Cell-surface glycosyltransferases in cultured fibroblasts: Increased activity and release during serum stimulation of growth

(galactosyltransferase/ α -lactalbumin/NIL and BHK/C13 fibroblast strains)

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ABSTRACT Cell-surface galactosyltransferase was studied in suspensions of intact baby hamster kidney fibroblasts with both endogenous and exogenous glycoprotein acceptors. The cell-surface location of galactosyltransferase was demonstrated in experiments with the enzyme modifier α -lactalbumin, which does not enter the cell. The addition of α -lactalbumin to the assay medium for galactosyltransferase resulted in accumulation of lactose in the medium but not in the cells. There was no detectable hydrolysis of UDP-galactose to free galactose by these cells, nor did a 100-fold molar excess of free galactose inhibit cell-surface galactosyltransferase. There was a marked increase in specific activity of cell-surface exogenous galactosyltransferase in serum-stimulated as compared to resting fibroblasts. Dividing but not resting fibroblasts released galactosyltransferase, but not sialyl- or fucosyltransferase, in soluble form into the tissue culture medium. The release of galactosyltransferase was greater from virally transformed than from nontransformed fibroblasts.

Glycosyltransferases are a family of membrane-associated enzymes found primarily in the smooth endoplasmic reticulum and Golgi apparatus that catalyze transfer of monosaccharide residues from nucleotide sugars to incomplete acceptor glycoproteins. A number of recent studies have demonstrated that these enzymes are also present on the plasma membrane of various mammalian cells, where they may mediate cell-to-cell adhesion or recognition (1, 2). Cell-surface glycosyltransferase has been demonstrated on human platelets and evidence has been presented from several laboratories (3, 4) that this enzyme may be directly involved in platelet–collagen adhesion, an important first step in blood coagulation. Roth and White (5) have suggested that surface galactosyltransferase on mouse 3T3 cells may glycosylate receptors on adjacent cells after intercellular contact is made and that this transglycosylation may be important in contact inhibition of growth. Others have questioned the interpretation that glycosyltransferases measured in whole cell suspensions reflect cell-surface enzyme activity. Deppert *et al.* (6) observed hydrolysis of nucleotide sugars by suspensions of intact baby hamster kidney (BHK) cells with subsequent uptake of the labeled sugar and intracellular glycosylation. Keenan and Morre (7) have also suggested that the apparent surface transferase activity of whole cell suspensions may reflect intracellular glycosylation or secretion of soluble glycosyltransferases into the incubation medium. However, the bulk of experimental evidence supports the localization of glycosyltransferases to the plasma membrane as well as internal membranes (8).

In previous work from this laboratory (9) a correlation was observed between surface galactosyltransferase and concanavalin A agglutination in various normal and malignant mammalian cells. We also demonstrated a significant increase in

cell-surface galactosyltransferase on lectin-transformed thymic lymphocytes compared to resting lymphocytes (10). In the current study, the relationship between cell-surface galactosyltransferase and cell division was explored in serum-stimulated NIL and BHK/C13 fibroblasts and their polyoma-transformed counterparts. Rapidly dividing fibroblasts have increased levels of cell-surface galactosyltransferases, and release a soluble form of galactosyltransferase into the tissue culture medium.

MATERIALS AND METHODS

Cell Cultures. BHK/C13 and NIL strains of baby hamster kidney fibroblasts (originally obtained from M. Stoker, Imperial Cancer Research Fund Laboratories, London) and the polyoma-virus-transformed counterpart of NIL, NIL/Py, were used in this study. The cells were grown on plastic petri dishes or in roller bottles in Dulbecco's modified Eagle's medium (Microbiological Associates, Bethesda, Md.) supplemented with heat-activated fetal calf serum. Cells were harvested with EDTA and washed in cold calcium–magnesium-free 0.1 M sodium phosphate buffer, pH 7.4, containing 0.154 M NaCl (phosphate-buffered saline). Cultures were routinely checked for growth of mycoplasma and contaminated cultures were discarded.

Glycosyltransferase Assays. Cell surface galactosyltransferase was measured in suspensions of intact, washed fibroblasts immediately after harvesting. Cell-surface galactosyltransferase activity was expressed as the amount of radiolabeled monosaccharide transferred to cell-surface (endogenous) acceptors or to an added glycoprotein (exogenous) acceptor. The rate of galactosyltransferase activity was shown to be linear up to 1 hr, and to be directly proportional to the amount of enzyme in cells or medium. The pH optimum of cell-surface or medium galactosyltransferase was between 7.0 and 7.5; the optimum manganese concentration was 5 mM for whole cells, and 25 mM for medium. The washed wells were suspended in a concentration range of 2 to 8×10^6 cells per ml in 0.1 M sodium cacodylate/0.154 M sodium chloride buffer, pH 7.4 (saline/cacodylate buffer). The incubation mixture for cell-surface galactosyltransferase consisted of 0.05 ml of cell suspension, 0.39 nmol of UDP-[14 C]galactose (257 mCi/mmol), 0.5 μ mol of manganese chloride, and 250 μ g of fetuin free of sialic acid and galactose (11) in a final volume of 0.0725 ml. The incubation mixture for galactosyltransferase in the medium was identical except that 0.05 ml of medium (centrifuged at 1000 rpm in an International centrifuge for 10 min to remove floating cells) and 2.5 μ mol of manganese chloride were substituted. Following a 30 min incubation at 37° the assay was terminated and radioactivity was determined as described previously (10). The incubation mixture for sialyltransferase consisted of 0.05 ml, 0.46 nmol of CMP-[14 C]sialic acid (229 mCi/mmol), 0.5 μ mol

Abbreviation: Py, polyoma virus.

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Table 1. Lactose synthesis by surface galactosyltransferase in the presence of α -lactalbumin

Additions	Lactose production, cpm	
	Cells	Supernatant
None	50	25
α -Lactalbumin	150	4250

Lactose production by 1.5×10^6 BHK/C13 cells in the presence of α -lactalbumin and UDP- ^{14}C galactose was measured as described in *Materials and Methods*. After incubation the cells and supernatants were separated by centrifugation and the presence of ^{14}C lactose was determined by high-voltage electrophoresis.

of manganese chloride, and 250 μg of fetuin free of sialic acid. The incubation mixture for fucosyltransferase was identical except that 0.38 nmol of GDP- ^{14}C fucose (174 mCi/mmol), and 250 μg of orosomucoid free of sialic acid (12) were added. Assay of fucosyltransferase in culture medium was also performed by the method of Schenkel-Brunner *et al.* (13) using lactase as acceptor and an incubation time of 24 hr.

The assay for lactose synthetase (lactose synthase; UDP-galactose 4- β -galactosyltransferase, EC 2.4.1.22) contained 1.5×10^6 BHK/C13 cells, in saline/cacodylate buffer, 200 μg of α -lactalbumin (obtained from Nutritional Biochemicals Corp., Cleveland, Ohio and heated at 80° for 30 min to remove any contaminating galactosyltransferase), 0.8 nmol of UDP- ^{14}C galactose (257 mCi/mmol), 1.0 μmol of manganese chloride, and 20 μg of glucose in a total volume of 0.160 ml. After 30 min incubation at 37°, the cells and supernatant were separated by centrifugation at 1000 rpm for 10 min in an International centrifuge. The cells were washed in 10 volumes of buffer, and both the supernatant and cells were subjected to high-voltage paper electrophoresis in 1% tetraborate buffer (14) to separate the unreacted UDP- ^{14}C galactose from the product ^{14}C lactose, which was identified by comparison with an authentic standard. The lactose areas on the dried chromatograms were cut out and radioactivity was determined in a scintillation counter.

Hydrolysis of UDP-Galactose. UDP-galactose hydrolysis to free galactose by BHK cells was measured by the method of Deppert *et al.* (6). At the end of the 30 min incubation for assay of galactosyltransferase activity, the incubation medium was centrifuged at 1000 rpm for 10 min. Aliquots of the cell-free incubation media and authentic standards of galactose and UDP-galactose were subjected to descending paper chromatography on Whatman no. 1 paper in ethyl acetate/pyridine/water 12:5:4 (vol/vol) for 16 hr to separate UDP-galactose and galactose. The dried paper was cut into 1 cm strips and eluted for 24 hr in 1.0 ml of water, and its radioactivity was measured in 10 ml of Bray's solution (15).

RESULTS

Surface glycosyltransferase enzymes

It has been previously shown that the presence of α -lactalbumin alters the substrate specificity of galactosyltransferase, such that it leads to the combination of glucose and galactose to form lactose (16), while in the absence of α -lactalbumin glucose is not a substrate for the enzyme. As shown in Table 1, incubation of intact BHK/C13 cells with α -lactalbumin and UDP- ^{14}C galactose resulted in the production of ^{14}C lactose, which accumulated primarily in the medium and not in the cells. Because α -lactalbumin, UDP-galactose, and lactose are probably not taken up as such by cultured fibroblasts, and because the

FIG. 1. Cell-surface galactosyltransferase after serum stimulation of growth. NIL and NIL/Py cells were grown in 10% fetal calf serum and harvested at various times after subculturing. Cell-surface exogenous acceptor galactosyltransferase was determined in intact cell suspensions.

^{14}C lactose accumulated in the medium, it seems that these observations are consistent with the presence of a galactosyltransferase on the cell surface. It is unlikely that ^{14}C lactose synthesis was due to secreted or released galactosyltransferase because preincubation of an identical number of cells in buffer for 30 min at 37° did not result in a significant appearance of galactosyltransferase in the medium.

The formation of free galactose from hydrolysis of UDP-galactose by cell-surface nucleotide sugar hydrolases has been postulated (7) to explain apparent glycosylation at the cell surface. Following extracellular hydrolysis of UDP-galactose, free galactose would then enter the cell to be incorporated into glycoproteins and glycolipids by intracellular galactosyltransferases. However, under our experimental conditions, there was no demonstrable formation of free ^{14}C galactose in the incubation medium of either NIL or NIL/Py cells, suggesting that uptake of free galactose followed by intracellular glycosylation was probably not responsible for the observed results. It might also be expected that addition of free galactose to the incubation media for galactosyltransferase would inhibit intracellular uptake and incorporation of free galactose, but would have little effect on cell-surface galactosyltransferase, which requires UDP-galactose, not free galactose, as substrate. Addition of a 100-fold molar excess of free galactose to the incubation buffer for the galactosyltransferase reaction caused only a slight decrease in cell-surface exogenous galactosyltransferase from 2240 to 2040 cpm/10⁶ cells per 30 min. This observation also supports the contention that hydrolysis of UDP-galactose followed by intracellular uptake does not occur to any significant extent under these experimental conditions.

Addition of fetal calf serum to quiescent calls in 0.5% serum was associated with a significant increase in activity of cell-surface galactosyltransferase (Fig. 1). Duplicate cultures of NIL and NIL/Py cells were harvested immediately after subculturing in 10% fetal calf serum and at 10, 20, and 30 hr. A marked increase in cell-surface galactosyltransferase was observed in both the transformed and nontransformed cells. The decrease in enzyme activity at 30 hr in the NIL cultures may represent partial synchronization of these cells for at least one round of cell division, which was complete at 30 hr.

The effect of culture conditions on cell-surface galactosyltransferase activity was examined in nonconfluent and confluent cultures of NIL and NIL/Py cells (Table 2). NIL cells

Table 2. Surface galactosyltransferase activity in confluent and nonconfluent cultures of NIL and NIL/Py cells

Cell type	Serum concentration, %	Galactosyltransferase activity, cpm	
		Non-confluent cultures	Confluent cultures
NIL	0.5	700	—
NIL	10.0	2250	1250
NIL/Py	10.0	1400	2250

Surface galactosyltransferase activity is expressed as cpm/2.5 × 10⁶ cells and represents incorporation of [¹⁴C]galactose into acid-precipitable protein. Nonconfluent cultures were harvested 24 hr after serum addition; confluent cultures were harvested at 72 hr.

in 0.5% fetal calf serum have few, if any, cells in S or M phase and are probably arrested in G₁ (17). Under these conditions they have very low galactosyltransferase activity. NIL cells in 10% fetal calf serum undergo cell division that continues until confluency is reached, after which growth decelerates due to density-dependent (contact) inhibition. In nonconfluent, growing cultures there was marked elevation of surface galactosyltransferase compared to nondividing (resting) nonconfluent cultures in 0.5% serum. As these untransformed fibroblasts reached confluence, there was decreased surface galactosyltransferase concomitant with a reduction in growth. The NIL/Py cells are not contact inhibited; thus growth continues at confluency. This appears to be reflected in the fact that galactosyltransferase did not decrease (and actually increased somewhat) in confluent NIL/Py cells.

Because the galactosyltransferase enzymes within the cell are located in the Golgi apparatus and smooth endoplasmic reticulum, an increase in activity of these enzymes should occur when cells are homogenized, because more enzyme is exposed to the nucleotide sugars and protein acceptors, which do not enter intact cells (10, 14). Homogenization of NIL/Py cells caused an increase in both endogenous and exogenous galactosyltransferase activity. In a typical experiment (Table 3), the endogenous activity of intact cells constituted 75% of the total activity, while in homogenates the endogenous activity was 44% of the total. This may reflect an increased amount or availability of endogenous membrane acceptors on the cell surface as compared to internal membranes.

Release of galactosyltransferase into the medium

Galactosyltransferase was released from cultures of both transformed and nontransformed fibroblasts during serum-

Table 3. Increase of galactosyltransferase activity in NIL/Py cells upon homogenization

	Galactosyltransferase, cpm/10 ⁶ cells	
	Endogenous	Endogenous and exogenous
Whole cells	3075	4000
Homogenates	3750	8500

NIL/Py cells were ruptured in a glass-Teflon homogenizer. Galactosyltransferase activity was measured using endogenous acceptors or the exogenous acceptor fetuin from which sialic acid and galactose had been removed. The above results are those of a typical experiment.

FIG. 2. Release of soluble galactosyltransferase into medium. Exogenous acceptor galactosyltransferase was measured daily in cell-free medium from roller bottle cultures of NIL and NIL/Py cells. Results are expressed as cpm/50 μ l of medium.

stimulated growth. NIL cells were subcultured in roller bottles with either 0.5% or 10% fetal calf serum. Samples of media were removed at 24 hr intervals under sterile conditions and galactosyltransferase activity was measured (Fig. 2). NIL cells in 0.5% fetal calf serum did not divide and the cultures were not maintained past 4 days due to poor survival in low serum concentrations. The NIL and NIL/Py cells in 10% fetal calf serum continued to divide during the 7 days of the experiment, and the cell number increased approximately 3-fold. Contact inhibition of growth is not observed under these conditions because of the large surface area of the roller bottles. A progressive accumulation of galactosyltransferase activity in the medium was noted from day 1 to day 7 (Fig. 2). The nondividing NIL cells in 0.5% serum released only very small amounts of the enzyme into the medium. The galactosyltransferase activity in conditioned cell-free medium was measured as exogenous activity with fetuin from which sialic acid and galactose had been removed; no endogenous enzyme activity could be detected in the medium.

The amount of galactosyltransferase released into the medium was greater with the NIL/Py than with the NIL cells (Fig. 3). NIL and NIL/Py cells were subcultured in medium containing 10% fetal calf serum and aliquots of medium were removed at intervals for measurement of galactosyltransferase. Cells were also harvested and counted from duplicate plates in order to obtain an accurate cell count. The activity of soluble

FIG. 3. Release of soluble galactosyltransferase from NIL and NIL/Py cells. Galactosyltransferase was measured in medium from duplicate cultures of NIL and NIL/Py cells subcultured in 10% serum. Cells were harvested at 10 hr intervals to determine cell counts, and medium was assayed for exogenous galactosyltransferase.

galactosyltransferase expressed as cpm/ 10^4 cells was higher in the transformed than in the nontransformed NIL cells at both 20 and 30 hr.

The release of galactosyltransferase from intact, dividing fibroblasts in culture is in contrast to observations of other glycosyltransferases. Neither fucosyltransferase activity, using either lactose or desialylated orosomucoid, nor sialyltransferase activity, using desialylated fetuin as acceptor, could be detected in media from these experiments. Another membrane-bound enzyme, alkaline phosphatase, was also not detected in the medium. In order to determine if the galactosyltransferase activity in the medium was bound to a membrane fragment or was soluble, samples of conditioned medium with high galactosyltransferase activity were centrifuged at $200,000 \times g$ for 2 hr. More than 99% of the enzyme activity remained in the supernatant fraction, indicating that the enzyme was not associated with a membrane fragment that would be sedimented under these conditions.

DISCUSSION

We have shown that suspensions of fibroblasts have galactosyltransferase activity using endogenous and exogenous acceptors. Several observations support the claim that this enzyme is located on the external surface of the plasma membrane. (i) The addition of α -lactalbumin modified the galactosyltransferase of the fibroblast cell surface and caused formation of lactose that accumulated in the assay medium, but not within the cells. This would not occur if the enzyme catalyzing this reaction were intracellular, because α -lactalbumin will not enter the cell and would therefore, not alter the substrate specificity. (ii) Addition of free galactose to the assay system for galactosyltransferase did not significantly reduce galactosyltransferase activity. An excess of free galactose would be expected to inhibit this activity if the enzyme activity being measured were intracellular and were dependent upon hydrolysis of UDP-galactose to form galactose.

Our results are in basic agreement with the recently published studies of Patt and Grimes (18) showing active cell-surface glycosyltransferases on BHK cells. Both suspensions and monolayers of these fibroblasts had transferase activity with exogenous acceptors, while cells in monolayers could glycosylate only endogenous acceptors. As in the current study, these investigators were also unable to demonstrate significant extracellular hydrolysis of nucleotide sugar substrates as shown previously by Deppert *et al.* (6). Although Keenan and Morre (7) have challenged the existence of cell-surface glycosyltransferases, there is significant evidence from this and other studies (8) to support the concept that these enzymes do exist on the outer surface of the plasma membrane and are capable of glycosylating extracellular glycoprotein acceptors.

In the present study, surface galactosyltransferases of cultured fibroblasts have been shown to be markedly increased during stimulation of growth and cell division after addition of serum to resting cultures. Previous studies of serum stimulation of fibroblast growth have shown that untransformed cells rapidly enter S phase after addition of serum, and undergo mitosis beginning at 20–30 hr (19). The data reported here suggest that addition of serum to resting fibroblasts is accompanied by an increase in the specific activity of cell-surface galactosyltransferase, which presumably occurs as the cells enter S phase.

A similar increase of surface galactosyltransferase has been observed by Weiser (14) in dividing intestinal crypt cells as compared to nondividing villus cells. We have also shown that surface galactosyltransferase of rat lymphocytes is markedly

increased during *in vitro* blast transformation induced by concanavalin A (10). Elevation of surface glycosyltransferase during S phase has also been demonstrated by Bosmann (20) in synchronized mouse lymphoma cells. Webb and Roth (2) have recently shown that endogenous surface galactosyltransferase activity varies within the cell cycle. They found that intercellular contact was not a requirement for surface galactosyltransferase of mitotic nontransformed 3T3 cells, but that nonmitotic 3T3 cells showed a requirement for cell contact during the assay in order to demonstrate optimal galactosyltransferase activity. Transformed 3T12 cells did not require cell contact during the assay at any stage of the cell cycle. They have interpreted these findings as indicating that only during mitosis do nonmalignant cells exhibit a type of galactosyltransferase activity found on malignant cells during all phases of the cell cycle. Webb and Roth also showed a marked increase in the level of surface galactosyltransferase in dividing as compared to nondividing 3T3 cells, comparable to our observations of serum-stimulated BHK/C13 and NIL cells. It has been proposed (21, 22) that during S and G_2 phase there is increased formation of plasma membrane, which is produced in the Golgi apparatus. Because the glycosyltransferases are enriched in Golgi membranes (23), one might expect an increased plasma membrane activity of these enzymes as Golgi membrane is externalized prior to cell division. The present data support the hypothesis that entry into S phase in both transformed and nontransformed cells is accompanied by an increase in surface galactosyltransferase activity. It also appears from these experiments that contact inhibition of growth of nontransformed cells is accompanied by a depression of surface galactosyltransferase that parallels the reduction of cell division.

We have shown that fibroblasts release galactosyltransferase, but not fucosyl- or sialyltransferase, into the tissue culture medium. This release is markedly increased in both normal and polyoma-virus-transformed cells undergoing cell division. Under identical culture conditions transformed cells appear to have increased release of soluble galactosyltransferase per cell as compared to nontransformed cells. The observation that release of galactosyltransferase is increased in transformed cells may reflect accelerated membrane turnover or remodeling of surface membranes in these cells. Webb and Roth have also demonstrated release of galactosyltransferase into the tissue culture medium from 3T3 cells (2), but they did not study the effect of serum or viral transformation on release.

The mechanism of solubilization of this normally membrane-bound enzyme is not known. The galactosyltransferase in the medium did not appear to be associated with a particulate fraction, as evidenced by the observation that 99% or more of the enzyme activity remained in the $200,000 \times g$ supernatant. It seems unlikely, therefore, that under these conditions membrane fragments are shed from the cell surface or that the medium galactosyltransferase is membrane bound. The absence of sialyl- and fucosyltransferase and the lack of an endogenous acceptor for galactosyltransferase activity in the tissue culture medium indicate that galactosyltransferase, but apparently not other glycosyltransferases, are solubilized and released. It is possible that proteolytic enzymes in the fetal calf serum or from the cells themselves are involved in the solubilization of surface membrane galactosyltransferase. It has been demonstrated that treatment of virally transformed cells with protease inhibitors causes cessation of growth at saturation densities, a phenomenon usually associated with nontransformed cells (24). This observation suggests that transformed cells produce a proteolytic activity that is essential for continuation of growth after confluence is attained.

Weiser *et al.* recently reported that a soluble isoenzyme of galactosyltransferase can be detected in the sera of patients with a variety of malignancies, but only rarely in control sera (25). This isoenzyme has also been detected in the sera of tumor-bearing animals, and appears to be shed from the surface of the tumor cells (26). Our demonstration of soluble galactosyltransferase from nontransformed fibroblasts and the previous reports that this enzyme appears in soluble form in human (27) and rat (28) serum indicate that solubilization and release of this enzyme from mammalian cells occurs under normal conditions of growth both *in vitro* and *in vivo*.

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