ABNORMAL RESPONSE OF GLYCOLIPID SYNTHESIS TO TEMPERATURE IN TRANSFORMED BHK CELLS THERMOSENSITIVE FOR GROWTH CONTROL

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SUMMARY: In DMN4B cells, a line of chemically mutagenized BHK hamster cells which exhibit transformed behavior at 38.5°C but not at 32°C, [^{14}C]-palmitate incorporation into mono-, di-, and trihexosylceramides was unimpaired at 32°C when compared with incorporation rates in untransformed BHK cells. At 38.5°C, labeling of these glycolipids increased greatly in the BHK cells, but failed to increase comparably in the DMN4B cells. Assay of cell-free preparations of the galactosyltransferase which catalyzes trihexosylceramide synthesis revealed a stimulatory effect of increased temperature on activity of the BHK enzyme, but not the DMN4B enzyme. The results suggest that transformation can result from a mutation affecting glycolipid synthesis.

Hamster cells that have undergone spontaneous or virus-induced malignant transformation frequently exhibit deficiencies of membrane glycolipids or glycoproteins (1-9). The glycolipid deficiency typically involves a paucity of neutral glycolipids with carbohydrate chains longer than lactose (1-6), and appears to reflect a deficiency in the activity of UDP-galactose:lactosylceramide α-galactosyltransferase (α-galactosyltransferase), an enzyme catalyzing the addition of galactose to GL2[1] to form GL3[1] (3). Deficient GL3 synthesis is not invariably correlated with transformation, however (6,10,11), suggesting either that the enzyme deficiency is not directly involved in transformation, or that its involvement is limited to a restricted number of cell lines.

The present study demonstrates that hamster cells transformed by a chemical carcinogen may also display impaired glycolipid synthesis, and suggests that a mutation affecting α-galactosyltransferase activity may be the cause of transformation in these cells. Our approach exploits the existence of hamster cells transformed by the carcinogen, dimethylnitrosamine, which are temperature-sensitive for growth control (12). These cells behave normally at 32°C, but exhibit a transformed phenotype and form colonies in soft agar at 38.5°C, suggesting that a cellular component critical to growth control has undergone a mutation which renders it thermolabile. Putative identification of a suspected component as the critical one can be accomplished by demonstrating the component to be temperature-sensitive in a cell-free system.

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[1]Abbreviations: GL1 (monohexosylceramide), GL2 (dihexosylceramide), GL3 (trihexosyl ceramide).

MATERIALS AND METHODS

Cell culture: Untransformed BHK21/13 (BHK) cells and the dimethylnitrosamine-transformed line of BHK cells, DMN4B, were gifts of Dr. G. DiMayorca, University of Illinois, and were routinely grown at 32°C in Dulbecco's medium containing 10% fetal calf serum. For experiments at 38.5°C, cells were grown for at least 4 cell generations at this temperature to permit full expression of the transformed phenotype (12). Cells were used within the first 15 passages, and only after plating in soft agar confirmed the previously reported ability of the DMN4B cells to form colonies at 38.5°C but not 32°C and the inability of the BHK cells to form colonies at either temperature (12). All experiments were done with cultures that had reached confluence, a state associated with maximum rates of GL3 synthesis in untransformed cells (2-6,10,11).

Glycolipid analysis: Neutral glycolipids were analyzed by the method of Robbins and MacPherson (4). Briefly, cultures grown in 75 cm^2 Falcon plastic tissue culture flasks were incubated with 0.7 μCi of [^{14}C]-palmitate (49.2 mCi/mmole, from New England Nuclear, Boston, Mass.). Adequate labeling was achieved in 2 days at 38.5°C, but required 5-7 days at 32°C. The cultures were then washed with phosphate-buffered saline, and the cells were scraped from the flasks and suspended in methanol. The suspension was dried under nitrogen, and the residue was extracted twice with 2:1 chloroform:methanol. After the combined extracts were dried under nitrogen and redissolved in chloroform:methanol, the glycolipids were isolated by 2-dimensional thin layer chromatography (13), detected by autoradiography, and quantified by liquid scintillation spectrometry.

Enzyme assay: The activity of UDP-galactose:lactosylceramide α-galactosyltransferase (α-galactosyltransferase) was assayed by the method of Kijimoto and Hakomori (3), using the particulate fraction obtained from sonicated cells by ultracentrifugation for 1 hour at 105,000 x g. This fraction is preferable to whole cell homogenates for this assay since it has been shown in previous studies of normal and transformed BHK cells to contain essentially all the cellular α-galactosyltransferase activity but to lack α-galactosidase activity (3). Assays were run at 32°C, 38.5°C, or 42°C for 2.5 hours; product formation was linearly proportional to incubation time under these conditions.

RESULTS

At 32°C, glycolipid labeling in DMN4B cells approximately equaled (GL1 and GL3) or exceeded (GL2) that in BHK cells (Table 1). At 38.5°C, labeling in BHK cells was higher than at 32°C, despite the longer labeling interval used at 32°C. DMN4B cells did not respond with a comparable increase at 38.5°C, and thus exhibited labeling at only 26% (GL3) to 58% (GL2) of the level in BHK cells.

Differences between BHK and DMN4B cells were also observed when activity of α-galactosyltransferase was assayed directly. The most striking difference was

TABLE 1

Effect of temperature on glycolipid synthesis in BHK and DMN4B cells as measured by incorporation of [^{14}C]-palmitate.

Growth temperature		Glycolipid labeling*	
		BHK	DMN4B
32°C	GL1	6.31 ± 0.41	4.97 ± 0.73
	GL2	4.53 ± 0.58	9.36 ± 0.62
	GL3	2.82 ± 0.19	2.55 ± 0.31
38.5°C	GL1	10.44 ± 1.12	3.24 ± 0.21
	GL2	9.74 ± 0.71	5.65 ± 0.24
	GL3	7.80 ± 0.59	2.03 ± 0.24

*Values represent incorporation into individual lipids as a fraction of the total, and are expressed as counts/min x 10^3 divided by sum of counts/min in phospholipids plus glycolipids. The latter sum varied little between the 2 cell lines or the 2 temperatures (range 1.32-1.64 counts/min/10^2 cells). Each value represents the mean ± SE of 8 experiments. Significant differences between BHK and DMN4B cells, as determined by Student's t test, were in: GL2 at 32°C and GL1, GL2, and GL3 at 38.5°C ($p<.01$ in each case).

in the response of the two enzyme preparations to increases in the assay temperature. This effect was most conspicuous when enzyme activity from cells grown at 38.5°C was measured (fig. 1A). α-Galactosyltransferase activity from BHK cells was markedly stimulated by increases in temperature. In contrast, the enzyme activity from DMN4B cells responded in a significantly different manner, exhibiting a slight decline with increasing temperature. When cells grown at 32°C were compared (fig. 1B), α-galactosyltransferase activity from BHK cells was again stimulated by an increase in the assay temperature, while the activity from DMN4B cells was not; however, the stimulation was substantially less than that observed in enzyme preparations from BHK cells grown at 38.5°C.

DISCUSSION

The synthesis of GL1, GL2, and GL3, as measured by palmitate incorporation, was not impaired in DMN4B cells grown at 32°C, a temperature at which the cells exhibit normal growth regulation. In contrast, glycolipid labeling at 38.5°C, a temperature at which DMN4B cells exhibit a transformed phenotype, was deficient in these cells compared with the untransformed BHK parent line. This relative deficiency was greatest in the case of GL3, consistent with results reported previously for virally or spontaneously transformed cells (2-6), but was also observed with GL1 and GL2.

We wished to analyze this effect further by determining whether the aberrant

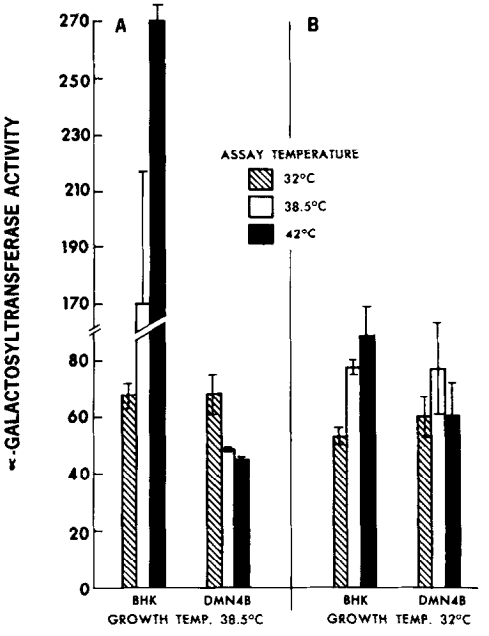

Fig. 1. Effect of in vitro assay temperature on activity of α-galactosyltransferase from BHK or DMN4B cells. Activity was expressed as pmoles of GL3 synthesized per hour per mg of protein in the enzyme suspension. The complete assay system (3) contained 0.05 μmoles of GL2 (Miles Laboratories, Elkhart, Indiana), 800 μg of Cutscum Detergent (Fisher Scientific Co., Medford, Mass.), 10 μl of 1 M cacodylate buffer, pH 6.1, 20 μl of 0.15 M $MnCl_2$, 20 μl of [^{14}C]-UDP-galactose in water (New England Nuclear, Boston, Mass.) diluted with unlabeled UDP-galactose to contain 1.4 x 10^5 counts/min in 80 mμmoles, and 100 μl of enzyme suspension in "Buffer A" (14) containing 0.5 to 1.0 mg protein. After the mixture was agitated in a water bath at the appropriate temperature for 2.5 hours, GL3 was isolated by one-dimensional thin layer chromatography on silica gel plates using chloroform: methanol:water (65:25:4), and quantified by scintillation spectrometry. Values for incomplete assay systems were: 12-16 pmoles/hour/mg minus GL2, and 0 pmoles/hour/mg minus enzyme. GL3 synthesis was unaffected by increasing the amount of UDP-galactose in the assay mixture. Each value shown is the mean ± SE of duplicate experiments, each representing cells pooled from 6-8 tissue culture flasks. Statistically significant results were a) increase in enzyme activity with temperature (42°C vs 32°C) in BHK enzyme preparations ($p<.01$ for Fig. 1A and $p<.05$ for Fig. 1B by Student's t test), and b) difference between the effects of temperature on BHK vs DMN4B enzyme activity, Fig. 1A ($p<.001$ by analysis of variance).

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response to increased temperature seen with intact DMN4B cells reflected an aberrant temperature response of some component of glycolipid-synthesizing microsomal preparations. Conceivably, the search for such an abnormal component might start by examining the composition or structure of microsomal lipids or proteins, or the activity of any of the glycolipid-synthesizing enzymes; α-galactosyltransferase was chosen for our initial study primarily because of previous reports implicating it or its product (GL3) in transformation (2-6). Analogous to GL3 labeling in

intact cells, DMN4B and BHK microsomal preparations exhibited similar α-galactosyltransferase activities at 32°C, but the DMN4B enzyme activity failed to increase appreciably (or even decreased) at higher temperatures, whereas the BHK enzyme activity was stimulated by increasing temperature. The stimulation was more apparent in preparations from BHK cells grown at 38.5°C than at 32°C, indicating that the metabolic state of the cell can influence the subsequent in vitro behavior of α-galactosyltransferase, conceivably by irreversibly affecting the enzyme itself, but more likely by influencing the concentration of co-factors, the composition of the lipoprotein matrix in which the enzyme is embedded, or some other microsomal constituent which affects enzyme activity.

The aberrant behavior of the DMN4B enzyme preparation indicates that a component of this preparation is abnormal (possibly thermolabile), thus rendering enzyme activity insusceptible to the normal stimulatory effect of temperature. In a cell which has undergone a mutation to a transformed state, abnormal behavior of an enzyme may signify that transformation is caused by a) a mutation in the enzyme protein, b) a mutation in some other cellular component affecting enzyme activity, c) a mutation in an intracellular process which influences enzyme synthesis or turnover, or d) a second mutation, coincidental but not causally related to the mutation affecting the enzyme. In the present study, alternative (c) is rendered unlikely by the finding that an abnormal response to temperature is demonstrable in cell-free enzyme preparations. Alternative (d) also appears unlikely, since the correlation between deficient α-galactosyltransferase activity and transformation has been observed previously with other transformed hamster cells (3). In addition, the probability that a particular function selected for study would have undergone a mutation purely by chance during the approximately 50 cell generations DMN4B cells have undergone between their origin and the present study can be computed directly to be very small ($p<.01$) on the basis of known mutation rates, even when the effects of potent mutagens are taken into account (15,16) It is even more unlikely that such a mutation would in addition display temperature-dependent characteristics. The results thus strongly suggest that a mutation affecting α-galactosyltransferase activity is a direct cause of transformation in DMN4B cells, but do not distinguish between alteration in the enzyme protein per se and a more general alteration (e.g., in microsomal membrane composition) which affects a multitude of microsomal functions. The latter alternative is favored by the observation that synthesis of GL1 and GL2, which are not products of α-galactosyltransferase activity, appears to be deficient in DMN4B cells, but a feedback effect of GL3 deficiency on these glycolipids cannot be excluded. Future analysis of additional enzymes, of other microsomal constituents, and when technically feasible, of purified preparations of α-galactosyltransferase may distinguish between the alternatives. Studies in other cell lines temperature-

sensitive for cellular functions mediating growth control will also be desirable because of the unique opportunity they offer for identifying biochemical alterations which can cause malignant transformation.

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