Extracellular and cellular proteins of rat cells with *O*-glycosidically linked fucose

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Fucose-labelled proteins were examined for the release of low- M_r *O*-linked fucose substituents after mild alkaline-borohydride treatment. A component tentatively identified as glucosylfucitol (DS) and an apparently higher- M_r component (TS), which also contained fucitol, were observed to be released over a broad molecular-size range of proteins. Approx. 90% of the DS-releasing proteins were in the particulate fraction, whereas only approx. 66% of the TS-releasing proteins were in that fraction. In addition to cell-associated proteins, a substantial proportion of DS-containing proteins were shed into the medium. For example, after 96 h of labelling there was 6-fold more of these components in the growth medium than were cell-associated. Moreover, the incorporation of labelled fucose into both the DS and TS appeared to be cell-population-density-dependent. Despite the apparent wide distribution of these novel fucose substituents in cellular proteins, it seems reasonable to suggest that they have not been routinely observed largely because each represents less than 0.5% of the fucose bound to protein.

Considerable evidence suggests that the fucose moiety of glycoproteins is in a terminal position. Hence the report of an amino acid fucoside in human urine (Hallgren *et al.*, 1975) in which the fucose was both internal and also attached to threonine (glucosylfucosylthreonine) was of particular interest. Likewise, we have observed glucosylfucosylthreonine, glucosylfucosylserine, fucosylthreonine and fucosylserine in rat tissue (Steiner *et al.*, 1978; Klinger *et al.*, 1981) and have examined (Via *et al.*, 1980) the extent of incorporation of radiolabelled fucose into these components in normal and in transformed rat cells. Our findings suggested that diminished incorporation of labelled fucose into glucosylfucosylthreonine (FL4a)/fucosylthreonine (FL3a) was a useful indicator of oncogenic transformation. Others (Respass *et al.*, 1981) have demonstrated that the decreased ratio of radiolabelled FL4a/FL3a was useful as a marker of both tumorigenic potential and metastatic potential in herpes-simplex-virus-transformed cells. The studies described above utilized fibroblasts. More recently, the relatively smaller incorporation of labelled fucose into FL4a as compared with that into FL3a was demonstrated to correlate positively with tumorigenic potential of transformed mouse mammary epithelial cells (Steiner *et al.*, 1983). We have also shown

that the incorporation of labelled fucose into FL4a and FL3a is cell-population-density-dependent in normal rat cells but independent of cell population density in mouse-sarcoma-virus-transformed rat cells (Gacto & Steiner, 1976).

The possibility that proteins might also contain *O*-linked glucosylfucose was examined in a preliminary study (Steiner *et al.*, 1978; Larriba, 1978). Mild alkaline-borohydride treatment of an ethanol-insoluble fraction from fucose-labelled rat cells was found to yield a component with the t.l.c. mobility of glucosylfucitol. The present study was undertaken to characterize further the fucose-labelled component(s) released after mild alkaline-borohydride treatment and to determine the molecular-size range and the cellular distribution of the putative parent proteins in the particulate and the supernatant fractions and in cells compared with those in the culture medium.

Experimental

Cell culture

Seed cultures of newborn-rat kidney fibroblasts (NRK cells) were originally obtained from Dr. K. Somers (Eastern Virginia Medical School, Richmond, VA, U.S.A.). Cells were cultured in BME medium (Grand Island Biological Co., Grand

Island, NY, U.S.A.) supplemented with 2mM-L-glutamine, 10% (v/v) foetal bovine serum and penicillin/streptomycin (50 units/ml and 100 µg/ml respectively) and in some cases L-[6-³H]fucose (10 µCi/ml; 16.8 Ci/mmol) (New England Nuclear, Boston, MA, U.S.A.). The labelling time varied from 48 to 72 h.

Preparation of cells and medium for analysis of O-fucose-linked substituents

Fucose-labelled cells were washed thrice, removed by scraping in water supplemented with 1mM-phenylmethanesulphonyl fluoride and subjected to Dounce homogenization until microscopic examination revealed approx. 95% breakage. The broken-cell suspension was centrifuged at 100000g for 60 min. The resultant high-speed supernatant and high-speed pellet material were fractionated by gel filtration on Sepharose CL-4B, and the amounts of O-linked fucose substituents were determined after mild alkaline-borohydride treatment. Alternatively, the amount of O-linked fucose substituents was determined in an ethanol-insoluble pellet of fucose-labelled cells. Cells were scraped into 60% (v/v) ethanol and heated at 100°C for 5 min, and the ethanol-insoluble residue was extracted twice with 60% ethanol at 100°C.

Culture medium was centrifuged at 100000g for 30 min to remove particulate material, made 60% with respect to ethanol and heated at 100°C for 5 min. Ethanol-insoluble material was pelleted by centrifugation at 5000g for 30 min and freeze-dried. A portion was treated with mild alkaline borohydride, and the low- M_r products were analysed.

Sepharose CL-4B gel-filtration chromatography

High-speed supernatant material was freeze-dried, suspended in 10mM-sodium phosphate buffer, pH 7.4, containing 0.1mM-EDTA and 1mM-2-mercaptoethanol and applied to a 2.5cm × 100cm Sepharose CL-4B column. The high-speed pellet material was solubilized in 25mM-Tris/HCl buffer, pH 7.4, containing 2% (w/v) sodium dodecyl sulphate and 7mM-2-mercaptoethanol at 37°C for 30 min (Meezan *et al.*, 1969). The high-speed supernatant material was eluted with the above phosphate buffer, whereas the high-speed pellet was eluted from the column with the phosphate buffer to which 0.1% sodium dodecyl sulphate had been added. The Sepharose CL-4B fractions were pooled as indicated in the Figures, concentrated by ultrafiltration (Amicon PM-10 filter that retains proteins of M_r 10000 or greater) and freeze-dried. A portion of each of the ultrafiltered pooled fractions was analysed by polyacrylamide-gel electrophoresis, and a second portion was subjected to alkaline-borohydride

treatment and the resultant low- M_r products were analysed by a combination of gel-filtration chromatography on Bio-Gel P-2 and by silicic acid t.l.c. in chloroform/methanol/water (60:35:8, by vol.).

Discontinuous-gradient polyacrylamide-gel electrophoresis

The pooled fractions from the Sepharose CL-4B columns were analysed by gradient sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the method of Laemmli (1970) as modified by O'Farrell (1975). After polyacrylamide-gel electrophoresis the gels were prepared for fluorography by the method of Bonner & Laskey (1974).

Mild alkaline-borohydride treatment and analysis of products

Mild alkaline-borohydride treatment was accomplished essentially by using the procedure of Carlson (1968). Briefly, samples were suspended in 1.0M-NaBH₄/50mM-NaOH and incubated for 16 h at 45°C. The excess of borohydride was removed by addition of 4M-acetic acid to pH 5.0. The acidified sample was chromatographed on a Dowex 50 (H⁺ form) column, and the material that was eluted with 10mM-formic acid was collected and repeatedly evaporated to dryness in a rotary evaporator with the addition of methanol to remove residual boric acid. The dried material was resuspended in water and chromatographed on a small anion-exchange column [Dowex 1X8 (formate form)], and the unbound fraction was analysed by t.l.c. in chloroform/methanol/water (60:35:8, by vol.) and by column chromatography on Bio-Gel P-2 as described previously (Morton *et al.*, 1982). When the base/borohydride mixture was neutralized before it was added to the sample, there was no demonstrable release of neutral products. In addition, mild base/borohydride treatment did not result in demonstrable cleavage of authentic glucosylfucose or glucosylfucitol. It is also worthwhile mentioning that glucosylfucitol is not released when glucosylfucosyl-threonine is subjected to mild base/borohydride conditions, this presumably being due to the presence of a free amino or free carboxy group. Furthermore, essentially the same amounts of low- M_r fucose-labelled substituents were released under a variety of base/borohydride conditions, including 50mM-, 0.1M- or 0.2M-NaOH and 0.25M-, 0.5M- or 1.0M-NaBH₄.

Acid hydrolysis and preparation of glucosylfucitol

Mild acid hydrolysis was accomplished in 0.1M-HCl at 100°C for 60 min. The sample was neutralized with solid Ag₂CO₃. Glucosylfucose, glucosylfucitol and fucitol were prepared as previously described (Klinger *et al.*, 1981).

Paper and thin-layer chromatography

The hydrolysis products, fucose and fucitol, were identified by descending chromatography on Whatman 3MM paper (50cm × 100cm) in butan-1-ol/ethanol/water (10:1:2, by vol.) for 18h. The position of the fucose-labelled material was determined by cutting 1.0cm strips from origin to the end of the paper and measuring the radioactivity by scintillation spectrometry. The position of authentic standards was determined by silver nitrate stain (Trevelyan *et al.*, 1950).

T.l.c. analysis of the low- M_r products released after alkaline-borohydride treatment was accomplished on Whatman K-5 t.l.c. plates in chloroform/methanol/water (60:35:8, by vol.). The t.l.c. plates were scraped in 0.5cm bands from origin to solvent front, and the radioactivity was measured by scintillation spectrometry.

Determination of trichloroacetic acid-insoluble radioactivity

Determination of the incorporation of labelled fucose into trichloroacetic acid-insoluble material was accomplished by the method of Atkinson & Summers (1971).

Protein determination

Protein was determined by the method of Hartree (1972), with bovine serum albumin as a standard.

Results

T.l.c. comparison of the mild-base/borohydride-releasable material from an ethanol-insoluble fraction of fucose-labelled cells consistently revealed a somewhat broader peak than was observed with authentic glucosylfucitol. Hence, after elution from the t.l.c. plate, this material was further analysed by gel-filtration chromatography on Bio-Gel P-2. Two distinct fucose-labelled peaks were observed (Fig. 1). One peak had the same elution volume as authentic glucosylfucitol (DS) (fractions 113–117) whereas the second peak was eluted at an apparently higher- M_r position (fractions 105–108; this fraction is termed TS). The elution volume of each compound, relative to the carbohydrate standards, was highly reproducible. Moreover, when these components were subjected to t.l.c. in parallel lanes, TS was observed to be slightly more mobile than DS (Fig. 2), which probably accounted for the originally observed slightly broader peak. Both compounds yielded fucitol after mild acid treatment (Fig. 3), which suggested that fucose was at the reducing end in each compound. Moreover, both the DS and the

Fig. 1. Bio-Gel P-2 column chromatography of mild-alkaline-borohydride-released material from a [^3H]fucose-labelled ethanol precipitate of NRK cells

The glucosylfucitol region of a t.l.c. chromatogram (chloroform/methanol/water, 60:35:8, by vol.) was scraped and eluted in methanol/water (1:1, v/v), dried and resuspended in 0.1M-pyridinium acetate, pH 5.0, and analysed on a Bio-Gel P-2 (200–400 mesh) column 1.5cm × 100cm). Fractions of volume 1.3ml were collected (flow rate 26.4ml/h), and the radioactivities of the fractions were measured by scintillation spectrometry. The designations S, R, L and F indicate the elution positions of stachyose, raffinose, lactose and fucose respectively. Blue Dextran was eluted at fractions 63–66; Phenol Red was eluted at fractions 147–151. The elution positions of authentic glucosylfucitol (Glc-FucOH) and fucitol (FucOH) are indicated. For additional details see the Experimental section.

TS were completely insensitive to treatment with a relatively non-specific α -L-fucosidase (results not shown), which is consistent with a non-terminal position for the fucose moiety.

To determine the size classes of proteins that contain the DS and TS components the fucose-labelled proteins from high-speed pellet (Fig. 4a) and high-speed supernatant fractions (Fig. 4b) of NRK cells were separated by gel filtration on Sepharose CL-4B, and various molecular-size ranges of fucoproteins were pooled as indicated. Each of the pooled fractions was analysed by polyacrylamide-gel electrophoresis (Figs. 5a and 5b) to ascertain the molecular-mass distribution of the fucoproteins. The polyacrylamide-gel electrophoresis profiles revealed that, although there was some overlapping, each pool represented an apparently different average molecular-size range of fucoprotein. Portions of the pooled gel-filtration fractions were subjected to mild alkaline-borohydride treatment, and the released TS and DS were analysed by a combination of t.l.c. and gel filtration on Bio-Gel P-2. The labelled fucose of

Fig. 2. T.l.c. analysis of the major low- M_r mild-base/borohydride-released components from a [^3H]fucose-labelled ethanol precipitate of NRK cells

(a) T.l.c. of material that was eluted from the Bio-Gel P-2 column (Fig. 1) with the same chromatographic mobility as authentic glucosylfucitol (fractions 113–117); (b) t.l.c. of material that was eluted from the Bio-Gel P-2 column (Fig. 1) before authentic stachyose (fractions 104–108). The solvent used was chloroform/methanol/water (60:35:8, by vol.). The positions of authentic glucosylfucitol (Glc-FucOH) and fucitol (FucOH) are indicated at the top of the Figure.

pool 4 of the high-speed supernatant was markedly decreased after ultrafiltration. Hence this material was not further analysed. As indicated in Table 1, the various fractions contained compounds, presumably proteins, that gave rise to DS and TS. Thus DS and TS are likely to be associated with a number of size classes of protein. However, there does appear to be enrichment of DS- and TS-containing proteins in particular fractions, e.g. the ratio of TS/DS is higher in the supernatant fraction, and pool 2 of the pellet fraction is enriched in DS and TS relative to the other particulate fractions.

The studies described above have focused on the cell-associated compounds with *O*-linked fucose. Since it has been observed that significant amounts of fucoprotein may be released from cells, the possibility that proteins containing *O*-linked fucose are released was examined. Table 2 indicates the results of studies in which the amount of [^3H]fucose in DS- and TS-containing glycoproteins was examined in both NRK cells and medium after 48 h of labelling (low cell population density), 72 h of labelling (cells subconfluent) and 96 h of labelling (cells confluent). The results indicate that a relatively high proportion of the DS-containing fucoprotein(s) are shed from the cells. In contrast, there was no detectable TS-containing fucoprotein in the medium. Additionally, the extent of incorporation of [^3H]fucose into cellular DS and TS relative to total [^3H]fucoprotein (trichloroacetic acid-insoluble material) increased approximately 2-fold in confluent cells. This suggests an enhanced synthesis of the *O*-linked fucoproteins as compared with total fucoproteins in confluent cells.

Table 1. Distribution of DS- and TS-releasing fucoproteins in Sepharose CL-4B fractions from the high-speed pellet and high-speed supernatant of fucose-labelled rat cells

The pooled fractions (see Fig. 4) were subjected to ultrafiltration, freeze-dried and resuspended in water, and portions were taken for scintillation spectrometry (total fucoprotein) and for mild alkaline-borohydride treatment as described in the Experimental section.

	Radioactivity in fucoprotein (c.p.m.)	Radioactivity in DS (c.p.m.)	Radioactivity in TS (c.p.m.)	DS (%)	TS (%)
High-speed supernatant					
Pool 1	75 000	< 20	349	—	0.47
Pool 2	300 000	487	2271	0.16	0.76
Pool 3	500 000	2334	4515	0.47	0.90
Total	875 000	2821	7135	0.32	0.82
High-speed pellet					
Pool 1	1 100 000	738	392	0.07	0.04
Pool 2	2 900 000	7288	5499	0.25	0.19
Pool 3	7 300 000	9488	6256	0.13	0.09
Pool 4	5 700 000	6049	1488	0.11	0.03
Total	17 000 000	23 563	13 635	0.14	0.08

Fig. 3. Paper-chromatographic analysis of the mild-acid-hydrolysis product of the mild-base/borohydride-releasable components from a [^3H]fucose-labelled ethanol precipitate of NRK cells

(a) Authentic fucitol subjected to acid treatment; (b) acid-hydrolysis product of the mild-base/borohydride-releasable component with the chromatographic properties of glucosylfucitol; (c) acid-hydrolysis product of the mild-base/borohydride-releasable component that is of apparently higher M_r than glucosylfucitol. The positions of authentic fucose (Fuc) and fucitol (FucOH), which were detected chemically, are indicated at the top of the Figure by the hatched ovals. For details of paper chromatography see the Experimental section.

Discussion

The observation of low- M_r components in rat tissue that contain fucose both *O*-linked to amino acid and in an internal position (Klinger *et al.*, 1981) prompted us to look for protein(s) with a similar type of oligosaccharide moiety. In a preliminary study we described the base/borohydride release of a component that had the chromatographic properties of glucosylfucitol (Steiner *et al.*, 1978). In the current study we have found a second base/borohydride-releasable component (TS) that has fucose at the reducing end and that on the basis of gel-filtration chromatography on Bio-Gel P-2

Fig. 4. Sepharose CL-4B gel-filtration column chromatography of the high-speed pellet and high-speed supernatant material from [^3H]fucose-labelled NRK cells

(a) Fucose-labelled high-speed pellet was solubilized in Tris buffer containing 2% sodium dodecyl sulphate and applied to a Sepharose CL-4B column. The fractions were combined as shown at the top of the Figure (pool 1, fractions 60–90; pool 2, fractions 91–108; pool 3, fractions 109–115; pool 4, fractions 116–140). BD, F and M indicate the elution positions of Blue Dextran (M_r 2000000), ferritin (M_r 200000) and myoglobin (M_r 18000). (b) Fucose-labelled high-speed supernatant was applied to the Sepharose CL-4B column, and 8.3 ml fractions were collected. The fractions were pooled as shown at the top of the Figure. The elution positions of Blue Dextran (BD) and myoglobin (M) are indicated at the top of the Figure. The radioactivity in each fraction was measured by scintillation spectrometry.

has an apparently higher M_r , i.e. larger than stachyose, whereas glucosylfucitol (DS) had the same elution volume as raffinose. The extent of incorporation of labelled fucose into TS as com-

Fig. 5. Autoradiogram of polyacrylamide-slab-gel electrophoresis of the pooled fractions from Sepharose CL-4B fractionation of [^3H]fucose-labelled NRK cells

The pooled fractions obtained from gel-filtration chromatography (see Fig. 4) of high-speed pellet (HSP) (right-hand side) or high-speed supernatant (HSS) (left-hand side) were subjected to ultrafiltration and freeze-dried, and approx. 100000 c.p.m. of each of the pooled fractions, representing less than 50 μg of protein, was applied to each of the above lanes. The pooled fraction number is indicated at the top of the lane. Approximate M_r values, based on authentic M_r standards, are indicated at the left-hand sides of the photographs. For additional details see the Experimental section. TD indicates the position of the tracking dye Bromophenol Blue.

Table 2. Distribution of DS- and TS-releasing proteins in culture medium and NRK cells

For experimental details see the Experimental section.

Time of fucose labelling ...	Radioactivity incorporated (c.p.m./mg of protein)		
	48 h	72 h	96 h
DS-containing proteins			
Cells	540	680	1800
Medium	1900	4300	11000
Cells/medium ratio	0.28	0.16	0.16
TS-containing proteins			
Cells	360	450	1300
Medium	<60	<60	<60
Trichloroacetic acid-insoluble material			
Cells	190000	330000	450000
Medium	118000	177000	244000
Cell/medium ratio	1.6	1.7	1.8

pared with DS was approx. 80%. Moreover, both the TS- and DS-releasing proteins were observed over a relatively broad molecular-size range. The distribution of TS- and DS-releasing proteins

between 'particulate' and 'cytosolic' fractions differed. Approx. 90% of the DS-releasing proteins were associated with the 'particulate' fraction, whereas only approx. 66% of the TS-releasing

proteins were associated with that fraction. The relative enrichment of the TS-releasing proteins in the 'cytosolic' fraction was consistently observed, and the percentage of these proteins relative to the major fucoproteins was approx. 5-fold in the cytosolic fraction. In contrast, the amount of labelled fucose incorporated into DS-releasing proteins as a percentage of the total label incorporated into fucoprotein was approximately the same in the 'particulate' and 'cytosolic' fractions. In addition to the cell-associated proteins, a substantial proportion of the DS-releasing proteins were shed into the medium. For example, the amount of DS-releasing proteins in the medium was 3.5- and 6.0-fold greater than was cell-associated after 48 and 96h of labelling respectively. In contrast, there were no detectable TS-releasing proteins observed in the medium. The lack of TS-releasing proteins in the medium probably indicates that these proteins were not shed or that they were shed and subsequently broken down in the growth medium by proteolytic or glycolytic enzymes. In addition to DS- and TS-releasing proteins, base/borohydride treatment resulted in the release of labelled fucitol. On the basis of our previous observations that there is incorporation of labelled fucose into fucosylthreonine and fucosylserine (Klinger *et al.*, 1981), it seemed reasonable to consider that some or all of the fucitol was derived from fucose that was O-linked to protein. Alternatively, it was conceivable that some of the fucitol (and possible DS or TS) was produced by the 'peeling' reaction of larger oligosaccharides (Lloyd *et al.*, 1968). However, comparable amounts of these components were released over a concentration range of base and borohydride, which makes it seem less likely that they were derived by 'peeling'. Furthermore, even if the DS and/or TS components were released by 'peeling', these compounds would be of interest since the fucose is in an internal position.

Despite the relatively broad distribution of the TS- and DS-releasing proteins, the total amount of labelled fucose into these components relative to other proteins is quite low, with each representing less than 0.5% of the total labelled fucose bound to protein. The low content of these components probably accounts for the fact that they have not been observed in other studies. When the amount of the DS- and TS-releasing proteins was examined

in NRK cells grown at different cell densities, it was observed that the incorporation of labelled fucose into both types of oligosaccharides was greater in cells at high cell population density. If the DS-containing proteins are the metabolic precursors of FL4a, then the cell-density-dependent incorporation of labelled fucose into these proteins could explain the cell-density-dependent incorporation of labelled fucose into FL4a (Gacto & Steiner, 1976).

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