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Topography of Glycosyltransferases Involved in the Initial Glycosylations of Gangliosides*

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We attempted to establish within which organelle UDP-Glc:ceramide $\beta1\rightarrow1'$glucosyltransferase (GlcT) is located and moreover to obtain information about its orientation on intracellular membranes as well as that of UDP-Gal:glucosylceramide $\beta1\rightarrow4$galactosyltransferase (GalT-2) and CMP-NeuAc:lactosylceramide $\alpha2\rightarrow3$sialyltransferase (SAT-1). An extremely purified Golgi apparatus fraction was the only liver fraction where a ceramide-dependent formation of glucosylceramide could be demonstrated. This Golgi fraction, mainly constituted by stacks of intact cisternae which retained the same topographical orientation as *in vivo*, was then incubated with liposomal dispersions of glycosphingolipid-glycosyltransferase acceptors in reaction mixtures containing all the requirements for enzyme activity but no detergent. Under such conditions, SAT-1 and other late acting glycosyltransferases were over 90% latent, while both GlcT and GalT-2 were just as active as in the detergent-containing assay; they were still inhibited by EDTA. Sepharose-immobilized ceramide and Sepharose-immobilized glucosylceramide were found to be suitable acceptors for GlcT and GalT-2, respectively, still using intact Golgi cisternae as the enzyme source. Moreover, a part of GlcT and GalT-2 activity was released from intact Golgi cisternae upon cathepsin D treatment. These results provide strong evidence that GlcT and GalT-2 face the cytoplasmic side of the Golgi apparatus, whereas SAT-1 and the other late acting enzymes face the luminal side.

It is well known that the initial assembly of Asn-linked oligosaccharides takes place in the endoplasmic reticulum by the action of different glycosyltransferases facing both the luminal and the cytoplasmic side of this organelle (1-3). Recently, it has also been reported that the initiation of O-glycosylation reactions takes place in the Golgi apparatus (4, 5), probably in the lumen of this organelle. Conversely, little information is available on the subcellular aspects of the initial glycosylations of glycosphingolipids. Once ceramide (Cer)[1] is biosynthesized in the endoplasmic reticulum (10), it serves as the substrate for UDP-Glc:Cer $\beta1\rightarrow1'$glucosyltransferase (GlcT), which then produces GlcCer, the substrate of UDP-Gal:GlcCer $\beta1\rightarrow4$galactosyltransferase (GalT-2,) which forms LacCer, the common substrate of the different glycosyltransferases involved in the biosynthesis of many glycosphingolipid classes, such as gangliosides and globosides (11). In the case of ganglio-series gangliosides, the LacCer processing enzyme is CMP-NeuAc:LacCer $\alpha2\rightarrow$3sialyltransferase (SAT-1), which produces G_{M3}. While this enzyme is localized to the Golgi apparatus (12), where we recently demonstrated that rat liver GalT-2 is also localized (13), the subcellular site of GlcT is not well established. The previous results on the Golgi distribution of SAT-1 (14), together with those obtained on the subcellular glycosylation of exogenous LacCer (15), led us to the working hypothesis of a sub-Golgi processing of each individual ganglioside, which involves either chain elongation or sorting to the plasma membrane. In agreement with this concept, we found that SAT-1 is resolved from the late acting glycosyltransferases (9), and other authors reported that brefeldin A does not inhibit G_{M3} biosynthesis, but instead its further glycosylation (16, 17). On the other hand, it is unclear whether such a processing involves Cer or GlcCer as the first compound and on which side of the Golgi membranes it begins. In this paper, we attempt to identify and characterize GlcT in rat liver, with the aim of establishing its subcellular localization. In addition, we aim to determine the topographical orientation of GlcT, GalT-2, and SAT-1 on the membrane where they are located. For the first purpose, we tested GlcT activity toward exogenous Cer using different subcellular fractions prepared from the liver, including an extremely purified Golgi apparatus fraction (18), as the enzyme source. We also studied its kinetic parameters and characterized the reaction product. Since we found GlcT on the Golgi apparatus fraction, we used this fraction for the determination of topographical orientation. Liposomal dispersion of the acceptor substrates was utilized for determining whether any glycosyltransferase could be detected with sealed, "right side out" Golgi cisternae or only after their permeabilization. A similar approach was recently reported to be successful in determining the orientation of the *N*-acetylglucosaminyltransferases involved in the dolichol pathway (3). We also incubated these intact Golgi cisternae with Sepharose-immobilized sphingolipids to assess whether acceptor availability into the Golgi lumen is a prerequisite for the activity of such glycosphingolipid glycosyltransferases. In addition, we used different proteases for determining the accessibility of these enzymes in the intact *versus* permeabilized Golgi membranes.

EXPERIMENTAL PROCEDURES

Materials—Detergents, asialofetuin, CDP-choline, nucleotide sugars, phosphatidylcholine from fresh egg yolk (type XI-E), pronase E, trypsin (type III), cathepsin D (from bovine spleen), sphingosine, and

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[1] Glycosphingolipids and gangliosides are coded according to the nomenclature of Svennerholm (6) and the IUPAC-IUB Recommendations (7). Designation of glycosyltransferases is according to Basu *et al.* (8) with the reported extensions (9). The abbreviations used are: HPTLC, high performance thin layer chromatography; Chaps, 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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CNBr-Sepharose 4B were obtained from Sigma. HPTLC plates, Silica Gel 100, sucrose for density gradient ultracentrifugation, stearic acid, and common chemicals were obtained from Merck. UDP-[*U*-^{14}C]glucose, UDP-[*U*-^{14}C]galactose, UDP-*N*-acetyl[*U*-^{14}C]galactosamine, CMP-*N*-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid, and [1-^{14}C]stearic acid were obtained from Amersham International (Amersham, Bucks, United Kingdom).

Sphingolipid Preparation—GlcCer and LacCer (18), G_{M3} and G_{M1} (19), and G_{M2} (20) were prepared according to the given references. Cer was prepared by coupling sphingosine, 8.0 mM, to stearoyl chloride (21), 10.0 mM, both dissolved in chloroform, in the presence of 12.0 mM triethylamine, overnight at room temperature. Final purification of Cer was accomplished by column chromatography on Silica Gel 100, using chloroform/methanol, 18:1, v/v, as the eluting solvent system. Deacylated GlcCer (lyso-GlcCer) and deacylated LacCer (lyso-LacCer) were prepared by alkaline treatment (1-butanol, 5 M aqueous KOH, 9:1, v/v, 110 °C, 5 h) of GlcCer and LacCer, respectively. After addition of 1 volume of water and 2 volumes of chloroform/methanol, 2:1, v/v, the hydrolysates were partitioned; each chloroform phase was collected, dried, and applied to a Silica Gel 100 column. Lyso-GlcCer and lyso-LacCer were eluted with chloroform/methanol/water, 55:20:3, v/v. Deacylated G_{M1} (lyso-G_{M1}) was prepared according to the reported method (22). Stearic acid-labeled Cer ([*stearoyl*-^{14}C]Cer) was prepared as described for cold Cer but using [1-^{14}C]stearic acid for generating [1-^{14}C]stearoyl chloride. Standard radiolabeled GlcCer (23), LacCer, and GalCer (24) were prepared according to the given references.

Preparation of Sepharose-immobilized Sphingolipids—Sphingolipids were attached to Sepharose by coupling the amino group of 12-aminododecanoic acid-containing sphingolipids to CNBr-activated Sepharose 4B. Such derivatized sphingolipids were obtained by substituting the naturally occurring fatty acid with 12-aminododecanoic acid. The amino group of 12-aminododecanoic acid was protected by a 9-fluorenylmethylchloroformyl residue, and the carboxyl group was activated in the presence of disuccinimidyl carbonate, as reported (25). The obtained product was coupled to sphingosine, lyso-GlcCer, lyso-LacCer, and lyso-G_{M1} according to the procedure reported for G_{M1} (25). The compounds obtained at the end of the coupling reactions were purified by Silica Gel 100 column chromatography using chloroform/methanol, 9:1, v/v (product of the reaction with lyso-GlcCer) or chloroform/methanol/water, v/v, in two different ratios: 125:30:2 (product of the reaction with lyso-LacCer) and 12:7:1 (product of the reaction with lyso-G_{M1}). The obtained purified compounds, as well as the whole mixture of the coupling reaction with sphingosine, were allowed to stand under an ammonia flow (25) in order to remove the protection from the amino group. The 12-aminododecanoic acid-containing sphingolipids were finally purified by silica gel column chromatography using chloroform/methanol/water, v/v, in the following ratios: 55:20:3 (12-aminododecanoic acid-containing Cer and 12-aminododecanoic acid-containing GlcCer), 12:6:1 (12-aminododecanoic acid-containing LacCer), and 60:35:8 (12-aminododecanoic acid-containing G_{M1}). Bovine testes β-galactosidase treatment (20) of the last compound was used to prepare the 12-aminododecanoic acid-containing G_{M2}, which was purified by silica gel column chromatography, using chloroform/methanol/water, 60:35:7, v/v, as the eluting solvent system. The identity of each 12-aminododecanoic acid-containing sphingolipid was assessed by GLC (19). Coupling to the activated Sepharose was done according to the manufacturer's instructions, but using methanol/water, 3:1, v/v, as the buffer solvent.

Subcellular Fractionation—Subcellular fractions were prepared from a single rat liver homogenate as previously reported (13, 18, 26). Briefly, the homogenate was spun at 6,000 × *g* for 15 min, and the upper two-thirds of the obtained pellet was resuspended and layered on 25 ml of 1.2 M sucrose and spun at 100,000 × *g* in a Beckman SW-28 rotor for 30 min. Material from the homogenate/sucrose interface was collected, made 40% (w/v) in sucrose, placed at the bottom of a nitrocellulose tube, overlayered with 10 ml of both 35% and 20% (w/v) sucrose, and spun at 90,000 × *g* for 1 h in the same rotor. Material at the 35/20% sucrose interface was collected, diluted to an isotonic solution, and pelleted at 30,000 × *g* for 15 min in a Kontron TFT 75 rotor. The obtained pellet was resuspended in 0.25 M buffered sucrose to obtain the Golgi apparatus fraction which was immediately used as the enzyme source in the regular assay and in all assays where sealed cisternae were required. For assessing whether or not cisternae were sealed and of the same topographical orientation as *in vivo*, the latency of CMP-NeuAc:asialofetuin sialyltransferase (asialofetuin-SAT) was determined under the different experimental conditions. Such a latency was calculated as the amount of enzyme activity recovered with the detergent-free assay with respect to the activity found in the presence of detergent. Asialofetuin-SAT was assayed according to Briles *et al.* (27). The same fraction was incubated at 4 °C for 10 min in the presence of 0.1 mg/ml Triton X-100 (28) to obtain Golgi cisternae permeabilization. After this treatment, the latency of asialofetuin-SAT, determined as described above, was less than 4%. For electron microscopy, the material collected from the last sucrose interface was fixed and processed as reported (18). The plasma membrane and endoplasmic reticulum fractions, from the lower one-third of the first pellet and from the supernatant of the first centrifugation, respectively, were prepared without modification of the reported method (18, 26).

Glycosphingolipid Glycosyltransferase Assays—The regular assay of glycosphingolipid glycosyltransferases was carried out according to the general procedure of Basu *et al.* (8). GlcT was assayed in a reaction mixture containing, in a final volume of 0.04 ml, 0.25 mM acceptor Cer, 6.0 mg/ml Triton CF-54, 0.2 M cacodylate buffer, pH 7.0, 10 mM $MnCl_2$, 0.5 mM donor UDP-[^{14}C]Glc (specific radioactivity 2.0 mCi/mmol), and the enzyme source, about 0.5 mg of protein/ml in the case of the Golgi apparatus. Incubation was for 1 h at 37 °C. GalT-2 (13), SAT-1 and CMP-NeuAc:G_{M1} $\alpha2\rightarrow3$sialyltransferase (SAT-4) (14), CMP-NeuAc:G_{M3} $\alpha2\rightarrow8$sialyltransferase (G_{M3}-SAT), UDP-GalNAc:G_{M3} $\beta1\rightarrow4N$-acetylgalactosaminyltransferase (GalNAcT-1), and UDP-Gal:G_{M2} $\beta1\rightarrow3$galactosyltransferase (GalT-3) (9), were assayed according to the given references. Blanks were regularly prepared by omitting the acceptors in the reaction mixtures. At the end of the incubation, the reaction mixtures were spotted on Whatman 3MM paper and assayed by descending chromatography in 1% tetraborate (8). The radioactivity of the appropriate areas was quantitatively determined by liquid scintillation counting, and blank values were subtracted.

In some experiments, the acceptors were not mixed with detergent but added as a liposomal dispersion (liposomal-acceptor assay). For this, phosphatidylcholine and acceptors were dissolved in chloroform/methanol, 4:1, v/v, in the proper molar ratio: 3:1 in the case of Cer, LacCer, and G_{M3}, 4:1 in the case of G_{M2}, 5:1 in the case of GlcCer and G_{M1}. After drying, liposomes were prepared according to the reported method (29). After sonication, the suspensions were centrifuged in 2-ml Eppendorf tubes for 30 min at 12,000 rpm, and the obtained supernatant was used immediately for the enzyme assay. The acceptor concentrations in this type of assay were 0.35 mM Cer, 0.50 mM GlcCer, and 0.25 mM and 0.20 mM G_{M3} for G_{M3}-SAT and GalNAcT-1, respectively, 0.2 mM LacCer, G_{M2} and G_{M1}. Blanks were prepared using liposomal suspensions without acceptors. After a 1-h incubation at 37 °C, the reactions were stopped by chilling on ice and kept for 15 min in the presence of 1.0 mg/ml Triton X-100 in order to obtain membrane disruption, as required for descending chromatography.

Some enzyme activities were also assayed using Sepharose-immobilized sphingolipids as the acceptors. For this, immobilized acceptors (0.07 ml of settled Sepharose, about 0.3 μmol of immobilized acceptor) were incubated in the same reaction mixtures as the liposomal acceptors, but in a final volume of 0.3 ml. The specific radioactivity of the nucleotide sugars employed was 10 mCi/mmol for UDP-[^{14}C]Glc, 5 mCi/mmol for UDP-[^{14}C]Gal, and 2 mCi/mmol for CMP-[^{14}C]NeuAc. Blanks were prepared by omitting the Golgi protein in the reaction mixture. Incubation was for 1 h at 37 °C under continuous gentle shaking. The reactions were stopped by chilling on ice and kept for 15 min on ice in the presence of 1.0 mg/ml Triton X-100. Each reaction mixture was placed on the sintered glass filter of a small disposable column (Bio-Rad) and washed with 5 ml each of water, bicarbonate buffer, pH 8.5, acetate buffer, pH 4.0, 1% borate, 5% Triton X-100, methanol, and water. Known amounts of washed Sepharose were then placed into 20-ml vials with 15 ml of Ultima Gold (Packard) for counting. Blank values were subtracted for calculation. The remaining material was placed in a reaction tube and submitted to alkaline hydrolysis and partitioned as reported above for cold lysosphingolipid preparation.

Reaction Product Characterization—In the case of GlcT, the glucose-labeled product was obtained using UDP-[^{14}C]Glc (specific radioactivity 5 mCi/mmol) as the donor and cold Cer as the acceptor in a 0.5-ml incubation. Glc-^{14}C-labeled product was eluted from the paper with chloroform/methanol, 6:1, v/v, and submitted to extraction and partitioning (30). The final product obtained in the dried organic phase was analyzed by HPTLC and visualized by fluorography (31). [*stearoyl*-^{14}C]GlcCer was obtained with cold UDP-Glc as the sugar donor and [*stearoyl*-^{14}C]Cer (specific radioactivity 10 mCi/mmol) as the acceptor, under the same conditions described for [*Glc*-^{14}C]GlcCer. It was then extracted from the whole reaction mixture

and partitioned (31). The obtained organic phase was dried and analyzed as reported above for [*Glc*-^{14}C]GlcCer. In the case of GalT-2 (13), SAT-1 and SAT-4 (14), G_{M3}-SAT, GalT-3, and GalNAcT-1 (9), the reaction products were obtained and analyzed as reported. In addition, the reaction products of GlcT and GalT-2 were prepared using liposomal dispersion of the acceptors, freshly prepared as described above for the corresponding enzyme assays, in reaction mixtures containing no detergent. The final radioactive products were obtained and analyzed in the same manner as those prepared in the presence of detergent.

Protease Treatment of the Golgi Apparatus—Intact Golgi cisternae and permeabilized Golgi membranes were incubated at 37 °C for 30 min in the presence of 5 μg/mg Golgi protein of either pronase E or trypsin, according to Coste *et al.* (32). After washing with 350 volumes of buffer and pelleting, the obtained membranes were assayed for glycosyltransferase activity. In the case of trypsin treatment, soybean trypsin inhibitor (50 mg/mg trypsin) was also added. This intact Golgi cisternae, as well as the permeabilized Golgi membranes, was also treated with cathepsin D (2 μg/mg Golgi protein) for 15 min at 37 °C at pH 5.6, according to Lammers and Jamieson (33). After increasing the solution pH to 7.0, the reaction mixtures were pelleted at 100,000 × *g* for 1 h, and the collected supernatants were assayed for glycosyltransferase activity. In both cases, enzyme activity was determined using the regular assay described above.

RESULTS

Characterization and Subcellular Distribution of GlcT—Using the Golgi apparatus fraction (see below) as the enzyme source, we detected an activity that was able to transfer Glc from UDP-Glc to exogenous Cer. This activity required high detergent concentration (Table I). Triton CF-54 was found to be the most effective among the common commercial detergents tested; Chaps (75% effective), Triton X-100 (69% effective), and Nonidet P-40 (62% effective) were also suitable. GlcT required Mn^{2+} and was not stimulated by CDP-choline. The activity was detectable at pH values ranging from 6.8 to 7.5, and the maximum activity was found at pH 7.0. GlcT dependence on the acceptor substrate was clearly demonstrated with Cer (Fig. 1*A*). Saturation occurred at values over 0.25 mM. Apparent calculated K_m and V_{max} were 0.17 mM Cer and 3.8 nmol/mg/h transferred Glc, respectively. The activity was also dependent on UDP-Glc as the sugar donor (Fig. 1*B*). Saturation occurred at concentrations over 0.5 mM, and the apparent calculated K_m for UDP-Glc was 0.42 mM. Activity was linear in a protein concentration range from 0.3 to 0.6 mg/ml and increased linearly with time from 40 to 80 min.

The above-mentioned Golgi apparatus fraction was 159-fold enriched in ovalbumin-GalT (19% yield of total homogenate activity), 131-fold enriched in asialofetuin-SAT (16% yield), 1.2-fold enriched in Glc-6-phosphatase (0.15% yield), 2.4-fold enriched in 5′-nucleotidase (0.34% yield), and 1.0-fold enriched in acid phosphatase (0.12% yield). It was mainly made up of intact cisternal stacks, as assessed by electron microscopy. The latency of asialofetuin-SAT in freshly prepared fractions was over 93%, indicating that cisternae were sealed and retained the natural topographical orientation (right side out). This was the only liver fraction where GlcT was actually measurable. In fact, a Cer-dependent formation of true GlcCer could not be demonstrated either in the whole homogenate or in other subfractions, including an endoplasmic reticulum fraction 4.2-fold enriched in Glc-6-phosphatase (3.5% yield) concurrently prepared from the same homogenate.

TABLE I

Requirements of rat liver Golgi GlcT

The complete reaction mixture contained acceptor Cer, detergent, donor UDP-[^{14}C]Glc, $MnCl_2$, Golgi protein, and buffer as described under "Experimental Procedures." Results are the mean for four experiments. Values are expressed as a percent of the "complete" assay condition. One hundred percent activity corresponds to 4.5 nmol/mg of protein/h transferred Glc. Added salts were 10.0 mM.

Assay conditions	Activity
Complete	100.0
Minus acceptor	48.7
Minus detergent	45.3
Minus Mn^{2+}	47.9
Minus Mn^{2+}, plus Mg^{2+}	53.4
Minus Mn^{2+}, plus EDTA	48.3
Plus Mg^{2+}	64.4
Plus CDP-choline	94.1

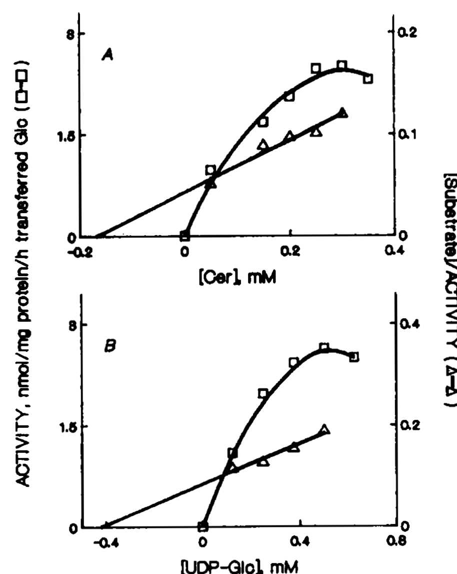

FIG. 1. **Characterization of rat liver Golgi GlcT.** Enzyme activity measurement and Golgi apparatus preparation were carried out as described under "Experimental Procedures." The effect of acceptor Cer (*panel A*) and donor UDP-Glc (*panel B*) is presented. The amount of radioactive Glc transferred to endogenous acceptors was subtracted to each point. Values are the mean for three experiments. Standard deviations are within the size of the symbols used. The *right scale* shows the transposition in a Hanes-Woolf plot of the activity values.

Reaction Product Characterization—When cold Cer was used as the acceptor and UDP-[^{14}C]Glc as the sugar donor, two major radioactive spots were obtained after partitioning in the organic phase. The first one (about 60%), co-migrated, by HPTLC, with standard GlcCer and the second one (about 35%) slightly slower than LacCer and differently from the other reference glycosphingolipids. This unknown spot was the only one detected when the incubation was performed by omitting exogenous Cer in the reaction mixture. When [*stearoyl*-^{14}C]Cer was used as the acceptor and cold UDP-Glc as the sugar donor in the same conditions as above, two spots were obtained. The first one (about 96%) co-migrated with reference standard Cer and the second one (about 2.3%) with standard GlcCer. This second spot was not found in a control incubation lacking exogenous UDP-Glc (Fig. 2).

Latency of Glycosyltransferase Activities toward Exogenous Sphingolipids in Rat Liver Golgi Apparatus—Glycosyltransferases involved in the initial glycosylations of glycosphingolipids required a high detergent concentration for acceptor substrate solubilization. Moreover, the presence of the proper detergent, in a specific concentration (see "Experimental Procedures"), allowed one to obtain the highest specific activity values (Table II, first column) for each activity, probably due to the formation of detergent-substrate aggregates which provide the optimal interaction with the catalytic site. Under such conditions, the integrity of the Golgi membranes is completely disrupted. In the absence of detergent, the specific

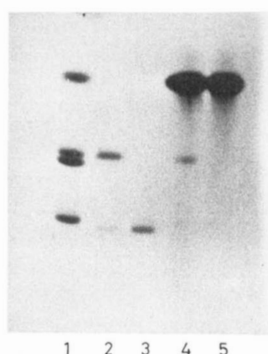

FIG. 2. **Reaction product characterization of GlcT.** The reaction product of GlcT was obtained as described under "Experimental Procedures." *Lane 1*, standard radiolabeled Cer, GlcCer, GalCer, and LacCer (from the *top*). *Lane 2*, reaction products of GlcT obtained using UDP-[^{14}C]Glc as the donor and cold Cer as the acceptor. *Lane 3*, reaction product of GlcT obtained using UDP-[^{14}C]Glc as the donor and no exogenous acceptor. *Lane 4*, reaction products of GlcT obtained using cold UDP-Glc as the donor and [*stearoyl*-^{14}C]Cer as the acceptor. *Lane 5*, reaction products of GlcT obtained using [*stearoyl*-^{14}C]Cer as the acceptor and no exogenous donor. The HPTLC plate was developed with chloroform/methanol/water (55:20:3, by volume) as the eluting solvent system and visualized by fluorography.

TABLE II

Latency of glycosphingolipid glycosyltransferases in rat liver Golgi apparatus

The regular assay of glycosphingolipid glycosyltransferases was performed as described under "Experimental Procedures" in the presence of high detergent concentration (disrupted Golgi) or in the absence of detergent (sealed cisternae). The liposomal-acceptor assay was carried out using liposomal dispersions of sphingolipids, freshly prepared by ultrasonication of phosphatidylcholine, and acceptors mixed in the proper molar ratios. Golgi cisternae permeabilization was obtained as described under "Experimental Procedures." Latency of asialofetuin-SAT was calculated at the end of the incubation using a control reaction mixture, lacking specific acceptor and donor, as the Golgi apparatus source. Values are expressed as nanomoles/mg of protein/h transferred sugar. Results are the means for two independent experiments.

Enzyme	Regular assay		Liposomal-acceptor assay	
	Disrupted Golgi	Sealed cisternae	Sealed cisternae	Permeabilized cisternae
GlcT	2.6	0.1	2.4	2.5
GalT-2	8.5	0.4	7.7	8.1
SAT-1	18.7	1.0	1.9	15.8
GM3-SAT	13.6	0.5	1.2	11.6
GalNAcT-1	20.2	0.6	1.2	10.4
GalT-3	65.4	15.6	7.1	74.3
SAT-4	72.3	15.2	7.5	60.2
Asialofetuin-SAT	1988	176.9	280.3	1922

activity values of GlcT, GalT-2, SAT-1, G_{M3}-SAT, and GalNAcT-1 were very low. Conversely, GalT-3 and SAT-4 provided up to 25% of the specific activity values obtained with the detergent (Table II, second column). When the acceptors were added as a liposomal dispersion, about 95% of the maximum specific activity values of GlcT and GalT-2 were recovered (Table II, third column). The latency of asialofetuin-SAT, calculated at the end of this incubation, was still over 85%. The dependence of GlcT and GalT-2 on the liposomal-acceptor concentration, as well as on the concentration of Golgi protein and on time, resembled that obtained in the regular assay. Moreover, both activities were over 85% inhibited by EDTA. The reaction products of both activities were identical with those obtained with the corresponding detergent-containing incubations. In the same experiment (liposomal-acceptor assay with sealed cisternae), the specific activity values of SAT-1, G_{M3}-SAT, and GalNAcT-1 increased very little with respect to the regular assay with sealed cisternae, whereas those of GalT-3 and SAT-4 decreased. When the glycosyltransferases were assayed using the same liposomal dispersions of the acceptors but with permeabilized cisternae (asialofetuin-SAT latency less then 4%), the specific activity values of GlcT and GalT-2 remained almost unchanged, whereas those of the other glycosyltransferases increased close to the maximum values (Table II, last column).

Biosynthesis in Vitro of Immobilized GlcCer and Immobilized LacCer by Intact Golgi Cisternae—The activity of GlcT and GalT-2 toward Sepharose-immobilized Cer and Sepharose-immobilized GlcCer as the acceptors, respectively, was well detected using sealed Golgi cisternae as the enzyme source. After extensive washing out, the insoluble material retained a significant amount of radioactivity, which was then released (about 85%) by alkaline hydrolysis at 110 °C. HPTLC analysis of the hydrolysates revealed a major radioactive spot in each case, co-migrating with reference standard lyso-GlcCer (GlcT incubation) and lyso-LacCer (GalT-2 incubation) (Fig. 3). The latency of asialofetuin-SAT, calculated at the end of the incubation, was 81.1% and 84.3% in two independent experiments. While the transfer of radioactive Glc to immobilized Cer and of radioactive Gal to immobilized GlcCer was almost identical using either sealed or permeabilized cisternae (Table III), that of radioactive sialic acid to immobilized LacCer and of radioactive Gal to immobilized G_{M2} was about 5-fold enhanced by permeabilization.

Partial Release of GlcT and GalT-2 from Intact Golgi Cisternae by Proteolysis—Attempts to determine glycosphingolipid glycosyltransferase latency in partially reversed ("right side in") Golgi vesicles, prepared by ultrasonication, were ineffective because many enzymes were quickly inactivated by such treatment. On the other hand, we obtain additional results on glycosphingolipid glycosyltransferase orientation by testing their susceptibility to proteolysis. Since preincubation of our Golgi fraction with either pronase or trypsin provided a complete loss of all glycosyltransferase activities, we attempted to obtain the release of catalytically active glycosyltransferases using cathepsin D. Upon the treatment of intact Golgi cister-

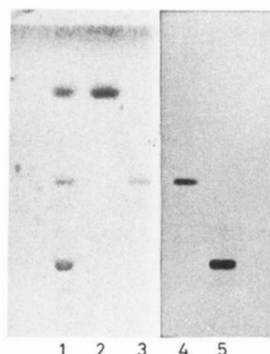

FIG. 3. **Characterization of GlcCer- and LacCer-Sepharose biosynthesized *in vitro*.** The immobilized radioactive products formed by the action of GlcT and GalT-2 with Cer-Sepharose and GlcCer-Sepharose, respectively, were isolated from the reaction mixture and hydrolyzed as described under "Experimental Procedures." *Lane 1*, standard sphingosine, lyso-GlcCer, and lyso-LacCer (from the *top*). *Lane 2*, hydrolysate from Cer-Sepharose previously incubated with GlcT. *Lane 3*, hydrolysate from GlcCer-Sepharose previously incubated with GalT-2. *Lanes 4 and 5*, fluorography detection of *lanes 2* and *3*, respectively. The HPTLC plate was developed with chloroform/methanol/water/concentrated ammonia (350:150:21:4, by volume) as the eluting solvent system and visualized by fluorography (*lanes 4–5*) or by ninhydrin (*lanes 1–3*).

TABLE III

Activity of glycosphingolipid glycosyltransferases toward Sepharose-immobilized acceptors

Preparation of intact cisternae from the Golgi apparatus, Golgi cisternae permeabilization, and asialofetuin-SAT latency determination (calculated at the end of the incubation) were performed as described under "Experimental Procedures." Eighty μg of Golgi protein were incubated with 70 μl of settled Sepharose (containing about 0.3 μmol of immobilized acceptors) in the presence of the proper requirements of each enzyme activity but not detergent, in a final volume of 0.3 ml. Blanks were prepared by omitting the Golgi protein in the reaction mixture. After extensive washing out of the Sepharose, bound radioactivity was determined by liquid scintillation counting (for details see text). Results are the means for two independent experiments.

Enzyme	Activity	
	Sealed cisternae	Permeabilized cisternae
	pmol transferred sugar	
GlcT	15.8	12.9
GalT-2	226.4	193.9
SAT-1	150.1	766.5
GalT-3	165.4	689.3

TABLE IV

Partial release of glycosyltransferases upon cathepsin D treatment of the Golgi apparatus

Intact Golgi cisternae and permeabilized Golgi membranes, prepared as described under "Experimental Procedures," were treated at pH 5.6 with cathepsin D (2 μg/mg Golgi protein) for 15 min at 37 °C. After increasing the solution pH to 7.0, the mixture was spun at 100,000 × *g* for 1 h. The collected supernatant was assayed for glycosyltransferase activity using the regular assay system. Results are the mean for two independent experiments. Values are expressed as nanomoles/h transferred sugar and refer to 1 mg of protein of the starting Golgi apparatus.

Enzyme	Starting Golgi apparatus	Supernatant from intact cisternae		Supernatant from permeabilized membranes	
		Control	Plus cathepsin D	Control	Plus cathepsin D
GlcT	2.3	Undetectable	0.44	0.11	0.52
GalT-2	7.9	0.01	1.26	0.32	1.51
SAT-1	16.1	0.02	0.03	0.44	1.77

nae with such protease, a little but well measured amount of GlcT (19% of the activity measured in the starting Golgi apparatus) and GalT-2 (16%) was recovered in soluble form, while a comparable amount of SAT-1 (11%) was released by cathepsin D only starting from permeabilized Golgi membranes (Table IV).

DISCUSSION

In this paper, we present evidence that GlcT is mainly located in the Golgi apparatus and that both GlcT and GalT-2 face the cytoplasmic side of the Golgi whereas SAT-1 and the other ganglioside glycosyltransferases face the luminal side of this organelle.

We found a Cer-dependent formation of GlcCer from UDP-Glc only using an extremely purified Golgi apparatus fraction as the enzyme source. The requirements and kinetic parameters of the activity were similar to those of other glycosphingolipid glycosyltransferases. GlcT was inactivated after 80 min of incubation at 37 °C and not stimulated by Golgi protein concentrations over 0.6 mg/ml. Moreover, a significant transfer of Glc to an unknown endogenous substance (10) made the blank values high. In a previous report (10), GlcT was not detected at all in rat liver homogenate and subcellular fractions, and we also failed to detect it in the whole homogenate, being therefore unable to calculate the recovery of the enzyme in the Golgi apparatus with respect to that of specific Golgi markers. Consequently, we cannot exclude the presence of a small amount of GlcT in other membranes which have been lost during our subfractionation procedure.

In the complete absence of detergent, GlcT, GalT-2, and SAT-1, which utilize water-insoluble acceptors, were almost undetectable. Conversely, GalT-3 and SAT-4 were over the expected latency of these enzymes, probably because G_{M2} and G_{M1} form micelles which allow partial penetration of the acceptor into the Golgi (34). G_{M3}-SAT and GalNAcT-1 were poorly detected under these conditions, even though G_{M3} is water-soluble. In this regard, it has recently been reported that G_{M3} does not form micelles but vesicles (35), which probably do not allow acceptor penetration into the Golgi. Incubation of intact Golgi cisternae with liposomal dispersion of the acceptors provides a good detection of only GlcT and GalT-2. Under such conditions, both activities were inhibited by EDTA, a membrane-impermeable reagent not affecting nucleotide sugar transport (2). After cisternae permeabilization, their specific activity values remained unchanged, indicating almost no latency of these activities. Conversely, the other glycosyltransferases recovered the bulk of their maximum specific activity, indicating that they are detectable, but latent, with the liposomal-acceptor assay. For assessing whether a selective penetration of acceptors from the liposomes into the Golgi may invalidate these results, we determined glycosyltransferase activity toward immobilized acceptors. For this purpose, sphingolipids were derivatized by substituting the natural fatty acid with a shorter fatty acid chain ending with an amino group, which allowed easy attachment to activated Sepharose. We found that the activity of both GlcT and GalT-2 toward immobilized acceptors was present, without latency, in intact Golgi cisternae, while that of SAT-1 and GalT-3 was strongly impaired by the integrity of the Golgi. These results indicate that GlcT and GalT-2 do not require the availability of their acceptors into the Golgi and corroborate the hypothesis that they face the cytoplasmic side of this organelle, whereas SAT-1 and late acting enzymes face the luminal side. The possibility of such a topographical orientation was already suggested for GlcT in porcine submaxillary Golgi membranes (32), due to its differential susceptibility to pronase and trypsin treatment. In rat liver Golgi, we found that these two proteases inactivate also glycosphingolipid glycosyltransferases which face the lumen of the Golgi. Conversely, catalytically active GlcT and GalT-2 were selectively released from intact Golgi cisternae upon the action of cathepsin D, while SAT-1 was released only from permeabilized Golgi membranes. This finding further substantiates the claim of a cytoplasmic orientation of GlcT and GalT-2. The membrane orientation of GalT-2 was never directly studied. Incubation with radioactive Gal of mutant cell lines (27, 36) lacking the translocator for UDP-Gal transport into the Golgi lumen (36) provided radioactive glycosphingolipid patterns which would suggest a luminal orientation of GalT-2 (38). Conversely, results on the intracellular localization of LacCer in neutrophils (39) and renal tubular cell (40), as well as the influence of low density lipoproteins on GalT-2 (41), are in agreement with a cytoplasmic orientation of such a transferase. Even though a tissue specificity may exist for the topographical orientation of glycosyltransferases, as it exists for their subcellular and sub-Golgi localization (42, 43), dedicated experiments are presumably required for establishing this point. In conclusion, we propose the following model for the

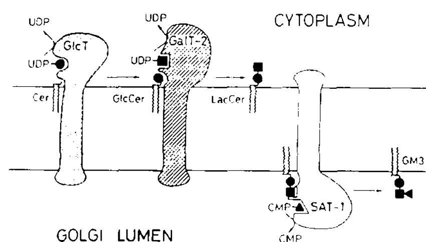

FIG. 4. **Proposed model for the topography of the initial glycosylations of gangliosides in the Golgi apparatus.** *Circle*, Glc; *square*, Gal; *triangle*, NeuAc.

topography of the initial glycosylations of ganglioside. Cer is made available on the cytoplasmic side of the Golgi, where it acts as the acceptor for GlcT. The formed GlcCer acts as the acceptor for GalT-2, still on the cytoplasmic side. The formed LacCer is then made available on the luminal side of the Golgi, where SAT-1 is present (Fig. 4). As in the case of *N*-glycosylation, the mechanisms for the putative transmembrane movement of lipid-linked oligosaccharide are largely unknown (44). Since phosphatidylcholine:Cer phosphocholinetransferase (sphingomyelin synthase) faces the luminal side of the Golgi apparatus (45), it is reasonable to assume that Cer is made available on both sides of the Golgi membranes.

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REFERENCES

1. Hirschberg, C. B., and Snider, M. D. (1987) *Annu. Rev. Biochem.* **56,** 63–87
2. Abeijon, C., and Hirschberg, C. B. (1990) *J. Biol. Chem.* **265,** 14691–14695
3. Kean, E. L. (1991) *J. Biol. Chem.* **266,** 942–946
4. Abeijon, C., and Hirschberg, C. B. (1987) *J. Biol. Chem.* **262,** 4153–4159
5. Piller, V., Piller, F., and Fukuda, M. (1990) *J. Biol. Chem.* **265,** 9264–9271
6. Svennerholm, L. (1964) *J. Lipid. Res.* **5,** 145–155
7. IUPAC-IUB Recommendations (1977) *Lipids* **12,** 455–468
8. Basu, M., De, T., Das, K., Kyle, J. W., Chon, H., Schaper, R. J., and Basu, S. (1987) *Methods Enzymol.* **138,** 575–607
9. Trinchera, M., Pirovano, B., and Ghidoni, R. (1990) *J. Biol. Chem.* **265,** 18242–18247
10. Walter, V. P., Sweeney, K., and Morrè, D. J. (1983) *Biochim. Biophys. Acta* **750,** 346–352
11. Makita, A., and Taniguchi N. (1985) in *New Comprehensive Biochemistry* (Wiegandt, H., ed) Vol. 10, pp. 1–100, Elsevier Science Publishers B. V., Amsterdam
12. Keenan, T. W., Morré, D. J., and Basu, S. (1974) *J. Biol. Chem.* **249,** 310–315
13. Trinchera, M., Fiorilli, A., and Ghidoni, R. (1991) *Biochemistry* **30,** 2719–2724
14. Trinchera, M., and Ghidoni, R. (1989) *J. Biol. Chem.* **264,** 15766–15769
15. Trinchera, M., and Ghidoni, R. (1990) *Biochem. J.* **266,** 363–369
16. van Echten, G., Iber, H., Stotz, H., Takatsuki, A., and Sandhoff, K. (1990) *Eur. J. Cell Biol.* **51,** 135–139
17. Young, W. W., Jr., Lutz, M. S., Mills, S. E., and Lechler- Osborn, S. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6838–6842
18. Trinchera, M., Carrettoni, D., and Ghidoni, R. (1991) *J. Biol. Chem.* **266,** 9093–9099
19. Ghidoni, R., Sonnino, S., Tettamanti, G., Baumann, N., Reuter, J., and Schauer, R. (1980) *J. Biol. Chem.* **255,** 6990–6995
20. Cahan, L. D., Irie, R. F., Singh, R., Cassidenti, A., and Paulson, S. C. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 7629–7633
21. Dubois, G., Zalc, B., Le Saux, F., and Baumann, N. (1980) *Anal. Biochem.* **102,** 313–317
22. Sonnino, S., Kirschner, G., Ghidoni, R., Acquotti, D., and Tettamanti, G. (1985) *J. Lipid Res.* **26,** 248–257
23. McMaster, M. C., and Radin, N. S. (1976) *J. Labelled Compd. & Radiopharm.* **23,** 353–357
24. Trinchera, M., Ghidoni, R., Sonnino, S., and Tettamanti, G. (1990) *Biochem. J.* **270,** 815–820
25. Sonnino, S., Chigorno, V., Acquotti, D., Pitto, M., Kirschner, G., and Tettamanti, G. (1989) *Biochemistry* **28,** 77–84
26. Morrè, D. M., Morrè, D. J., Bowen, S., Reutter, W. and Windel, K. (1988) *Eur. J. Cell Biol.* **46,** 307–315
27. Briles, E. B., Li, E., and Kornfeld, S. (1977) *J. Biol. Chem.* **252,** 1107–1116
28. Capasso, J. M., Abeijon, C., and Hirschberg, C. B. (1988) *J. Biol. Chem.* **263,** 19778–19782
29. Masserini, M., Sonnino, S., Giuliani, A., Tettamanti, G., Corti, M., Minero, C., and Degiorgio, V. (1985) *Chem. Phys. Lipids* **37,** 83–97
30. Tettamanti, G., Bonali, F., Marchesini, S., and Zambotti, V. (1973) *Biochim. Biophys. Acta* **269,** 160–170
31. Ghidoni, R., Trinchera, M., Venerando, B., Fiorilli, A., Sonnino, S., and Tettamanti, G. (1986) *Biochem. J.* **237,** 147–155
32. Coste, H., Martel, M. B., and Got, R. (1986) *Biochim. Biophys. Acta* **858,** 6–12
33. Lammers, G., and Jamieson, J. C. (1988) *Biochem. J.* **256,** 623–631
34. Yusuf, H. K., Pohlentz, G., and Sandhoff, K. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 7075–7079
35. Sonnino, S., Cantù, L., Acquotti, D., Corti, M., and Tettamanti, G. (1990) *Chem. Phys. Lipids* **52,** 231–241
36. Brändli, A. W., Hansson, G. C., Rodriguez-Boulan, E., and Simons, K. (1988) *J. Biol. Chem.* **263,** 16283–16290
37. Deutscher, S. L., and Hirschberg, C. B. (1986) *J. Biol. Chem.* **261,** 96–100
38. Sasaki, T. (1990) *Experientia* **46,** 611–616
39. Symington, F. W., Murray, W. A., Bearman, S. I., and Hakomori, S. (1987) *J. Biol. Chem.* **262,** 11356–11363
40. Chatterjee, S., Kwiterovich, P. O., Jr., Gupta, P., Erozan, Y. S., Alving, C. R., and Richards, R. L. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 1313–1317
41. Chatterjee, S., Ghosh, N., Castiglione, E., and Kwiterovich, P. O., Jr. (1988) *J. Biol. Chem.* **263,** 13017–13022
42. Roth, J., Taatjes, D. J., Weinstein, J., Paulson, J. C., Greenwell, P., and Watkins, W. M. (1986) *J. Biol. Chem.* **261,** 14307–14312
43. Taatjes, D. J., Roth, J., Weinstein, J., and Paulson, J. C. (1988) *J. Biol. Chem.* **263,** 6302–6309
44. Pagano, R. E. (1990) *Curr. Opin. Cell Biol.* **2,** 652–663
45. Futerman, A. H., Stieger, B., Hubbard, A. L., and Pagano, R. E. (1990) *J. Biol. Chem.* **265,** 8650–8657