

Metabolism of a Transformation-sensitive Amino Acid Fucoside in Normal and Simian Virus 40-transformed Human Embryonic Lung Cells¹

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ABSTRACT

We have demonstrated previously that there is a marked decrease in the level of labeled fucose incorporated into an amino acid fucoside, *i.e.*, fucosyl-*N*-acetylglucosaminylasparagine (FL4c) in SV40-transformed human embryonic lung cells (SV40-WI-38) as compared to WI-38 cells (Morton, P. A., Klinger, M. M., and Steiner, S. *Cancer Res.*, 42: 3022-3027, 1982). In the current study, we have observed that the reduction in labeled fucose incorporated into FL4c in the SV40-WI-38 cells is paralleled by reduced chemical quantity of that component. [³H]-Fucose pulse/chase and long-term fucose labeling/chase studies, in some instances in the presence of tunicamycin, have revealed that FL4c is a relatively stable end product of *N*-linked-type glycoprotein metabolism. The relative metabolic stability argues against breakdown of FL4c as the basis for the markedly reduced level in the SV40-WI-38 cells. However, the transformed cells manifested an almost 3-fold higher level of α -L-fucosidase activity when *p*-nitrophenyl- α -fucoside was used as substrate. These results raise the possibility that the parent glycoprotein of FL4c might be more rapidly catabolized in the SV40-WI-38 cells than in the WI-38 cells. Whatever the biochemical basis for the decrease in level of FL4c in transformed human cells, it would seem to underlie a difference between normal and transformed cells in membrane glycoprotein catabolism.

INTRODUCTION

Recent studies in this laboratory have elucidated the composition and carbohydrate sequence of the amino acid fucoside designated as FL4c.³ These studies also revealed that FL4c represents a significant portion of the labeled fucose that was incorporated into cellular material in human embryonic lung cells (WI-38), while there was a striking decrease in the incorporation of [³H]fucose into FL4c in SV40-transformed WI-38 cells (SV40-WI-38) (9). In addition, there is a relatively low level of this component in cell lines derived from human tumors (12), and the incorporated of labeled fucose into FL4c is cell population density-dependent in WI-38 cells but independent of cell population density in human cancer cells (8). Thus, it appears as though the level of labeled fucose incorporated into FL4c might prove to be a useful marker of transformation in human cells. However, little is known about the metabolic origin of FL4c or the basis for its marked reduction in transformed human cells. In the current study, we have used [³H]fucose in labeling experiments to examine the metabolism of FL4c in WI-38 and SV40-WI-38 cells

as well as to determine whether the basis of the reduction of this component in SV40-WI-38 cells is due to enhanced shedding or catabolism. Moreover, since the decreased level of FL4c in SV40-WI-38 cells as compared to the WI-38 cells has been based solely on the level of incorporation of [³H]fucose, we have compared the chemical level of this compound in the 2 cell types.

MATERIALS AND METHODS

Cell Culture. The following seed cultures of human embryonic lung (WI-38) cells and SV40-transformed WI-38 cells were obtained from the American Type Culture Collection, Rockville, MD: ATCC CCL 75, WI-38 cells; ATCC CCL 75.1, WI-38 VA3 subline 2 RA (SV40-transformed WI-38 cells); and ATCC CCL 95.1, WI26 VA4 (SV40-transformed WI-38 cells). Cells were cultured in minimal essential medium supplemented with 10% fetal calf serum and twice the standard concentration of nonessential amino acids and vitamins.

[³H]Fucose-labeling Experiments. For long-term labeling/chase experiments, sparsely growing cells (approximately 5×10^4 cells/75-sq cm tissue culture flask) were cultured in medium supplemented with [³H]-fucose (2.5 μ Ci/ml; 16.8 Ci/mmol; New England Nuclear, Boston, MA) for 48 hr. For [³H]fucose pulse/chase experiments, medium supplemented with [³H]fucose (3.5 μ Ci/ml) was added to subconfluent cultures for 3 hr. Following the long-term labeling or the "pulse" period, the medium was replaced with fresh medium supplemented with 50 mM "cold" fucose. Cells were harvested at the indicated time intervals and analyzed for the level of radioactivity incorporated into FL4c and into the TCA-insoluble cellular material. In some experiments, the incorporation of labeled fucose into FL4c was measured in cells incubated in medium which was also supplemented with tunicamycin (1 μ g/ml). The tunicamycin was prepared in stock solutions of 500 μ g/ml in 25 mM NaOH. The incorporation of [³H]-2-mannose (2 μ Ci/ml; 16.8 Ci/mmol; New England Nuclear) and of [³H]leucine (3 μ Ci/ml; 8.2 Ci/mmol; New England Nuclear) into the TCA-insoluble fraction of tunicamycin-treated cells was also determined.

Analysis of the Amino Acid Fucosides. The methods for harvesting of the cells and analysis of the amino acid fucosides are as described previously (9). Briefly, monolayers washed 3 times with buffered 0.9% NaCl solution (saline) were scraped into ice-cold distilled water, and ethanol was added to 60%. The cells were extracted 3 times with 60% ethanol at 100°. The ethanol extract was taken to dryness and resuspended in water, the amino acid fucosides were bound to Ag50(H⁺) (Bio-Rad Laboratories, Richmond, CA), and the bound material eluted with 0.5 M ammonium hydroxide. The ammonium hydroxide-eluted material was taken to dryness by rotary evaporation and chromatographed on K-5 thin-layer plates (Whatman Industries, Inc., Clifton, NJ) in System I: chloroform/methanol/concentrated ammonium hydroxide (40/80/15, v/v/v). The components were analyzed by scraping the thin-layer plates, in 0.5-cm strips, from origin to solvent front, and quantitating the level of radioactivity by scintillation spectrometry.

Determination of TCA-insoluble Radioactivity. Radioisotopically labeled cells were scraped into ice-cold water, a small aliquot of the suspension was applied in triplicate to Whatman No. 3MM filter discs (diameter, 2.1 cm), and the discs were allowed to dry at room temperature overnight. The filter discs were placed in ice-cold 10% TCA sequentially for three 20-min periods and then rinsed twice in ice-cold 95%

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³ The abbreviations used are: FL4c, fucose-labeled amino acid fucoside (fucosyl-*N*-acetylglucosaminylasparagine); TCA, trichloroacetic acid; α -L-fucosidase, α -L-fucoside fucosylase (EC 3.2.1.51).

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Table 1

Determination of the specific activity of FL4c in WI-38 cells as compared to SV40-WI-38

The assumption is made that the specific activity of the [^3H]fucose derived from the TCA-insoluble pool is comparable to the specific activity of the [^3H]fucose of FL4c. This assumption is based on the fact that FL4c has been shown to be derived from the TCA-insoluble pool and that the cells used to determine the specific activity were labeled with [^3H]fucose for long-term, presumably to "steady-state."

	A. TCA-insoluble [^3H]fucose (cpm/nmol fucose)	B. [^3H]Fucose (cpm in FL4c/mg protein)	B/A
WI-38	2,840	15,000	5.3
SV40-WI-38	1,510	786	0.5

ethanol. The filters were then dried at 50° for 30 min or at room temperature overnight. The amount of radioactivity was determined by scintillation spectroscopy.

Determination of the Specific Activity of FL4c. Cells were cultured in medium supplemented with [^3H]fucose (2.5 $\mu\text{Ci/ml}$; specific activity, as above) for 72 hr. The cells were washed 3 times with saline buffer and then scraped into the buffer. The mixture was subjected to precipitation with 5% TCA under ice-cold conditions, and the pellet was washed with ice-cold TCA and then with ethanol/ether, 2/1, v/v. The TCA pellet was subjected to hydrolysis in 0.1 N HCl at 100° for 45 min and neutralized with barium carbonate, and the hydrolysate was subjected to ion-exchange chromatography on AG 50 (H^+) followed by chromatography on AG 1 (formate). The neutral fraction which contained the free fucose was subjected to chromatography on borate-treated K-5 plates (Whatman) essentially as described by Yurchenko *et al.* (17). The [^3H]fucose was located by use of authentic standards, excised and eluted from the TLC plate, and subjected to quantitation by gas-liquid chromatography analysis.

Gas-Liquid Chromatography Analysis. Samples were prepared for gas-liquid chromatography analysis essentially by the method of Clamp *et al.* (6). Briefly, the dried samples plus 50 nmol of D-mannitol were methanolized in anhydrous 1.0 N methanolic-HCl at 100° for 3 hr, neutralized with solid silver carbonate, and re-N-acetylated with acetic anhydride, and the solid material was removed by low-speed centrifugation. The combined supernatant fraction was dried in a vacuum over phosphorus pentoxide for 16 hr and silylated at ambient temperature for at least 30 min in hexamethyldisilazane/trimethylchlorosilane/pyridine (1/2/5, v/v/v; Regis Chemical Co., Morton Grove, IL).

Analysis for α -L-Fucosidase Activity. Cells were harvested in 0.05% trypsin-2 mM EDTA, placed in ice-cold medium immediately after being dislodged, pelleted at 600 $\times g$ for 10 min, and broken by brief sonication in 0.2 M phosphate buffer adjusted to pH 5.8 with 0.1 M citric acid. *p*-Nitrophenyl- α -L-fucoside (1.5 mM), in the same buffer, was added to approximately 0.5 mg protein of the above homogenate and the mixture was incubated at 37°. In parallel, [^3H]FL4c was added to a comparable cell mixture. The reaction was stopped with the addition of TCA, *i.e.*, a final concentration of 3%, and the enzyme activity was estimated either colorimetrically as compared to a boiled enzyme blank or by thin-layer analysis for release of labeled fucose.

RESULTS

The observation of a decreased level of FL4c in SV40-transformed WI-38 cells as compared to WI-38 cells is based on differences in the incorporation of radioisotopically labeled fucose. An estimate of the chemical level of FL4c in WI-38 and SV40-transformed WI-38 cells (Table 1) revealed that there was approximately 10-fold more FL4c in the WI-38 cells, which is similar to our findings based on the incorporation of labeled fucose.

The structure of FL4c suggests that the component is derived from N-linked glycoproteins. [^3H]Fucose pulse/chase and long-term fucose labeling/chase experiments were undertaken to ascertain whether the metabolism of FL4c is consistent with its being a product of fucoprotein catabolism. "Chase" of WI-38 cells (Chart 1) revealed that there was a decrease in the level of labeled fucose in the TCA-insoluble fraction and, up to 24 hr of chase, an almost stoichiometric increase in labeled fucose in FL4c. From 24 to 48 hr, there was a more marked decrease in radioactivity in the TCA-insoluble fraction as well as a decrease in FL4c. In a similar [^3H]fucose pulse/chase experiment with SV40-transformed WI-38 cells (Chart 2), the decreased label from the TCA-insoluble pool during the entire 48-hr chase period was paralleled by an increase in the labeled fucose incorporated

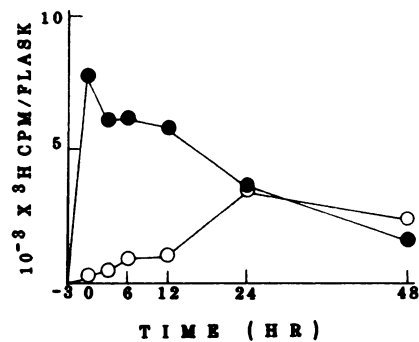


Chart 1. [^3H]Fucose pulse/chase labeling of WI-38 cells. ●, [^3H]fucose incorporation into the TCA-insoluble fraction of WI-38 cells; ○, [^3H]fucose incorporation into FL4c. For experimental details, see "Materials and Methods."

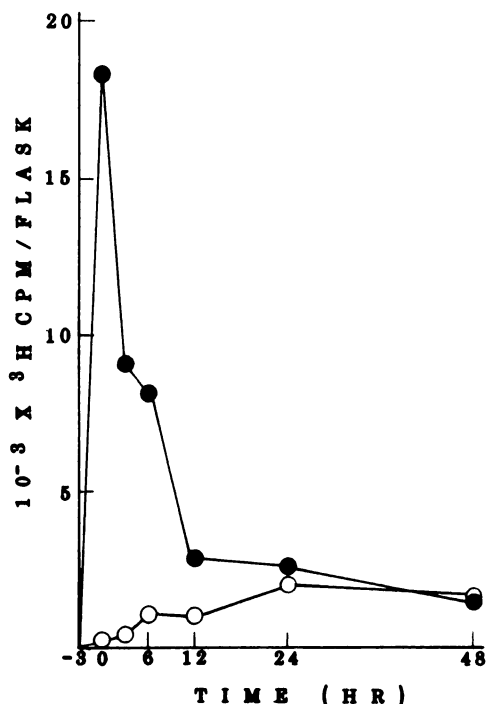


Chart 2. [^3H]fucose pulse/chase labeling of SV40-transformed WI-38 cells. ●, [^3H]fucose incorporation into the TCA-insoluble fraction of SV40-transformed WI-38 cells; ○, [^3H]fucose incorporation into FL4c. For experimental details, see "Materials and Methods."

into FL4c. To obtain additional information about the metabolic stability of FL4c as well as its relationship to fucoprotein metabolism, long-term fucose-labeling/chase experiments were carried out. WI-38 cells labeled for 48 hr were subjected to 72 hr of chase (Chart 3). The decreased level of labeled fucose associated

with the TCA-insoluble fraction throughout the 72 hr of "chase" is paralleled by an increase in FL4c. Similarly, the results with the SV40-transformed WI-38 cells (Chart 4) revealed a decrease in TCA-insoluble labeled fucose throughout 48 hr of "chase" with a concomitant increase in labeled fucose into FL4c throughout the entire "chase" period. Interestingly, from 48 to 72 hr of "chase" of the transformed cells, the TCA-insoluble fraction appears to manifest little loss of labeled fucose.

The combined metabolic labeling experiments were consistent with FL4c being a relatively stable catabolic end product of fucoprotein metabolism. To further explore the relationship between FL4c and glycoprotein metabolism, WI-38 cells were grown in the presence of tunicamycin, an inhibitor of the synthesis of *N*-linked oligosaccharide chains (7). The results (Table 2) indicated a preferential inhibition of incorporation of label into FL4c as compared to the *O*-linked amino acid fucosides FL4a [glucosyl β -(1 \rightarrow 3)fucosyl α -1 \rightarrow threonine] and FL3a (fucosyl α -1 \rightarrow threonine) (Table 2). These results supported the idea that FL4c is derived from *N*-linked-type glycoprotein. The metabolic labeling experiments indicated that the reduced level of FL4c in the transformed WI-38 cells was not likely to be due to breakdown of that component. Alternatively, enhanced release of fucose from the parent fucoprotein(s), due to elevated fucosidase activity, might lower the level of precursor available for subsequent catabolism to FL4c. One approach to explore this possibility is to determine whether there was increased fucosidase activity in the transformed cells. It can be seen that the SV40-WI-38 cells manifest an increased level of α -L-fucosidase activity as compared with the WI-38 cells (Table 3). In contrast, no α -L-fucosidase activity was detectable with [3 H]FL4c as substrate with either cell type.

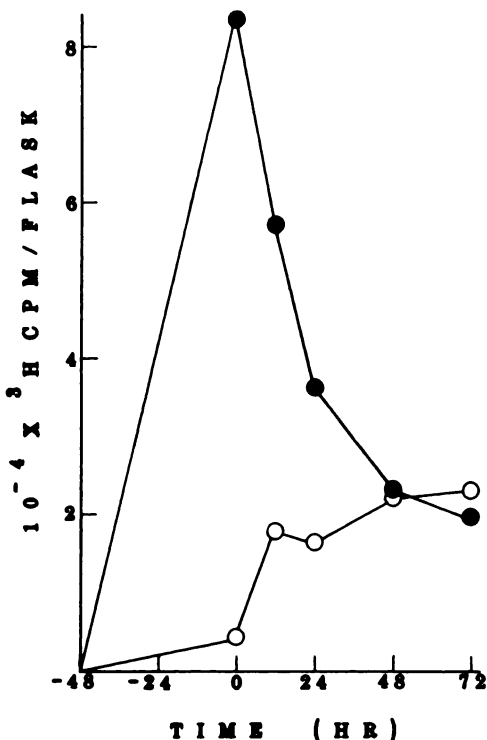


Chart 3. Long-term [3 H]fucose labeling/chase of WI-38 cells. ●, [3 H]fucose incorporated into the TCA-insoluble fraction of WI-38 cells; ○, [3 H]fucose incorporated into FL4c. For experimental details, see "Materials and Methods."

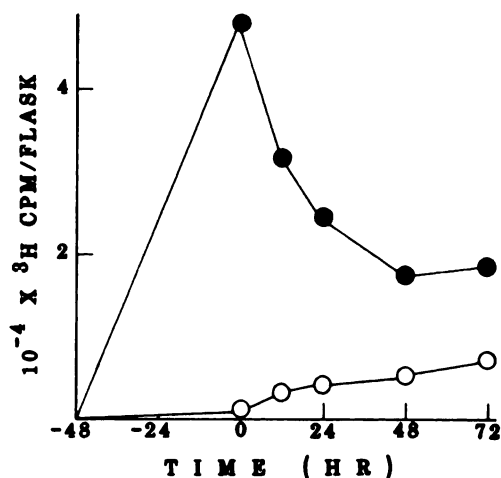


Chart 4. Long-term [3 H]fucose labeling/chase of SV40-transformed WI-38 cells. ●, [3 H]fucose incorporated into the TCA-insoluble fraction of WI-38 cells; ○, [3 H]fucose incorporated into FL4c. For experimental details, see "Materials and Methods."

Table 2
Effect of tunicamycin on the incorporation of [3 H]fucose into FL4c in WI-38 cells

The medium of the HEL cultures was supplemented with tunicamycin at the time of addition of the radioisotopically labeled precursor. Cells were harvested and analyzed 18 hr after the addition of the tunicamycin.

	[3 H]cpm/mg protein		% of decrease in incorporation of tunicamycin-treated cells ^a
	No addition	Tunicamycin supplement (1 μ g/ml)	
FL4c	10,980	347	97
FL4a	1,475	1,387	6
FL3a	1,976	2,338	-18
Leucine	163,400	91,400	43
Mannose	30,400	2,400	92

^a (Control - tunicamycin)
Control

Table 3
 α -L-fucosidase activity of cell-free preparations from WI-38 and SV40-transformed WI-38 cells

Incubation period (hr)	WI-38		SV40-WI-38	
	nmol fucose released/hr/mg protein ^a	% of [3 H]fucose released from FL4c/mg protein/hr	nmol fucose released/hr/mg protein ^a	% of [3 H]fucose released from FL4c/mg protein/hr
24	1.5	<0.01	3.5	<0.01
48	1.6	<0.01	4.8	<0.01

^a Determined colorimetrically using *p*-nitrophenyl α -L-fucoside as substrate.

DISCUSSION

The [3 H]fucose pulse/chase, the long-term fucose labeling/chase experiments, and the studies with tunicamycin suggest that FL4c is a relatively stable metabolic end product of *N*-linked fucoprotein metabolism in both the normal and transformed human embryonic lung cells. These results would argue against the idea that the basis for the reduced level of FL4c in the SV40-transformed WI-38 cells is due to breakdown or to shedding of that component. Thus, it seems reasonable to speculate that the basis of the reduced level of FL4c is either: (a) that the parent fucoprotein(s) is shed at an enhanced rate in the transformed cells as, for example, has been shown with fibronectin (10, 14, 15); (b) that the parent fucoprotein(s) is structurally different, *i.e.*,

less "core-type" fucose; or (c) that the fucose is cleaved from the parent glycoprotein at a relatively early stage in the catabolism of the fucoprotein(s) such as might result from increased fucosidase activity. With regard to the second possibility, Santer and Glick (11) have demonstrated the presence of a significant number of fucosyl residues linked α 1 \rightarrow 3 or 4 to *N*-acetylglucosamine in human tumor cells of neuroectodermal origin but not on membrane glycopeptides from human fibroblasts. These studies suggest the possibility of varying types of fucose linkages between normal and transformed human cells. In addition, a comparison of the fucoproteins derived from like fucoproteins of normal as compared to transformed cells revealed that a high percentage of the fucoproteins manifested differences in the oligosaccharide chains (13). Furthermore, Ceccarini (5) has shown that there is an enrichment in fucoproteins with a higher average molecular weight in transformed WI-38 cells as compared to nongrowing ones. With regard to the last possibility, enhanced glycosidase activity has been demonstrated to follow transformation of mouse (2) or chicken cell lines (4) and that higher levels of sialidase, β -galactosidase, and α -mannosidase have been found in human malignant breast and colon tissue homogenates as compared to normal tissue in surrounding areas (3). Furthermore, it has been demonstrated that there is a striking increase in α -L-fucosidase activity in a rapidly growing Morris hepatoma (1). Also, Yurchenko and Atkinson (16) observed that 75 to 80% of the protein-bound radioisotopically labeled fucose released from the surface fucoproteins of HeLa cells over a 22-hr period was in the form of free fucose. One interpretation of their data is that there is an active fucosidase at the HeLa cell surface. We have observed that FL4c is approximately 100-fold lower in HeLa cells than in WI-38 cells (9). It is intriguing to speculate that the relatively low level of FL4c in HeLa cells and in SV40-transformed WI-38 cells is due to increased fucosidase activity. In fact, we have observed⁴ a relatively higher proportion of low-molecular-weight catabolic products in the cytosol and medium of SV40-WI-38 cells as compared to WI-38 cells. Also consistent with this speculation is the observation that broken-cell preparations of SV40-transformed WI-38 cells have almost a 3-fold-higher level of α -L-fucosidase activity with *p*-nitrophenyl α -fucoside as substrate. However, there was no demonstrable fucosidase activity against [³H]FL4c. We have demonstrated previously that there is quantitative release of [³H]fucose from [³H]FL4c when α -L-fucosidase with broad enzymatic activity was used (9). The metabolic labeling experiments with WI-38 cells which demonstrate elevated levels of labeled fucose "chased" into FL4c are consistent with the previously reported observation of a relatively high amount of label in FL4c in steady-state fucose-labeled WI-38 cells (9). Moreover, we have demonstrated that the relatively high level of labeled fucose incorporated into FL4c

⁴ S. M. Steiner and P. A. Morton, unpublished observations.

of the WI-38 cells as compared with their SV40-transformed counterparts is paralleled by a comparatively high chemical level of FL4c. Whatever the biochemical basis for the decrease in the level of FL4c, it would seem to underlie an important difference between normal and transformed cells in membrane glycoprotein catabolism. Whether such differences contribute to the loss of growth regulation often associated with oncogenic transformation remains to be seen and is the object of current study in this laboratory.

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