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AN UNCOMMON FUCOSYL LINKAGE IN SURFACE MEMBRANES OF HUMAN NEUROBLASTOMA CELLS

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SUMMARY

The surface membranes of human neuroblastoma cells contain a fucosyl linkage, defined by using an α-L-fucosidase from almond emulsin specific for the cleavage of Fucα1→3GlcNAc and Fucα1→4GlcNAc. These linkages are not found in significant amounts on the surface of mouse neuroblastoma cells, or human or hamster fibroblasts. The enzyme released fucose from glycoproteins as well as glycopeptides, making it particularly useful for in vivo studies.

Carbohydrate components unique to the surface of particular cells can be defined by utilizing the exquisite specificities of certain glycosidases. Continuing our studies on the membranes of human neuroblastoma cells (1,2), we have utilized an α-L-fucosidase from almond emulsin. This enzyme, originally described by Kobata and his colleagues (3,4), will cleave L-fucose linked α1→3 or α1→4 to GlcNAc, but not Fucα1→2Gal, Fucα1→6GlcNAc, or the usual synthetic fucosides (3). With the enzyme we have demonstrated a fucosyl linkage in the membrane glycoproteins from human neuroblastoma cells (5), which was not present to a significant extent in the membranes of other cell types examined.

METHODS AND MATERIALS

Cell culture and harvest. Details of cell culture and harvest have been described for human neuroblastoma cells CHP-134 (2), and IMR-5 (6), mouse neuroblastoma clonal cell lines NIE-115, N-18, and N-1 (7,8), human skin fibroblasts CF-10 and C-16 (9), hamster fibroblasts BHK_{21}/C_{13} and the RSV-transformed clone C_{13}/B_4 (10); and human skin fibroblasts CHP-134-F, which were from the patient with the neuroblastoma tumor, CHP-134 (9). Forty-eight hours before harvest, the cells were made radioactive by growth in L-[^{3}H]- or -[^{14}C]fucose added in fresh medium at 5 μCi/75 cm^2 flask.

Preparation of cell fractions. Radioactive glycopeptides were removed from the cell surface with trypsin under conditions of maintaining cell viability (10). The glycopeptides were then digested exhaustively with Pronase (10), passed over Biogel P-2 in water and the material in the void volume lyophilized. Glycoproteins were obtained by homogenizing the remaining cells in PBS[1] containing

[1] PBS, 0.01 M sodium phosphate, pH 7.0, in 0.14 M NaCl; LNF II, lacto-N-fucopentaose II; LNF III, lacto-N-fucopentaose III; MP-NeuAc, 2-(3-methoxyphenyl)-N-acetyl-α-D-neuraminic acid.

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0.1% Triton X-100, centrifuging at 1000xg for 5 min and passing the supernatant material over Sephadex G-100 in PBS containing 0.02% Triton X-100. The highest molecular weight material was concentrated with polyethylene glycol 20,000.

Preparation of α-L-fucosidase. α-L-Fucosidase I from almond emulsin was isolated and assayed as described (3), with the exception that the second purification was on Sephacryl S-200. One unit of enzyme was defined arbitrarily as that amount which releases 50% of the fucose from LNF II[1] or LNF III[1] in 1 h at a 1:6 dilution. The purification of the α-L-fucosidase from rat testes has been described (11).

Release of fucose by almond emulsin α-L-fucosidase. Membrane glycopeptides or cell glycoproteins, metabolically labeled with fucose were incubated with 2 U of almond emulsin α-L-fucosidase in 0.1 M Na_2HPO_4, 0.05 M citric acid pH 5.0 for 24 h or 72 h. Water (100 µl) was added and the incubation mixture boiled and applied to a precalibrated column of Biogel P-2 or Sephadex G-100, as appropriate. The radioactivity of each fraction was determined and the percentage radioactivity which eluted in the position of monosaccharides was calculated. Controls were glycopeptides or glycoproteins incubated with boiled enzyme. To verify that the released radioactivity was fucose, ^{3}H-labeled-glycopeptides from CHP-134 cells (10,000 cpm) were incubated with excess almond emulsin α-L-fucosidase for 72 h and the entire incubation mixture chromatographed on Whatman #1 cellulose using Gal, Man, Glc, GlcNAc and Fuc as monosaccharide markers (12). All radioactivity which migrated away from the origin comigrated with fucose under conditions which separated all the monosaccharide markers.

Materials. L-[^{3}H]fucose, 4.3 or 50 Ci/mmol, or L-[^{14}C]fucose, 50.8 mCi/mmol were obtained from New England Nuclear Corp. Trypsin, 3X crystallized, and soy bean trypsin inhibitor were from Worthington Biochemicals. Vibrio cholerae neuraminidase was from Boehringer, Arthrobacter ureafaciens neuraminidase was from E.Y. Labs, and Pronase was from Calbiochem. Almond emulsin was obtained as "β-glucosidase" from Sigma. Borotritide-reduced LNF II and LNF III were a gift from Dr. Victor Ginsburg, NIH, and ovalbumin and IgM derivatives were from Dr. Michiko Fukuda, Yale University.

TABLE I

Release of radioactivity from fucose-containing glycopeptides by almond emulsin α-L-fucosidase

CELL TYPE	RADIOACTIVITY[a] TOTAL GLYCOPEPTIDE[b]	RELEASED FUCOSE[c]	
	cpm	cpm	% of total
Human neuroblastoma			
CHP-134	1290	430	33
IMR-5	1040	190	18
Mouse neuroblastoma			
NIE-115	1760	10	0.6
N-18	1370	10	0.7
N-1	1090	0	0
Human fibroblasts			
CHP-134-F	1170	23	2.0
C-16	2230	60	2.7
CF-10	1575	30	1.9
Hamster fibroblasts			
BHK_{21}/C_{13}	1080	5	0.5
C_{13}/B_4	1340	0	0

a) Cells were labeled metabolically with L-[^{3}H]- or - [^{14}C] fucose.
b) Pronase digested membrane glycopeptides treated with almond emulsin α-L-fucosidase.
c) Monosaccharide separated on Biogel P-2 (See Fig. 1).

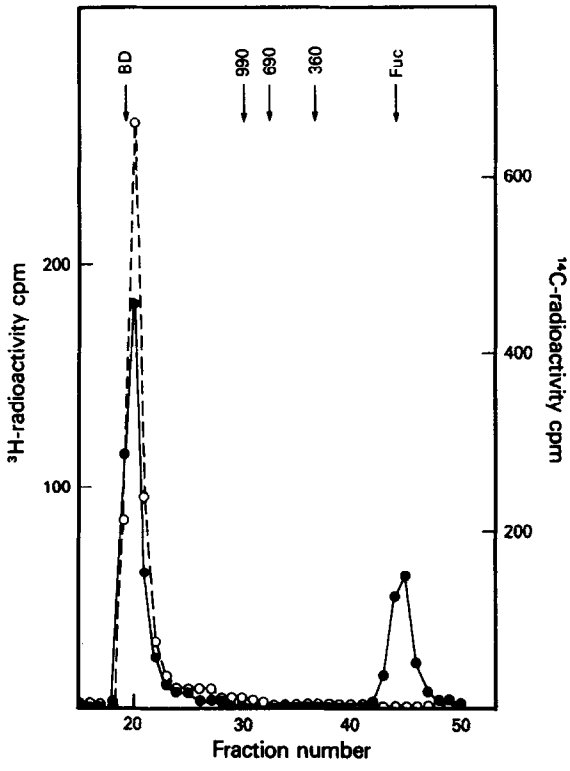

Figure 1. Action of almond emulsin α-L-fucosidase on membrane glycopeptides. Pronase-digested membrane glycopeptides from (●——●) CHP-134 cells and (o---o) N-1 cells, metabolically labeled with L-[^{3}H]-or-[^{14}C] fucose, respectively, were mixed, incubated with enzyme and chromatographed on Biogel P-2 (0.9 x 58 cm). Fractions of 0.8 ml were collected. The arrows indicate the position of (BD) Blue Dextran 2000. $(Man)_5GlcNAc$-OT; $(Man)_3GlcNAc$-OT; ManGlcNAc-OT; and Fuc in that order, with approximate molecular weights for the marker oligosaccharides.

RESULTS

Specific fucosyl linkages in membrane glycopeptides. A fucosyl linkage which was sensitive to almond emulsin α-L-fucosidase was demonstrated in two human neuroblastoma cell lines, CHP-134 and IMR-5. When [^{3}H]fucose-containing glycopeptides from the surface of cells labeled metabolically were incubated with 2 U of enzyme for 72 h, the released fucose was approximately 33% and 17%, respectively, of the total radioactivity in the membrane glycopeptides. In contrast, less than 3% of the fucosyl residues contained in the membrane glycopeptides of any of the other cell types which were examined were linked α1→3 or α1→4 to GlcNAc (Table I). Figure 1 illustrates the contrasting effect of treatment with almond emulsin enzyme on [^{3}H]fucose-labeled glycopeptides from human neuroblastoma cells

and [^{14}C]fucose-labeled glycopeptides from mouse neuroblastoma cells. Moreover, since only 2% of the membrane fucose was released from the skin fibroblasts, CHP-134-F, by almond emulsin α-L-fucosidase, it is not likely that the presence of a particular blood or tissue type accounts for the finding of the enzyme-sensitive linkage in the neuroblastoma cells, CHP-134.

The conditions chosen for treatment with almond emulsin α-L-fucosidase were limiting, that is, in no case did a second enzyme treatment of the remaining high molecular weight material release additional radioactivity. More than 90% of the remaining [^{3}H]-or [^{14}C]fucose was terminal, however, since it was released with the broad spectrum α-L-fucosidase from rat testes.

Independent of NeuAc. Even though a relationship between sialic acid and fucose on terminal branches of the glycopeptides has been reported (13,14), the action of the almond emulsin α-L-fucosidase was unaffected by the pretreatment of membrane glycopeptides with neuraminidase from V. cholerae or A. ureafaciens. In a number of experiments with glycopeptides from human neuroblastoma cells, the same percentage of radioactive fucose was released with or without prior treatment with neuraminidase. No additional fucose was released from CHP-134-F, NIE-115, or N-18 after neuraminidase pretreatment. The activity of neuraminidase preparations was demonstrated by the change in the elution profile of the glycopeptides from Biogel P-4 after the enzyme treatment, and with the use of the synthetic substrate MP-NeuAc[1] (15).

Independent of size. Almond emulsin α-L-fucosidase removed fucose from oligosaccharides attached to polypeptide chains of a spectrum of molecular weights. The percentage of fucose released was similar for intact glycoproteins represented by Triton-extractable material, glycopeptides removed by trypsin, and the Pronase-digested material.

The surface membrane glycopeptides were removed by trypsin from CHP-134 cells, chromatographed on Biogel P-10 in 0.05 M ammonium acetate, and the material just included in the gel ($\geq$ 50,000 M_r) was collected, lyophilized, and incubated with almond emulsin α-L-fucosidase. After rechromatography on Biogel

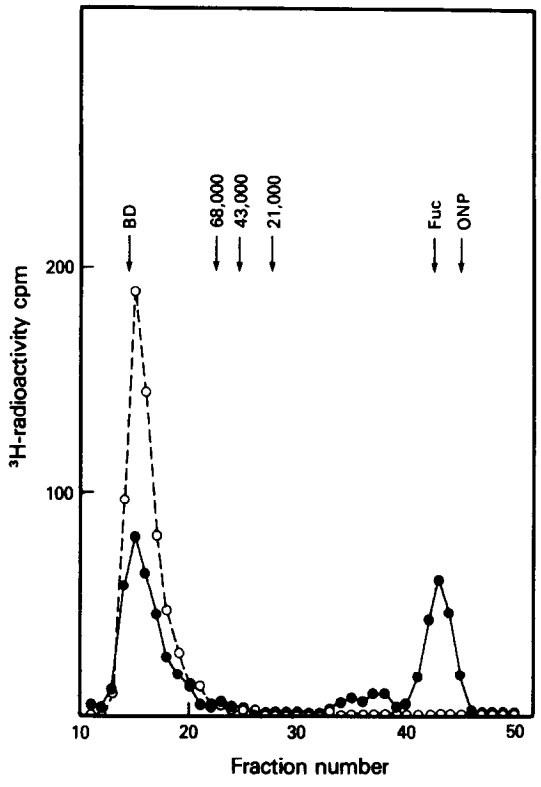

Figure 2. Action of almond emulsin α-L-fucosidase on glycoproteins. A 0.1% Triton X-100 extract of CHP-134 cells, metabolically labeled with L-[^{3}H] fucose was chromatographed on a Sephadex G-100 column (0.9 x 55 cm) and 1 ml fractions collected. Fractions 13-17 were combined, reduced in volume, incubated with (●——●) α-L-fucosidase or (o---o) boiled enzyme, and rechromatographed on Sephadex G-100. All solutions contained 0.02% Triton X-100. The arrows indicate the position of (BD) Blue Dextran 2000; bovine serum albumin (68,000); ovalbumin (43,000); soybean trypsin inhibitor (21,000); Fuc; and (ONP) o-nitrophenol in that order.

P-10, 31% of the radioactivity comigrated with free fucose and the remaining radioactivity eluted with the void volume.

Almond emulsin α-L-fucosidase also released fucose from glycoproteins (Fig. 2). An average of 30% of the radioactivity was released as monosaccharide from glycoproteins ≥ 100,000 M_r. When the total glycoproteins of CHP-134 cells were digested with Pronase, again 30% of the radioactivity was released by the almond emulsin enzyme.

Other properties. Several other properties of the almond emulsin α-L-fucosidase were examined in order to determine the suitability for whole cell experiments. Using either glycopeptides from CHP-134 cells or LNF III as substrates,

it was shown that the almond emulsin enzyme was stable to heating at 60°C, but not 65°, for 20 min. The α-L-fucosidase activity was not inhibited by 200 mM γ-D-galactolactone, a concentration which effects 95% inhibition of almond emulsin β-D-galactosidase. Nor was there any inhibition of enzymatic hydrolysis by exogenous fucose up to 25 mM. The enzyme retained 62% of the maximum activity at pH 6.5 when membrane glycopeptides served as substrate.

DISCUSSION

The membrane glycoproteins of human neuroblastoma cells contained fucose in a terminal linkage not found to the same extent in those of mouse neuroblastoma cells or human fibroblasts. This linkage may be either Fucα1→3GlcNAc or Fucα1→4GlcNAc as determined by the specificity of almond emulsin α-L-fucosidase (3). Since the enzyme has been tested on only a limited number of compounds of known structure, other uncommon fucosyl linkages may also serve as substrates. Therefore, further studies are required to determine the exact linkage. Mild acid hydrolysis preferentially removed the fucose which was sensitive to the almond emulsin enzyme[2] suggesting that the linkage may be Fucα1→3GlcNAc. The lability of this linkage to mild acid in comparison to other fucosyl residues of glycopeptides has been described (16).

Fucα1→3GlcNAc has been demonstrated on a number of glycoproteins and glycopeptides (17-20) where it is often found on the branches of structures characteristic of the complex asparagine-linked glycopeptides with (21) or without (13) sialic acid on the same branch. Preliminary studies[2] have shown that the almond emulsin enzyme-susceptible linkage of human neuroblastoma cells is frequently positioned on the branches of complex glycopeptides, although prior treatment with neuraminidase did not alter the release of fucose from any of the cell types examined. Furthermore, in human neuroblastoma cells, the linkage is not limited to any specific glycopeptide or glycoprotein, since after separation by size and charge (10), the linkage was present in almost all groups[2].

[2]Santer, U.V. and Glick, M.C., unpublished observations

In contrast to α-L-fucosidase from rat testes and other mammalian sources (11) almond emulsin α-L-fucosidase was active on large glycopeptides and glycoproteins, making it feasible to examine in vivo the effect of the removal of fucose in a specific linkage. This is of particular interest since the Fucα1→3GlcNAc linkage has been shown to vary in several cellular systems. For example, in mouse melanoma cells, the loss of metastatic properties was correlated with a decrease of Fucα1→3GlcNAc (20); other studies using the mouse teratocarcinoma cell system (22,23) can be interpreted to indicate a loss, with differentiation, of the linkage cleaved by almond emulsin α-L-fucosidase. The presence of the latter fucosyl linkage on human neuroblastoma cells but not fibroblasts (Table I) points to a correlation with tumorigenicity or differentiation. However, the absence of the linkage on mouse neuroblastoma cells or virus transformed hamster cells shows that neither correlation is true for all species. Thus, while cell surface oligosaccharides are generally altered in tumor cells (5,24), individual monosaccharide alterations may be specific to each cell type and species.

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