O-GLYCOSIDICALLY LINKED FUCOSE IN HIGH MOLECULAR WEIGHT GLYCOPROTEINS, IN NORMAL AND VIRUS-TRANSFORMED RAT CELLS

G. LARRIBA

Departamento de Microbiología, Instituto de Microbiología, CSIC, Facultades de Ciencias y Farmacia, Universidad de Salamanca, Salamanca, Spain

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1. Introduction

Fucose metabolism in animal cells has been the subject of various biological studies for two main reasons.

(1) It seems to be incorporated directly in glycoproteins without undergoing further anomeric alterations as shown in He-La cells [1].

(2) Fucose-labeled glycopeptides present alterations upon viral transformation [2,3].

A small portion of the radioactive fucose is incorporated into two compounds of low molecular weight by NRK (normal rat cells) and MSV-NRK (murine sarcoma virus-transformed rat cells). These compounds have been recently characterized as unusual aminoacylfucosides and are composed of threonine and fucose (FL3) and threonine, fucose and glucose (FL4) [4,5]. Another two similar components belonging to the serine series were also found in rat tissue but not in cell culture [4].

Alterations in the level of radioactive fucose incorporated in FL4 in oncornavirus-transformed cells has been reported when they were thought to be fucolipids [6–8]. However in view of the real nature of FL3 and FL4, a re-examination of their status in normal and transformed cells was considered.

This communication presents data indicating that the structures of FL3 and FL4 also occur as part of a high molecular weight protein(s), fucose being the internal *O*-glycosidically linked sugar. The new methodology used and the results obtained in studies undertaken to disclose the actual levels of FL3 and FL4 in NRK and MSV-NRK cells are also presented.

2. Materials and methods

2.1. *Cell growth and harvesting*

The cultured cells employed were NRK and MSV-NRK [9]. Cells were grown in Eagle's medium plus 10% fetal calf serum (v/v) and supplemented with 3 μCi/ml [6-^{3}H]fucose (25 Ci/nmol; Amersham Searle Corp.). Perchloric acid (PCA) 0.5 N, was added to confluent monolayers and cells were harvested, pelleted and washed twice with 0.5 N PCA.

2.2 *Determination of FL3 and FL4*

Supernatants were pooled, neutralized with KOH, the insoluble salt discarded, and chromatographed on AG-50H$^+$. FL3 and FL4 were eluted with 0.5 N NH_4OH and analyzed by thin-layer chromatography (TLC) (Q-5 plates, Quantum Industries, Fairfield, NJ) in sequential double run (solvent A followed by solvent B). Radioactivity was measured by scraping the plate in 0.5 cm bands and counting them by scintillation spectrometry.

2.3. *β-Elimination of insoluble protein*

Pellets of PCA-extracted cells were washed with ice-cold methanol–water (1:1, v/v) till neutral. β-Elimination was performed in presence of $NaBH_4$ as in [10]. Briefly, samples were resuspended in 0.8 M $NaBH_4$ in 0.1 N NaOH at 37°C. After 48 h they were placed in an ice-bath, diluted with 10-fold vol. cold water, brought to pH 5 with acetic acid and passed through a column of AG-50 H$^+$. Columns were washed with 4 column vol. 0.01 N formic acid. The efluents were combined and the boric acid volatized as

methyl borate. Products were analyzed by TLC in solvents A and C. They were purified by preparative TLC in solvent A. Radioactive bands were eluted with solvent B and repurified by TLC in solvent B.

2.4. *Derivatization of FL3 and FL4*

N-Acetylation of isotopically pure FL3 and FL4 was carried out by the method in [11]. Methylation was performed with thionyl chloride and amidation by treating the methyl derivatives with methanolic ammonia [12]. [^{14}C]Serine controls were prepared in an analogous manner. The compounds were acetylated overnight in 0.2 ml acetic anhydride in 0.3 ml pyridine.

2.5. *Chromatography*

The following solvents were used in TLC: (A) $CHCl_3$–CH_3OH–H_2O (60:35:8, by vol.); (B) 2-propanol–NH_4OH–H_2O (7:2:1, by vol.); (C) 1-butanol–CH_3OH–H_2O (10:1:2, by vol.); (D) 1-butanol–propionic acid–H_2O (6:3:4, by vol.). Solvents A and B were used in silica gel plates and solvents C and D in cellulose plates. Gel filtration was performed as in [4]. The identification procedure for the presence of either [^{3}H]fucose or [^{3}H]fucitol in the samples was carried out by TLC in systems A, C and D in parallel lines, one containing [^{14}C]fucose and the other [^{14}C]-fucitol as internal standards.

3. Results

3.1. *Release of sugars from FL3 and FL4*

The β-elimination reaction proceeds unsatisfactorily if the glycosilated amino acid residue is in terminal position on the peptide chain [13].

For this reason the β-elimination of FL3 and FL4 was tried as their derivatives in which either the amine *O*-carboxyl group or both had been blocked. So, in addition to the parent compounds, their amidated, *N*-acetylated carboxymethylated, *N*-acetylated, and *N*-acetylated amidated derivatives were subjected to β-elimination. Alkaline treatment removed the amide or methyl groups but it did not affect the hypothetical *O*-glycosidic bond nor the *N*-acetyl group.

However after *N*-acetylation, followed by total acetylation and final subjection to alkaline treatment in presence of $NaBH_4$, FL3 and FL4 produced compounds tentatively identified as fucitol and glucose–fucitol, respectively (see below). Although the mechanism of breakage is unknown, it occurred after the *N*-acetylation step as the treatment of *N*-acetylated compounds with $NaBH_4$ alone followed by acid hydrolysis (1 N HCl at 100°C for 1 h) liberated fucose and not fucitol. But when totally acetylated compounds were treated in the same way they produced fucitol and not fucose. Two alternative explanations are possible:

(1) Sometime during the total acetylation step, the sugar–amino acid bond is cleaved and the C1 of fucose become accessible for posterior reduction;
(2) During the acetylation step a lactame involving the carboxyl group of threonine and a hydroxyl group of the sugar moiety is formed [14].

The blockage of the carboxyl group would facilitate a β-elimination reaction even in the slightly alkaline conditions exhibited by $NaBH_4$ solutions in water.

3.2. *β-Elimination of PCA-insoluble fucose-labeled protein*

When the PCA-insoluble protein was subjected to β-elimination and the resulting soluble neutral products analyzed by TLC (solvent A) most of the radioactivity stayed at the origin but two minor though well defined radioactive spots were visible. The label found in these spots accounted for ~2% of the total PCA-insoluble counts.

The upper component cochromatographed with the compound liberated from FL3 under the conditions described before and with authentic fucitol standard in systems A, C and D.

The less mobile spot cochromatographed with the compound liberated from FL4 in the same three systems (fig.1,a,a′,b,b′,c,c′). In order to further compare both products they were sized in Sephadex G-10 columns calibrated with standard sugars (stachyose, raffinose, lactose and fucose). Both compounds co-eluted with lactose (fig.1,d,d′). These results pointed to the fact that a compound with the properties of a disaccharide had been liberated from FL4 as well as from the protein.

Internal sugar in the parent compounds (protein and FL4) was determined hydrolyzing (1 N HCl at 100°C for 1 h) the disaccharide released either from insoluble protein or FL4 under the conditions described before. The liberation of either radioactive fucose or fucitol would indicate the presence of terminal or

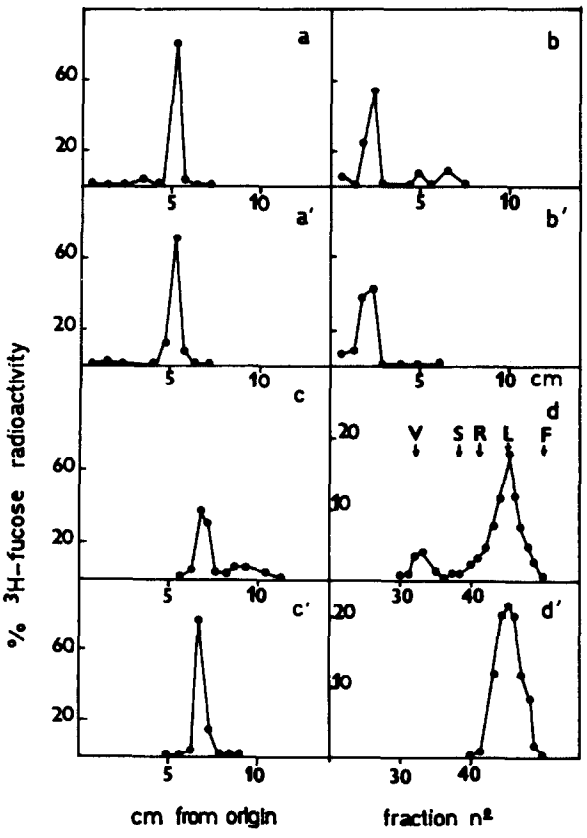

Fig.1. Comparison of disaccharides released from FL4 (a,b,c,d) and from PCA-insoluble material (a',b',c',d') by TLC in systems A, C and D (a,b,c) and gel filtration (d). Radioactivity is given as percent distribution. V_O, void volume; S, stachyose; R, raffinose; L, lactose; F, fucose. Small peaks in samples of disaccharide released from FL4 are small contaminants present in the original sample.

internal fucose, respectively. In both cases fucitol was identified as the only radioactive hydrolytic product. On the contrary, when PCA-insoluble protein treated with $NaBH_4$ alone was hydrolyzed, all the radioactivity was recovered as fucose and no fucitol was detected. This indicates that fucose is the internal sugar in FL4 and as well as being internal is *O*-glycosidically bound to the peptide moiety in the glycoprotein(s). To rule out the possibility that some terminal fucose was cleaved under the mild alkaline conditions used in the β-elimination treatment, 200 mg tyroglobulin, a protein containing terminal fucose [15], were treated in the same way and the neutral soluble products analyzed by GLC as alditol acetate derivatives. No traces of fucose were found.

The presence of *O*-glycosidically bound fucose to protein was more carefully examined by kinetic studies as shown in fig.2. Almost all the fucitol as well as the disaccharide were liberated under reductive alkaline conditions during the first 24 h. Controls treated with $NaBH_4$ alone showed very small amounts of both compounds and their liberation appeared to be linear. This might be due to the slightly alkaline pH of $NaBH_4$ solutions.

3.3. *Levels of fucose O-glycosidically bound to protein in NRK and MSV-NRK cells*

Significant differences in the incorporation of isotopically-labeled fucose in FL3 and FL4 in NRK and MSV-NRK cells have been reported in the past [9,16] when a lipidic nature was attributed to these compounds [7]. Table 1 shows that these differences are rather insignificant. No differences were found in the levels of fucose and disaccharide bound to high molecular weight protein either.

Differences in the levels of FL3 and FL4 in the pellets of scraped monolayer seem to be due to the extension of cell breakage during harvesting, transformed cells being tougher; in addition FL3 is preferentially lost in the supernatant in normal and transformed cells (unpublished observations).

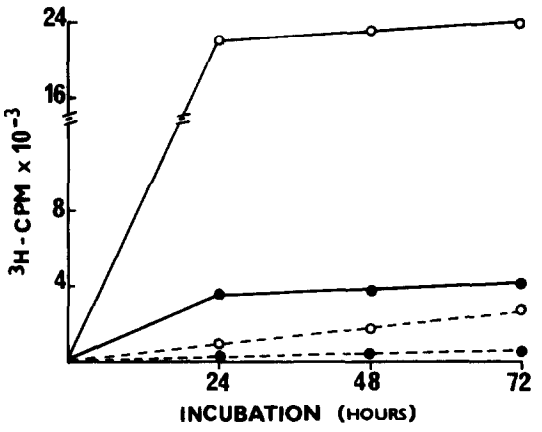

Fig.2. Kinetics of release of disaccharide (•) and fucitol (○) from PCA-insoluble material of NRK cells by β-elimination. Aliquots were taken at the indicated times, processed as indicated in section 2 and analyzed by TLC in system C. Controls treated with $NaBH_4$ were carried out (dotted lines).

Table 1
Incorporation of fucose into FL3, FL4 and its *O*-glycosidic incorporation into protein(s) in NRK and MSV-NRK cells

	FL3			FL4			Fucitol			Disaccharide		
	Sup.	Pell.	Total	Sup.	Pell.	Total	Sup.	Pell.	Total	Sup.	Pell.	Total
NRK	828	406	1234	192	708	900	30	816	846	46	240	286
NSV-NRK	633	516	1149	190	422	612	30	714	744	52	151	203

The cell monolayers were scraped in distilled water, homogenized in a glass homogenizer (50 strokes), pelleted by high speed centrifugation (100 000 × *g*) and compounds of interest determined as indicated in section 2. Total incorporation in each compound (cpm/mg protein) was determined by addition of supernatant and pellet. Comparable results were obtained when the cells monolayers were scraped in saline isotonic solutions or PCA. Protein was measured in aliquots by the method in [17]

4. Discussion

Two products, one identified as fucitol and the other tentatively identified as glucose–fucitol, have been liberated from the protein under alkaline reductive conditions known to liberate sugars which are *O*-glycosidically bound to protein [13]. The hypothesis that FL3 and FL4 might be degradation products of such a protein(s) appears attractive but as yet remains to be established.

Significant differences were not found between the levels of FL3 and FL4 in NRK and NRK-MSV cells, though it has been claimed that they exist [9,16]. The most rational explanation for these discrepancies is that FL3 and FL4 appear also in the supernatant of scraped cells and accordingly they were partially lost by centrifugation of the cells in [9,16]. After the results reported in this paper it appears necessary to reconsider the status of FL3 and FL4 in other cell lines.

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