washed Whatman 3MM paper for 3 days with pyridine–ethyl acetate–acetic acid–water[41] (5:5:1:3). Non-radioactive glycopeptide, detected with fluorescamine, migrated more rapidly than the radioactive material, detected by a radioactivity scanner. The latter was eluted from the paper with water, and the eluate was concentrated and passed through a column (1.5 × 27 cm) of Sephadex G-10 equilibrated with 25mM ammonium hydroxide. The final yield of product was 0.42 μmol (~52% yield); 35% of the product was present in peaks I and II from the Bio-Gel P-6 column, whereas ~13% was lost during purification. Further characterization was carried out by methylation analysis and ^{1}H-n.m.r. spectroscopy at 360 MHz (see below).

N.m.r. study. — Trace metals which interfere with the anomeric proton signals were removed from the sample by passage through a 1.0-mL column of Chelex 100 which had been extensively washed with water. The sample was concentrated to dryness either by lyophilization or flash evaporation from 99.5% deuterium oxide three times, and dissolved in 99.96% deuterium oxide for analysis. The spectra were taken with a Nicolet 360-MHz spectrometer operated with Quadrature detection in the Fourier Transform mode at 25° and 70°. Acetone was used as the internal standard, assuming a chemical shift of 2.225 p.p.m. relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). Spectra were recorded and interpreted through the courtesy of Drs. A. A. Grey and J. P. Carver (University of Toronto, Faculty of Medicine NMR Center).

Methylation analysis. — An aliquot of glycopeptide (at least 100–200 nmol) was placed in a 6-mL vial, dried overnight over P_2O_5 in an evacuated desiccator, and then permethylated by the procedure of Hakomori[42]. The vial was sealed with a Teflon-lined cap, 0.7 mL of dry dimethyl sulfoxide was added, and the vial was flushed with dry nitrogen. The glycopeptide was dissolved by sonication at 40° for 30 min, 0.7 mL of sodium methylsulfinylmethanide was added, and sonication was continued at 40° for 4 h under a stream of dry nitrogen. The sample was solidified on ice, 0.7 mL of methyl iodide was slowly added, and sonication was continued for 1 h. The permethylated product was extracted with chloroform and applied to a column (0.5 × 5.0 cm) of silicic acid (Unisil, 200–325 mesh) equilibrated in chloroform. The column was washed with 10 mL of chloroform to remove dimethyl sulfoxide, and the glycopeptide was eluted with chloroform–methanol (95:5); sialic acid-containing glycopeptides require 100% methanol for elution. The permethylated glycopeptide was dried in a nitrogen stream, and hydrolyzed with 4M trifluoroacetic acid for 4 h at 100°. Neutral and basic residues can be separated[18] prior to analysis, but were usually analyzed together. The hydrolysate was dried *in vacuo* over P_2O_5 and acetylated with 0.75 mL of pyridine–acetic anhydride (1:1) at 100° for 1 h. The solvents were evaporated and toluene was distilled several times from the residue

which was then treated with 25 mg of sodium borodeuteride in 0.75 mL of water at pH 10 and 40° for 18 h. After evaporation of water, borate was removed as the methyl ester by repeated distillation from the residue of methanol acidified with 1% of acetic acid. The residue was dried *in vacuo* over P_2O_5 and re-acetylated with 0.75 mL of pyridine–acetic anhydride (1 : 1) at 100° for 4 h, as described above. A solution of the dry product in 5 mL of chloroform was extracted with water (3 × 5 mL), dried (Na_2SO_4), filtered, and analyzed by g.l.c.–m.s. with a Hewlett-Packard 5985B gas chromatograph–quadrupole mass spectrometer operating in the electron-impact mode and coupled to a Hewlett-Packard HO-1000 E series computer. Basic and neutral residues were analyzed in a single run on an SP-2100 glass-capillary column (10.0 m) with helium as carrier gas (10 mL/min) and a temperature program from 120° to 280° at 4° or 8° per min with a 6-min delay at 120° prior to the temperature program. Ion monitoring was usually carried out at *m/z* 118, 161, 189, and 190 for neutral sugar derivatives and 117 and 159 for basic sugar derivatives. Mass spectra were taken over all peaks detected by ion monitoring, to identify the structures of the partially methylated alditol acetates. Spectra were corrected for variation of total ion current[43]. Molar ratios were estimated from the total ion currents[44].

High-voltage electrophoresis. — The analytical and preparative procedures using 1% aqueous sodium tetraborate have been described in detail[18]. Radioactive samples were detected with a Packard model 7201 radiochromatogram scanner, and non-radioactive glycopeptides were stained[18] with fluorescamine. Runs were stopped when the marker dye (phenolindo-2,6-dichlorophenol; BDH Chemicals Ltd.) had migrated 10 inches from the origin. Glycopeptides were eluted from the paper by a series of washes using a centrifugation method[33]. The paper was washed 5 times with methanol containing 1% of acetic acid, 5 times with methanol, and finally 5 times with water to elute the glycopeptide.

Fucosyltransferase assays. — The assay conditions previously described[8] were used to study the substrate specificity of the 6-α-fucosyltransferase, except that Golgi-rich membranes (39 μg/assay tube) from porcine liver were used as the enzyme source. Glycopeptides were initially tested as substrates at 0.2 and 0.5mM. Inactive glycopeptides were incubated at 0.1mM for 18 h at 37° with 2 additions of Golgi membranes to rule out an active substrate having a high K_m or low V_{max}, or both. Active glycopeptides were assayed at four sub-saturating concentrations, in duplicate, to determine K_m and V_{max} values by the Lineweaver–Burk plot.

Glycosidase activity of Golgi-rich membranes. — Since glycosidase action on glycopeptides being tested as fucosyltransferase substrates would interfere with the accurate interpretation of the data, [^{14}C]-*N*-acetylated glycopeptides (~8000 c.p.m.) were incubated in a total volume of 0.030 mL containing 10mM MES (pH 6.7), 10mM $MnCl_2$, 0.1% of Triton X-100, and 39 μg of Golgi-membrane protein, at 37° for at least 1 h. The reaction was terminated by heat at 100° for 2 min, endo-β-*N*-acetylglucosaminidase C_{II} or H was added as previously described[36], and incubation was continued for 3 h at 37°. Cleavage by endo-glycosidase was measured by high-voltage paper electrophoresis at pH 3.6 in pyridine–acetic acid–water (10 : 100 : 890);

the cleaved [^{14}C]-*N*-acetyl-Asn-GlcNAc fragment moves faster towards the anode than the uncleaved glycopeptide. Each assay was carried out with a paired control using heat-inactivated Golgi membranes. This endo-glycosidase susceptibility assay tests for α-D-mannosidase activity, since removal of mannose residues by Golgi-rich membranes has been demonstrated[36] and results in loss of susceptibility to either endo-β-*N*-acetylglucosaminidase C_{II} or H. The facts that glycopeptides having terminal GlcNAc residues are excellent substrates for the Golgi 6-α-fucosyltransferase from porcine liver and that a terminal GlcNAc is essential for enzyme activity (see below) indicate that β-*N*-acetylglucosaminidase does not interfere with the detection of transferase activity.

In fucosyltransferase incubations involving substrates susceptible to Golgi α-D-mannosidase action, D-mannono-1,4-lactone was added to the reaction at 20mM[45,46]. A control experiment showed that the lactone at 20mM inhibited by 96% the action of porcine liver, Golgi α-D-mannosidase on GlcNAc(Man)$_5$(GlcNAc)$_2$Asn-*N*-[^{14}C]-acetyl, the product of UDP-GlcNAc: α-D-mannoside 2-β-acetamidodeoxyglucosyltransferase I action[35,36] on ovalbumin glycopeptide V; this inhibitory effect was maintained for at least 1 h under the conditions of fucosyltransferase action.

Carbohydrate analysis. — This was carried out by g.l.c. following methanolysis (0.5M methanolic HCl at 80° for 20 h) of the glycopeptide and trifluoroacetylation (0.5 mL of trifluoroacetic anhydride at 80° for 30 min) according to the method of Zanetta *et al.*[19]. Inositol was used as the internal standard. Analyses were carried out on a Varian Aerograph model 2100 gas chromatograph coupled to a Varian CDS111 Data System with a temperature program from 110→190° at 1°/min and a glass column (180 × 0.2 cm) packed with 3% of OV-210. Glucosamine analysis of glycopeptides was carried out with a Durrum D500 analyzer following hydrolysis with 4M HCl at 100° under nitrogen for 4 h. Samples contained 10 nmol of ε-aminocaproic acid as internal standard.

Other analyses. — Glycopeptides were quantified on the basis of asparagine content determined after acid hydrolysis (4M HCl, 4 h, 100°) by means of a Durrum D500 analyzer, using 10–20 nmol of ε-aminocaproic acid as internal standard. Protein was determined by the method of Lowry *et al.*[47] with bovine serum albumin as standard.

RESULTS

Identification of fucosyltransferase product

A large-scale incubation of Golgi-rich membranes from porcine liver with GDP-[^{14}C]-L-fucose and glycopeptide from α_1-acid glycoprotein yielded ~0.8 μmol of glycopeptide product. The fucosylated glycopeptide was cleaved with endo-β-*N*-acetylglucosaminidase C_I after β-*N*-acetylglucosaminidase treatment, and a fragment was purified in 58% yield. Amino acid analysis of this material indicated a 3:1 mixture of GlcNAc-Asn and [^{14}C]-L-Fuc-GlcNAc-Asn. Preparative paper chromato-

graphy was used to remove GlcNAc-Asn from the radioactive product, which was recovered in 51% yield (0.42 μmol).

The radioactive product was analyzed by ^{1}H-n.m.r. spectroscopy at 360 MHz. The anomeric proton region contained peaks at δ 5.10 ($J_{1,2}$ 8 Hz) consistent with a β-linked GlcNAc residue[48], 4.91 ($J_{1,2}$ 4 Hz) consistent with α-L-fucose in (1→6) linkage[48], and 4.68 (sharp peak) which was attributed to an unidentified contaminant. Integration of the first two peaks indicated a ratio of 2:1, consistent with a residual 50% content of GlcNAc-Asn which had not been removed by paper chromatography. Upfield peaks were detected at δ 2.02 (singlet due to *N*-acetyl protons) and 1.23 (doublet due to the CH_3 protons of L-fucose); the ratio of these peaks was 2:1, in agreement with a 1:1 mixture of GlcNAc-Asn and Fuc-GlcNAc-Asn. N.m.r. spectra have been reported[26] for compounds with α-Fuc (1→3)-linked to the outer (antennary) GlcNAc residue of complex oligosaccharides; signals for this fucose were at δ 5.11 (H-1), 4.83 (H-5), and 1.17 (CH_3). The H-5 signal was not detected in the spectrum of our product; the data are not conclusive, since Fuc(α1-3)GlcNAc-Asn was not available for testing. However, methylation analysis (see below) confirmed the absence of a Fuc(α1-3)GlcNAc linkage. N.m.r. analysis conclusively establishes that the Fuc is α-linked.

The fucosylated product (260 nmol) was permethylated and 78 nmol (30%) were recovered. After hydrolysis and reduction with borodeuteride, g.l.c.–m.s. gave a single peak in the partially methylated, neutral alditol acetate region and four peaks in the partially methylated 2-deoxy-2-*N*-methylacetamidohexitol acetate region (basic sugars). The mass spectra clearly identified the neutral sugar as 1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methylhexitol (presumably derived from a terminal fucose residue), the first basic peak as a 2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamidohexitol acetate (presumably derived from terminal GlcNAc residues), and the third basic peak as a 2-deoxy-3,4-di-*O*-methyl-2-*N*-methylacetamidohexitol acetate (presumably derived from 6-substituted GlcNAc). The second and fourth basic peaks were relatively minor components whose spectra did not correspond to any of the known, partially methylated alditol acetates; they were, perhaps, undermethylated sugars. The methylation analysis therefore supports the n.m.r. data and indicates the product sample to contain a mixture of GlcNAc-Asn and Fuc(α1-6)GlcNAc-Asn. No evidence for a 3-substituted GlcNAc was found by methylation analysis or n.m.r. spectroscopy (see above), although minor amounts of the Fuc(α1-3)GlcNAc-Asn linkage (13%) were reported[49] for urinary glycopeptides isolated from fucosidosis patients. It is possible that our techniques may have missed such small proportions of material, but it is clear that the enzyme from porcine liver is primarily a 6-α-fucosyltransferase.

Substrate-specificity studies

A series of glycopeptides were tested as substrates for the porcine liver 6-α-fucosyltransferase. The names, structures, and substrate activities of these glyco-

peptides are summarized in Table II; eight inactive glycopeptides are shown on the left side of the Table and four active preparations on the right.

IgG glycopeptide MM was inactive both before and after mild hydrolysis with acid to remove fucose. IgG glycopeptides MGn and MGn(Gn), however, both became active after mild hydrolysis with acid. The only observable effect of mild, acid hydrolysis on MM and MGn was partial removal of fucose, and it can therefore be concluded that a β-GlcNAc (1→2)-linked to the Man(α1-3) arm is required for fucosyltransferase activity. However, mild hydrolysis with acid removes some of the intersecting GlcNAc residues of MGn(Gn), and therefore α-L-fucosidase was used to remove fucose from this compound. N.m.r. analysis (Table I) indicates that the spectrum of the fucosidase-treated MGn(Gn) has no detectable signals for H-1 or CH_3 of fucose and shows decreases in the chemical shifts for H-1 and the N-acetyl protons of GlcNAc(IV), as expected for a fucose-free glycopeptide[50]. This material was totally inactive as an enzyme substrate; further, mixed-substrate experiments with MGn and MGn(Gn) indicated no inhibitory activity in the MGn(Gn) preparation. Thus, the intersecting GlcNAc residue appears to inhibit fucosyltransferase activity.

Glycopeptide GnGn (Table II) appears to be an even better fucose acceptor than MGn (K_m = 0.08 and 0.6mM respectively). However, the corresponding glyco-

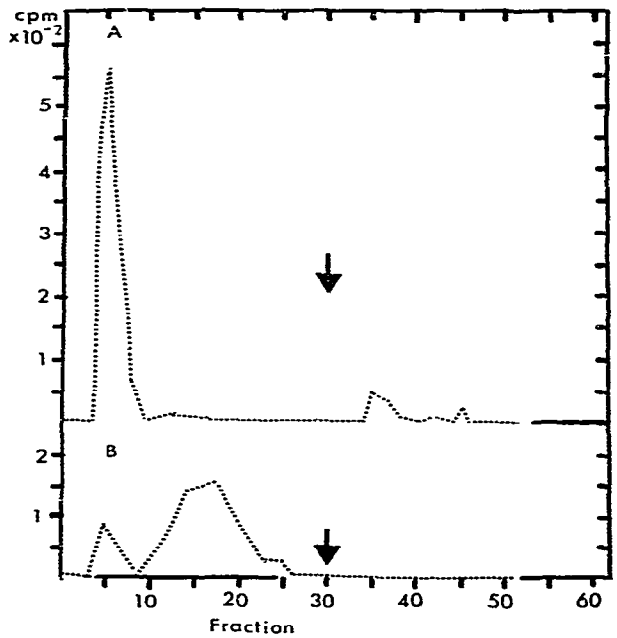


Fig. 1. Chromatography on Concanavalin A/Sepharose columns (0.7 × 7 cm), as previously described[17]: fraction size, 1.0 mL; arrows show when elution with 0.2M methyl α-D-mannopyranoside commenced. A, Product of fucosyltransferase action on glycopeptide GnGn(Gn) (ovalbumin glycopeptide V-1); B, chromatography of *N*-[^{14}C]acetylated glycopeptide GnGn(Gn), showing an impurity which passes rapidly through the lectin column.

peptide with an intersecting GlcNAc, *i.e.*, GnGn(Gn), was completely inactive. This glycopeptide was isolated from ovalbumin and was characterized primarily by n.m.r. spectroscopy (Table I). The changes in the n.m.r. spectrum due to an intersecting GlcNAc residue[50] are seen in the spectrum of GnGn(Gn), *i.e.*, decreased chemical shifts for H-1 of mannose residues (1) and (2) and H-2 of mannose residue (1), increased chemical shifts for H-1 of mannose (3) and H-2 of mannoses (2) and (3), and an unusually low-field signal for H-1 of the intersecting GlcNAc(III). Although glycopeptide GnGn(Gn) appeared homogeneous by n.m.r. analysis, it showed a low fucose-acceptor activity which was suspected to be due to a minor contaminant in the preparation. Analysis of glycopeptide GnGn(Gn) by chromatography on Concanavalin A/Sepharose (Fig. 1) showed that most of the preparation was retarded, as expected for a glycopeptide with an intersecting GlcNAc residue[36]. The identity of the small amount of material that was unretarded is not known, but it was responsible for all of the fucose-acceptor activity (Fig. 1). It can be concluded that GnGn(Gn) is not a fucose acceptor and that, for a second pair of glycopeptides, the intersecting GlcNAc residue inhibits fucosyltransferase activity.

The glycopeptide fraction from α_1-acid glycoprotein which did not adhere to a preparative Concanavalin A/Sepharose column was also an effective fucose acceptor (Table II). This is a rather complex mixture of ~1:1 of tri-antennary GnGnGn and tetra-antennary GnGnGnGn with ~10% of fucosylated antennae; thus, although extensive conclusions cannot be drawn, the experiment shows that glycopeptides do not have to be bi-antennary to accept fucose. As pointed out in the Experimental, the preparation may contain ~2% of material that has suffered some β-*N*-acetylglucosaminidase degradation during preparation; further, there is the possibility of β-*N*-acetylglucosaminidase degradation during incubation with Golgi membranes. To assess the effects of this degradation, the radioactive product formed by fucosyltransferase action was analyzed on Concanavalin A/Sepharose (data not shown); all of the radioactivity was unretarded, indicating[17] that the product was neither fucosylated GnGn nor MGn, but contained 4- or 6-linked β-GlcNAc residues (or both). Thus β-*N*-acetylglucosaminidase degradation to GnGn or MGn cannot account for the fucosyltransferase activity observed with this acceptor. It is interesting that GnGnGn(Gn), isolated from ovalbumin, does not accept fucose (Table II), again suggesting that the intersecting GlcNAc residue is an inhibitor of the transferase. The tri-antennary structure of GnGnGn(Gn) is indicated by the n.m.r. spectrum of this glycopeptide (Table I); the chemical shift for H-2 of mannose residue (1) is decreased and of mannose residue (2) is increased relative to the bi-antennary structure GnGn(Gn). The spectrum of GnGnGn(Gn) also shows the changes characteristic of an intersecting GlcNAc residue (see above).

The remaining glycopeptides tested were of the "high mannose" type, *i.e.*, they contained more than three residues of mannose. Golgi-rich membranes contain an α-D-mannosidase[36] which can act on some of these compounds. We tested mannose loss from our glycopeptides by determining whether incubation with Golgi-rich membranes converts endo-β-*N*-acetylglucosaminidase C_{II}- or H-sensitive material

into non-cleavable glycopeptide (Table III). Only the product of GlcNAc-transferase I action on ovalbumin glycopeptide V, GlcNAc(Man)$_5$(GlcNAc)$_2$Asn, was susceptible to α-D-mannosidase. This degradation could be inhibited by 95% for at least 1 h by 20mM D-mannono-1,4-lactone (Table III). Fig. 2 shows the results of fucosyltransferase action on GlcNAc(Man)$_5$(GlcNAc)$_2$Asn, in the presence of 20mM D-mannono-1,4-lactone. All of the panels in Fig. 2 show high-voltage electrophoretograms in borate of radioactive glycopeptides which had been incubated with Golgi-rich membranes at 37° for 30 or 60 min in the presence of 20mM D-mannono-1,4-lactone at pH 6.7. Panels A and B represent glycopeptide markers (as indicated) which had been incubated in the absence of radioactive GDP-L-fucose. Panel C shows the product formed after fucosyltransferase action on glycopeptide MGn. Panels D and E show the action of the fucosyltransferase on glycopeptide GlcNAc-(Man)$_5$(GlcNAc)$_2$Asn. It is clear that a double-labelled product is formed with a mobility higher than that of the (Man)$_3$-containing product and equivalent to that of a (Man)$_5$-containing glycopeptide; the broad nature of the product peak indicates that some (Man)$_4$-containing material may also be present, presumably due to α-D-mannosidase activity during the incubation. The incubations were also subjected to endo-β-*N*-acetylglucosaminidase C$_{II}$ followed by high-voltage paper electrophoresis at pH 3.7; the results are shown in Table IV. Three radioactive products were resolved by high-voltage electrophoresis: (*a*) a ^{3}H-labelled oligosaccharide cleavage-product at the origin; (*b*) a double-labelled, uncleaved glycopeptide product; and (*c*) a rapidly

TABLE III

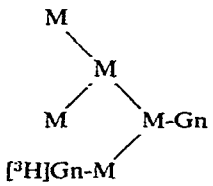
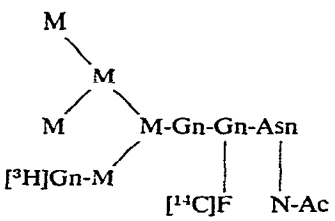
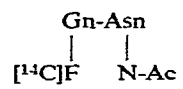
α-D-MANNOSIDASE ACTIVITY[a] OF GOLGI-RICH MEMBRANE

Glycopeptide[a]	N-[^{14}C]*Acetyl-Asn-GlcNAc formed (%)*				
	Endo-C$_{II}$		*Endo-H*		
	Intact Golgi	*Heat-inactivated Golgi*	*Intact Golgi*	*Intact Golgi + lactone*	*Heat-inactivated Golgi*
MM	0	—[b]	0	—	—
MGn	0	—	0	—	—
Ovalbumin III-A	90	90	95	—	95
Ovalbumin III-B[c]	—	—	95	—	95
Ovalbumin III-C	2	2	90	—	90
Ovalbumin V	96	96	96	—	96
GlcNAc(Man)$_5$(GlcNAc)$_2$-Asn	—	—	6	96	100

[a]Radioactive (*N*-[^{14}C]acetylated) glycopeptides (see Table II for nomenclature and structures) were incubated for 1 h with Golgi-rich membranes from porcine liver, followed by a 3-h exposure to either endo-β-*N*-acetylglucosaminidase C$_{II}$ (endo-C$_{II}$) or H (endo-H). Cleavage was detected by measuring the % formation of *N*-[^{14}C]acetyl-Asn-GlcNAc after electrophoresis at pH 3.6. Some incubations contained 20mM D-mannono-1,4-lactone. [b]—, Not done. [c](1→2)-Linked α-Man residues may be removed; this will not be detected by the method used.

TABLE IV

ENDO-GLYCOSIDASE TREATMENT[a] OF 6-α-FUCOSYLTRANSFERASE PRODUCT

Radioactive species[b]	*Analysis (nmol)*			
	30 Min		*60 Min*	
	^{14}C	^{3}H	^{14}C	^{3}H
	—	1.6	—	1.4
	0.8	0.6	1.8	1.2
	0.06	—	0.1	—

[a]The incubations described in panels D and E of Fig. 2 were also treated with endo-β-*N*-acetylglucosaminidase C_{II} and analyzed for cleavage products after electrophoresis at pH 3.7. [b]Code: M, Man; Gn, GlcNAc; F, Fuc; Ac, acetyl.

migrating peak of [^{14}C]-L-Fuc-GlcNAc-Asn-NAc. The small proportion of [^{14}C]-L-Fuc-GlcNAc-Asn-NAc formed indicates that endo-β-*N*-acetylglucosaminidase C_{II}, like endo-β-*N*-acetylglucosaminidase H[51], is inhibited by the presence of a fucose residue linked to the Asn-linked GlcNAc. The cleavage of unfucosylated substrate (~2 nmol) by endo-β-*N*-acetylglucosaminidase C_{II} is complete for both incubations, since the molar ratio of [^{14}C]-L-Fuc to [^{3}H]GlcNAc in uncleaved product is ~1, *i.e.*, there is no excess of ^{3}H to ^{14}C. The cleavage of product, on the other hand, is only 7%.

The other "high mannose" glycopeptides tested, namely, ovalbumin glycopeptides III-A, III-B, III-C, and V, were all inactive (Table II) as fucosyltransferase substrates. Glycopeptide III-A is of special interest, since it differs from the active acceptor GlcNAc(Man)$_5$(GlcNAc)$_2$Asn only by having an additional, intersecting GlcNAc residue. Thus, in four pairs of glycopeptides (Table II) differing only by the absence or presence of an intersecting GlcNAc, the latter residue inhibits fucosyltransferase activity.

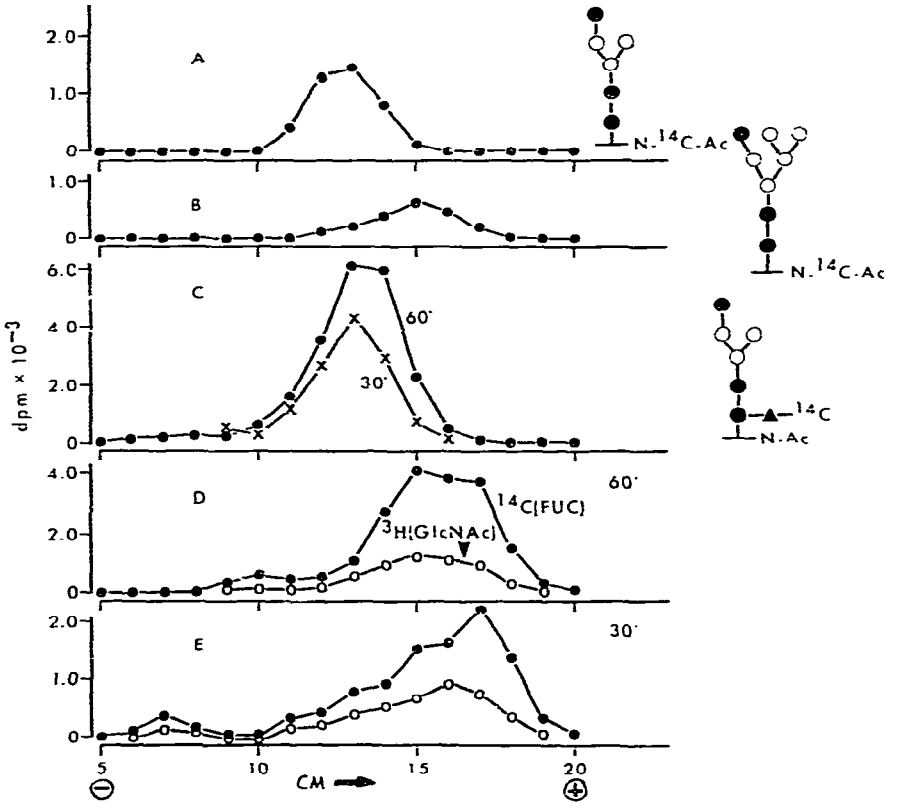


Fig. 2. High-voltage electrophoretograms in 1% tetraborate. All incubations contained radioactive glycopeptide, Golgi-rich membranes, 20mM D-mannono-1,4-lactone, and buffer at pH 6.7. Incubations were at 37° for 30 or 60 min, as indicated. A, *N*-[^{14}C]Acetyl-glycopeptide MGn. B, GlcNAc-(Man)$_5$(GlcNAc)$_2$-Asn-*N*-[^{14}C]acetyl. C, Acetylated MGn after incubation in the presence of GDP-L-[^{14}C]Fuc (9000 d.p.m./nmol); —●—, 60 min; —×—, 30 min. D, [^{3}H]GlcNAc(Man)$_5$(GlcNAc)$_2$-Asn-NAc (2000 d.p.m./nmol) after incubation in the presence of GDP-L-[^{14}C]Fuc (9000 d.p.m./nmol) for 60 min; —●—, ^{14}C; —○—, ^{3}H. E, Same as D, incubated for 30 min. Code: ●, GlcNAc; ○, Man; ▲, Fuc.

Another conclusion to be drawn from Table II is that all active glycopeptides possess a terminal β-GlcNAc residue (1→2)-linked to the (1→3)-linked α-Man of the (Man)$_3$(GlcNAc)$_2$ core. Substitution of this GlcNAc residue by a galactose residue inhibits fucosyltransferase activity, since asialo-α_1-acid glycoprotein is inactive as an acceptor, whereas asialo,agalacto-α_1-acid glycoprotein is highly effective[10]. A second (1→2)-linked β-GlcNAc residue on the (1→6)-linked α-Man arm seems to enhance activity and a third terminal GlcNAc residue on the (1→3)-linked α-Man arm does not seem to affect fucosyltransferase activity. However, the addition of an intersecting GlcNAc residue to an effective acceptor causes inhibition of activity.

DISCUSSION

Golgi-rich membrane preparations from various tissues are enriched[10] in a fucosyltransferase that transfers L-fucose from GDP-L-Fuc to acceptors having terminal GlcNAc residues such as asialo,agalacto derivatives of fetuin and α_1-acid glycoprotein. Studies with rat-liver preparations[10] lacking a fucosyltransferase which acts on asialo-α_1-acid glycoprotein showed that the fucosyltransferase acting on GlcNAc-terminal acceptors did not act on Gal-terminal acceptors; competition studies with porcine liver also indicated[8] that the two fucosyltransferase activities were due to different enzymes. The initial studies of the enzyme acting on GlcNAc-terminal acceptors did not establish the nature of the product formed; it was assumed that L-fucose was transferred to the terminal GlcNAc residues on the antennae of complex *N*-glycosyl oligosaccharides. However, Wilson *et al.*[12] showed that the rat-liver enzyme did not attach Fuc to a terminal GlcNAc but rather to the Asn-linked GlcNAc residue of the $(Man)_3(GlcNAc)_2Asn$ core; the linkage formed was not determined.

The present report shows that the porcine liver enzyme also attaches Fuc to the Asn-linked GlcNAc residue, that the linkage is predominantly (1→6)-α, that a GlcNAc in (1→2)-β linkage to the Man(α1-3)Man(β1- terminus is essential for enzyme activity, and that an intersecting GlcNAc residue attached in (1→4)-β linkage to the β-linked Man residue prevents enzyme action.

The product identification was carried out in a manner analogous to the earlier work with rat liver[12]. An acceptor glycopeptide was prepared from α_1-acid glycoprotein, the product was prepared, and β-*N*-acetylglucosaminidase and endo-β-*N*-acetylglucosaminidase C_I were used to cleave the product to produce the [^{14}C]Fuc-GlcNAc-Asn fragment. It was necessary to treat the product with β-*N*-acetylglucosaminidase, prior to endo-glycosidase action, to remove GlcNAc residues blocking the Man(α1-3)Man(β1- terminus; endo-β-*N*-acetylglucosaminidase C_I does not act if this terminus is blocked[15].

The Fuc-GlcNAc-Asn fragment was purified in 52% yield, and the linkage between Fuc and GlcNAc was identified to be (1→6)-α by high-resolution, ^{1}H-n.m.r. spectroscopy and permethylation analysis. No evidence for the alternative (1→3)-α linkage was detected; the presence of 13% of (1→3)-α linkages in glycopeptides isolated from the urine of fucosidosis patients has been reported[49]. The porcine liver enzyme appears to be predominantly a 6-α-fucosyltransferase.

Various glycopeptides were tested as fucosyl acceptors for the 6-α-fucosyltransferase from porcine liver. Only four of these were active acceptors, namely, glycopeptides MGn, GnGn, $GlcNAc(Man)_5(GlcNAc)_2Asn$, and a mixture of tri- and tetra-antennary glycopeptides with terminal GlcNAc residues (Table II). These active glycopeptides all have a β-GlcNAc (1→2)-linked to the Man(α1-3)Man(β-1 terminus. It has been possible to prepare four analogous glycopeptides differing from the active acceptors only by the presence of an intersecting GlcNAc residue attached in (1→4)-β linkage to the β-linked Man residue; these four glycopeptides

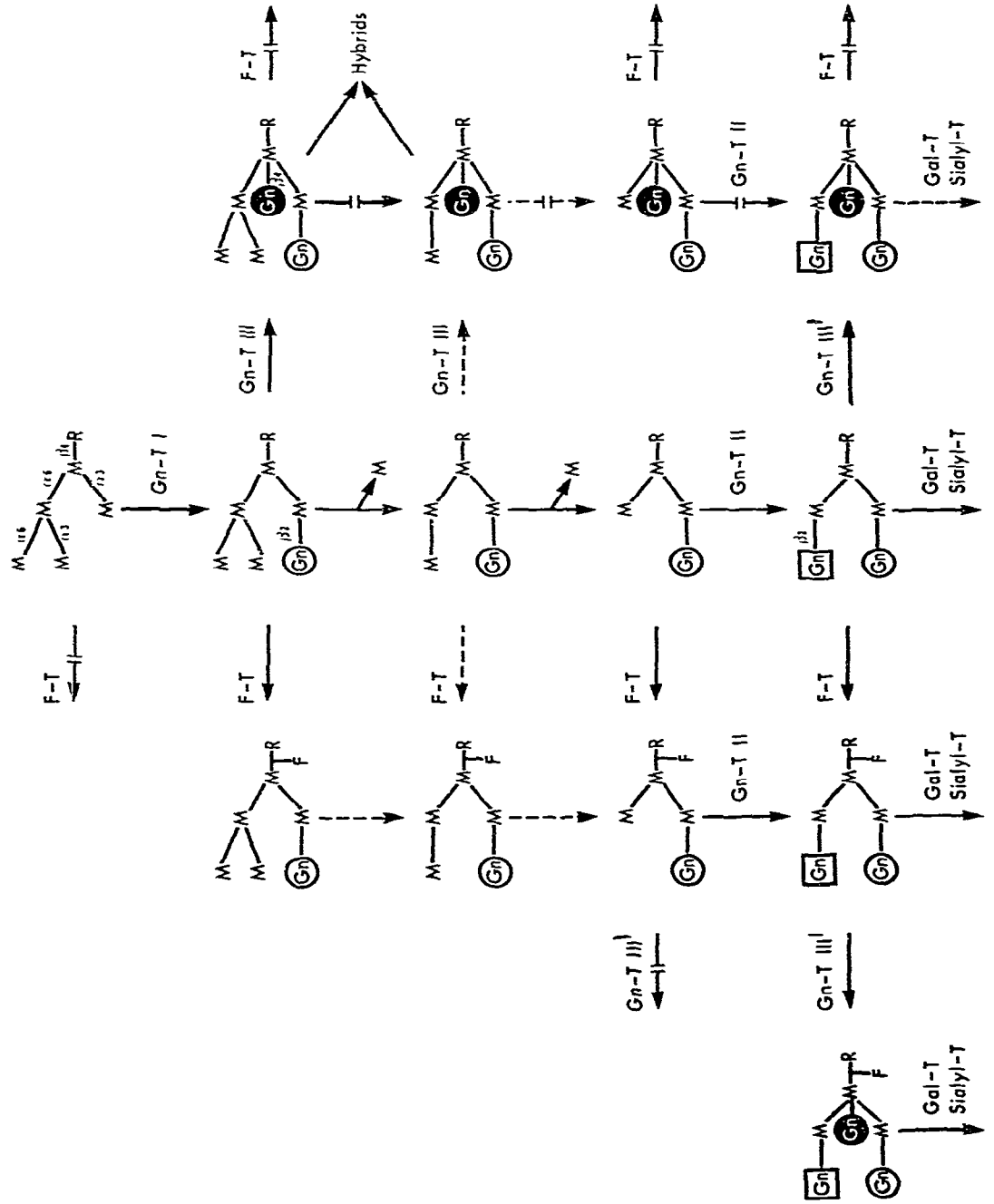


Fig. 3. Scheme showing the Golgi-localized reactions involved in the assembly of bi-antennary *N*-glycosyl oligosaccharides. Key: M, Man; Gn, GlcNAc; F, Fuc; T, transferase; R, GlcNAc(β1-4)-GlcNAc-Asn-X; →, reactions which have been demonstrated *in vitro*; ----→, postulated reactions not

were all inactive (Table II), indicating that the intersecting GlcNAc residue prevented fucosyltransferase action. The other inactive glycopeptides either lacked a GlcNAc(β1-2)Man(α1-3)Man(β1- terminus (MM, ovalbumin glycopeptides III-B and V) or carried an intersecting GlcNAc (ovalbumin glycopeptide III-C). The presence of more than two antennae does not appear to interfere with fucosyltransferase action, although we have not determined whether tri- or tetra-antennary structures, or both, are active.

Glycopeptides with terminal Gal residues are not included in Table II, since it has previously been shown[8,10] that Gal-terminal structures are excellent acceptors for a different fucosyltransferase in porcine liver. Gal-terminal acceptors can be effective for at least 3 fucosyltransferases[2-6], a 2-α-fucosyltransferase making the human blood-group H determinant Fuc(α1-2)Gal, a 3-α-fucosyltransferase making the Gal(β1-4)[Fuc(α1-3)]GlcNAc or Gal(β1-4)[Fuc(α1-3)]Glc structure, and a 4-α-fucosyltransferase making the human blood-group Lewis determinant Gal-(β1-3)[Fuc(α1-4)]GlcNAc. All of these enzymes require a Gal-terminal acceptor and are therefore not involved in the activities demonstrated in Table II.

Some of these conclusions are incorporated into the biosynthetic scheme shown in Fig. 3. The biosynthesis of *N*-glycosyl oligosaccharides involves[52] transfer, within the rough endoplasmic reticulum, of a large oligosaccharide containing 3 Glc, 9 Man, and 2 GlcNAc residues from dolichol pyrophosphate oligosaccharide to suitable Asn residues in the polypeptide chain. If a complex oligosaccharide is being made, the 3 Glc residues and 4 of the 9 Man residues are removed as the glycoprotein moves towards the Golgi apparatus. The scheme in Fig. 3 begins at this point with the structure $(Man)_5(GlcNAc)_2$Asn-X. The immediate product is the addition of a GlcNAc in (1→2)-β linkage to the Man(α1-3)Man(β1- terminus by a Golgi-located enzyme that we have called[35] UDP-GlcNAc: α-D-mannoside 2-β-GlcNAc-transferase I. Mutant cell-lines lacking GlcNAc-transferase I accumulate the $(Man)_5(GlcNAc)_2$-Asn-X structure[53-55], indicating that there are no alternative routes.

If GlcNAc-transferase I acts, a variety of possible products can be made from this key intermediate. Bi-antennary *N*-glycosyl oligosaccharides are made by the removal of 2 Man residues followed by the action of UDP-GlcNAc: α-D-mannoside 2-β-GlcNAc-transferase II (Fig. 3). The removal of the 2 Man residues cannot occur until GlcNAc-transferase I has acted[36]. Bi-antennary structures can occur[1] either with or without a Fuc in the core or with or without an intersecting GlcNAc residue. The relevant paths are shown in Fig. 3. The core Fuc cannot be attached until GlcNAc-transferase I has acted, as we have shown in the present report; after transferase I action, Fuc can be attached either at the $(Man)_5$ or $(Man)_3$ stage, and possibly also at the $(Man)_4$ stage, although the latter compound was not available for testing. Fuc can be attached either before (glycopeptide MGn) or after (glycopeptide GnGn) GlcNAc-transferase II action (Fig. 3).

The intersecting GlcNAc residue has important control functions. We have demonstrated[56] a GlcNAc-transferase III′ which transfers β-GlcNAc in (1→4) linkage to the β-linked Man of glycopeptide GnGn (Fig. 3); this enzyme allows

synthesis of bi-antennary, bisected oligosaccharides, structures shown[1] to occur in human multiple-myeloma IgG. Such bi-antennary, bisected oligosaccharides can occur either with or without a core Fuc; if a core Fuc is present, it must be incorporated before GlcNAc-transferase III′ action (Fig. 3), since the intersecting GlcNAc residue prevents fucosyltransferase action (see above).

The intersecting GlcNAc appears to control the synthesis of hybrid oligosaccharides[22] that contain complex antennae on the Man(α1-3) arm and high mannose structures on the Man(α1-6) arm (Fig. 3). We have demonstrated[56] a GlcNAc-transferase III, which may or may not be identical to GlcNAc-transferase III′, and which incorporates an intersecting GlcNAc residue into $GlcNAc(Man)_5(GlcNAc)_2$-Asn-X (Fig. 3) and possibly also into $GlcNAc(Man)_4(GlcNAc)_2$Asn-X, although the latter was not tested. The effect of this intersecting GlcNAc is to inhibit the α-D-mannosidase which converts $(Man)_5$ compounds into $(Man)_3$ compounds[36], to inhibit 6-α-fucosyltransferase action, and to inhibit GlcNAc-transferase II action[35]. This shunts the biosynthetic path towards hybrid structures (Fig. 3) and explains why hybrid structures usually carry an intersecting GlcNAc and lack a core Fuc. Rhodopsin has been reported to contain the hybrid structures $GlcNAc(Man)_5(GlcNAc)_2$Asn-X and $GlcNAc(Man)_4(GlcNAc)_2$Asn-X, which do not contain an intersecting GlcNAc residue[57]; this apparent exception to the rule can be explained by postulating that the synthesis of rhodopsin involves an interference with processing of $(Man)_5$ structures to $(Man)_3$ structures and with the action of GlcNAc-transferase II (see Fig. 3). Our scheme predicts that the two rhodopsin hybrid-structures can occur with a core Fuc residue; such oligosaccharides have not as yet been described.

The synthesis of tri- and tetra-antennary oligosaccharides is not shown in Fig. 3. We have preliminary evidence for GlcNAc-transferases which incorporate a second GlcNAc residue on the Man(α1-3)Man(β1- arm of glycopeptides with the GlcNAc(β1-2)Man(α1-3)terminus. We have shown in this paper that fucose incorporation is not inhibited by such additional GlcNAc residues.

Other predictions of the scheme in Fig. 3 are that oligosaccharides having a high content of mannose should not contain a core Fuc and that mutant cell-lines lacking GlcNAc-transferase I should be unable to make oligosaccharides with a core Fuc. To date, the first prediction has not been violated. Further, a GlcNAc-transferase I-deficient cell-line of Chinese hamster ovary has been found[58] to be deficient in fucose-containing complex oligosaccharides; this finding explains a previously paradoxical observation[59] that such GlcNAc-transferase I-deficient lines are resistant to the *Lens culinaris* agglutinin, since recent experiments[60] have shown that the *Lens culinaris* agglutinin binds to the core Fuc of complex oligosaccharides.

Various problems remain unresolved. It is not clear why some glycoproteins (for example, human-serum α_1-acid glycoprotein or transferrin) lack a core fucose residue, whereas other glycoproteins (for example, human multiple-myeloma IgG) are rich in core-fucose residues; the presence or absence of the enzyme in the relevant tissues is an obvious explanation which requires a thorough tissue survey for verification. The 6-α-fucosyltransferase in human serum has also been widely studied as a

possible diagnostic tool in leukemias and other cancers[61-63], and this area of research requires further work. Finally, the enzyme has not as yet been purified; our preliminary attempts at purification have not been successful. A purified enzyme would enable detailed studies into the enzyme's binding site, which apparently recognizes a specific sugar residue [GlcNAc(β1-2)] that is three hexose residues removed from the site of covalent-bond formation (Asn-bound GlcNAc).

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