Surface Glycoproteins of Normal and Transformed Cells: A Difference Determined by Sialic Acid and a Growth-Dependent Sialyl Transferase

(fucose-labeled surface components/malignant cells/temperature-sensitive oncogenic virus mutants/
hamster/*N*-acetylneuraminic acid/chick)

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ABSTRACT The pattern of elution from a column of Sephadex G-50 of a fucose-labeled carbohydrate component derived from the surface glycoproteins of transformed cells can be altered to resemble that from untransformed cells by enzymatic removal of the sialic acid. These results indicate that the consistent differences found between control and virus-transformed cells may depend upon a relatively specific sialyl transferase that is found in greater amounts (2.5- to 11-times) in transformed cells than in control cells, and in dividing cells as compared to nondividing cells.

There have been several studies on the surface glycoproteins of normal and malignant cells (1-4). In previous work (5-8), it has been shown that a fucose-containing glycopeptide is present in relatively high concentrations in the surface membrane of virus-transformed cells, as compared to their untransformed counterparts.

In these studies with [^{14}C]- and L-[^3H]fucose used in a double-label technique, either solubilized material from cells treated with trypsin or isolated surface membranes from normal and transformed cells were mixed, and they were exhaustively digested with Pronase; the products were chromatographed on columns of Sephadex G-50. The macromolecular materials chromatographed are essentially intact carbohydrate moieties that bear labeled L-fucose in a terminal position. These large oligosaccharides had been attached to glycoproteins residing in the surface structure of the cell. Upon comparison of glycopeptides from the surface membranes of virus-transformed cells and control cells by gel filtration, it was found that transformed cells were enriched in a population of relatively large, early-eluting molecules that were present in only small quantities in material derived from control cells. Enrichment of this material occurred in cells derived from mouse, chick, or hamster that were transformed by DNA- or RNA-containing oncogenic viruses (7). The occurrence of this material is dependent upon growth (6); it is not detected in control cells that are not growing, and is markedly reduced in transformed cells that are not growing.

Recently, it has been shown (8) that the early-eluting material is present in relatively large quantity in the surface of T5-transformed chick-embryo fibroblasts only when they are grown at a permissive temperature (35°). Cells transformed by this temperature-sensitive mutant of Rous sarcoma virus

manifest malignancy when grown at 35°, but appear normal by several criteria when grown at 40°. The process is reversible. Virus is produced at both temperatures (9).

In this report, we describe what we believe to be the basis for the difference between the normal and malignant patterns. The early-eluting material that increases in quantity in dividing, transformed cells contains an extra complement of sialic acid. Further, we have found a specific sialic acid transferase (sialyl transferase), which is increased in activity in transformed cells and is greatly reduced in activity in nongrowing control cells, that transfers *N*-acetylneuraminic acid (NeurAc) from CMP-NeurAc to an acceptor—desialylated, early-eluting material derived from the surface of growing, virus-transformed cells. This transferase activity is present in greater amount in T5-transformed chick-embryo fibroblasts grown at 36° (permissive temperature) than in those grown at 41°, where the cells retain their normal fibroblast morphology.

MATERIALS, METHODS, AND RESULTS

The culture and processing of BHK₂₁-C₁₃ cells, those transformed with the Bryan strain of Rous sarcoma virus, (C₁₃-B₄), and those transformed by polyoma virus (Py Y) have been described in detail (5-8). Briefly, control cells were grown in roller bottles for 3 days in the presence of L-[^{14}C]fucose, while transformed cells were allowed to divide for 3 days in the presence of L-[^3H]fucose. The cells were treated with trypsin to remove surface components of the cells; the soluble fractions after centrifugation of treated cells were mixed and digested with Pronase (5). Pronase digests were chromatographed on columns of Sephadex G-50 (fine) and each fraction was counted for both ^{14}C and ^3H (5). The culture and handling of chick-embryo fibroblasts and their transformation by the Schmidt-Ruppin strain of Rous virus and by T5, a temperature-sensitive mutant derived from this virus, has also been described (8, 9).

For the preparation of enzymes, cells were harvested in the logarithmic and in the stationary phase of growth. Medium was decanted and the cells were gently rinsed 3 times with 10 ml of a solution containing 0.8% NaCl-0.05% KCl-1 mM EDTA-1 mM KPO₄, pH 7.4 (buffer I) (10). The cells were scraped off the surface with a rubber policeman; 5 ml of buffer I was added and the cells were centrifuged at 600 × *g* for 5 min. The supernatant solution was removed. For every 10⁷ cells, 0.15 ml of buffer II (0.01 M KPO₄, pH 6.5-1 mM

Abbreviation: NeurAc, *N*-acetylneuraminic acid.

FIG. 1. Elution patterns from a column of Sephadex G-50 (fine) of large-scale Pronase digests from trypsin-treated BHK 21- C_{13} cells and C_{13} -B $_4$ cells. Preparative Pronase digests were run separately, but are recorded here on the same graph. BHK $_{21}$ - C_{13} , ^{14}C : $\bigcirc$ — $\bigcirc$; C_{13} -B $_4$, 3H : $\bullet$ — $\bullet$.

MgCl $_2$ -0.1% Triton X-100) was added and the cells were transferred to a small Potter-Elvehjem homogenizer with a Teflon pestle. The cells were broken by about 100 vigorous strokes at 4°. The homogenate was centrifuged at 600 $\times$ g for 10 min. The opalescent supernatant was removed and 0.05 ml of buffer II was added to the pellet for each 10 7 cells in the original preparation. The pellet was stirred and then centrifuged as before. The resulting supernatant was combined with the first supernatant; this mixture was the enzyme preparation. All steps were performed at 2–4°. Surface membranes were isolated from BHK $_{21}$ - C_{13} and C_{13} -B $_4$ cells by the zinc ion method (11).

Cytidine-5'-monophospho-*N*-acetyl- ^{14}C neuraminic acid (CMPNeurAc), ([4,5,6,7,8,9- ^{14}C]sialic acid, 223 Ci/mol) was purchased from New England Nuclear Corp. L-[1- ^{14}C]fucose (50 Ci/mol) and L- 3H fucose (generally labeled, 4 Ci/mmol) were purchased from Calbiochem., Los Angeles, Calif. and New England Nuclear Corp., Boston, Mass., respectively.

Sialic acid was removed from bovine submaxillary gland mucin and fetuin by acid hydrolysis, as described by Grimes (10). Purified fetuin was purchased from Grand Island Biological Co., Rock Island, N.Y. Bovine submaxillary gland mucin was purchased from Sigma Chemical Co., St. Louis, Mo.

"Specific acceptor" was prepared from Pronase digests of the trypsin-soluble fraction of cells derived from lots of 40 roller bottles (4×10^9 cells) of C_{13} -B $_4$ cells harvested in the logarithmic phase of growth. The cells in six of these bottles were grown for 3 days in the presence of L- 3H fucose (5). Trypsin-soluble fractions were obtained as described (5). They were digested with Pronase and applied to a column of Sephadex G-50 fine, (180 $\times$ 3.5 cm). The column was developed with 0.04 M Tris-acetate, pH 8.0, at a flow rate of 0.8 ml/min. 8-ml Fractions were collected. Aliquots were counted (Fig. 1), and appropriate tubes were pooled to obtain fractions A and B; which were lyophilized. 1–2 ml of water was added to each fraction; these were desalted by passage through a column of Sephadex G-10 (30 $\times$ 2 cm). The eluates were again lyophilized and redissolved in about 1 ml of water. Fraction A (tubes 30–49) consists of material that is greatly increased in amount after viral transformation. In order to convert this material to a form lacking sialic acid, half of the solution containing peak A (0.5 ml) was incubated with 50 μ mol of Na acetate, pH 5.2, 10 μ mol of CaCl $_2$, and 20 units of *Vibrio cholerae* neuraminidase (Calbiochem) for 2 hr at 37°. After incubation, the solution was heated for 5 min at 100° to

inactivate the enzyme. This preparation (desialylated peak A), labeled with L- 3H fucose, served as substrate for the sialyl transferase to be described.

The incubation mixture used to detect and measure sialyl transferase activity was essentially as described by Grimes (10). A typical reaction mixture contained 10 μ l of 0.25 M K-phosphate buffer, pH 6.9-mM MgCl $_2$ -0.1% Triton X-100 and about 2 nmol (0.4 μ Ci) of CMP- ^{14}C NeurAc. Acceptors added were 20 μ l of desialylated peak A (from about 4×10^7 cells) or 0.5 mg of desialylated fetuin or bovine submaxillary gland mucin. About 0.2–0.5 mg of enzyme protein was added, in a volume of 30 μ l. Volumes were made to 70 μ l with water. Incubations took place in 12-ml stoppered conical centrifuge tubes at 37° for 1 or 2 hr. The tubes were then stored at –20° until assayed.

To determine the level of sialyl transferase, 0.02 ml of a mixture of Blue dextran 2000 and phenol red was added to each tube, and the entire contents were placed on a column (60 $\times$ 0.8 cm) of Sephadex G-50 fine. The column was de-

FIG. 2. Assay for sialyl transferase. The results of three assays. In the first, no acceptor is present and there is no 3H (acceptor) peak or ^{14}C NeurAc peak in tubes 5–15. The enzyme extract was derived from C_{13} -B $_4$. In the second and third assays, desialylated peak-A material (3H) ($\bullet$ — $\bullet$) is present. $\bigcirc$ — $\bigcirc$ indicates transfer of ^{14}C NeurAc to acceptor. 0.2 mg of particulate enzyme protein was used in each assay, and incubation was for 1 hr. a, no acceptor; b, enzyme from BHK $_{21}$ - C_{13} cells; c, enzyme from C_{13} -B $_4$ cells.

FIG. 3. Double-label elution patterns of Pronase digests of trypsin-soluble preparations eluted from columns of Sephadex G-50 (fine). The procedure has been described in detail (5-8). (a and b) Material derived from BHK 21-C₁₃ cells labeled in log phase (O—O) for 3 days with L-[^{14}C]fucose and from C₁₃-B₄ cells labeled with L-[^3H]fucose (●—●). In b, the Pronase digests were treated with 5 units of neuraminidase for 1.5 hr at pH 5.2 in 3 mM CaCl before application to the column. Controls were also incubated, but without enzyme.

TABLE 1. Transfer of [^{14}C]NeurAc to various acceptors by extracts from control and virus-transformed cells

[^{14}C]NeurAc acceptor	Extract from BHK 21-C ₁₃ cells	Extract from C ₁₃ -B ₄ cells
	cpm/mg of protein per hr.	
None	0	0
Endogenous	1,690	2,170
Peak B	1,600	2,030
Peak A	0	0
Desialylated peak A	1,790	5,450
Desialylated fetuin	14,800	13,500
Desialylated bovine mucin	17,700	18,000

veloped with 0.1 M Tris-acetate buffer, pH 9.0–0.1% sodium dodecyl sulfate–0.1% 2-mercaptoethanol–0.01% EDTA. Nineteen 0.8-ml fractions were collected; each was counted for ^3H and ^{14}C in 10 ml of Aquasol (New England Nuclear) in a liquid scintillation counter. A typical series of results is seen in Fig. 2. The product of the transferase reaction is found in tubes 5–15. There is a great excess of CMP-NeurAc and NeurAc that begins to elute from the columns in tube 18. Endogenous acceptor, desialylated fetuin, and bovine submaxillary mucin that had accepted [^{14}C]NeurAc eluted in tubes 1–3, along with the marker of Blue dextran-2000.

In Fig. 3 can be seen elution patterns of the carbohydrate components of glycoproteins derived from the surface of control and virus-transformed cells chromatographed before and after digestion with neuraminidase. As previously reported, there is a large, early-eluting material that contains fucose in transformed cells (*a, c, e*). However, when these preparations are treated with neuraminidase from *V. cholerae* (or *Clostridium perfringens*) before gel filtration, the profiles of the glycopeptide from transformed and control cells are virtually identical (*b, d, f*). Upon treatment with neuraminidase, only peak B remains. These results suggest that transformed cells might contain a relatively specific sialyl transferase, capable of transferring NeurAc from CMP-NeurAc to fucose-containing acceptors residing in a protein on the surface of transformed cells.

To assay for such an enzyme, we used as a sialic acid acceptor the desialylated material in peak A (Fig. 1) derived from C₁₃-B₄ cells that had been cultured in the presence of L-[^3H]fucose. Typical results of the enzyme assay are seen in Fig. 2. A peak of ^{14}C activity just preceding the ^3H peak indicates that [^{14}C]NeurAc has been transferred to a desialylated acceptor present in the peak-A fraction from transformed cells.

There is 3-times more transferase activity in extracts of C₁₃-B₄ than there is in BHK₂₁-C₁₃ cells (Table 1). In various experiments where normal and virus-transformed cells were compared [BHK₂₁-C₁₃ and C₁₃-B₄, BHK₂₁-C₁₃ and polyoma transformed (Py Y)] there was 2.5- to 11-times more activity

TABLE 2. Transfer of [^{14}C]NeurAc by fractions of cells in the logarithmic and stationary phase of growth

Cell	Growth phase	Fraction	^{14}C Incorporated into desialylated peak A material cpm/mg of protein per hr
BHK 21-C ₁₃	Log	Supernatant	2,140
BHK 21-C ₁₃	Plateau	Supernatant	546
C ₁₃ -B ₄	Log	Supernatant	10,850
C ₁₃ -B ₄	Plateau	Supernatant	1,900
C ₁₃ -B ₄	Log	Crude homogenate*	9,700
C ₁₃ -B ₄	Plateau	Crude homogenate*	2,860
C ₁₃ -B ₄	Log	Surface membrane	7,400
C ₁₃ -B ₄	Plateau	Surface membrane	2,840

* Cells were treated with ZnCl_2 (1 mM) for 2 hr before centrifugation, then surface membranes were prepared (11), and a crude homogenate was made of an aliquot of the cells treated with zinc ions. Incubation was for 1 hr.

in extracts from transformed cells. Transferases that sialylate nonspecific acceptors of unknown nature in the enzyme extract itself or desialoylated fetuin and bovine submaxillary gland mucin were present at similar activities in extracts of control and transformed cells. In all cases, neuraminidase could remove the [^{14}C]NeurAc transferred to the acceptors. Material in peak A that was not desialylated did not accept [^{14}C]NeurAc (Table 1), while peak-B material could accept [^{14}C]NeurAc without prior treatment with neuraminidase.

Control and transformed cells in the stationary phase of growth had considerably less activity than did those actively dividing (Table 2). When enzyme preparations derived from BHK₂₁-C₁₃ and C₁₃-B₄ cells were combined in incubation vessels, the resultant activity was the same as the sum of the separate transferase activities. Similarly, the sum of activities in extracts from dividing and nondividing cells were the same whether or not the extracts were combined in incubation vessels.

The enzyme activity was particulate. Surface membranes isolated from C₁₃-B₄ cells by the zinc ion method (11) had good sialoyl transferase activity with desialoylated peak A as an acceptor, although as seen in Table 2 the specific activity is not greater than that of the crude homogenate when cells were treated with zinc ions. Our unpublished work suggests that the accessory peak A, seen in the surface structure of transformed cells, is present in internal membranes as well (mitochondria, nucleus, and endoplasmic reticulum). This finding would indicate that the sialyl transferase that is probably responsible for the formation of this material is also present within the cell. Thus, an increase in specific activity in the surface membrane might not be expected. Surface membranes from cells in the stationary phase of growth (3–4 days without any increase in cell number) had

(*c* and *d*) Material derived from BHK 21-C₁₃ cells (○—○) and its polyoma virus-transformed counterpart (Py Y) (●—●). Note that the pattern, though slightly different from that of C₁₃-B₄, is almost identical to that described (7). (*d*) Material treated with neuraminidase before application to the column.

(*e* and *f*) Material derived from chick-embryo fibroblasts before (○—○) and after (●—●) transformation with the Schmidt-Ruppin (SR) strain of Rous sarcoma virus. (*f*) Material treated with neuraminidase.

correspondingly less activity than those from cells in the log phase (Table 2). The pH optimum of the enzyme is 6.9, which is higher than the optimum for transfer of NeurAc by cell extracts to endogenous acceptor, desialylated fetuin, or bovine submaxillary gland mucin.

We have found that there is 3- to 4.5-fold more activity in extracts of T5-transformed cells than in control cells grown at 36°, while activities were at about the same values in extracts from control and transformed cells grown at 41°. (For details see ref. 17.)

DISCUSSION

We have reported (5-8) that there is a consistent difference in certain carbohydrate components of glycoproteins located on the surface of control and virus-transformed cells, whichever the species of cells and the oncogenic virus responsible for transformation. While a relatively large amount of this polymer is present in the transformed cells, there is clearly only a small amount in material from control cells. It may, therefore, be a normal component that is present in far greater amount in the transformed, malignant cell. Its appearance is also dependent on cellular growth, since it increases in amount in rapidly dividing normal or transformed cells.

Treatment of the surface component with neuraminidase causes it to elute much more slowly from columns of Sephadex G-50, in a region containing lower molecular weight material characteristic of control or nondividing cells.

The data presented here suggest that the appearance of this surface component may depend upon the presence of a specific sialyl transferase. This transferase shows considerable specificity, is relatively low in amount in nondividing cells, and is present in much greater quantity in transformed than in control cells. Cells transformed by T5 virus contain more of the transferase activity when they are grown at 36°, a permissive temperature at which transformation is manifested, than at 41°, where the cells appear normal. The isolated surface membrane contains some of the total transferase activity. It is of interest that a galactosyl transferase has been found in the surface of chick-embryo retinal cells that may be involved in the process of intercellular adhesiveness (12). A sialyl transferase has also been observed in isolated surface membranes of rat liver (13).

It is of particular interest that while we find sialyl-transferase activity of unknown specificity in greater amounts in transformed cells, three other reports provide evidence for considerable reductions in sialyl-transferase activity (10, 14, 15). These reductions would appear to be due to other sialyl transferases. However, other sugar transferases may be increased in activity in virus-transformed cells (16).

It must be kept in mind that the resolving power of Sephadex G-50 is not great, and that the materials we are working with are not homogeneous.

There appears to be a consistent difference between control and virus-transformed cells that is essentially quantitative in nature. It may be that an initial genetic alteration results in a sustained perturbation in which all the components of the normal cell are present, but the relative amount of each is altered. The slight alterations could allow viability, but might interfere with crucial control mechanisms required to regulate cell division.

The biological function of the carbohydrate moiety that we are studying is unknown. We can only speculate on its involvement in the process of malignancy. However, the consistency with which it is found in various transformed cells (5-8), the changes it undergoes in cells transformed by temperature-sensitive mutants of Rous sarcoma virus (8), and the fact that it increases in quantity in rapidly dividing—as compared to nongrowing—cells encourages us to believe that it is of importance in the malignant process—a disorder of cell division and growth that probably involves the surface of the cell.

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