THE CONTROL OF GLYCOPROTEIN SYNTHESIS: N-ACETYLGLUCOSAMINE LINKAGE TO A MANNOSE RESIDUE AS A SIGNAL FOR THE ATTACHMENT OF L-FUCOSE TO THE ASPARAGINE-LINKED N-ACETYLGLUCOSAMINE RESIDUE OF GLYCOPEPTIDE FROM α_1-ACID GLYCOPROTEIN.

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Summary. Rat liver microsomes catalyze the incorporation of L-fucose from GDP-L-fucose into a glycopeptide prepared from human plasma α_1-acid glycoprotein; the glycopeptide must have terminal mannose-linked β-N-acetylglucosamine to be active as an acceptor but fucose does not become linked to this N-acetylglucosamine residue. Endo-β-N-acetylglucosaminidase treatment of the product of the reaction shows that the fucose is incorporated into the asparagine-linked N-acetylglucosamine residue. Competition studies show that this fucosyltransferase is the same enzyme as a GDP-fucose:β-N-acetylglucosaminide fucosyltransferase previously described to be present in rat liver Golgi apparatus.

We have previously described the presence in pork liver of two fucosyltransferases which transfer L-fucose from GDP-L-fucose into various derivatives of human plasma α_1-acid glycoprotein (1); one enzyme required acceptors with terminal β-galactoside residues, while the other required acceptors with terminal β-N-acetylglucosaminide residues. The role of the GDP-L-fucose:β-N-acetylglucosaminide fucosyltransferase has until recently been obscure. The enzyme is enriched in the Golgi apparati of rat and pork liver (2) and of rat and mouse testis (3). Although a terminal N-acetylglucosamine residue on the acceptor is essential for enzyme activity, it has not been proven that L-fucose becomes attached to this residue. Such a linkage has, in fact, not been found in Asn-GlcNAc-type glycoproteins (4). However, L-fucose has been found as a branched residue attached to the asparagine-linked N-acetylglucosamine residue of Asn-GlcNAc-type glycoproteins such as mouse histocompatibility-2 alloantigen (H-2) (5) and immunoglobulins

IgG, IgM, IgE and IgA (6-10). The recent availability of endo-β-N-acetylglucosaminidases capable of cleaving between the two internal GlcNAc residues of Asn-GlcNAc-type glycopeptides (12-16) has enabled us to prove that the rat liver fucosyltransferase catalyzes the attachment of a branched L-fucose residue to the asparagine-linked GlcNAc residue of glycopeptides from human α_1-acid glycoprotein, as we had previously suggested (11).

Materials. Human plasma α_1-acid glycoprotein was either prepared in this laboratory (17) or was obtained from the American Red Cross National Fractionation Center (NIH grant HE1388). Sialidase (18), a mixture of β-galactosidase and β-N-acetylglucosaminidase (19) and endo-β-N-acetylglucosaminidase C_I (15) were prepared from Cl. perfringens culture medium. Pronase, grade B, was obtained from Calbiochem. Jack bean α-mannosidase was obtained from Boehringer. Uniformly labelled GDP-[^{14}C]-L-fucose, 143 μCi per μmole, was obtained from New England Nuclear and diluted with non-radioactive GDP-L-fucose (1) to a specific activity of 5 μCi per μmole. Acetic anhydride-1-[^{14}C], 9.3 mCi per μmole, was purchased from New England Nuclear. α-L-fucosidase from C. lampus was purchased from Miles Labs. All other reagents were of commercial origin.

Preparation of α_1-acid glycoprotein (AGP) glycopeptide. Purified AGP (3.0 g) was incubated with 2.9 units of sialidase in 60 ml 0.33 M potassium acetate, pH 4.5, at 37° for 15 hours, over chloroform; free sialic acid (20) was equal to total sialic acid (21). The protein was incubated with 14 units β-galactosidase and 10 units β-N-acetylglucosaminidase in 40 ml 0.2 M potassium phosphate, pH 6.0, at 37° for 30 hours under toluene; 43% of the total GlcNAc was released. The digest was concentrated and applied to Sephadex G-150 (5 x 110 cm) equilibrated with water. A peak of protein free of glycosidase activity was obtained. The latter (1.4 g) was dissolved in 560 ml 0.1 M sodium tetraborate, pH 7.9, containing 0.01 M calcium acetate, 32 mg Pronase was added and the digest incubated at 37° for 48 hours under toluene (22); an additional 32 mg Pronase was then added and incubation continued for a further 48 hours. The digest was concentrated and applied to Sephadex G-25 (5 x 76 cm) equilibrated with 0.1 N glacial acetic acid. A glycopeptide peak was obtained by assaying fractions for hexose (23) and was further purified on Biogel P-10 (2.4 x 79 cm) eluted with water followed by passage through Dowex 50-X2, pyridinium form, 2.4 x 80 cm, equilibrated with 1 mM pyridinium formate, pH 3.0. The glycopeptide was desalted with Sephadex G-25, 5 x 76 cm, equilibrated with water. Recovery of hexose was 62%. High voltage paper electrophoresis at pH 1.9 (formic acid-acetic acid-water, 39:74:1250), 2.5 KV, for 1.5 hours revealed a single band moving towards the cathode by both ninhydrin and periodate-benzidine stains. A single ninhydrin-positive band near the origin was detected after descending paper chromatography in 3 different systems. High voltage paper electrophoresis at pH 6.5 (pyridine-acetic acid-water, 100:4:896) resolved the glycopeptide into 3 bands (A,B,C) moving towards the anode. Amino acid (Beckman Model 121) and carbohydrate (24) analyses showed B and C were derived from the Glu-Asn(carbohydrate)-Gly-Thr position of the AGP sequence (25) whereas A was mainly from the Asn(carbohydrate)-Thr-Thr sequence. The only sugars present were mannose and glucosamine in the ratio of 3.0 to 2.2 residues. Additional GlcNAc (0.3 residues per mole of glycopeptide) was removed by treatment with β-N-acetylglucosaminidase at 37° for 20 hours. Treatment with β-N-acetylglucosaminidase and α-mannosidase released 1.9 residues of mannose determined enzymatically (26). The AGP glycopeptide therefore appears to be a mixture of structures I and II shown in Figure 1 (27).

Preparation of rat liver microsomes. Livers from male Wistar rats (150-200 g) were homogenized in 2.5 volumes of 0.05 M Tris-maleate (pH 7.4) containing 0.25 M sucrose using a motor-driven glass-Teflon Potter-Elvehjem homogenizer. Homogenate was centrifuged at 20,000 g (20 min) and 105,000 g (60 min) to yield a microsome pellet. The pellet was suspended in 0.05 M Tris-maleate (pH 7.4) containing 10 mM KCl at a protein concentration of about 50 mg per ml.

Fucosyltransferase assays. The standard incubation contained the following (in μmoles) in a final volume of 0.05 ml: Tris-maleate (pH 7.4), 1.0; $MgCl_2$, 2.0; GTP, 0.125; GDP-[^{14}C]-fucose, 0.02 (213,000 dpm); glycopeptide, 0.10; 0.5 μl Triton X-100; and 0.5 to 1.2 mg enzyme protein. Incubations were at 37° for 2 hours. Aliquots were subjected to high voltage paper electrophoresis at pH 6.5 (2 KV for 2 hours) and the origins were washed by descending chromatography with 80% ethanol. Radioactive product remained at the origin and was counted by liquid scintillation techniques (28).

Endo-β-N-acetylglucosaminidase assay. "Side chain-free" glycopeptide was prepared from human multiple myeloma IgG (6) and was acetylated with acetic anhydride-1-^{14}C (12), for use in the endoglycosidase assay (12,15).

Identification of product. The standard incubation was scaled up 10 fold. The incubation was carried on at 37° for 7 hours; 37,600 dpm of product were formed. The digest was fractionated on Biogel P-10 (2.5 x 68 cm) equilibrated with water; fractions were assayed, by combined high voltage electrophoresis and paper chromatography, for the presence of radioactive fucose, fucose-1-phosphate, GDP-fucose and glycopeptide product. The glycopeptide product was recovered from this column free of protein, fucose-1-phosphate and GDP-fucose; the product was, however, contaminated with 320,000 dpm of radioactive fucose. This fraction was incubated in 1.0 ml 5% (w/v) sodium bicarbonate containing 0.020 ml acetic anhydride at room temperature for 1 hour, was then diluted with 10 ml water and Dowex 50-X8 (hydrogen ion form), 20-40 mesh, was added until evolution of carbon dioxide ceased. The supernatant was subjected to preparative high voltage paper electrophoresis at pH 6.5; radioactive fucose remained at the origin whereas the acetylated glycopeptide moved towards the anode and was eluted with water. Recovery was 32,000 dpm (85%). Product (2400 dpm) was incubated with endo-β-N-acetylglucosaminidase (app. 8 μg protein) in 0.040 ml 0.05 M potassium phosphate (pH 6.5) at 37° for 24 hours. The digest was analyzed by high voltage paper electrophoresis at pH 6.5; radioactive peaks were detected by liquid scintillation techniques. Product (1850 dpm) was also subjected to mild acid hydrolysis (0.8 N HCl, 70°, 1 hour) or to the action of α-L-fucosidase and digests were analyzed by descending paper chromatography with ethyl acetate-pyridine-water(40:11:6)for the release of radioactive fucose(12).

Protein. Protein was measured by the procedure of Lowry et al. (29) using bovine serum albumin as standard.

RESULTS

Competition studies. Our previous work using rat and pork liver (1,2) as a source of GDP-fucose:β-N-acetylglucosaminide fucosyltransferase was carried out with sialidase-, β-galactosidase-treated α_1-acid glycoprotein [AGP(-SA,Gal)] When we found that AGP glycopeptide was also an excellent acceptor for fucose, we carried out competition experiments between these two acceptors to ensure that we were dealing with the same enzyme. With either acceptor the enzyme

TABLE 1

Mixed substrate experiments

Substrate concentration (mM)		Fucosyltransferase activity (cpm/2 hours)		
AGP(-SA,Gal)	AGP glycopeptide	Experimental	Calculated 1 enzyme	Calculated 2 enzymes
4.5	0	2250		
8.9	0	3050		
17.8	0	3960		
0	0.4	3080		
0	0.8	4820		
0	1.6	6580		
4.5	0.4	4180	3750	5330
8.9	0.8	5360	4980	7870
17.8	1.6	6420	5940	10500

The standard incubation mixture was used in this experiment. The calculated value for the situation in which separate transferases act on the two substrates is the sum of the activities for each substrate alone. The calculated value for the case in which the two substrates compete for a common enzyme was derived from the expression:

$$v = \left[V_1(A_1/K_1) + V_2(A_2/K_2)\right] \Big/ \left[1 + A_1/K_1 + A_2/K_2\right]$$

where V_1 and V_2 are maximal velocities for the two substrates (5260 and 11400 cpm respectively), K_1 and K_2 are the respective Michaelis constants (6.5 and 1.1 mM) and A_1 and A_2 are the respective substrate concentrations.

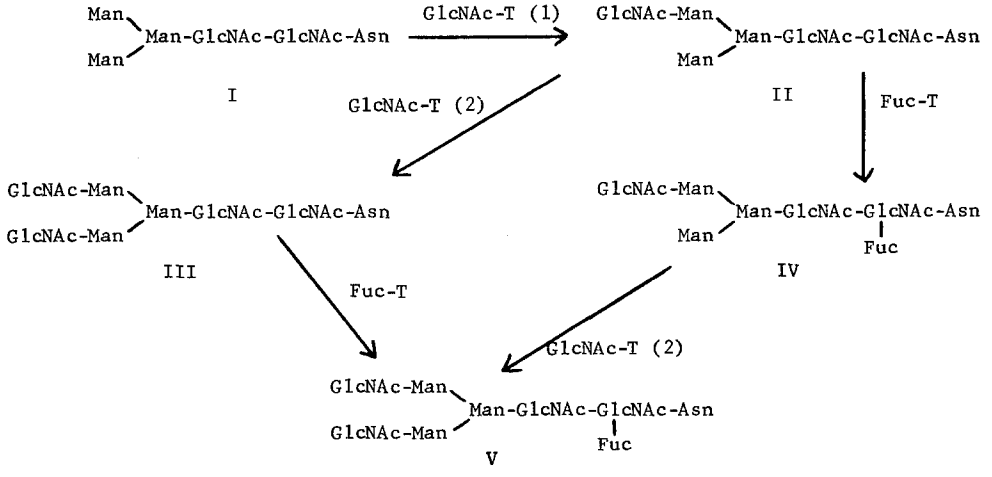

Figure 1. Evidence for all the above reactions has been obtained (30,31 and unpublished data). Abbreviations: GlcNAc-T, N-acetylglucosaminyltransferase; Fuc-T, fucosyltransferase.

assay showed proportionality with time and enzyme protein concentration and reciprocal velocity-substrate concentration plots were linear. The results of mixed acceptor assays are shown in Table 1; it is clear that both acceptors compete for common enzyme active sites.

Acceptor specificity. Since α_1-acid glycoprotein from which only sialic acid and galactose were removed was found to be an excellent acceptor for the fucosyltransferase, structure III (Fig.1) is believed to be a substrate

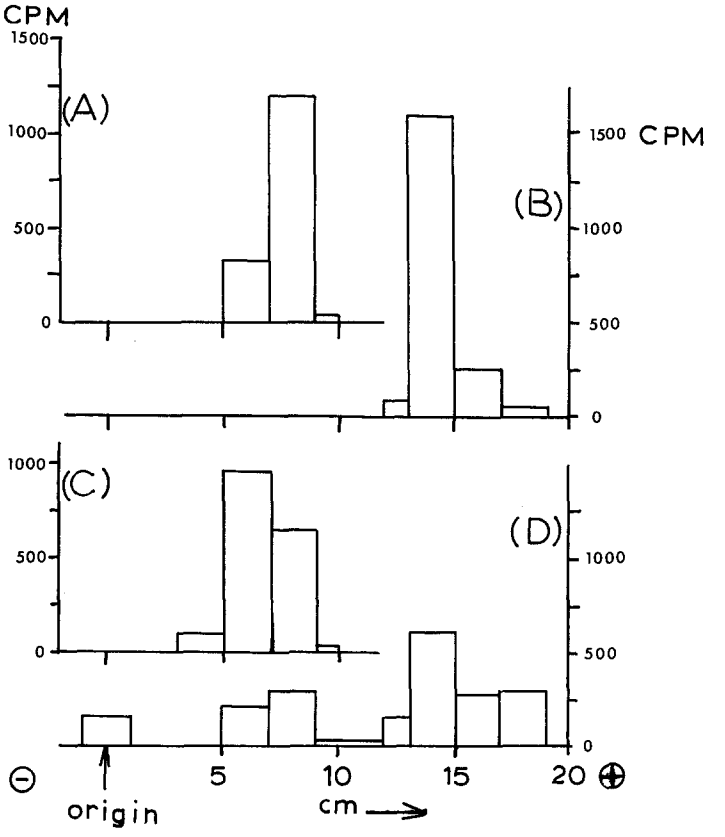

Figure 2. High voltage electrophoretograms (pH 6.5) were cut into sections and counted. The four patterns are: (A) "Side chain-free" IgG glycopeptide acetylated with acetic anhydride-1-[^{14}C]. (B) The [^{14}C]-acetyl "side chain-free" IgG glycopeptide after digestion with endo-β-N-acetylglucosaminidase. (C)[^{14}C]-fucose labelled glycopeptide product after acetylation with non-radioactive acetic anhydride. (D) Acetylated [^{14}C]-fucose glycopeptide after digestion with endo-β-N-acetylglucosaminidase.

for this enzyme. AGP glycopeptide treated with β-N-acetylglucosaminidase was found to have only 9% of the activity of the untreated material; thus, structure I (Fig.1) is inactive as an acceptor whereas structure II (Fig.1) is active. Definitive proof of the latter conclusion has been obtained by the use of glycopeptides analogous to structures I and II (Fig.1) prepared from human multiple myeloma IgG (J.R. Wilson and H. Schachter, in preparation).

Product identification. Figure 2 shows the results of endo-β-N-acetylglucosaminidase digestion of radioactive glycopeptide product. It is apparent that digestion yields a radioactive product which is negatively charged and smaller than the original acetylated glycopeptide; this product is probably peptide-Asn-GlcNAc-[^{14}C]-Fucose (15). There is minimal radioactivity at the origin where the oligosaccharide moiety would be expected. Treatment of product with either mild acid hydrolysis or α-L-fucosidase resulted in the release of app. 60 % of the radioactivity as radioactive fucose.

DISCUSSION

The data presented in this paper shows that the previously-described rat liver GDP-fucose:β-N-acetylglucosaminide fucosyltransferase (1,2) transfers fucose only to acceptors with a terminal GlcNAc residue but little or no fucose becomes attached to this terminal residue; this is shown by the relative absence of radioactivity in the neutral oligosaccharide region after endo-β-N-acetylglucosaminidase cleavage. Rather, the fucose remains with the Asn-GlcNAc moiety after endoglycosidase cleavage. Several glycoproteins have been shown to have a fucose residue attached to the asparagine-linked GlcNAc (5-10) and it appears that the GDP-fucose:β-N-acetylglucosaminide fucosyltransferase is the enzyme responsible. These results together with recent studies from this laboratory on N-acetylglucosaminyltransferases in Chinese hamster ovary cells (30,31) indicate that the attachment of a GlcNAc residue to a mannose in the $(Man)_3(GlcNAc)_2$-Asn core of Asn-GlcNAc-type

glycoproteins is an essential pre-requisite for both the attachment of a fucose residue to the internal GlcNAc residue and for the attachment of a second GlcNAc residue to the core (Figure 1). The attachment of the internal fucose is therefore a late step in the assembly process and this is compatible with the fact that the fucosyltransferase carrying out the process is a Golgi-situated enzyme (2). It is obvious that any interference with the incorporation of the first external GlcNAc residue into the core, as occurs, for example, in a lectin-resistant mutant line of Chinese hamster ovary cells (31), will prevent fucose incorporation and the addition of the sialyl-galactosyl-N-acetylglucosamine trisaccharide commonly found in Asn-GlcNAc-type glycoproteins.

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