UDP-galactose:globotriaosylceramide α-galactosyltransferase activity in rat pheochromocytoma (PC12h) cells

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Abstract The activity of α-galactosyltransferase in cultured rat pheochromocytoma subcloned (PC12h) cells was examined using Gb3 as the acceptor for the galactose from UDP-galactose. The major reaction product was identified as galα1-3Gb3 based on its mobility on thin-layer chromatographic (TLC) plates and susceptibility to specific galactosidases. The enzyme activity in PC12h cells was the highest at pH 7.0 while the presence of Triton CF-54 (0.1%) and Mn^{2+} (5 mM) was required for its full activity. The apparent K_m values for Gb3 and UDP-galactose were 57 and 17 μM, respectively. The enzyme activity in PC12h cells was compared with that in parent PC12 cells, in which galα1-3Gb3 is not expressed in an appreciable amount. In the enzyme reaction with exogenous Gb3, the enzyme activity in PC12h cells was about 1.5-fold higher than that in PC12 cells. In the absence of exogenous Gb3, this difference became even more pronounced; galα1-3Gb3 was generated from endogenous Gb3 at a much higher rate in PC12h cells than in PC12 cells. These findings suggest that the higher level of the α-galactosyltransferase activity in PC12h cells may, at least in part, be responsible for the accumulation of unique neutral glycosphingolipids having galα1-3 terminal residues in the cells.—**Pal, S., M. Saito, T. Ariga, and R. K. Yu.** UDP-galactose:globotriaosylceramide α-galactosyltransferase activity in rat pheochromocytoma (PC12h) cells. *J. Lipid Res.* 1992. **33:** 411–417.

Supplementary key words glycosphingolipids • galα1-3Gb3 • differentiation

Glycosphingolipids are important constituents of the plasma membrane and constitute part of the glycocalyx network of the cell surface. They have been implicated to participate in diverse cellular functions: receptors for hormones, virus, and bacteria, cell–cell interactions, proliferation, and differentiation (1, 2). Recently, we found that unique neutral glycosphingolipids having galα1-3 terminal residues, including galα1-3Gb3, accumulated in a subclone of cultured rat pheochromocytoma (PC12h) cells (3, 4). These glycolipids are not expressed in appreciable amounts in the parent PC12 cells (3). Since PC12h cells are considered to be more differentiated morphologically and functionally compared with PC12 cells (5; T. Ariga and S. Pal, unpublished observations), this suggests that these unique glycosphingolipids may be related to the differentiation process of this cell type. To examine the functional role of these glycosphingolipids, it would be important to clarify the regulatory mechanism for their metabolism in PC12h cells. In this investigation, we characterized the activity of UDP-galactose:Gb3 α-galactosyltransferase in PC12h cells, and demonstrated that the higher level of the enzyme activity in PC12h cells might, at least partially, be responsible for the accumulation of these unique glycosphingolipids in the cells.

MATERIALS AND METHODS

Materials

UDP-[^{14}C]galactose (325 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). Triton CF-54, Triton X-100, CHAPS, Nonidet P-40, UDP-galactose, CDP-choline, galactonolactone, DEAE-Sephadex (A-25), α-galactosidase (from green coffee beans, 12 units/mg protein) and β-galactosidase (from *Aspergillus niger*, 27 units/mg protein) were purchased from Sigma Chemical Co. (St. Louis, MO). High performance thin-layer chromatographic (HPTLC) plates were obtained from E. Merck (Darmstadt, Germany). Gb3 was isolated from porcine liver, as previously

Abbreviations: Gb3 (globotriaosylceramide), Gal α1-4Gal β1-4Glc β1-1′Cer; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonate; DEAE-Sephadex, diethylaminoethyl-Sephadex; UDP-galactose, uridine 5′-diphosphogalactose; NEDH, New England Deaconess Hospital; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; HPTLC, high performance thin-layer chromatography; GlcCer (glucosylceramide), Glc β1-1′Cer; LacCer (lactosylceramide), Gal β1-4Glc β1-1′Cer; Gb4 (globoside), GalNAc β-1-3Gal α1-4Gal β1-4Glc β1-1′Cer.

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reported (6). Galα1-3Gb3 was purified from PC12h cell tumors that were produced by transplanting the cells into rats (3). All other reagents used were of analytical grade.

Cell culture

PC12 cells were kindly provided by Dr. L. A. Greene (Department of Pathology, Columbia University College of Physicians and Surgeons, New York, NY) and cultured according to the method of Greene and Tischler (7). PC12h cells were cultured under the conditions described by Hatanaka (5). To produce PC12h cell tumors, the PC12h cells (approximately 1×10^7 cells) were injected subcutaneously into New England Deaconess Hospital (NEDH) rats (3). When the tumors grew to approximately 2.5 cm in diameter, the animals were killed under anesthesia for collecting the tumor tissues.

Assay of UDP-galactose:Gb3 α-galactosyltransferase activity

The α-galactosyltransferase activity in cell or tissue homogenates was determined using Gb3 as the acceptor for the galactose of UDP-galactose. The reaction mixture consisted of 25 μg (220 μM) of Gb3, 50 μM UDP-galactose containing 0.1 μCi [^{14}C]UDP-galactose, 0.1% Triton CF-54, 5 mM $MnCl_2$, 50 mM HEPES buffer (pH 7.0), and an enzyme preparation in a total volume of 100 μl. CDP-choline and galactonolactone (5 mM each) were also included in the reaction mixture to inhibit activities of endogenous nucleotide pyrophosphatase and α-galactosidase, respectively (8). Blanks were prepared without enzyme preparation. After incubation of the mixture at 37°C for 1 h, total lipids were extracted from the mixture with chloroform–methanol 2:1 (v/v), followed by base treatment to hydrolyze phospholipids in the sample. The lipids were subjected to Folch's partitioning by adding 2.4 ml of chloroform–methanol 2:1 and 0.5 ml of 0.9% NaCl (9). After removing the upper phase, the lower phase was rinsed with theoretical upper phase of chloroform–methanol–water 1:50:49 without mixing. The glycolipids recovered in the lower phase were mixed with a certain amount (0.5–1.0 μg) of authentic galα1-3Gb3 and were separated on HPTLC with a solvent system of chloroform–methanol–2.5 N NH_4OH 55:50:10. The lipids were visualized by brief heating of the plate after spraying with the orcinol–sulfuric acid reagent. The silica gel corresponding to the galα1-3Gb3 band was scraped off the plate and the radioactivity was measured. For the characterization of the enzyme activity in PC12h cells, the assay conditions were appropriately altered.

The recovery of galα1-3Gb3 in the lower phase on Folch's partitioning was examined as follows. A mixture containing a fixed amount of purified, nonradiolabeled galα1-3Gb3 was subjected to Folch's partitioning in a similar manner as described above. The lipids recovered in the lower phase were separated on a TLC plate and were visualized with the orcinol–sulfuric acid reagent. Galα1-3Gb3 was quantitated by densitometric scanning of the plate.

Characterization of the reaction product

The reaction product(s) in the α-galactosyltransferase assay was analyzed by autoradiography of the glycolipids isolated from the reaction mixture. The reaction mixture was subjected to Folch's partitioning, as described above. The glycolipids recovered in the lower phase were concentrated under nitrogen, dissolved in chloroform–methanol–water 30:60:8, and subjected to DEAE-Sephadex column chromatography (10). The neutral glycolipids were eluted with the same solvent while residual radioactive UDP-galactose was trapped by the column. The lipids were separated on TLC plates using three different solvent systems: chloroform–methanol–water 65:35:8, chloroform–methanol 2.5 N NH_4OH 55:50:10, and propanol–water 80:20. The plate was then exposed to an X-ray film. The mobility of the radiolabeled reaction product(s) was compared with that of authentic galα1-3G3 that was developed on the same plate and visualized with the orcinol–sulfuric acid reagent.

The major reaction product was isolated and characterized using specific galactosidases. The radiolabeled product was extracted from the TLC plate, followed by further purification by DEAE-Sephadex column chromatography. The purified radiolabeled product was treated with α- or β-galactosidase. For treatment with α-galactosidase, the enzyme product (3,500 dpm) was incubated with 0.0192 unit of the enzyme in 0.15 ml of 0.1 M citrate buffer (pH 5.0) containing 10% sodium taurocholate (3). For treatment with β-galactosidase, the same amount of the product was incubated with 0.0192 unit of the enzyme in 0.15 ml of 50 mM citrate–phosphate buffer (pH 4.3) containing 0.3% sodium taurocholate (11). After incubation at 37°C up to 3 h, the reaction mixtures were subjected to Folch's partitioning. The extent of the degradation of the product by the enzyme was calculated by measurement of the radioactivity in the lower phase.

RESULTS

Quantitative measurement of enzyme reaction

The assay method for UDP-galactose:Gb3 α-galactosyltransferase was evaluated. The method consists of two steps: Folch's partitioning of the reaction mixture

and TLC of glycolipids recovered in the lower phase. On Folch's partitioning, almost all (95 ± 3%) of galα1-3Gb3 in the reaction mixture was recovered in the lower phase. Galα1-3Gb3 was successfully separated from other glycolipids such as Gb3 and globoside on TLC with the solvent system chloroform–methanol–2.5 N NH_4OH 55:50:10 (3).

The standard error for the measurement of enzyme activity by the present method was ± 5.4% of the mean value (n = 6).

Characterization of the reaction product(s)

The reaction product(s) in the enzyme assay was analyzed by autoradiography of the neutral lipids isolated from the reaction mixture. When the lipids were separated on TLC, one major band and several very faint ones were observed (**Fig. 1**). In all the cases with three different solvent systems, the major band showed the same mobility as galα1-3Gb3 and contained over 90% of the total radioactivity in the lipid fraction.

The major reaction product was purified and treated with specific galactosidases. As shown in **Table 1**, the linkage of radiolabeled galactose in the reaction product appeared to be specifically susceptible to α-galactosidase, but not to β-galactosidase. These results suggest that the enzyme product is galα1-3Gb3 which is one of the major neutral glycolipid in PC12h cells.

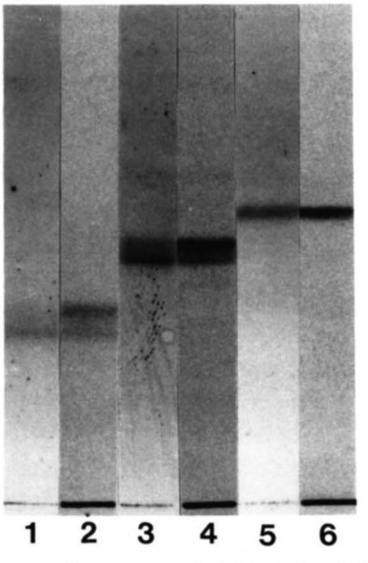

Fig. 1. Autoradiography of neutral lipids isolated from the reaction mixture for the UDP-gal:Gb3 α-galactosyltransferase reaction with PC12h cell homogenates. The neutral lipids were separated on a TLC plate with authentic galα1-3Gb3 using three different solvent systems, followed by autoradiography of the plate. The authentic glycolipid was visualized by the orcinol-sulfuric acid reagent. Lanes 1 and 2, developed with chloroform–methanol–water 65:35:8; lanes 3 and 4, developed with chloroform–methanol–2.5 N NH_4OH 55:50:10; and lanes 5 and 6, developed with propanol–water 80:20. Lanes 1, 3, and 5 for authentic galα1-3Gb3 and lanes 2, 4, and 5 for radiolabeled neutral glycolipids.

TABLE 1. Susceptibility of the reaction product to α- and β-galactosidases

Galactosidase	Incubation Time	Degradation of the Product
	min	%
α-Galactosidase	30	13.4
α-Galactosidase	60	17.6
α-Galactosidase	180	49.6
β-Galactosidase	30	0.7
β-Galactosidase	60	3.3
β-Galactosidase	180	9.1

The purified reaction product was treated with 0.0192 U of α- or β-galactosidase. The extent of the degradation of the product was calculated based on the radioactivity in the lower phase on Folch's partitioning of the reaction mixtures.

Effects of pH and detergent on enzyme activity

The pH dependency of the α-galactosyltransferase activity in PC12h cells was examined using two different buffers. As shown in **Fig. 2**, the maximal activity for the enzyme in PC12h cells was obtained at pH 7.0. **Table 2** shows the detergent requirement for the enzyme activity. In the presence of Triton CF-54, the enzyme activity was the highest, reaching about 300% of the control without detergent. Triton X-100 or Nonidet P-40 also showed stimulatory effects while the other detergents were less effective (CHAPS) or rather inhibitory (sodium deoxycholate). The optimal concentration of Triton CF-54 for the enzyme activity was about 0.1% (**Fig. 3**).

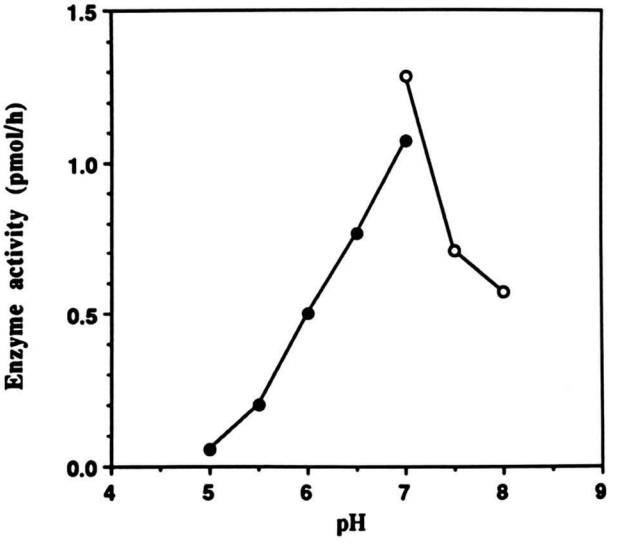

Fig. 2. Effects of varying pH on the UDP-gal:Gb3 α-galactosyltransferase activity in PC12h cells. The enzyme activity was assayed with two different buffers: cacodylate (from pH 5 to 7, ●) and HEPES buffer (from pH 7 to 8, ○) to cover a pH range from 5 to 8.

TABLE 2. Detergent requirement of the UDP-gal:Gb3 α-galactosyltransferase in PC12h cells

Detergent	Enzyme Activity
	pmol/h/mg protein
(None)	62.7
CHAPS	96.5
Triton X-100	170.4
Triton CF-54	186.2
Sodium deoxycholate	50.6
Nonidet P-40	142.3

The final concentration of each detergent in the reaction mixture was 0.1% (w/v). The data are averages of two experiments.

TABLE 3. Effect of divalent cations on the UDP-gal:Gb3 α-galactosyltransferase activity in PC12h cells

Metal Ion	Enzyme Activity
	pmol/h/mg protein
(None)	7.0
Mn^{2+}	132.3
Mg^{2+}	24.0
Ca^{2+}	13.7
Co^{2+}	45.0
Ni^{2+}	14.1

Various divalent metal ions (as chloride salts) were used at the final concentration of 5 mM. The data are averages of two experiments.

Effect of divalent metal ions

The effect of divalent cations (5 mM each) on the α-galactosyltransferase activity in PC12 cells is shown in **Table 3.** While the highest enzyme activity was attained in the presence of Mn^{2+}, only 5% of the activity was observed without metal ions. Other divalent cations examined had much less stimulatory effect compared with Mn^{2+}. The minimal concentration of Mn^{2+} required for full enzyme activity was around 5 mM (**Table 4**).

Linearity of enzyme reaction and K_m values

The relationship between the enzyme reaction and the amount of protein (**Fig. 4**) or incubation time (**Fig. 5**) was examined. The enzyme reaction was proportional to the amount of protein up to 300 μg; the reaction rate decreased with larger amounts of protein. The α-galactosyltransferase reaction proceeded in a fairly linear manner up to 3 h. The apparent K_m values for Gb3 (**Fig. 6A**) and UDP-galactose (Fig. 6B) were 57 μM and 17 μM, respectively.

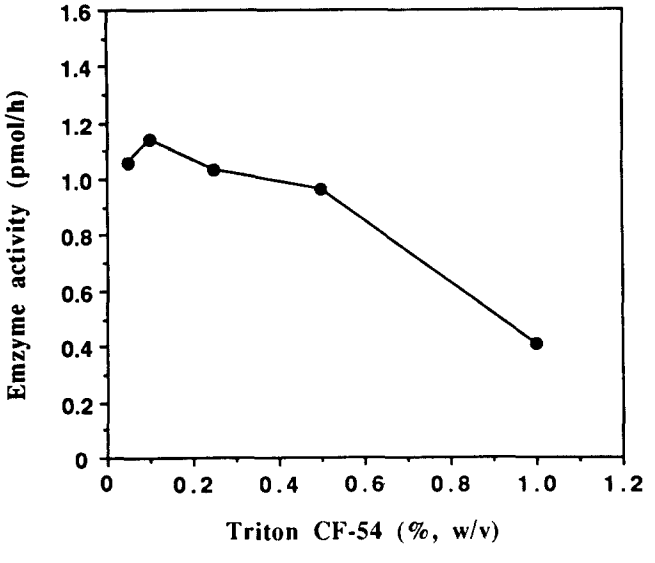

Fig. 3. Effect of Triton CF-54 concentration on the UDP-gal:Gb3 α-galactosyltransferase activity in PC12h cells.

α-Galactosyltransferase activities in PC12 cells, PC12h cells, and transplanted PC12h cell tumor

The α-galactosyltransferase activities in PC12 and PC12h cells were measured with or without exogenous Gb3. The enzyme activity measured with exogenous Gb3 in PC12h cells was about 1.5-fold higher than that in PC12 cells (**Table 5**). In the absence of exogenous Gb3, this difference became even more pronounced; galα1-3Gb3 was generated from endogenous Gb3 in a much more efficient manner in PC12h cells than in PC12 cells (**Fig. 7**).

The enzyme activity in PC12h cells was well preserved in the transplanted PC12h tumors (Table 5).

DISCUSSION

Our recent studies (3) have demonstrated that PC12h cells, a subclone of PC12 cells, accumulate unusual globo-series neutral glycosphingolipids containing galα1-3 terminal residues, including galα1-3Gb3. To clarify the metabolic basis for the accumulation of these glycolipids in PC12h cells, it is essential to examine and characterize the α-galactosyltransferase activity that is involved in the synthesis of the glycolipids in the cells. In this study, we characterized the enzyme

TABLE 4. Effects of Mn^{2+} concentration on the UDP-gal:Gb3 α-galactosyltransferase activity in PC12h cells

Mn^{2+}	Enzyme Activity
mM	*pmol/h/mg protein*
0	5.2
1	69.0
5	99.3
20	96.0

The data are averages of two experiments.

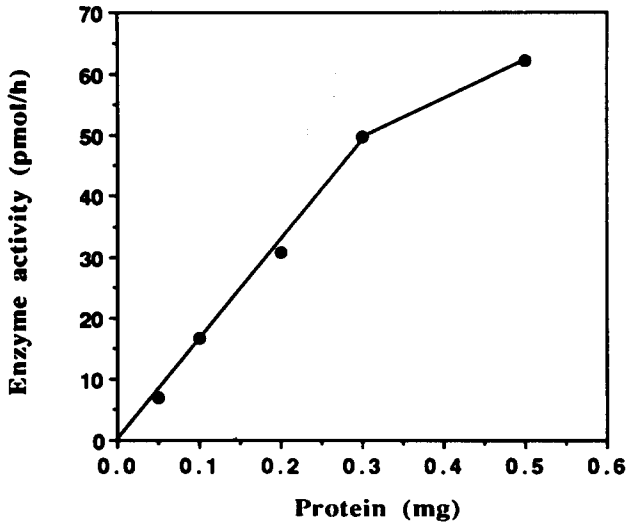

Fig. 4. Effect of amounts of protein on the UDP-gal:Gb3 α-galactosyltransferase activity in PC12h cells.

activity that catalyzes the reaction from Gb3 to galα1-3Gb3 using PC12h cell homogenates.

We first established an accurate and reproducible assay method for UDP-galactose:Gb3 α-galactosyltransferase activities in cells and tissues. The reaction product, which was assumed as galα1-3Gb3, was quantitatively recovered in the lower phase on Folch's partitioning while practically all radioactive UDP-galactose was removed from the lower phase. Galα1-3Gb3 was clearly separated from other lipids on TLC (see Fig. 7, lanes 1 and 4) (3).

When crude enzyme preparations such as cell homogenates are used for galactosyltransferase assays, there is the possibility that an endogenous membrane lipid may serve as the major acceptor for the reaction

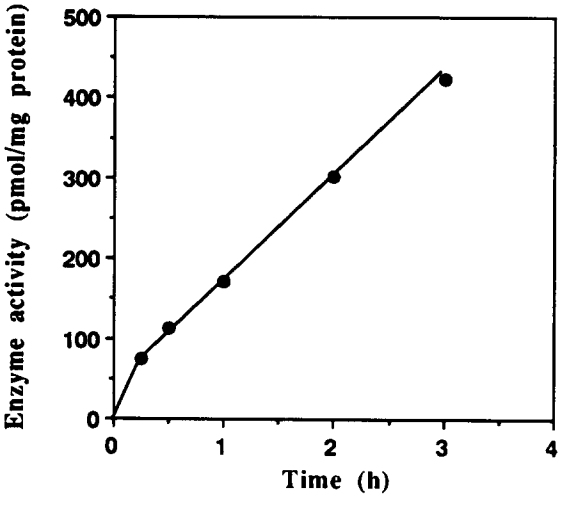

Fig. 5. Effect of incubation time on the UDP-gal:Gb3 α-galactosyltransferase activity in PC12h cells.

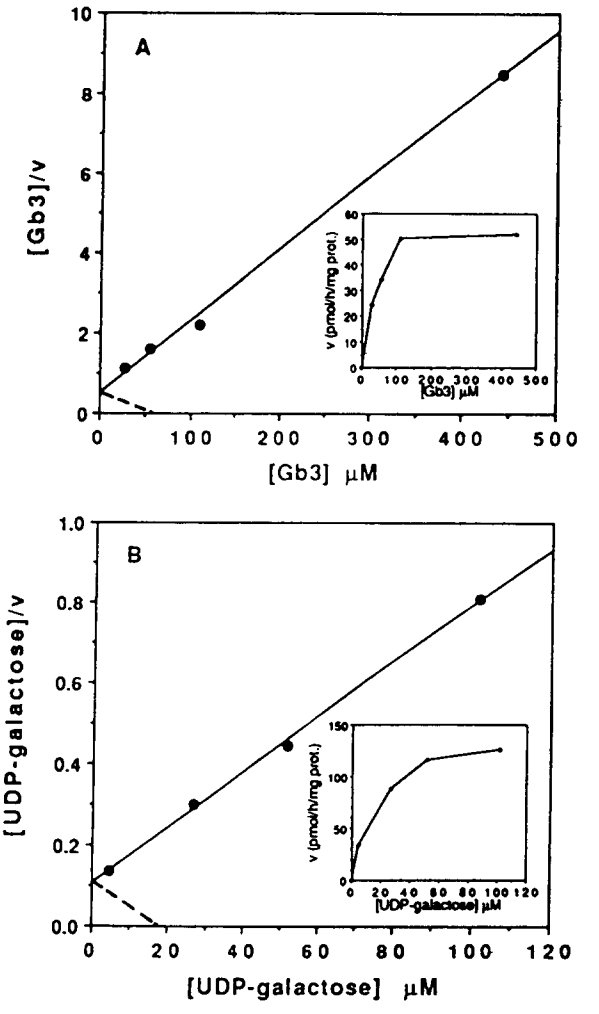

Fig. 6. Dependency of the UDP-gal:Gb3 α-galactosyltransferase activity on concentrations of Gb3 (A) and UDP-galactose (B). Figures show the Hanes plots.

despite the presence of exogenous lipid substrates. This, however, is not the case in our assay system. The enzyme reaction generated a single major radioactive band, in which over 90% of the radioactivity in the lipid fraction was recovered. The incorporation rate of the radioactive galactose into this glycolipid product depended on the concentration of exogenous Gb3 (Fig. 6A), suggesting that this glycolipid is likely produced directly from Gb3.

This enzyme product was characterized by examining its mobility on TLC and susceptibility to α- and β-galactosidases. The mobilities of the reaction product and authentic galα1-3Gb3 were identical in three different solvent systems. Susceptibility of the product to α-galactosidase, but not β-galactosidase, suggests that the radiolabeled galactose may be linked to Gb3 through an α-linkage. To more completely characterize the structure, more chemical studies, including

TABLE 5. UDP-Gal:Gb3 α-galactosyltransferase activities in PC12 cells, PC12h cells, and transplanted PC12h tumor tissue

Cells or Tissues	Enzyme Activity
	pmol/h/mg protein
PC12h	210 ± 19
PC12	143 ± 11
PC12h tumor	206 ± 17

The values represent the means ± SEM (n = 3).

methylation analysis and NMR studies have to be done.

The α-galactosyltransferase activity in PC12h cells showed similarities to α1-3galactosyltransferases in other cells and tissues in many respects (8, 12–17). The optimal pH for these enzyme activities was within the range of pH 6 and 7. The enzyme activities were stimulated most efficiently by nonionic detergents, except one enzyme where activity was activated by sodium deoxycholate (8). Mn^{2+} was required for full enzyme activity in all the cases. The apparent K_m values for lipid substrates as the acceptor ranged from 5 to 100 μM except the very high value (1670 μM) reported by Basu and Basu (12). The K_m values for UDP-galactose for the various α-galactosyltransferase preparations fell into a range from 10 to 140 μM. These similarities in properties of "different" α-galactosyltransferase activities may suggest some structural homology among these enzymes.

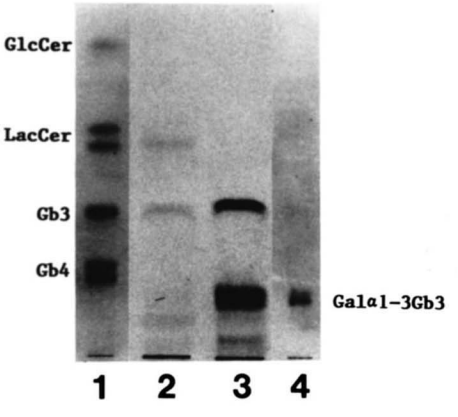

Fig. 7. Autoradiography of the reaction products from endogenous substrates in PC12 and PC12h cells. A fixed amount of cell homogenate (250 μg as protein) was incubated with [^{14}C]UDP-galactose in the absence of exogenous Gb3. Neutral lipids were isolated from the reaction mixture and developed on a TLC plate, followed by autoradiography of the plate. Standard glycolipid mixture (lane 1), radioactive products obtained with PC12 cells (lane 2) and PC12h cells (lane 3), and authentic galα1-3Gb3 (lane 4). Lanes 1 and 4 were visualized by the orcinol–sulfuric acid reagent.

While PC12h cells accumulate galα1-3Gb3 as one of the major neutral glycosphingolipids, the content of this glycolipid in PC12 cells appears to be extremely low (3). Comparison between the α-galactosyltransferase activities in PC12h and PC12 cells suggests that the content of galα1-3Gb3 is likely regulated at the level of the enzyme activity in the cells. On the other hand, the pronounced difference between the synthetic rates for galα1-3Gb3 from endogenous Gb3 in both cell types cannot be explained only by the observed difference between the enzyme activities in them, suggesting that the endogenous Gb3 concentrations might also be an important factor. The finding that the enzyme activity was well preserved in the transplanted PC12h cell tumors indicates that the tumor tissues can be used as a suitable source for isolation and purification of this enzyme in further studies.

In summary, we have characterized the UDP-galactose:Gb3 α-galactosyltransferase activities in PC12h and parent PC12 cells. This enzyme is expected to play an important role in the synthesis of unique neutral glycosphingolipids having galα1-3Gb3 terminal residues. The possibility that induction of this enzyme may be involved in the possible differentiated status of the PC12h cells is currently under investigation.

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