Lactosylceramide molecular species specificity of rat liver CMP-N-acetylneuraminate:lactosylceramide sialyltransferase

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Abstract Six naturally occurring and three synthetic molecular species of lactosylceramide (LacCer) were used to examine the molecular species specificity of CMP-N-acetylneuraminate:lactosylceramide α2,3-sialyltransferase in a Golgi-rich fraction of rat liver. The enzyme molecular species specificity was determined either in the presence of nonspecific lipid transfer protein or in the presence of detergents. Assays performed in the presence of transfer protein showed that for those lactosylceramide molecular species with either d18:1 or d18:0 long chain base the enzyme activity decreased linearly as the effective carbon number of the fatty acid increased. An increase in the carbon number of the long chain base decreased the activity of the enzyme twice as much as a corresponding increase in the carbon number of the fatty acid. On the other hand, when the enzyme activity was assayed in the presence of detergents, there was no significant difference in activity among the various molecular species of lactosylceramide based upon the carbon number of the fatty acid or on the presence of a double bond in the long chain base. However, the decrease in enzyme activity with an increase in the carbon number of the long chain base persisted. These results demonstrate that sialyltransferase has binding specificity with respect to the long chain base, but not the fatty acid. The apparent molecular species towards the fatty acid is related to the aqueous solubility of the various LacCer molecular species. — **Kadowaki, H., L. A. Symanski, K. E. Rys-Sikora, and R. S. Koff.** Lactosylceramide molecular species specificity of rat liver CMP-N-acetylneuraminate:lactosylceramide sialyltransferase. *J. Lipid Res.* 1989. **30:** 1789–1797.

Supplementary key words rat liver Golgi apparatus

Glycosphingolipids are synthesized in the Golgi membrane and endoplasmic reticulum (1), and are subsequently transported to the plasma membrane, where they participate in a variety of cell surface-related events (2–6). Glycosphingolipid biosynthesis proceeds by the sequential addition of a carbohydrate, first to ceramide and subsequently to an increasingly complex carbohydrate chain. Although each tissue has a characteristic glycosphingolipid class and ceramide molecular species pattern, it is a general phenomenon that there is no obvious relationship between the molecular species composition of those glycosphingolipids with more complex carbohydrate chains and their simpler (shorter carbohydrate chain) glycosphingolipid precursors (7). Moreover, several investigators have reported that there is no precursor–product relationship between the simple and more complex glycosphingolipid classes (8, 9). This has led to suggestions that each glycosphingolipid class is synthesized by its own multienzyme complex (10) or that the actual precursor pool of simple glycosphingolipids represents only a small fraction of the total cellular pool of these glycolipids (7, 8).

Kannagi, Nudelman, and Hakomori (11) have suggested that, at least for glycosphingolipids with complex carbohydrate chains, the observed variation in molecular species composition of the glycosphingolipid classes could be due to the fatty acid specificity of the glycosyltransferases that synthesize the various glycosphingolipid classes. However, no study has yet been reported examining the molecular species specificity of any of the glycosyltransferases using defined naturally occurring molecular species of glycosphingolipid as substrate. We, therefore, examined the molecular species specificity of sialyltransferase (CMP-N-acetylneuraminate:lactosylceramide α2,3-sialyltransferase (EC 2.4.99.9)) which catalyzes the transfer of N-acetylneuraminic acid (NeuAc) from CMP-NeuAc to lactosylceramide (LacCer) to form the simplest ganglioside, GM3. Sialyltransferase was chosen for this study because LacCer is the precursor not

Abbreviations: LacCer, Galβ1–4Glc-ceramide; $GbOse_4Cer$, GalNAcβ1-3Galα1-4Galβ1-4Glc-ceramide; $GgOse_3Cer$, GalNAcβ1-4Galβ1-4-Glc-ceramide; $GgOse_4Cer$, Galβ1-3GalNAcβ1-4Galβ1-4Glc-ceramide; GM3, NeuAcα2-3Galβ1-4Glc-ceramide; CMP-NeuAc, CMP-N-acetylneuraminic acid; RRT, relative retention time; GLC, gas–liquid chromatography; HPLC, high performance liquid chromatography. The long chain base abbreviations as suggested by Breimer, Karlsson, and Samuelsson (39) are used throughout. For example, in the notation d18:1–18:0, the d18:1 represents the long chain base sphingosine (1,3-dihydroxy-2-aminooctadecene) and 18:0 represents the fatty acid (octadecanoic acid).

only of GM3 and the higher gangliosides, but also of several other glycosphingolipid classes including the lacto, lactoneo, and globo series, each of which has its own characteristic molecular species pattern. It is therefore likely that the enzymes that metabolize LacCer will exhibit some specificity towards the various LacCer molecular species.

EXPERIMENTAL PROCEDURES

Materials

Cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-NeuAc), N-palmitoyl-DL-dihydrolactocerebroside (d18:0-16:0), N-stearoyl-DL-dihydrolactocerebroside (d18:1-18:0), N-lignoceroyl-DL-dihydrolactocerebroside (d18:0-24:0), β-galactosidase (from jack beans), β-N-acetylhexosaminidase (from jack beans) were obtained from Sigma (St. Louis, MO); CMP-[4,5,6,7,8,9-^{14}C]NeuAc (273 mCi/mmol) was from du Pont, NEN Products (Boston, MA); hog blood globoside ($GbOse_4Cer$), purified mixed bovine brain gangliosides, and methanolic-HCl were from Supelco (Bellefonte, PA); and Unisil was from Clarkson Chemical (Williamsport, PA). GM3 was prepared from human red blood cells and rabbit brain (Pel Freeze, Rogers, AR) as previously described (12).

Preparation of Golgi-rich fraction

A Golgi-rich fraction of rat liver was prepared as previously described (13) by a modification of the procedure of Schachter et al. (14). Briefly, male Sprague-Dawley rats (Charles River, Kingston, NY), weighing between 200 and 230 g were anesthetized with sodium pentobarbital. The livers were perfused with saline to remove blood, quickly excised, and homogenized in 0.1 M Tris-HCl (pH 7.6) containing 0.5 M sucrose, 0.01 M magnesium chloride, and 1% dextran (mol wt, 2×10^6). The Golgi-rich fraction was isolated by discontinuous sucrose density gradient centrifugation (13). In order to achieve maximum sialyltransferase activity, the suspended Golgi pellet was sonicated on ice for a total of 2 min using a microtip sonicator (Branson, Danbury, CT). Protein was quantitated by the method of Lowry et al. (15).

Preparation of Golgi lipids

The Golgi-rich fraction was extracted with 19 vol of chloroform-methanol 2:1 and partitioned with 0.2 vol of saline according to the procedure of Folch, Lees, and Sloane Stanley (16). The lower-phase was washed once with 0.2 vol of methanol-saline 1:1 and the entire lower-phase was used for the preparation of liposomes. Lipid phosphorus was determined as described by Bartlett (17).

Preparation of specific molecular species of LacCer

LacCer was prepared either from $GbOse_4Cer$ (13) or from brain gangliosides. Gangliosides (10 mg) were incubated with 2 ml of 0.05 N sulfuric acid at 80°C for 2 h to cleave the sialic acid. The resulting hydrolysate was neutralized with sodium hydroxide, and 10 ml of chlorform-methanol 2:1 was added. After vigorous mixing the two phases were separated by centrifugation. The lower phase, which contained gangliotetraosylceramide ($GgOse_4Cer$) as the major component, was dried under nitrogen gas. The terminal galactose of the $GgOse_4Cer$ was cleaved by β-galactosidase, and then the terminal N-acetylgalactosamine of the resulting gangliotriaosylceramide ($GgOse_3Cer$) was cleaved by β-N-acetylhexosaminidase as previously described (18). LacCer was separated from the other neutral glycosphingolipids by chromatography on a Unisil column (13).

The purified LacCer was then separated into individual molecular species (**Fig. 1**) by HPLC using a 4.6 mm × 25 cm Ultrasphere ODS column (Beckman, San Ramon, CA) with a mobile phase of methanol-water 96:4. The effluent was monitored at 205 nm. Molecular species of LacCer were identified by the retention time of their perbenzoyl derivatives (19) on a reversed-phase column (18). The identity of the LacCer molecular species and the amounts of each molecular species in those peaks which contained two molecular species of LacCer were also determined by GLC (20) after methylation of the fatty acids with methanolic-HCl

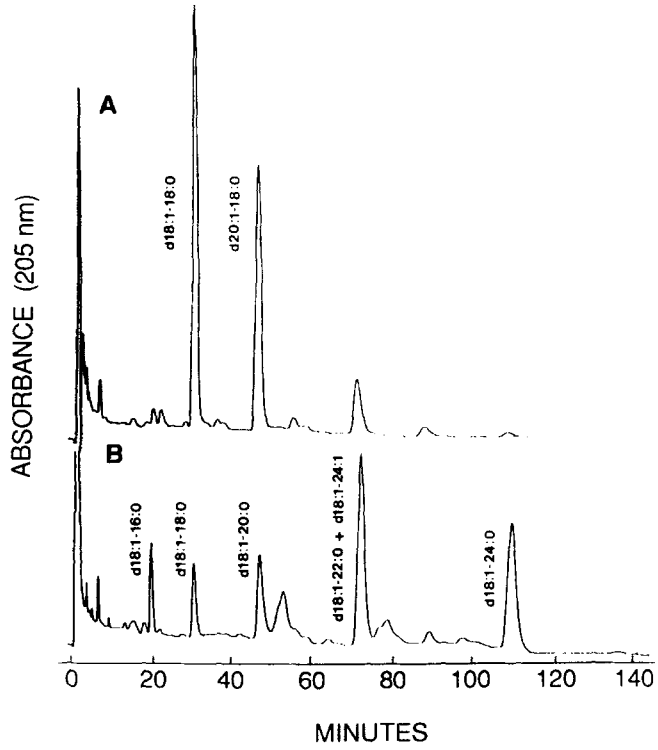

Fig. 1. HPLC separation of the molecular species of intact LacCer. LacCer dissolved in ethanol was applied to a 4.6 mm × 25 cm, 5 μm, Ultrasphere ODS column (Beckman, San Ramon, CA). The column, maintained at 30°C, was eluted with methanol-water 96:4 at a flow rate of 1.0 ml/min. Detection was by absorption at 205 nm with an attenuation of 0.16 absorbance unit full scale. (A) LacCer prepared from brain gragliosides (50 μg) and (B) LacCer from $GbOse_4Cer$ (40 μg).

(21). LacCer containing d18:1–18:0 and d20:1–18:0 (this LacCer also contains 15.4% of d18:1–20:0) ceramide (Fig. 1A) were isolated from LacCer prepared from brain gangliosides as described above. LacCer containing d18:1–16:0, d18:1–20:0, d18:1–24:1 (60.1%) plus d18:1–22:0 (39.9%), and d18:1–24:0 ceramide (Fig. 1B) were isolated from LacCer prepared from $GbOse_4Cer$. Individual LacCer molecular species were collected from the column, dried under nitrogen, redissolved in 10 ml of chloroform–methanol 2:1, and partitioned with 0.2 vol of water. The LacCer was recovered in the lower phase. An aliquot was perbenzoylated (19) and quantitated by normal-phase HPLC (13).

Preparation of unilamellar liposomes containing defined molecular species of LacCer

Liposomes were prepared with Golgi lipids and a single molecular species of LacCer. LacCer and Golgi lipids (10 nmol of LacCer/200 nmol of Golgi lipid phosphorus) were evaporated to dryness; the dried lipid mixtures were suspended in 0.05 M Tris-HCl (pH 7.4) containing 3 mM sodium azide and sonicated under a nitrogen atmosphere for 10 min at 20–25°C with a microprobe sonicator. The sonicated liposomes were centrifuged at 10,000 rpm for 20 min at room temperature. LacCer and lipid phosphorus were quantitatively recovered in the supernatant.

Preparation of transfer protein

Pure nonspecific lipid transfer protein was prepared from bovine liver as described by Crain and Zilversmit (22). Some experiments were performed with partially purified nonspecific lipid transfer protein, i.e., the final octylagarose column was omitted. Both preparations contain both the CM-I and CM-II transfer proteins. The transfer protein was concentrated just prior to use with an Amicon YM5 membrane. The high ionic strength buffer used to elute the transfer protein from the CM-cellulose column or to neutralize the effluent from the octylagarose column has been shown to inhibit the activity of transfer protein (22). Therefore, while concentrating the transfer protein, when the volume was reduced to 1 ml, 5 ml of 5 mM sodium phosphate (pH 7.4) containing 5 mM β-mercaptoethanol and 3 mM sodium azide was added in order to reduce the ionic strength. The transfer protein was then concentrated to the desired volume. Any protein that precipitated during the concentration procedure was removed by centrifugation. The purity of both the purified and partially purified transfer protein was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (**Fig. 2**) according to the procedure of Laemmli (23).

Enzyme assays

Sialyltransferase activity was determined by two incubation methods, with transfer protein and with detergents.

Incubation with transfer protein. Unless otherwise stated, the incubation mixture was prepared on ice with 10 nmol of LacCer in liposomes (200 nmol of phosphate), 50 μg of Golgi protein, 120 to 150 μg (13) of the concentrated partially

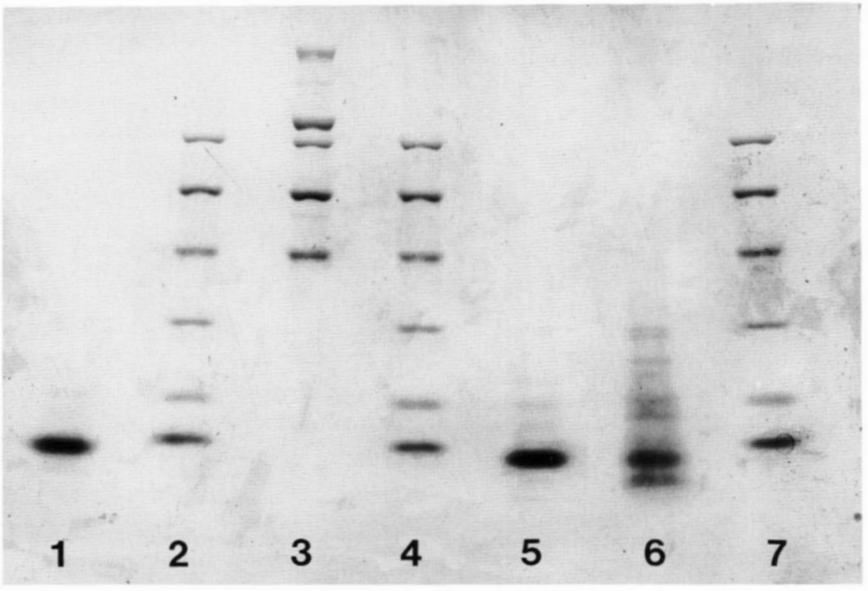

Fig. 2. Polyacrylamide gel electrophoresis of partially purified and purified bovine liver nonspecific lipid transfer proteins. Proteins were treated with SDS and applied to gradient gel of a 3–20% polyacrylamide with a 4% stacking gel. The protein was stained with Coomassie brilliant blue. Electrophoresis was for 16 h at 40 V. Bio-Rad molecular weight standards (lanes 2, 3, 4, and 7) are from top to bottom: myosin (200,000), *E. coli* β-galactosidase (116,000), rabbit muscle phosphorylase (97,400), bovine serum albumin (66,200), chicken ovalbumin (42,699), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400). Lane 6 shows partially purified transfer protein (15 μg of protein), and lanes 1 and 5 show two separate preparations of purified transfer protein (15 μg of protein each). Molecular weight of bovine liver nonspecific lipid transfer protein (CM-I and CM-II) is approximately 14,000 (22).

purified transfer protein preparation or 24 to 28 μg of purified transfer protein, and sodium cacodylate buffer. The mixture was preincubated at 37°C for 10 min, and the reaction was then initiated by adding 50 nmol of CMP-[^{14}C]-NeuAc. The final volume was 50 μl and the final sodium cacodylate concentration was 50 mM. The pH of the reaction mixture was 6.2. The reaction mixture was incubated at 37°C for 60 min and the reaction was terminated by adding 3 ml of chloroform–methanol 2:1. After partitioning with 0.2 volume of water, the lower phase was washed 4 times with methanol–water 1:1 as described by Yu and Lee (24). When pure molecular species of [^{14}C]GM3 were used as standards, 96.1% of the d18:1–18:0, 94.4% of the d18:1–24:1 plus d18:1–22:0, and 94.8% of the d18:1–24:0 molecular species was recovered in the upper phase. The reaction product, [^{14}C]GM3, was purified from the combined upper phases as previously described (13).

Incubation with detergent. The detergent assay procedure was adapted from the method described by Richardson, Keenan and Morre (25). One nmol of LacCer (in ethanol), 100 μg of Triton CF-54, and 50 μg of Tween 80 (both in methanol) were mixed and the solvent was evaporated. Sodium cacodylate buffer and 50 μg of Golgi protein were added and the mixture was preincubated at 37°C for 10 min. The reaction was initiated by adding 50 nmol of CMP-[^{14}C]-NeuAc. The final volume was 50 μl and the final sodium cacodylate concentration was 150 mM. The pH of the reaction mixture was 6.2. The reaction mixture was incubated at 37°C for 60 min and the reaction was terminated by adding 3 ml of chloroform–methanol 2:1 and the product, [^{14}C]GM3, was analyzed as described above.

Separation of Golgi vesicles from liposomes and quantitation of LacCer and GM3 molecular species

Golgi vesicles were incubated in the presence of transfer protein as described above. After incubation the samples were immediately chilled in ice water to stop further reaction and layered on ice-cold discontinuous sucrose gradients consisting of 0.3 ml of 1.3 M, 0.2 ml of 0.7 M, and 3.2 ml of 0.5 M sucrose containing 50 mM Tris-HCl (pH 7.4), 10 mM magnesium chloride, and 3 mM sodium azide in an 11 × 60 mm ultra-clear centrifuge tube (Beckman, Palo Alto, CA). The gradients were centrifuged at 4°C in a Spinco SW60Ti head at 40,000 rpm for 60 min. Golgi vesicles were recovered in the 0.7 M sucrose layer and the liposomes remained in the sample layer at the top of the sucrose gradient. Tubes were immediately transferred to dry ice and after freezing were cut with a blade to obtain Golgi and liposome fractions. Lipids were extracted from both fractions with chloroform–methanol as described by Folch et al. (16). After partitioning with 0.2 vol of water, the lower-phase was washed 4 times with 0.2 vol of methanol–water 1:1.

All upper phases were combined and [^{14}C]GM3 was isolated as previously described (13). The radioactivtiy was determined in half the sample. A small amount of GM3 isolated from rabbit brain or human red blood cells was added to the other half of the sample as carrier. The samples were converted to the 2,4-dinitrophenylhydrazide derivatives (26) and separated into molecular species by HPLC on a 2 mm × 25 cm Ultrasphere ODS column at 30°C with a mobile phase of methanol–water–acetic acid 96:4:0.02 at a flow rate of 0.3 ml/min (18). The radioactivity of each peak was determined by liquid scintillation counting. Only the GM3 molecular species corresponding to the substrate LacCer molecular species were radiolabeled.

The lower phase was used for the analysis of LacCer. Isolation, perbenzoylation (19), and quantitation by HPLC (13) were performed as previously described. The benzoylated LacCer was also separated into individual molecular species by reversed-phase HPLC at 30°C with methanol as the mobile phase (18), and the molecular species were quantitated by digital integration.

RESULTS

In order to determine whether sialyltransferase exhibits specificity with respect to LacCer molecular species, the activity of the enzyme towards each of six different naturally occurring LacCer molecular species (d18:1–16:0, d18:1–18:0, d18:1–20:0, d18:1–24:1 plus d18:1–22:0, d18:1–24:0, and d20:1– 18:0) and three synthetic molecular species (d18:0–16:0, d18:0–18:0, and d18:0–24:0) was determined in a Golgi-enriched fraction of rat liver. Since there was some variation in the specific activity of sialyltransferase among the Golgi preparations, the results were normalized by dividing the activity observed with each LacCer molecular species by the activity observed with d18:1–18:0 LacCer which were determined for each Golgi preparation. When sialyltransferase activity was measured in the presence of partially purified nonspecific lipid transfer protein, among those LacCer molecular species with the same long chain base (d18:1) in combination with different fatty acids (16:0, 18:0, 20:0, 22:0, 24:1, or 24:0) the activity decreased linearly as the effective carbon number (12, 27) of the fatty acid increased (**Fig. 3A**). Similar results were obtained with the three synthetic LacCer molecular species (d18:0–16:0, d18:0–18:0, and d18:0–24:0). Indeed, the activity of the enzyme towards all the molecular species with either a d18:1 or d18:0 long chain base was directly proportional to the log of the relative retention time (RRT)[1] of the molecular species on a C18 reversed-phase column (Fig. 1).

An increase in the carbon number of the long chain base (d20:1–18:0), on the other hand, decreased the activity of the enzyme more than a similar increase in the carbon number

[1]RRT is calculated by dividing the absolute RT of each molecular species of LacCer by the RT of d18:1–18:0 LacCer. The log of the RRT is also used to determine the effective carbon number of the fatty acid, long chain base or the entire molecular species (12, 18).

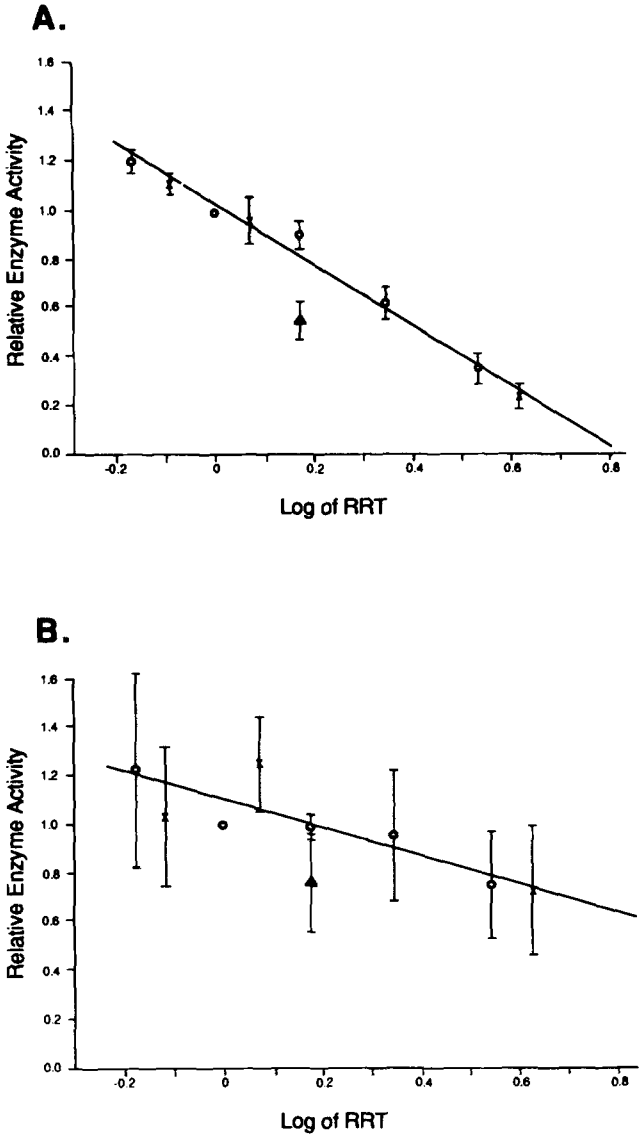

Fig. 3. The relative activity of sialyltransferase towards various molecular species of LacCer. The relative enzyme activities were determined by dividing the activity of the various LacCer molecular species by the activity of d18:1-18:0 LacCer. RRT is calculated by dividing the absolute RT of each molecular species of LacCer eluted from a C18 reversed-phase column by the RT of d18:1-18:0 LacCer (Fig. 1). (A) Enzyme activity assayed in the presence of partially purified transfer protein and (B) in the presence of detergents. The enzyme specific activity (nmol/mg Golgi protein/h) with d18:1-18:0 LacCer as substrate in the presence of transfer protein is 1.88 ± 0.25 (mean ± SD of five determinations) and in the presence of detergents is 1.40 ± 0.49 (four determinations). (O) LacCer containing d18:1 long chain base; log of RRT = −0.175, 0, 0.180, 0.363, and 0.544 for 16:0, 18:0, 20:0, 24:1 + 22:0, and 24:0 fatty acid, respectively. (x) d18:0 long chain base; log of RRT = −0.120, 0.070 and 0.626 for 16:0, 18:0 and 24:0 fatty acid. (△) d20:1-18:0, log of RRT = 0.180. The values are mean ± SD of three to five determintions in each LacCer molecular species.

of the fatty acid. Specifically, an increase of 2 in the carbon number of the fatty acid resulted in a 20% decrease in enzyme activity, while an increase of 2 in the carbon number of the long chain base resulted in decrease of 40% in the enzyme activity. Thus, the difference in activity between d20:1-18:0 and its lower homologue (d18:1, d18:0) was much greater than the difference in the log of their respective RRTs and, consequently, d20:1-18:0 does not fall on the line of enzyme activity versus log of RRT, which is established for all of the molecular species containing a d18 long chain base.

When the activity of the enzyme was measured in the presence of detergents, and the activity towards each molecular species was plotted against the log of the RRT of that molecular species, a straight line was also obtained which suggests that there could be a relationship between enzyme activity and the RRT of the substrate (Fig. 3B). However, the strength of that relationship, if any, (as judged by the slope of the line) is much less than that observed in the presence of transfer protein. In fact, when determined in the presence of detergent, there was no statistically significant difference in the activity of the enzyme towards any of the LacCer molecular species tested except between d18:0-18:0 and d20:1-18:0 ($P < 0.01$).

Since sialyltransferase exhibited virtually no molecular species specificity in the presence of detergents, it seemed possible that the molecular species specificity observed when assayed in the presence of nonspecific lipid transfer protein could be due to the molecular species specificity of the transfer protein. This possibility was excluded by another experiment in which various proportions of d18:1-18:0 LacCer (reference molecular species) and each of three different molecular species of LacCer (d20:1-18:0, d18:1-24:1 plus d18:1-22:0, or d18:1-24:0) were incubated together with partially purified transfer protein, the Golgi-rich fraction, and CMP-[^{14}C]NeuAc under the same conditions used for the enzyme assay. At the end of the incubation the Golgi and liposomes were separated by ultracentrifugation and the amount of each LacCer molecular species in the Golgi fraction and the liposomes was determined. The recovery of LacCer (Golgi fraction plus liposomes) was 96.3 ± 9.0% (mean ± SD of 36 observations). As shown in **Table 1**, for each of the three experimental LacCer molecular species the ratio of the molecular species recovered in the Golgi fraction was precisely the same as the ratio originally added to the incubation mixture. Furthermore, the total amount of LacCer recovered in the Golgi fraction did not vary significantly with changes in the proportions of the molecular species (data not shown). However, the ratio of the GM3 molecular species synthesized during the incubation was significantly different from the ratio of the LacCer molecular species found in the Golgi. In each case the molecular species with the shorter RRT was more rapidly converted to GM3 than the molecular species with a longer RRT. Moreover, the total amount of GM3 synthesized decreased as the proportion of the more reactive molecular species (d18:1-18:0) decreased.

Since it is also possible that some other protein in the partially purified transfer protein preparation was interacting with the sialyltransferase to produce the apparent molecular species specificity, the molecular species specificity of sialyl-

TABLE 1. Molecular species specificity of sialyltransferase in the presence of partially purified transfer protein

Experimental LacCer Molecular Species	Exp. LacCer Added to Incubation Mixture	Exp. LacCer Found in Golgi Fraction	GM3 Synthesized Exp. Molecular Species	Sialyltransferase Activity
	%	%	%	*nmol/mg protein/h*
I. d20:1-18:0	0	0	0	1.89
	25	23.7	18.2	1.76
	50	49.3	35.6	1.74
	62.5	63.2	46.0	1.76
	75	72.1	57.9	1.71
	100	100	100	1.42
II. d18:1-24:1 d18:1-22:0	0	0	0	1.16
	25	24.1	11.5	0.99
	50	50.7	25.4	1.07
	62.5	60.9	34.6	0.76
	75	76.1	50.6	0.88
	100	100	100	0.64
III. d18:1-24:0	0	0	0	1.50
	25	23.7	4.4	1.22
	50	51.7	11.6	1.08
	62.5	62.3	20.6	1.10
	75	75.9	33.6	0.83
	100	100	100	0.43

The incubation mixture contained liposomes (20 nmol LacCer/400 nmol lipid phosphorus) consisting of various proportions of reference molecular species (d18:1-18:0 LacCer) and experimental (Exp.) molecular species of LacCer as listed in the table, 100 μg of Golgi protein, 100 nmol of CMP-[^{14}C]NeuAc, and transfer protein preparation (266 μg of protein for experimental I, and 317 μg for experiments II and III) in a final volume of 100 μl. The lipid phosphorus content of the Golgi fraction in experiment I was 117 nmol and in experiments II and III was 148 nmol. After incubation at 37°C for 60 min, the Golgi fraction was separated from liposomes by ultracentrifugation and LacCer was quantitated as described in Experimental Procedures. The amount of GM3 synthesized was determined in both the Golgi and liposome fractions; 80.8 ± 2.3%, 49.0 ± 6.8%, and 51.1 ± 5.4% (mean ± SD) of the total GM3 was recovered in the liposomes of experiments I, II, and III, respectively. GM3 synthesized and sialyltransferase activity are the sum of both fractions.

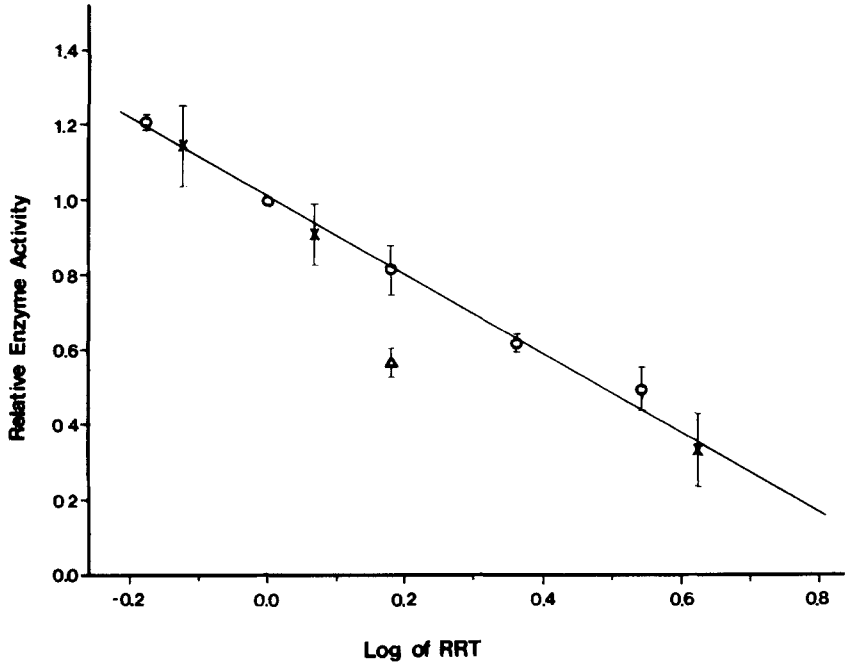

Fig. 4. The relative activity of sialyltransferase towards various molecular species of LacCer assayed in the presence of purified transfer protein (24-28 μg). The enzyme specific activity (nmol/mg Golgi protein/h) with d18:1-18:0 LacCer as substrate is 1.58 ± 0.18 (mean ± SD of three determinations). Log of RRT of each LacCer molecular species is described in Fig. 3. The values are mean ± SD of three determinations in each LacCer molecular species.

transferase was also determined using highly purified transfer protein. The purity of the transfer protein was verified by SDS-polyacrylamide gel electrophoresis (Fig. 2). When 50 μg of Golgi protein was incubated with various amounts of purified transfer protein, maximum GM3 synthesis was achieved with 22 μg of purified transfer protein preparation. Addition of more purified transfer protein had no effect on the rate of GM3 synthesis. When the molecular species specificity of sialyltransferase was assayed with purified transfer protein, virtually the same results were obtained (**Fig. 4**) as when the enzyme was assayed with partially purified transfer protein (Fig. 3A). Furthermore, as was the case with partially purified transfer protein, when Golgi and liposomes containing d18:1–18:0 LacCer and either d18:1–16:0, d18:1–24:0, or d20:1–18:0 LacCer were incubated together with purified transfer protein under the condition of the enzyme assay, and the Golgi vesicles and liposomes were reisolated by untracentrifugation, the ratio of LacCer molecular species found in the Golgi fraction was the same as the ratio of LacCer molecular species added in the liposomes.

These experiments show that the transfer protein has no molecular species specificity (the ratio of LacCer molecular species in liposomes and Golgi is the same) and that the differences in GM3 synthesis observed among the molecular species are due to differences in activity of sialyltransferase towards the molecular species.

DISCUSSION

Sialyltransferase is an intrinsic membrane enzyme and one of its substrates, LacCer, is also an integral component of the membrane. Traditionally, glycosphingolipid substrates, which are not water-soluble, have been introduced into enzyme reactions solubilized in detergents. However, detergents are known to change the lipid environment of the membrane and, as a consequence (or by independent mechanisms), to change the conformation of membrane proteins (28) and substrate specificity (29). In order to avoid these detergent effects, we have recently described a procedure for introducing glycosphingolipid substrates into the Golgi membrane using nonspecific lipid transfer protein and liposomes that contain the glycosphingolipid substrate (13). Nonspecific lipid transfer protein facilitates the establishment of an equilibrium in lipid composition between the Golgi membrane and the liposomes (13). Nonspecific lipid transfer protein is known to transfer phospholipids and cholesterol as well as glycosphingolipids (22, 30). Therefore, for these present experiments the liposomes were prepared with rat liver Golgi lipids in order to minimize extraneous changes in the lipid composition of the Golgi membrane.

The molecular species specificity of nonspecific lipid transfer protein towards glycosphingolipids has not been examined. It was possible that the transfer protein had a molecular species specificity that could mask the molecular species specificity of the sialyltransferase. We examined that possibility (Table 1) and were able to demonstrate that, under the conditions of the sialyltransferase assay, nonspecific lipid transfer protein exhibits no LacCer molecular species specificity, i.e., the relative ratio of LacCer molecular species found in the Golgi is identical to the ratio added in the liposomes.

From the data presented in Figs. 3A and 4 and Table 1, it is obvious that sialyltransferase shows differential activity towards the various molecular species of LacCer when the enzyme is assayed using transfer protein. On the other hand, when sialyltransferase is assayed using detergent-solubilized substrates, the enzyme does not, with one exception, show any molecular species specificity (Fig. 3B).

The differences in activity among the molecular species could be based upon either the specificity of the enzyme towards the long chain base, the fatty acid, or the physical properties of the molecular species as a whole. Sialyltransferase clearly shows specificity towards the long chain base. In particular, for those three molecular species with the same fatty acid (18:0) in combination with a different long chain base (d18:0, d18:1, and d20:1), it appears that the enzyme discriminates strongly based on the carbon number of the long chain base, but only modestly, if at all, on the presence or absence of a double bond in the long chain base. Since the effect of an increase in the carbon number of the long chain base is observed even when the enzyme is assayed in the presence of detergent (d18:0–18:0 vs. d20:1–18:0), it seems safe to assume that the specificity for the long chain base is an inherent property of the enzyme. From the results obtained with those molecular species containing d18:1 long chain base in combination with different fatty acids, (16:0, 18:0, 20:0, 24:1 + 22:0, or 24:0), it is also clear that the relative activity of sialyltransferase varies inversely as the equivalent chain length of the fatty acid. Similar results are obtained with the synthetic substrates d18:0–16:0, d18:0–18:0, and d18:0–24:0.

Considering both detergent and transfer protein data, it appears that the enzyme does not recognize either the carbon number or the presence of a double bond in the fatty acid. Rather the results imply that the activity of the enzyme towards a particular d18:0 or d18:1 molecular species is based upon a physical property of the molecular species that can be characterized by the log of the RRT on a reversed-phase column. The RRT is a function of the partition coefficient of a LacCer molecular species between an oil phase (the hydrocarbon chain of the reversed-phase column) and an aqueous phase (methanol–water mobile phase). In other systems, i.e., the spontaneous exchange of phosphatidylcholine molecular species between membranes, the same linear relationship between activity and log of RRT has been observed (31–33). In these studies and in

similar systems, i.e., the spontaneous transfer of phospholipids (34), cholesterol (35), and glycosphingolipids (36–38), the rate-limiting step has been shown to be the desorption of the molecule from the lipid phase (i.e., membrane) into the aqueous phase. If a similar conclusion is warranted in this case, then it can be concluded that for LacCer with either d18:0 or d18:1 long chain base the rate-limiting step in the sialyltransferase reaction is the release of the substrate from the Golgi membrane either directly to the active site of the enzyme or to the aqueous phase from which it can gain access to the active site. In either case, substrate binding specificity is not responsible for the effect of the substrate fatty acid composition on the activity of sialyltransferase. (It is even possible that the enzyme has no binding site for the fatty acid or that it recognizes only that portion of the fatty acid involved in the amide bond.)

The conclusions are supported by the data obtained with detergent-solubilized substrate in which there is no significant difference among the LacCer molecular species based upon the equivalent chain length of the fatty acids, because the detergent-solubilized substrates are already and equally desorbed from the membrane. Even in the presence of detergent, however, the specificity of the enzyme with respect to the carbon number of the long chain base persists since that specificity is a property of the enzyme binding site and not a function of the partition coefficient (or aqueous solubility) of the substrate.

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