Partial Structure of a Membrane Glycopeptide from Virus-Transformed Hamster Cells†

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ABSTRACT: The predominant surface glycopeptide from a clone of baby hamster kidney cells transformed by Rous sarcoma virus (C_{13}/B_4), metabolically labeled with L-[^{14}C]fucose, has been characterized for the first time. This glycopeptide represents 19% of the total radioactivity removed by trypsin from the cell surface of the transformed fibroblasts and is more abundant in the transformed cells than in the normal counterpart. Purification of the glycopeptide after digestion with Pronase was by successive chromatography on DEAE-cellulose and Sephadex G-50. The monosaccharide content of the glycopeptide was 42, 127, 138, 114, and 243 nmol of fucose, sialic acid, galactose, mannose, and glucosamine, respectively. A partial structure of the glycopeptide was proposed from the results of sequential enzymatic degradation coupled with gas–liquid chromatographic analysis of the resultant monosaccharides. All of the enzymes used were purified and pretested on natural substrates and found to remove terminal monosaccharides of the correct configuration, quantitatively. The purification and properties of an α-L-fucosidase from rat testes were described. All of the radioactivity in the glycopeptide, recovered as fucose, was present at the core and was removed by treatment with this α-L-fucosidase. The proposed structure is a triantennary, completely sialylated, complex glycopeptide containing a core region of β-D-mannose, β-D-*N*-acetylglucosamine, and α-L-fucose.

The role of oligosaccharide specificities in membrane phenomena has been amply documented and reviewed (Hughes, 1976; Glick, 1976b; Poste & Nicholson, 1977; Glick & Flowers, 1978); however, with the exception of the erythrocyte (Marchesi et al., 1976), there is a paucity of structural information concerning membrane glycoproteins from mammalian cells. In part this is due to the complex nature of the membranes and the problems of isolating sufficient material for detailed analysis.

Sequential enzymatic degradation with the appropriate purified exoglycosidases, when coupled with quantitation of the released monosaccharides, yields considerable information about the structure of oligosaccharides or glycopeptides (Kornfeld & Kornfeld, 1976; Muramatsu, 1976; Li & Li, 1977; Flowers & Sharon, 1978). This methodology, using exoglycosidases specific for a single anomeric configuration of a particular monosaccharide, circumvents the small quantities of purified membrane glycopeptides which are available. Partial structures of a few glycopeptides have recently been described [Kornfeld, 1978; see Glick & Flowers (1978) for a review], but as yet none have been reported from purified glycopeptides of tissue-culture cells.

It is possible to remove the glycopeptides which are representative of the glycoproteins of the surface membrane by treating whole cells with trypsin. After subsequent digestion with Pronase, these glycopeptides can be separated into distinct groups (Glick, 1979). A comparison of these glycopeptides from virus-transformed cells to those of their normal counterparts has revealed that some of these glycopeptides are altered in the transformed cells (Glick, 1974a; Warren et al., 1978). We have begun a detailed study of these glycopeptides (Glick, 1974a, 1979) and report here the isolation, purification, and partial structure of one of the major glycopeptides from the surface of a clone of virus-transformed baby hamster kidney fibroblasts, C_{13}/B_4.

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Materials and Methods

Cell Growth and Harvest. Low-passage (10–11) baby hamster kidney fibroblasts, transformed by the Bryan strain of Rous sarcoma virus (C_{13}/B_4), were grown to the upper logarithmic phase in the presence of 125 μCi of L-[1-^{14}C]fucose (50.8 mCi/mmol; New England Nuclear Corp.) for 72 h, and 2.5×10^8 cells were harvested from five Bellco roller bottles (654.51-cm^2 growth area). The harvesting was done by controlled trypsinization, and the material removed from the cells with trypsin (trypsinate) was processed and lyophilized (Glick, 1979).

Purification of Glycopeptides. Three lyophilized radioactive trypsinates from a total of 7.7×10^8 cells grown under similar conditions were combined and digested exhaustively with Pronase (Calbiochem), precipitated with 5% trichloroacetic acid, dialyzed, and lyophilized as described (Glick, 1979). Columns of DEAE-cellulose (1.3 × 22.5 cm) and of Sephadex G-50 (2.5 × 120 cm) were used to separate the fucose-containing glycopeptides with eluting agents as described (Glick, 1979). Aliquots were assayed for radioactivity, and fractions were combined according to radioactivity.

Sequential Degradation of TG-V. Table I lists the enzymes used and the sources thereof. Each enzyme was added stepwise to the purified glycopeptide which will be designated TG-V. All enzyme incubations were carried out on lyophilized material, resuspended to a total volume of 0.2 mL in an atmosphere of toluene at 37 °C. The following conditions for degradation of the glycopeptide were chosen on the basis of pretests on natural substrates to maximize release of terminal monosaccharides while minimizing hydrolysis of the radioactive fucose from the glycopeptide. These conditions did not necessarily reflect the pH optima of the enzymes.

β-D-Galactosidase and β-D-*N*-acetylhexosaminidase, 0.1 and 2 units, respectively, were added in 0.05 M citrate buffer, pH 5.0, in the presence of 0.2 mg of bovine serum albumin (Pentex grade, Miles Laboratories). A subsequent addition of 20% of the original amount of enzyme was made after 48 h and the reaction was terminated at 72 h. α-D-Mannosidase, 2 units, was added to 0.05 M citrate buffer, pH 5.0, and the reaction mixture was incubated for 48 or 72 h. β-D-Mannosidase (0.015 unit) was added in 0.05 M citrate buffer, pH 4.0, and the reaction mixture was incubated for 16 h. α-L-Fucosidase, 2.5

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Table I: Characteristics of Enzymes Used in Sequential Enzymatic Degradation of TG-V

enzyme	source	sp act.[a]	natural substrates	contaminants[b]	prepn method
α-L-fucosidase	rat testes	16	LNF I, II, and III[c] membrane trypsinates	none	Materials and Methods
β-D-*N*-acetyl-hexosaminidase	porcine epididymis	176	fetuin ovalbumin	<0.01% β-galactosidase <0.005% α-mannosidase	Findlay & Levvy (1960)
α-D-mannosidase	jack bean meal	9.6	fetuin ovalbumin	<0.1% β-*N*-acetyl-glucosaminidase	Li & Li (1968) Snaith & Levvy (1968)
β-D-galactosidase	suckling rat ileum	38	fetuin lacto-*N*-tetraose[c] lacto-*N*-neotetraose[c]	<0.1% β-*N*-acetyl-glucosaminidase	*d*
β-D-mannosidase[e]	*Polyporus sulfureus*	4.2	cores of ovalbumin Taka amylase A and α_1 acid glycoprotein	2% α-galactosidase	Wan et al. (1976)

[a] Micromoles of *p*-nitrophenol released per minute per milligram of protein from the appropriate *p*-nitrophenyl glycoside at the optimal pH. [b] Unless otherwise noted, the following *p*-nitrophenyl glycosides were not hydrolyzed in 64 h with 2 times the amount of enzyme used on TG-V: α- and β-D-galactoside, α- and β-D-*N*-acetylglucosaminide, α- and β-D-mannoside, α- and β-D-glucoside, and α-L-fucoside. The conditions used would detect 0.5 nmol of PNP. No glycosyl asparaginase was noted in any of the enzymes. [c] The [^{3}H]sorbitol derivatives (Kobata & Ginsburg, 1972). [d] The enzyme was obtained from Drs. Palmeiri and O. Koldovsky.[2] [e] The enzyme and data were from Wan et al. (1976).

units, was added to 0.05 M citrate buffer, pH 5.8, and the reaction mixture was incubated for 96 h.

All incubation mixtures were terminated by heating at 100 °C for 3 min and centrifuged at 1100*g*, and the supernatant solution was removed. The precipitate was washed with 0.3 mL of water, and the supernatant solutions were combined and applied to a column (1.0 × 20 cm) of Bio-Gel P-2 (Bio-Rad) or a column (1 × 80 cm) of Fractogel PGM 2000 (E. M. Labs, Inc.) previously calibrated for the elution of mono- and disaccharides. The column was eluted with water and aliquots of each fraction were tested for radioactivity. Any material which was eluted in the monosaccharide region was pooled, lyophilized, and analyzed by gas–liquid chromatography. Higher molecular weight fractions containing radioactivity were pooled, lyophilized, and subsequently incubated with the next enzyme.

When sialic acid was removed from the total glycopeptide, TG-V was dissolved in 1 mL of 0.1 N H_2SO_4, heated at 80 °C for 1 h, and diluted with 4 volumes of water, and the sialic acid was separated from the bulk glycopeptide by eluting from a column (0.8 × 2 cm) of Dowex 1-X8 with 0.3 N formic acid. Less than 1% of the total radioactivity was recovered in this fraction. The remaining glycopeptide was recovered in the water wash of the column and, after lyophilization, used for the subsequent removal of monosaccharides by enzyme degradation.

Quantitation of Monosaccharides. To determine the carbohydrate composition of TG-V, aliquots were hydrolyzed in duplicate with 0.02 N HCl in the presence of Dowex 50 H^+. The neutral monosaccharides and hexosamines were quantitated by gas–liquid chromatography (Hewlitt-Packard Model 5830 A) as alditol acetate derivatives, using 2-deoxyglucose and allosamine as the internal standards (Glick, 1974b). The monosaccharides released from each step of sequential enzymatic degradation were also quantitated by gas–liquid chromatography. The sialic acid content of TG-V was determined by a microadaptation of the thiobarbituric acid assay after hydrolysis of duplicate aliquots with 0.1 N H_2SO_4 for 1 h at 80 °C (Glick, 1974b). Aliquots of sialic acid released from the bulk glycopeptide were also quantitated by this procedure. A correction factor, introduced after each enzymatic treatment, was based on the amount of radioactivity recovered in the partially degraded glycopeptide. This was possible since no contaminating α-L-fucosidase was detected in any of the enzymes and little or no radioactivity was found in the released monosaccharide fractions. The specific activity of fucose was determined by collection of the radioactivity through a stream-splitting device on the gas–liquid chromatograph. All radioactivity was recovered with the retention time of fucose.

Enzymes. Unless otherwise noted, enzymes were assayed with PNP[1] glycoside substrates (Research Products International and Sigma). One unit of enzyme was that amount which released 1 μmol of *p*-nitrophenol per min. The specific activities and other characteristics of all the enzymes are given in Table I. The enzymes were assayed for contaminants at both the conditions used for degradation of TG-V and at the presumed pH optimum of the contaminants, by using 2 times the amount of enzyme used in sequential enzymatic degradation. Radioactive natural substrates were also used to ascertain the absence of α-L-fucosidase activity in the other enzymes. None of the enzymes showed contamination with other proteins when 20 μg or more of protein was examined by $NaDodSO_4$–polyacrylamide gel electrophoresis (Laemmli, 1970).

β-D-Galactosidase was prepared by affinity chromatography from the ileum of suckling rats.[2] β-D-*N*-Acetylhexosaminidase was purified from porcine epididymis by the method of Findlay & Levvy (1960). Like other mammalian enzymes purified by their ability to hydrolyze PNP-β-D-*N*-acetylglucosaminide, the enzyme also hydrolyzes PNP-β-D-*N*-acetylgalactosaminide. In addition to the characteristics listed in Table I, there is slight release of *p*-nitrophenol (10 nmol) from PNP-α- and -β-glucoside and PNP-α-galactoside after 64-h incubation. The enzyme from the same source has been used for sequential enzyme degradation of several glycoproteins (Spiro, 1972).

α-Mannosidase from jack bean meal (A. H. Thomas Co.) was extracted by the method of Li & Li (1968) and then purified according to Snaith & Levvy (1968). The treatment with pyridine was repeated, resulting in a preparation with high specific activity (Table I). The properties of jack bean α-mannosidase have been extensively described (Li & Li, 1968, 1977; Tai et al., 1975). The β-mannosidase[3] used has been characterized (Wan et al., 1976).

[1] Abbreviations used: PNP, *p*-nitrophenyl; $NaDodSO_4$, sodium dodecyl sulfate; LNF I, lacto-*N*-fucopentaose I; LNF II, lacto-*N*-fucopentaose II; LNF III, lacto-*N*-fucopentaose III; GlcNAc-Asn, 2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose.

[2] M. Palmeiri and O. Koldovsky, unpublished experiments.

[3] This preparation of β-mannosidase included a final passage through hydroxyapatite, which freed it of the contaminating endoglycosidase.

Purification of α-L-Fucosidase. α-L-Fucosidase was purified from testes of mature male rats, removed immediately after killing, and stored in 30% glycerol at −40 °C. The covering membrane and adhering blood vessels were removed. The initial steps in the purification followed the method of Carlsen & Pierce (1972) for the purification of α-L-fucosidase from rat epididymis. The testes were homogenized in a Waring blender for 2 min at 0 °C in 0.1 M sodium acetate buffer, pH 6.0, containing 0.1 M NaCl. The homogenate was incubated at 37 °C for 1 h and then adjusted to pH 5.5 with 4 N HCl and heated at 60 °C for 10 min. The pH of the solution was adjusted to pH 6.4 with 4 N NaOH, and the precipitate was removed by centrifugation.

The crude extract was precipitated with ammonium sulfate and the material which precipitated between 35 and 50% saturation was further purified by chromatography on CM-cellulose as described by Carlsen & Pierce (1972). The fractions containing α-L-fucosidase were pooled, precipitated with 70% ammonium sulfate, dissolved in 0.01 M NaH_2PO_4 buffer, pH 5.5, and 0.02% sodium azide, and dialyzed against the same buffer for 20 h. The dialyzed material was applied to a column (0.35 × 12.5 cm) of agarose–ε-aminocaproyl-fucosamine (Miles Laboratories). The α-L-fucosidase, which was eluted with 50 mM fucose (Alhadeff et al., 1975), was precipitated with 70% ammonium sulfate, redissolved in 0.1 M citrate buffer, pH 6.0, and dialyzed extensively against the same buffer. If the enzyme was used to release fucose for quantitation, additional purification was by filtration on a column (1 × 25 cm) of Sephadex G-100 (Pharmacia) before dialysis. The enzyme was stored at 4 °C and was stable for more than 6 months. The conditions used for gradient polyacrylamide slab gel electrophoresis in $NaDodSO_4$ (Laemmli, 1970) and isoelectric focusing of the purified enzyme before and after treatment with *Vibrio cholerae* neuraminidase (Calbiochem) have been described (Turner et al., 1975).

Action of α-L-Fucosidase on Milk Oligosaccharides. The sodium borotritide reduced products of the milk oligosaccharides lacto-*N*-fucopentaose I, lacto-*N*-fucopentaose II, lacto-*N*-fucopentaose III, and lacto-*N*-tetraose (Kobata & Ginsburg, 1972) will be referred to as LNF I, LNF II, LNF III, and LNT, respectively. The enzyme, 0.05 M citrate buffer, pH 5.2, and radioactive milk oligosaccharide were incubated at 37 °C in a capillary pipet. At the end of the incubation period, the mixture was separated by descending chromatography as described (Nishigaki et al., 1974). The chromatogram was cut into 1-cm strips and counted in a scintillation counter. The percentage of fucose released was equivalent to the total radioactivity migrating with LNT over the total radioactivity recovered.

Action of α-L-Fucosidase on Membrane Glycopeptides. Radioactive glycopeptides were prepared from cells labeled metabolically with L-[^{14}C]- or L-[^{3}H]fucose (Glick, 1979). An aliquot of glycopeptides from both sources was made 0.05 M with respect to citrate buffer, pH 5.8, and the enzyme was added. The mixture was incubated in an atmosphere of toluene at 37 °C. Aliquots were removed at various times, and radioactive fucose was recovered from a precalibrated column (0.9 × 20 cm) of Bio-Gel P-2. Aliquots taken at zero time served as controls.

Results

Purification and Characterization of TG-V. The Pronase-digested radioactive fucose-containing glycopeptides from C_{13}/B_4 trypsinates were separated on DEAE-cellulose as shown in Figure 1. The material which was eluted with 21–26 mM sodium phosphate buffer, pH 6.8 (fractions 83–103), was combined, dialyzed, and lyophilized prior to rechromatography on a column of DEAE-cellulose. Fractions which were eluted from the second column of DEAE-cellulose with 24–25 mM phosphate buffer, pH 6.8 (fractions 94–103), were combined, dialyzed, and lyophilized. This latter radioactive fraction was further chromatographed over Sephadex G-50 and was eluted as a single peak between fractions 26 and 35 (Figure 2). After dialysis and lyophilization, this radioactive material was used for sequential enzyme degradation. The designation TG-V was used to mark the fifth glycopeptide from the transformed fibroblasts which was eluted from DEAE-cellulose (Figure 1). Additional characteristics concerning the purity of this material have been described (Glick, 1979).

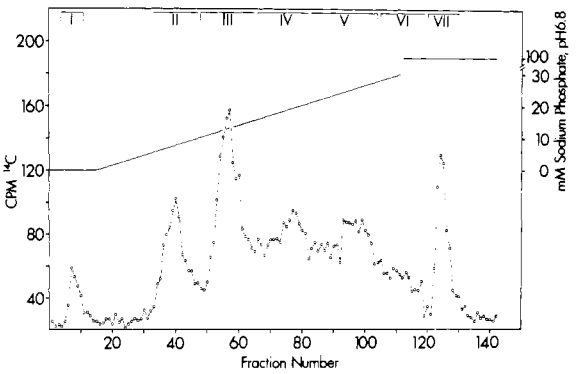

FIGURE 1: Chromatography on DEAE-cellulose of Pronase-digested trypsinates from C_{13}/B_4 cells metabolically labeled with L-[^{14}C]fucose. The glycopeptides were eluted from the column with increasing molarity of sodium phosphate buffer, pH 6.8, and were combined as shown, areas I–VII, according to radioactivity. Area V was the material used for further purification. All details are described under Materials and Methods.

The total radioactivity recovered in the glycopeptide fraction was 110550 cpm and represented 19% of the total radioactivity in the starting trypsinates. The trypsinates of C_{13}/B_4 cells contain 20% of the radioactivity of the whole cells when radioactive fucose was the glycoprotein precursor (Glick, 1979). The fucose content of TG-V was 42 nmol, yielding a specific activity of 2630 cpm/nmol. The sialic acid content was 127 nmol and represented 25% of the total sialic acid in the trypsinate and 5% of the sialic acid content of the whole cells (Glick, 1979). The mannose, galactose, and glucosamine contents of TG-V were 114, 138, and 243 nmol, respectively. In addition, 220 nmol of glucose was detected but was not considered as components of TG-V because of the purification procedures. The molar ratios of fucose/sialic acid/mannose/galactose/glucosamine found in TG-V were 1:3.0:2.7:3.3:5.8.

Sequential Degradation of TG-V. The results of the degradation of TG-V are shown in Table II. The glycopeptide was treated with β-D-galactosidase and β-D-*N*-acetylhexosaminidase, and no detectable monosaccharides were released under conditions which would detect 1 nmol. After this incubation, intact TG-V was recovered in the void volume of Bio-Gel P-2. Sialic acid (102 nmol) was released by mild acid hydrolysis and was equal to 3 molar equiv of fucose. Subsequent incubation of TG-V with β-D-galactosidase and β-D-*N*-acetylhexosaminidase released 3 molar equiv each of galactose and *N*-acetylglucosamine (Table II). No further monosaccharides were released by a second incubation with the same enzymes. TG-V was recovered from Bio-Gel P-2 where it was included just within the gel (Figure 3). The next incubation was with α-D-mannosidase, releasing 1.6 molar equiv of mannose. After reincubation with β-D-galactosidase

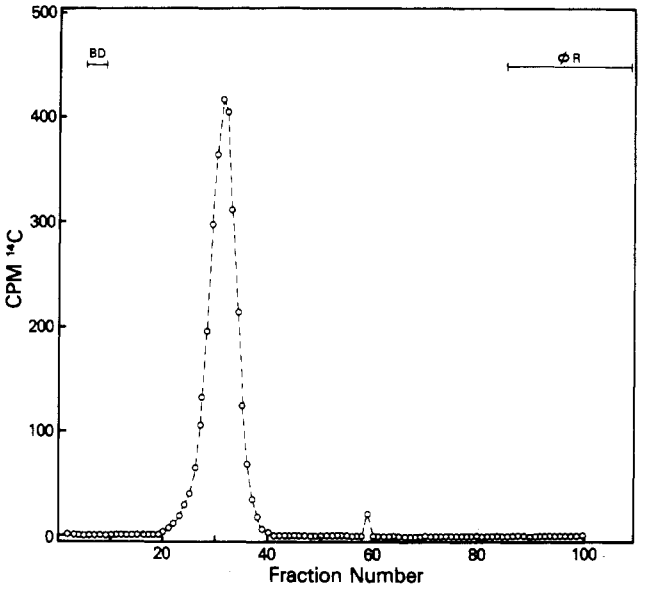

FIGURE 2: Further purification of glycopeptides by filtration on Sephadex G-50. The radioactive fucose containing glycopeptides from area V, Figure 1, after an additional purification over DEAE-cellulose (see Results), were chromatographed on Sephadex G-50. Fractions 26–35 were combined, dialyzed, and lyophilized, and this material was designated TG-V. BD, Blue Dextran 2000, and ϕR, phenol red, served as markers. All details are described under Materials and Methods.

Table II: Sequential Removal of Monosaccharides from TG-V

	nmol recovered				
	NeuAc	Gal	GlcNAc	Man	Fuc
intact glycopeptide[a]	106	116	204	96	35
treatment					
(1) β-D-galactosidase and β-D-*N*-acetylhexosaminidase		0	0	0	0
(2) 0.1 N H_2SO_4, 1 h, 80 °C	102				
(3) repeat (1) 2 times		97	101	0	2
(4) α-D-mannosidase		0	0	56	0
(5) repeat (3) and (4)		0	18	0	0
(6) β-D-mannosidase		0	0	19	0
(7) repeat (6) and β-D-*N*-acetylhexosaminidase		0	0	12	0
(8) α-L-fucosidase					
(a) from higher molecular weight peak		0	0	0	24.5
(b) from lower molecular weight peak		0	0	0	14.5

[a] Actual material used for sequencing after removal of aliquots for carbohydrate analysis.

and β-D-*N*-acetylhexosaminidase, followed again by α-D-mannosidase, less than 1 molar equiv of *N*-acetylglucosamine was recovered. The remaining mannose residues (1 molar equiv) were released by two successive incubations with β-D-mannosidase; the second incubation contained β-D-*N*-acetylhexosaminidase, but no detectable *N*-acetylglucosamine was released (Table II).

The remaining radioactive material eluted from Bio-Gel P-2 in two areas, one just included in the gel, M_r <2000, and the other in the region of an oligosaccharide marker, M_r 855 (Figure 3). The lower molecular weight material constituted 64% of the total radioactivity and was collected separately. An aliquot of this material was rechromatographed on Fractogel PGM 2000, where it was eluted again as a single peak in the region of the marker, M_r 855. After treatment of this core material with α-L-fucosidase, all detectable radioactivity was recovered as monosaccharide. Treatment of the higher molecular weight core with α-L-fucosidase also resulted in the recovery of 95% of the radioactivity as monosaccharide. The released radioactivity from both fractions was characterized as fucose by gas–liquid chromatography. The fucose from the smaller glycopeptide had a specific activity of 3440 cpm/nmol, while that of the larger glycopeptide had a specific activity of 1740 cpm/nmol. The summation of the specific activities calculated from both fractions was 2830 cpm/nmol, comparable to the specific activity of the starting material. The material which remained was nonradioactive and was not recovered.

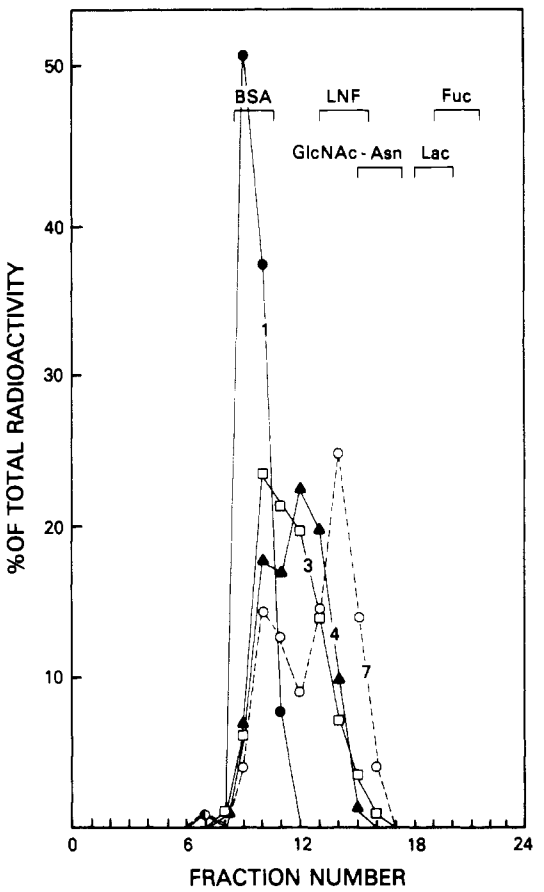

FIGURE 3: Bio-Gel P-2 gel filtration of TG-V and the radioactive products of sequential enzymatic degradation. Products of enzymatic digestion were eluted from Bio-Gel P-2 with water. Radioactivity was determined on aliquots of each fraction and was recorded as the percent of the total recovered after each enzyme treatment. The column separations are after the following enzyme treatments (see Table II): (1, ●) β-D-galactosidase and β-D-*N*-acetylhexosaminidase on intact TG-V; (3, □) β-D-galactosidase and β-D-*N*-acetylhexosaminidase after desialyzation; (4, ▲) α-D-mannosidase; (7, ○) second β-D-mannosidase treatment, including β-D-*N*-acetylhexosaminidase, the last treatment before removal of fucose. The following are the marker substances, shown in brackets: BSA, bovine serum albumin; LNF, lacto-*N*-fucopentaose reduced with sodium [^{3}H]borohydride; GlcNAc-Asn, 2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose; Lac, lactose; Fuc, fucose. Glucose, mannose, galactose, and *N*-acetylglucosamine comigrated with fucose. See Materials and Methods for details.

Partial Structure of TG-V. The results of the sequence analysis were compatible with the partial structure for TG-V given in Figure 4. The release of molar equivalents of fucose and mannose by α-L-fucosidase and β-D-mannosidase, respectively, after 3 molar equiv of β-*N*-acetylglucosamine, galactose, and sialic acid suggested the triple-branched structure. Since only 2 equiv of mannose was released by α-mannosidase treatment, two branches were attached to one

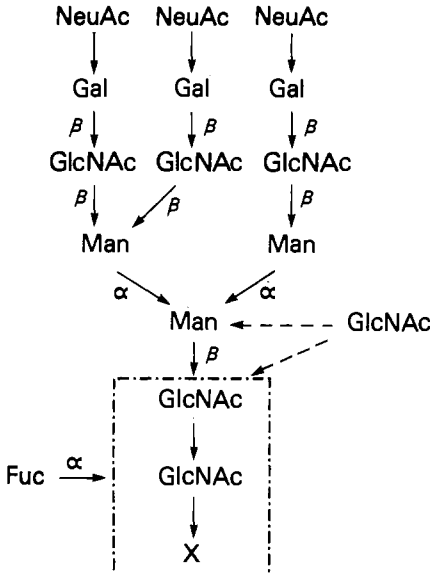

FIGURE 4: Proposed partial structure of TG-V.

α-linked mannose. The partial release of *N*-acetylglucosamine after α-mannosidase treatment suggested an additional *N*-acetylglucosamine near the core region and is therefore shown as a dotted line. Attempts to release *N*-acetylglucosamine before the release of fucose suggested that fucose could be positioned on the *N*-acetylglucosamine next to β-mannose, and therefore, fucose is shown outside a dotted line. Alternate structures are compatible with data (Table II); however, all include a three-branched structure with fucose at the core.

Verification of Methodology on Substrates of Known Structure. Trial experiments on the complex glycopeptide of fetuin, ovalbumin glycopeptides, [^{3}H]sorbitol derivatives of milk oligosaccharides, and mixed membrane glycopeptides had demonstrated the suitability of these enzymes for sequential degradation. The results of these experiments showed that the enzymes: (1) removed only the monosaccharides predicted; (2) gave quantitative release of these monosaccharides from the linkages tested (Table I); (3) by using substrateless control, either in single incubations or in multiple steps simulating the exact conditions of enzyme degradation, released no monosaccharides; (4) contained no contaminants which interfered with quantitation. In addition, since the monosaccharides released by sequential enzymatic degradation were quantitated by gas–liquid chromatography, any unpredicted monosaccharides would be noted.

For example, fetuin was digested with Pronase and the trichloroacetic acid soluble material dialyzed extensively against water. Only the acidic glycopeptide (Spiro & Bhoyroo, 1974) was retained in this procedure. Incubation with β-D-galactosidase, β-D-*N*-acetylhexosaminidase, and α-D-mannosidase released no detectable monosaccharides from the total glycopeptide. After removal of sialic acid by mild acid hydrolysis, incubation with β-D-galactosidase removed galactose in molar equivalents to sialic acid removed and to *N*-acetylglucosamine when β-D-*N*-acetylhexosaminidase was used either sequentially or simultaneously with the β-D-galactosidase treatment. The remaining glycopeptide core was free of galactose. Further treatment of the fetuin glycopeptide with α-D-mannosidase released 1–1.5 molar equiv of mannose.

The glycopeptide remaining after enzymatic digestion of fetuin was eluted more slowly from columns of Bio-Gel P-2 and resolved into two peaks, as did TG-V after similar enzyme treatments. The two peaks were more clearly separated on a column of Fractogel PGM 2000. Rechromatography showed that each peak retained its original elution profile. Both peaks of fetuin core had similar carbohydrate composition (1 molar equiv of mannose to 1 molar equiv of *N*-acetylglucosamine). The larger contained an average of three amino acids when examined by an amino acid analyzer. The apparent molecular weight on Bio-Gel P-2 or Fractogel PGM 2000 was thus not indicative of the calculated molecular weight. Other model compounds showed similar anomalous behavior with water elution of the molecular sieving gels. For example, GlcNAc-Asn, M_r 319, was eluted more rapidly than lactose, M_r 342, while GlcNAc coeluted with the fucose and hexoses (Figure 3). The elution behavior of GlcNAc-Asn, acetylated by the method of Riordan & Vallee (1972), was even less representative of the molecular weight; the ^{14}C compound was eluted in the molecular weight range of more than 1200 in water but in the molecular weight range of 600 in a buffered elution system.

Kinetic experiments showed that 0.005 units of β-D-galactosidase could remove all of the galactose from 100 nmol of fetuin glycopeptide in 72 h. Both β-D-galactosidase and β-D-*N*-acetylhexosaminidase gave quantitative release with 3-day incubation, but only 83% of the available *N*-acetylglucosamine was released after 24-h incubation.

β-D-*N*-Acetylhexosaminidase and α- and β-D-mannosidase were further tested on a glycopeptide mixture obtained from ovalbumin after Pronase digestion. The sequential release of *N*-acetylglucosamine and mannose confirmed the results of others (Li, 1966, 1967; Huang et al., 1970).

Using the [^{3}H]sorbitol derivatives of lacto-*N*-tetraose and lacto-*N*-neotetraose, it was shown that the rat β-D-galactosidase hydrolyzed both Galβ1→3GlcNAc and Galβ1→4GlcNAc linkages. However, when fucose was linked to the penultimate *N*-acetylglucosamine (LNF II or LNF III), the enzyme did not remove the terminal galactose. In the latter respect, the enzyme resembled the β-D-galactosidase from jack bean meal (Arakawa et al., 1974; Li et al., 1975), *Streptococcus pneumonia* (Glascow et al., 1977), beef testes, and rat jejunum.[4]

Characteristics of the Rat Testes α-L-Fucosidase. The activity of α-L-fucosidase in the rat testes exceeded that of other glycosidases, making it a unique source of the enzyme. The relative activities of the glycosidases from rat testes and epididymis, the rat organ with the highest specific activity of α-L-fucosidase (Carlsen & Pierce, 1972; Gossrau, 1977), are contrasted in Table III. This demonstrates an advantage of obtaining the enzyme from the rat testes.

The purified enzyme moved as a single band with an apparent molecular weight of 47 000–48 000 on $NaDodSO_4$–polyacrylamide gel electrophoresis. No other proteins were apparent after staining with Coomassie blue when 30 μg of protein were applied to the gel. The testes enzyme, purified following the method of Carlsen & Pierce (1972), had a specific activity which was half that of the enzyme prepared by affinity chromatography and was contaminated with lower molecular weight proteins. Isoelectric focusing on acrylamide gels revealed two major bands of activity with p*I* values of 5.9 and 6.1 and some minor components in both the crude testes extract and the purified enzyme. The isoelectric point of the enzyme was not affected by treatments with neuraminidase. The purified enzyme had a pH maximum at pH 5.0–5.2, with a broad peak extending from pH 4.3 to 5.8. The apparent K_m on PNP-α-L-fucoside was 0.23 mM and on 4-methylumbelliferyl-α-L-fucoside was 0.06 mM.

[4] U. V. Santer and M. C. Glick, unpublished experiments.

Table III: Comparison of Exoglycosidase Activities in Extracts of Rat Tissue[a]

enzyme	testis		epididymis	
	munit/mg of protein	% relative to α-L-fucosidase	munits/mg of protein	% relative to α-L-fucosidase
α-L-fucosidase	100	100	226	100
β-D-galactosidase	10	10	517	228
α-D-galactosidase	3	3	20	9
β-D-mannosidase	6	6	100	44
α-D-mannosidase	4	4	285	126
β-*N*-acetyl-glucosaminidase	43	43	1820	805
α-*N*-acetyl-glucosaminidase	0.7	0.7	1.3	0.5
α-D-glucosidase	1.4	1.4	5	2
β-D-glucuronidase	3.2	3.2	25	11

[a] Tests were made on a crude extract as defined under Materials and Methods. Neither organ had any detectable β-D-glucosidase activity.

Table IV: Action of Rat Testes α-L-Fucosidase on Membrane Glycopeptides

membrane glycopeptide[a]	α-L-fucosidase units[b]	% radioactivity released[c]	
		18 h	3 days or more
trypsinate (M_r >25 000)	5	8	10
pronase-digested trypsinate (M_r <6000)	5	57	82
core of trypsinate (M_r <2000)	5		97
core of TG-V (M_r <1000)	1.25	43	98–100

[a] Glycopeptides, removed as trypsinates from BHK_{21}/C_{13} and C_{13}/B_4 cells metabolically labeled with L-[^{3}H]- and L-[^{14}C]fucose, respectively, were treated as described under Materials and Methods to obtain the sequentially smaller glycopeptide units. The method of preparation of the core of TG-V is described in the text. [b] Units of α-L-fucosidase per mL of incubation mixture. [c] Recovered as monosaccharide on gel filtration.

The rate of hydrolysis by α-L-fucosidase of LNF I, II, and III showed that fucose was released most rapidly from Fucα1→2Gal (LNF I), next from Fucα1→4GlcNAc (LNF II), and most slowly from Fucα1→3GlcNAc (LNF III). These differences were not large enough to differentiate the linkages in enzymatic sequencing. PNP-α-L-fucoside was hydrolyzed 10^4–10^5 times more rapidly than any of the milk oligosaccharides. LNT, a tetrasaccharide, was the end point of the enzyme activity, verifying the lack of contaminating β-D-galactosidase in the α-L-fucosidase preparation.

The hydrolysis of membrane glycopeptides by α-L-fucosidase was dependent on the oligosaccharide structure and the number of amino acids (Table IV). When glycopeptides containing many amino acids were treated with enzyme, only 10% of the radioactivity was released after 72 h. After removal of most of the amino acids by Pronase digestion, 82% of the radioactivity was released. When the oligosaccharide branches were degraded to the core region, 100% of the radioactivity was recovered as monosaccharide.

Discussion

Altered membrane glycopeptides have been considered to be the most consistent biochemical parameter correlated with virus transformation and tumorigenesis (Glick, 1976a; Van Beek et al., 1977; Warren et al., 1978). In the preceeding paper (Glick, 1979) it was demonstrated that this alteration is the increased expression of three glycopeptides in the transformed cell membrane when compared with those of the control cells. The purification and structural analysis of one of these glycopeptides, TG-V, from transformed baby hamster kidney cells is described here. TG-V was a predominant membrane component and represented a recovery of 3×10^7 molecules per membrane. The structure is triantennary and in the category of complex asparagine-linked glycopeptides.

The structure presented in Figure 4 is consistent with the data from sequential enzymatic degradation and the quantitation of the monosaccharides. The assignment of 1 mol of fucose per mol of glycopeptide was in agreement with the carbohydrate analysis of TG-V and correlated with the quantity of mannose released by β-D-mannosidase. A triantennary structure, each branch terminating in sialic acid, was clearly indicated by the data. All the galactose residues of TG-V were inaccessible to β-galactosidase until the sialic acid was removed, suggesting that all galactose units were sialylated. Since β-D-galactosidase and β-D-*N*-acetylhexosaminidase were used simultaneously, it was not proven that galactose was penultimate on these branches. However, galactose was shown to be in this position in C_{13}/B_4 surface glycopeptides by the use of the galactose oxidase/sodium borotritide procedure on neuraminidase-treated cells.[5] The *N*-acetylglucosamine residue released after, but not before, α-mannosidase treatment (Table II) might be inserted between an α-mannose and the β-mannose residue. This, however, would be a most unusual linkage (Montreuil, 1975; Kornfeld & Kornfeld, 1976), and it is more likely that the *N*-acetylglucosamine was attached to the β-mannose core, terminal, but inaccessible to enzyme action until the outer branches were fully degraded. Indeed, one β-*N*-acetylglucosamine residue linked at the C-4 position of the β-mannose residue of an ovalbumin glycopeptide has been shown to be unusually resistant to periodate oxidation (Yamashita et al., 1978). TG-V contained 6 mol of *N*-acetylglucosamine per mol of fucose, and only 4 mol was released by the sequential enzymatic degradation. The remaining *N*-acetylglucosamine was not recovered after treatment of the fucose-containing core with β-D-*N*-acetylhexosaminidase, suggesting that fucose may be linked to the *N*-acetylglucosamine adjacent to the β-mannose residue. However, in a similar system (Ogata et al., 1976) it was shown that most of the fucose remained with the protein–carbohydrate linkage after endoglycosidase D hydrolysis. Thus, it is possible that the porcine β-D-*N*-acetylhexosaminidase, like most β-D-galactosidases, cannot hydrolyze a terminal monosaccharide when fucose is attached to the penultimate *N*-acetylglucosamine. It will require the preparation of additional material to define the exact core region of TG-V.

Alternative structures were compatible with the data (Table II), but most of the reasonable alternatives cannot be distinguished by sequential enzymatic degradation alone. For example, one or more of the *N*-acetylglucosamine-containing branches could be attached to the β-mannose residue, as in the hepatic membrane binding glycoprotein (Kawasaki & Ashwell, 1976), rather than the attachment of two branches to an α-mannose as shown in Figure 4. However, TG-V was not retained by Con A–Sepharose (Glick, 1979) and the structure proposed here was consistent with this property. Most triantennary acidic glycopeptides are not bound by concanavalin A (Krusius et al., 1976) since at least two α-mannose residues with free hydroxyl groups at C-3, C-4, and C-6 are required for the interaction with the lectin (Ogata et al., 1975). Thus, lectin affinity was used to discriminate

[5] H. Flowers and M. C. Glick, unpublished experiments.

between the alternative structures just discussed and ruled out the attachment of one branch to a β-mannose residue (Lis & Sharon, 1977).

The structure for TG-V differed from that proposed for the heterogeneous group of "large glycopeptides" from BHK_{21} cells transformed by polyoma virus (Ogata et al., 1976). The assignment of a four-branched structure to the latter membrane glycopeptides was based solely on molecular weight determination by gel filtration of glycopeptides, with no quantitative recovery of the released monosaccharides. Comparisons of molecular weights by gel filtration are especially unreliable when comparing sialylated with nonsialylated compounds. Moreover, the structure was deduced from studies of glycopeptides which have been shown in Rous sarcoma virus transformed cells to be separable into at least three groups (Glick, 1979). Because of the heterogeneity of the fraction, it may be predicted that a glycopeptide with a triantennary structure such as TG-V was a major contaminant in their fraction of "large glycopeptides".

Triantennary complex glycopeptide structures have been proposed for other glycopeptides derived from the surface of mammalian cells. Among these are the acidic glycopeptide of the hepatic membrane binding protein from rabbit liver (Kawasaki & Ashwell, 1976) and the glycoprotein of vesicular stomatitis virus grown in BHK_{21} cells (Reading et al., 1978). The latter studies used lytic virus probes which do not reflect the total membrane surface glycopeptides but rather utilize the biosynthetic capabilities of the cells in which they are grown (Keegstra & Burke, 1978). Nevertheless, a number of structures have been proposed for the acidic glycopeptides from mammalian viruses, and all contained a β-mannose–chitobiose fucosylated core, with variations in the number and completeness of the extending branches (Pesonen & Renkonen, 1976; Etchison et al., 1977; Robbins et al., 1977; Keegstra & Burke, 1978; Reading et al., 1978). Two quartantennary glycopeptides were found on the calf thymocyte plasma membrane in addition to a triantennary glycopeptide (Kornfeld, 1978). There was no evidence for a glycopeptide like TG-V, but this is not surprising as only small amounts may be found on a nontumorigenic cell. It remains to be shown if these variations of branching and monosaccharide components will account for some of the diverse biological phenomena attributed to the surface-membrane glycoproteins.

After each step of the sequential enzymatic degradation, the released monosaccharides were separated by column chromatography. The elution pattern of the subsequent glycopeptide was consistent with the stepwise removal of oligosaccharide units. The difficulty of sizing small, charged molecules by gel filtration is well-known (Brunngraber, 1972; Glick, 1979), and it is stressed that the marker compounds indicated in the elution patterns shown in Figure 3 defined only the characteristics of the column. Water elution of columns was used in these experiments since it eliminated any possibility of interference with the analysis of monosaccharides by gas–liquid chromatography. With successive enzyme treatments, greater retardation of the remaining glycopeptide was apparent, paralleling successive decreases in molecular weight. A portion of the remaining glycopeptide, however, even after removal of all the mannose residues (Figure 3, step 7), behaved like a somewhat larger molecule and was included just within the gel. Each of these two peaks, rechromatographed, behaved as a single peak, suggesting that separation on these columns was real. The same phenomenon was seen with the sequential enzymatic degradation of fetuin, a molecule with a homogeneous oligosaccharide structure (Spiro, 1973). This was shown to be due to differences in amino acid residues, in accord with the results obtained by Spiro (1962). However, the discrepancy between the calculated molecular weight and the apparent molecular weight by water elution of Bio-Gel P-2 or Fractogel PGM 2000 was quite clear. When the core molecules from fetuin were rechromatographed with 0.05 M ammonium acetate as eluant, the elution position more closely approximated the molecular weight, by using glycopeptide and oligosaccharide markers to calibrate the column. The elution behavior of the products of sequential enzymatic degradation from gel permeation columns with low exclusion limits is not usually reported, and it may be that any glycopeptide present at multiple sites on amino acid chains will show behavior similar to the TG-V glycopeptide.

TG-V, due to the original isolation procedure, came from a variety of external membrane glycoproteins, so it will not be surprising if different amino acids will be found near the oligosaccharide-linkage region. In fact, it has been shown that the glycopeptides characteristic of transformed cells were found on several partially purified glycoproteins therefrom (Van Nest & Grimes, 1977; Tuszynski et al., 1978). The fact that TG-V was from a number of membrane glycoproteins could also account for the difference in specific activity of the fucose recovered after α-L-fucosidase treatment of the two fractions (Table II, step 8). Modification of the trypsinization procedure to yield two classes of glycopeptides, from glycoproteins more and less loosely associated with the membrane, showed that these two groups contained fucose molecules with specific activities which differed by a factor of 10 or more[6] (Glick & Santer, 1978). Glycopeptides in different positions on the cell membrane may come from different pools or have different turnover rates. Although it may be coincidental, the populations of TG-V which probably had fewer amino acids attached after Pronase digestion contained fucose with a higher specific activity, and furthermore, preliminary results showed the same correlation for other glycopeptides from C_{13}/B_4.

Glycosidic enzymes are exquisitely specific for the target monosaccharide and anomeric linkage, although there is, in some cases, a lack of specificity for the linkage position on the adjacent monosaccharide. This latter characteristic is an advantage in sequential enzymatic degradation of small amounts of glycopeptides when the methods are dependent on quantitative recovery of monosaccharides. It is thus possible to obtain partial structures of oligosaccharide units which are available only in nanomole quantities and heretofore could not be defined.

Added in Proof

A recent report [Baenziger, J. U., & Fiete, D. (1979) *J. Biol. Chem. 254*, 789] that nonmammalian β-D-galactosidases failed to release 100% of the galactose from desialylfetuin glycopeptide illustrates the advantage of using mammalian glycosidases.

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[6] A. Fischer and M. C. Glick, unpublished experiments.

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