Transbilayer Movement of Monohexosylsphingolipids in Endoplasmic Reticulum and Golgi Membranes†

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ABSTRACT: The transbilayer movement of glycosphingolipids has been characterized in Golgi, ER, plasma, and model membranes using spin-labeled and fluorescent analogues of the monohexosylsphingolipids glucosylceramide and galactosylceramide and of the dihexosylsphingolipid lactosylceramide. In large unilamellar lipid vesicles, monohexosylsphingolipids underwent a slow transbilayer diffusion (half-time between 2 and 5 h at 20 °C). Similarly, the inward redistribution of these sphingolipids in the plasma membrane of the hepatocyte-like cell line HepG2 and of erythrocytes was slow. However, in rat liver ER and Golgi membranes, we found a rapid transbilayer movement of spin-labeled monohexosylsphingolipids (half-time of ≈3 min at 20 °C), which suggests the existence of a monohexosylsphingolipid flippase. The transbilayer movement of glucosylceramide in the Golgi and the ER displayed a saturable behavior, was inhibited by proteolysis, did not require Mg-ATP, and occurs in both directions. Treatment with DIDS inhibited the flip-flop of glucosylceramide but not that of phosphatidylcholine. These data suggest that the transbilayer movement of monoglucosylceramide in the ER and in the Golgi involves a protein that could be distinct from that previously evidenced for glycerophospholipids in the ER. In vivo, transbilayer diffusion should promote a symmetric distribution of monohexosylsphingolipids which are synthesized in the cytosolic leaflet. This should allow glucosylceramide rapid access to the lumenal leaflet where it is converted to lactosylceramide. No significant transbilayer movement of lactosylceramide occurred in both artificial and natural membranes over 1 h. Thus, lactosylceramide, in turn, is unable to diffuse to the cytosolic leaflet and remains at the lumenal leaflet where it undergoes the subsequent glycosylations.

Glycosphingolipids are usually minor components of the membranes of eukaryotic cells. They are mainly located in the exoplasmic leaflet of the plasma membrane and in the lumenal leaflet of endosomes and lysosomes (*1*). Glycosphingolipids and their breakdown products interact with signaling systems and cell receptors and play a key role in several biological phenomena such as signal transduction and cell−cell communication; in addition, glycosphingolipids often act as receptors for viruses (*2*−*5*). Finally, glycosphingolipids are thought to play a key role in lipid and protein sorting in epithelial cells by forming microdomains in the lumenal leaflet of the trans Golgi network (*6*, *7*).

The precursor of glycosphingolipids, ceramide, is synthesized at the cytosolic leaflet of the endoplasmic reticulum (ER)[1] (*8*−*10*) and reaches the Golgi apparatus via an unknown mechanism (for reviews see refs *11* and *12*). Because it lacks a polar headgroup, ceramide easily crosses the membrane and has access to both membrane leaflets. Sphingomyelin (SM) synthesis has been localized to the lumenal leaflet of the cis-medial Golgi apparatus (*13*, *14*), while glucosyltransferase (GlcT), which synthesizes the monohexosylsphingolipid glucosylceramide (GlcCer), is active at the cytosolic surface of Golgi (*11*, *12*, *15*−*19*). The second major monohexosylsphingolipid is galactosylceramide (GalCer). Depending on the fatty acid composition, GalCer may be synthesized at the cytosolic surface of the Golgi or the lumenal leaflet of the ER; the GalCer synthesized in the ER appears to have rapid access to the cytosolic surface of the ER (*19*, *20*). There is convincing evidence that the next step in glycosphingolipid synthesis, the galactosylation of GlcCer yielding lactosylceramide (LacCer) and the galac-

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[1] Abbreviations: SL, spin-labeled; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; ER, endoplasmic reticulum; DIDS, 4-acetamido-4′-isothiocyanostibene-2,2′-disulfonic acid; NBD, *N*-6-(7-nitro-2,1,3-benzoxadiazol-4-yl); GlcCer, 1-*O*-*β*-D-glucopyranosyl-*N*-(acyl)-sphingosine; GalCer, 1-*O*-*β*-D-galactopyranosyl-*N*-(acyl)sphingosine; LacCer, 1-*O*-[*β*-D-galactopyranosyl-(1−4)-*O*-*β*-D-glucopyranosyl]-*N*-(acyl)sphingosine; PC, phosphatidylcholine; SM, sphingomyelin; BSA, bovine serum albumin; RBC, red blood cells; TLC, thin-layer chromatography.

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tosylation of GalCer yielding galabiosylceramide (Ga_2Cer), occurs on the lumenal leaflet of the Golgi (*19*, *21*) as do all further steps in complex glycosphingolipid biosynthesis (*18*). It was also shown that the locations of successive glycosylation activities over the Golgi stacks reflect the position of glycosyltranserases; i.e., GlcT and GalT activities are largely restricted to the cis Golgi whereas later glycosylation steps predominantly occur in the trans Golgi (*20*, *22*).

Thus GlcCer and possibly GalCer have to be translocated to the lumenal leaflet of the cis-medial Golgi apparatus in order to be available for subsequent glycosylations. It is generally believed that the large polar headgroup of both glycosphingolipids will cause only slow spontaneous transbilayer movement. Faster transbilayer movement would require a passive or active GlcCer/GalCer-facilitated translocation system in the Golgi apparatus comparable to the glycerophospholipid flippase activity found in the ER (*23*−*28*) or the aminophospholipid translocase activity observed in the plasma membrane of most eukaryotic cells (*29*). Direct evidence for glycosphingolipid translocation in the Golgi has been obtained (*19*, *21*), but the kinetics, selectivity, and mechanism of translocation have not been studied so far.

Therefore, we have investigated the transbilayer movement of glycosphingolipids in rat liver Golgi and ER membranes and in the plasma membrane of the hepatocyte-like cell line HepG2 and red blood cells (RBC), as well as in large unilamellar vesicles (LUVs) prepared from egg PC in detail. For this purpose spin-labeled glucosyl-, galactosyl-, and lactosylceramide were synthesized (Figure 1A). We found that monohexosylsphingolipids can spontaneously, albeit slowly, cross the bilayer of LUVs by passive diffusion. In contrast, the higher order glycosphingolipid cannot. In both ER and Golgi, transverse diffusion of SL-GlcCer and SL-GalCer was found to be at least 30 times faster than in plasma and model membranes. Kinetic data as well as chemical modification and proteolysis experiments suggest that this rapid transbilayer movement might be protein mediated.

EXPERIMENTAL PROCEDURES

Materials. All commercial chemicals were from Sigma except uridine diphospho-D-[6-^{3}H]galactose from Amersham and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) from Aldrich. Microspin filters were from Polylabo, and AG1-X2 anion-exchange resin was from Bio-Rad. Silica gel 60 for column chromatography and TLC silica gel 60/F_{254} plates were from Merck. Spin-labeled 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphocholine (SL-PC) and -phosphatidylserine (SL-PS) and the corresponding sphingomyelin (SL-SM) (see Figure 1B) were synthesized as described previously (*30*). FAB mass spectra were recorded at the Institut de Pharmacologie et Biologie Structurale of Toulouse, France, using a MNBA/glycerol/trichloroacetic acid matrix.

Synthesis of Lactosylsphingosine. Following the procedure of Shapiro et al. (*31*, *32*), sphingosine was glycosylated with heptaacetyllactosyl bromide after successive protections of amine, primary alcohol, and secondary alcohol and subsequent deprotection of the primary alcohol. Alkaline hydrolysis led then to lactosylsphingosine. The product of each step was purified by chromatography on a silica gel column and checked by its migration on silica gel plates.

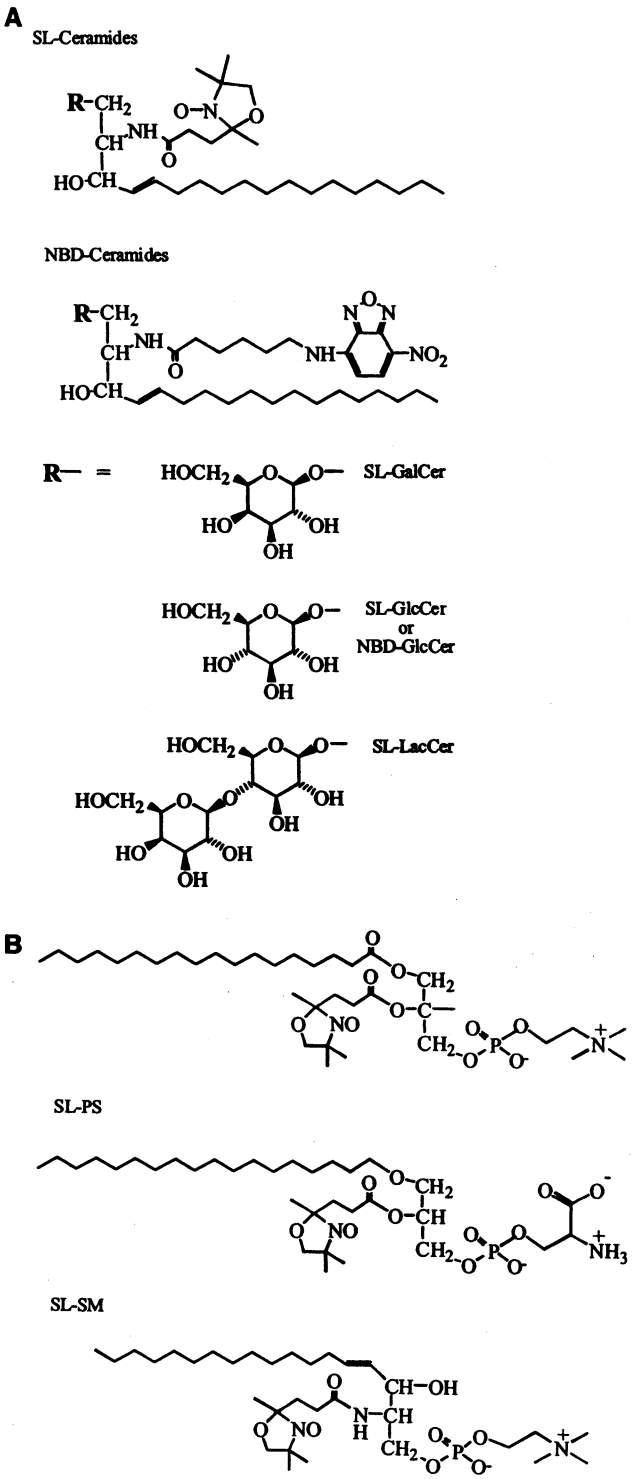

FIGURE 1: Formulas of the spin-labeled or fluorescent sphingolipids (A) and of the spin-labeled phospholipids (B) used to investigate lipid transmembrane movements.

Sphingosine (83 mmol) and methyl dichloroacetate gave *N*-(dichloroacetyl)sphingosine (A) with a yield of 67% after chromatographic purification; R_f = 0.50 (chloroform−methanol, 90:10) (R_f = 0.05 for sphingosine). Compound A (56 mmol) and triphenylmethyl chloride gave *N*-(dichloroacetyl)-1-*O*-tritylsphingosine (B) with a yield of 53%; R_f = 0.18 (chloroform−0.25% methanol). Compound B (30 mmol) and benzoyl chloride led to *N*-(dichloroacetyl)-3-*O*-benzoyl-1-*O*-tritylsphingosine (C): yield 75%; R_f = 0.43 (chloroform) (R_f = 0.09 for compound B). Compound C (22

mmol) reacted with 90% acetic acid to give *N*-(dichloroacetyl)-3-*O*-benzoylsphingosine (D): yield 93%; $R_f = 0.48$ (chloroform−methanol, 97:3) ($R_f = 0.84$ for compound C).

Glycosidation of compound D (20 mmol) with heptaacetyl-α-D-lactosyl bromide, prepared as described (*33*), afforded 1-(heptaacetyllactosyl)-*N*-(dichloroacetyl)-3-*O*-benzoylsphingosine (E): yield 84%; $R_f = 0.29$ (chloroform−methanol−water, 80:20:3) ($R_f = 0.80$ for compound D). Alkaline deprotection led to 1-lactosylsphingosine: yield 22%; $R_f = 0.08$ (chloroform−methanol−water, 65:25:4) ($R_f = 0.51$ for compound E).

The one-step preparation from commercial lactosylceramide by the procedure of Goda et al. (*34*) failed; the control of the base-catalyzed hydrolysis depends on the origin of the ceramide and probably upon the nature of the alkyl chains. This dependence can be shown by the use of the dichloroacetyl protecting group.

Synthesis of Spin-Labeled Glycosphingolipids. Glycoceramides were obtained by acylating the corresponding glycosphingosines with 4-doxylpentanoic acid (*30*), according to Belleau and Malek (*35*). Typically, EEDQ (11 mmol) in benzene (100 mL) was added to a solution of the acid (10 mmol) in ethanol (100 mL). After one night at room temperature, the reaction product was deposited on a 0.25 mm silica gel glass plate. Elution with chloroform−methanol−water (80:20:3) gave pure glycoceramide at the expected R_f. The spot was scraped and extracted with methanol. The yield was determined by ESR quantitation. We obtained 28% of SL-GalCer ($R_f = 0.62$), 44% of SL-GlcCer ($R_f = 0.66$), and 23% of SL-LacCer ($R_f = 0.36$). Structures were confirmed by FAB mass spectrometry: SL-GlcCer and SL-GalCer gave a molecular peak (MH^+) at a value of $m/z = 647.4$ and SL-LacCer at $m/z = 809.8$.

Synthesis of Fluorescent Glucosylceramide. The glycosylsphingosine was acylated with 6-NBD-aminohexanoic acid (*36*) in the same manner as described above for spin-labeled compounds. On TLC plates fluorescent glucosylceramide (NBD-GlcCer; Figure 1A) migrates slightly faster than the spin-labeled analogue (SL-GlcCer).

Endoplasmic Reticulum. Rough microsomes were isolated from rat liver as described previously and stored frozen at −80 °C (*25*, *37*). Membranes were resuspended in 1 mM $MgSO_4$, 250 mM sucrose, and 50 mM Tris-HCl buffer, pH 7.3 (buffer A). Protein concentration was determined by the Lowry method (*38*). The purity of rough microsome fractions was found to be similar to those of other preparations as assessed by glucose-6-phosphatase activity (*37*) with inorganic phosphate assayed according to Rouser et al. (*39*). The integrity of microsomal membranes was checked by determining the mannose-6-phosphatase activity in the presence and absence of taurocholate (*40*, *41*). A latency of 90% ($n = 5$) was found, in agreement with previous results (*40*−*42*).

Golgi Membranes. Liver Golgi membranes were prepared from male Wistar rats as previously described and stored frozen at −80 °C (*43*). Membranes were resuspended in buffer A. The purity of the fraction was determined by assaying the protein galactosyltransferase activity (*44*). A total of 55−73% of this activity was recovered in the heavy "cis-enriched" Golgi fraction used in this study, and the specific activity was similar to previous results (*45*). To determine the integrity of the Golgi fraction, we determined the latency of the protein galactosyltransferase using ovomucoid as the substrate; a latency of 83−88% was found. The glucose-6-phosphate assay showed that the Golgi fraction contained approximately 6−9% ER membrane.

A Golgi-enriched fraction was also isolated from the postnuclear supernatant of human liver-derived HepG2 cells at the interface between 0.6 and 1.25 M sucrose (1 mM EDTA and 10 mM Hepes−NaOH buffer, pH 7.2) as previously described (*17*). The Golgi fraction was diluted to 0.25 M sucrose (1 mM EDTA and 10 mM Hepes−NaOH buffer, pH 7.2) and stored frozen at −80 °C.

Vesicle Preparation. Large unilamellar vesicles were prepared by extruding four times a suspension of 2 mM egg PC or cellular organelle lipids in buffer A through 0.2 μm pore size 13 mm diameter polycarbonate filters (*46*). Lipids were extracted from Golgi membranes according to Folch et al. (*47*) or Bligh and Dyer (*48*).

Measurement of Glycosphingolipid Translocation in LUVs. In all cases, translocation was initiated by adding a concentrated suspension of the analogue in buffer A to a final concentration of 1 mol % of total phospholipid to LUVs (1 mM). Translocation of NBD-GlcCer was assayed at 20 °C using the dithionite method (*49*). Fluorescence measurements were made on a Perkin-Elmer LS-50-B fluorescence spectrometer. Spin-labeled sphingolipid translocation was measured using the classical ascorbate assay (*50*) with some modifications: at indicated times, 50 μL of spin-labeled LUVs was mixed with 30 μL of ice-cold BSA in buffer A (5% final concentration) and incubated for 30 s at 4 °C; then ascorbic acid (80 mM final concentration) was added. The extent of reduction was measured by ESR on a Bruker ER-200D-SRC spectrometer.

Measurement of PC, SM, and Glycosphingolipid Analogue Translocation. Translocation experiments were usually performed on thawed membranes that had been stored frozen. However, to explore whether storage at −80 °C and thawing affects lipid flip-flop, control experiments were also performed with freshly prepared membranes. To measure lipid flip-flop with a time resolution of ca. 30 s, the following procedure was used (*30*). Translocation was initiated by adding membranes (final protein concentration 0.8 mg/mL) to a concentrated suspension of spin-labeled lipids (2 mol % fraction of total phospholipid) in buffer. After various incubation times at 20 °C, 50 μL aliquots were taken and mixed by repetitive pipetting for 20 s with 50 μL of BSA, 20% (w/v), at 4 °C in the upper compartment of 0.45 μm nylon membrane microfilters precooled to 4 °C. The final temperature of the membrane−BSA mixture was measured to be 10 °C. The microfilter was then centrifuged in an Eppendorf tube for 10 s at 14000*g*. Control experiments indicated that 95% of the extravesicular medium containing the outer leaflet lipid analogue was recovered in the filtrate while 95% of the membranes containing the inner leaflet analogue was retained on the filter. To recover the latter, the filter was filled with 100 μL of 10% SDS, bath-sonicated at 37 °C for 30 min, and centrifuged. Control experiments indicated that 90% of the filter material was recovered. Both BSA (outer leaflet analogue) and SDS (inner leaflet analogue) fractions were then assayed by ESR using a Bruker ER-200D-SRC spectrometer. The total amount of probe was obtained from two control aliquots: one in which the membranes were incubated for 10 min with BSA before

filtration, allowing for total probe extraction, and another in which the membranes were mixed with buffer instead of BSA, allowing for total probe recovery in the SDS fraction. All data were corrected to take into account filter dead-volume effects.

Membrane Treatment by Trypsin and DIDS. In trypsin experiments, Golgi membranes (0.8 mg of protein/mL) were preincubated with trypsin (protein/protease = 5:1) for 20 min at 20 °C in 50 mM Tris-HCl and 250 mM sucrose, pH 7.3. Proteolysis was stopped by adding a 5-fold excess of soybean trypsin inhibitor. In control experiments, membranes were preincubated with a mixture of trypsin and soybean trypsin inhibitor. In the case of DIDS experiments, Golgi membranes were preincubated with 0.25 mM DIDS at 20 °C for 15 min in 50 mM NaCl, 10 mM EDTA, and 10 mM Tris−maleate, pH 7.3. In control experiments Golgi membranes were preincubated for 15 min in buffer alone. After these treatments, lipid translocation was assayed as previously described.

Cell Culture. Culturing and labeling of HepG2 cells were done as described recently (*51*). Monolayer cultures of HepG2 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal calf serum and antibiotics (10^5 units/L penicillin, 100 mg/L streptomycin). Cells were passaged and plated in 175 cm^2 plastic culture flasks (Nunc, Biochrom KG, Berlin, Germany) and grown in a 5% CO_2 atmosphere at 37 °C to confluence. Before use flasks were precoated with a 0.01% collagen A solution. The medium was changed every 2−3 days and a day before the experiment. Cell suspensions prepared in that cell monolayer were rinsed with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, harvested by treatment with 0.05% trypsin and 0.02% EDTA in PBS for 5 min at 37 °C, resuspended in culture medium, and dispersed by pipetting. After 30 min on ice the cell suspension was centrifuged and washed twice with PBS at 4 °C. Finally, the cell pellet was resuspended in PBS supplemented with 20 mM glucose, 1 mM sodium pyruvate, and 10 mM HEPES (mPBS) to prevent a decline in cellular ATP. Subsequently, cells were immediately used for spin-labeling experiments (see ref *51*).

Spin-Labeling of HepG2 Cells in Suspension and ESR Measurements. Appropriate amounts of spin-labeled phospholipids in chloroform/methanol (1:1 v/v) were transferred to a glass tube, dried under nitrogen, and vortexed with the desired volume of mPBS before addition to the cell suspension. To diminish hydrolysis of phospholipid analogues, 5 mM DFP (final concentration) was added to the cell suspension prior to labeling. For labeling, 2 volumes of cell suspension was mixed with 1 volume of label suspension representing time zero for all kinetic measurements. If not stated otherwise, the total amount of label was 6 nmol per 2 × 10^7 cells, which is about 0.4 mol % of the total cell phospholipid concentration. As shown previously (*51*), after addition to the HepG2 suspension all label became incorporated into the plasma membrane within 30 s at 20 °C.

For back-exchange of the analogues in the exoplasmic layer, 50 μL samples (2 × 10^6 cells) were transferred at given time points to 25 μL of 10% fatty acid-free BSA on ice and centrifuged after 1 min of incubation (30 s at 12000*g*). The amount of probe present in the supernatant was estimated from its ESR spectrum intensity (Bruker ECS 106 spectrometer). This was performed in the presence of the reoxidizing agent ferricyanide (10 mM) in order to exclude any intensity loss by reduction of the NO moiety, e.g., by cellular redox systems. Hydrolysis of the spin-labeled analogues into lyso derivatives became apparent from the appearance of three narrow peaks superimposed on the spectrum of BSA-bound spin-labels. This narrow component corresponds to the cleaved short-chain, spin-labeled fatty acid, which is water soluble. Correct estimation of the amplitude of the broad ESR component as well as of the extent of hydrolysis was obtained by a computer subtraction of the narrow spectrum.

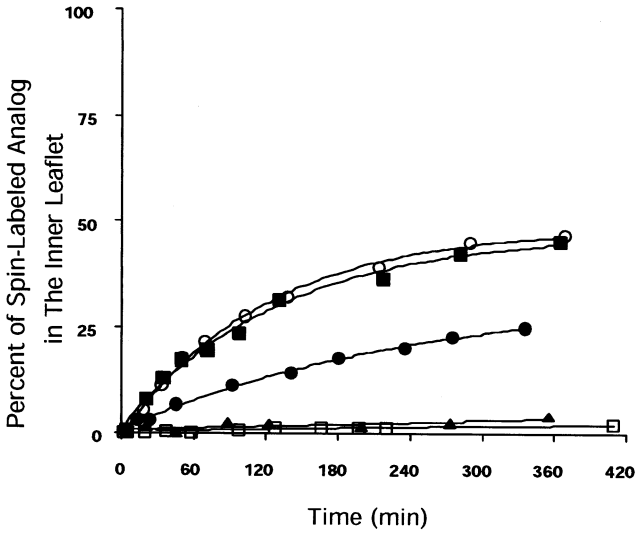

FIGURE 2: Transbilayer movement of lipids in LUVs. Experiments were carried out at 20 °C using 1 mM LUVs (100% egg lecithin) in 1 mM $MgSO_4$, 50 mM Tris-HCl, and 250 mM sucrose, pH 7.3. The flip-flop was assayed with the dithionite and ascorbic acid method for NBD-GlcCer and SL-lipids, respectively (see Experimental Procedures). Key: SL-GlcCer (○), SL-GalCer (■), SL-LacCer (▲), SL-PC (□), and NBD-GlcCer (●). The experiments were performed in triplicate with identical results.

Preparation and Spin-Labeling of Red Blood Cells (RBC). Blood was obtained from the local blood bank. For RBC, the following buffer was used: 20 mM Hepes, pH 7.5, 5 mM Na_2HPO_4, 145 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM inosine, 1 mM $MgSO_4$, and 0.1 mM EGTA. After removal of the buffy coat, erythrocytes were washed twice in buffer at 2000*g*. Spin-labeling of RBC and measurement of analogue translocation were performed as described by Morrot et al. (1989). Prior to labeling, cells were incubated with 5 mM DFP in order to diminish hydrolysis of phospholipid analogues (*52*). No hydrolysis was detected during translocation kinetics.

RESULTS

*Inward Translocation of Lipids in LUV*s. To evaluate the intrinsic ability of glycosphingolipids for spontaneous transbilayer movement, we have measured their flip-flop rates in egg PC LUVs in comparison with that of phosphatidylcholine. In agreement with earlier results (*29*), SL-PC transbilayer movement was found to be very slow; i.e., after an incubation of 4 h at 20 °C none of the SL-PC, initially incorporated in the outer leaflet, was detected in the inner leaflet (Figure 2). A similar absence of flip-flop was obtained for the dihexosylsphingolipid SL-LacCer, confirming prediction on its behavior. Monohexosylsphingolipids, SL-GlcCer

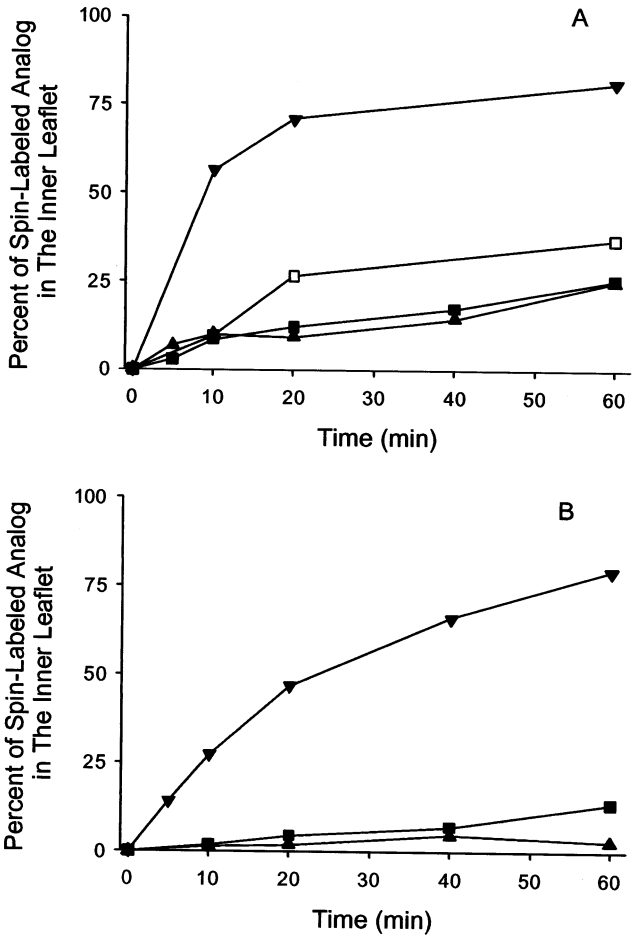

FIGURE 3: Transbilayer movement of lipids in the plasma membrane. Transbilayer movement of spin-labeled lipid analogues in suspended HepG2 cells (A) and RBC (B). The fraction of internalized label at 20 °C was assessed by the back-exchange assay (see Experimental Procedures). At $t = 0$ cells were labeled with SL-PS (▼), SL-PC (□) (only shown for HepG2 cells), SL-GalCer (■), and SL-LacCer (▲). Data represent the average of two independent experiments.

and SL-GalCer, underwent a significant transbilayer diffusion in LUVs with a half-time of 2 h. Control experiments were performed to assess the effect of lipid composition and probe structure on this flip-flop process. In LUVs from extracted Golgi lipids (*47*, *48*) we found a half-time of 2.1 h, i.e., similar to that found in egg PC LUVs. The transbilayer movement of NBD-GlcCer in LUVs was also assayed. The fluorescent glycosphingolipid also underwent transverse diffusion in LUVs albeit with a half-time of 5 h, i.e., significantly slower than its spin-labeled counterpart SL-GlcCer.

Transbilayer Movement of Glycosphingolipids in the Plasma Membrane. As we have already shown previously, aminophospholipids such as phosphatidylserine are rapidly transported to the cytoplasmic leaflet of the plasma membane of RBC (*53*) and the hepatocyte-like cell line HepG2 (*51*) by the ATP-dependent aminophospholipid translocase (Figure 3). In contrast, glycosphingolipids traverse very slowly the plasma membrane of both cell types (Figure 3). The kinetics of their transbilayer movement is comparable to that found for monohexosylsphingolipids in liposomes (see above) and is even slower than that of the respective phosphatidylcholine (PC) analogue (shown for HepG2; Figure 3A). PC (SL-PC) is not recognized by the aminophospholipid translocase and transverses the plasma membrane passively as has been shown for RBC (*53*) and other mammalian cells.

Transbilayer Movement of Glycosphingolipids in the ER and Golgi Apparatus Membranes. In a previous study (*25*) we have developed a phospholipid translocation assay that allows the measurement of rapid transverse diffusion processes in membranes of isolated cellular organelles (see Experimental Procedures for detail). We have used this method to assess the transbilayer movement of spin-labeled glycosphingolipids in ER and Golgi membranes. In the Golgi (Figure 4A) SL-GlcCer initially incorporated in the cytosolic leaflet translocated to the lumenal leaflet rapidly, with a half-time of ≈3 min and an initial rate of translocation 30 times greater than in pure lipid vesicles (Figure 2) or in plasma membranes (Figure 3). After approximately 20 min a steady state was reached with approximately 40% of SL-GlcCer in the lumenal leaflet. Experiments performed with SL-GalCer showed an identical behavior. In contrast, a small amount of SL-LacCer was detected in the lumenal leaflet after 1 h, indicating that SL-LacCer incorporated in the cytosolic leaflet cannot cross the Golgi apparatus membrane. The same results were observed in the "trans-enriched" Golgi subfraction (data not shown). A few experiments ($n = 5$) were carried out with fresh membranes and gave an identical pattern of transbilayer movement of spin-labeled glycosphingolipids (e.g., for SL-GalCer; Figure 5).

Very similar results were obtained with ER membranes (Figure 4B) where a rapid flip-flop of both monohexosylsphingolipids was found with a half-time of ≈4 min and a final distribution corresponding to 40% analogue in the lumenal leaflet. No transbilayer movement of SL-LacCer could be measured in the ER membranes.

In order to compare with intracellular membranes isolated from different cells, we have also investigated purified Golgi membranes from HepG2 cells. The transbilayer movement of SL-glycosphingolipids was comparable to that in rat liver Golgi with a half-time of ≈7 min and a plateau corresponding to the translocation of 30−34% of monohexosyl probes reached in 25 min, while no SL-LacCer was detected in the lumenal leaflet even after 1 h (Figure 4C).

In rat liver Golgi, the transbilayer movement of SL-glycosphingolipid was not influenced by the addition of castanospermin, an inhibitor of α- and β-glucosidases as well as β-glucocerebrosidase (*54*) (see Figure 6), suggesting that production of ceramide, a lipid which flips rapidly, is negligible. Enzymatic breakdown of SL-glycosphingolipids was also directly checked by thin-layer chromatography of extracted lipids and found to be nonsignificant. In some experiments incubation lasted for 3 h, and after lipid extraction and TLC analysis no breakdown product was detected, suggesting that ceramide formation, if any, was less than 5%.

In both the Golgi and ER these results were not influenced by the presence of ATP together with a regenerating system (data not shown). To assess whether the transbilayer movement of SL-glycosphingolipids is vectorial, the outward translocation of SL-GlcCer was investigated. The Golgi membranes were labeled with SL-GLcCer as in the experiment described above. After 40 min, the labeled membranes were mixed with BSA, which rapidly extracted SL-GlcCer

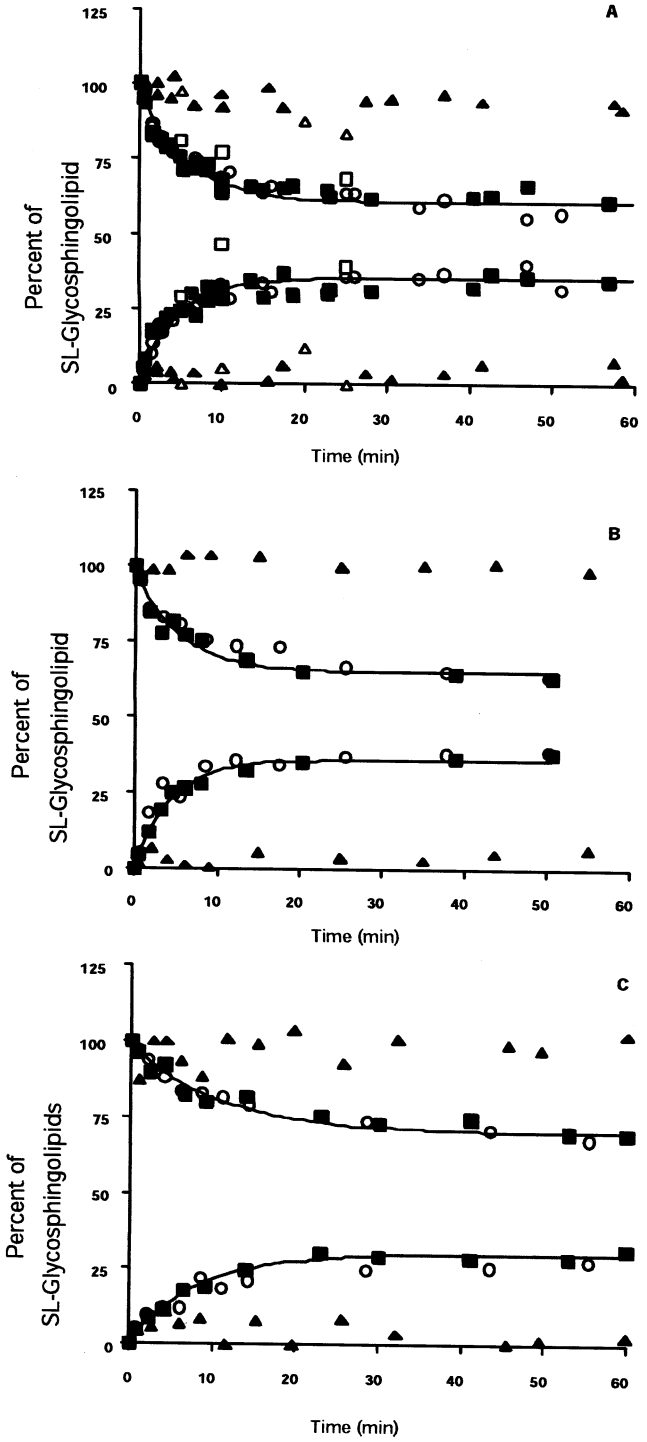

FIGURE 4: Cytosolic−lumenal movement of SL-glycosphingolipids in the ER and Golgi from rat liver and in the Golgi isolated from HepG2 cells. SL-glycosphingolipids (2 mol % of total phospholipids) were initially incorporated into the cytosolic leaflet of the Golgi (A) or ER membrane (B) isolated from rat liver or of the Golgi isolated from HepG2 cells (C). The amount of each analogue present in the lumenal (lower curves) and cytosolic (upper curves) leaflets was followed during 1 h at 20 °C: SL-GlcCer (○), SL-GalCer (■, frozen samples; □, fresh samples), and SL-LacCer (▲, frozen samples; △, fresh samples). Latencies of glucose-6-phosphatase in the ER and of UDP-galactose galactosyltransferase in the Golgi remained identical before and after the incubation and were 89% and 85%, respectively. For (A) and (B), experiments were performed four times; data corresponding to two of them are shown. For (C), experiments were performed twice; all data are shown. Kinetics were fitted to a monoexponential function (solid lines).

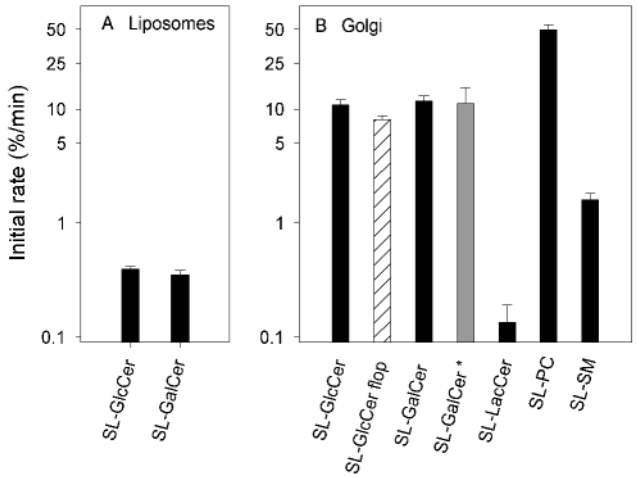

FIGURE 5: Initial rates of translocation of SL-glycosphingolipids in liposomes and Golgi membranes from rat liver. Initial rates of SL-glycosphingolipid inward redistribution in liposomes (A) and cytosolic−lumenal movement in Golgi membranes (B) were taken from monoexponential fits of the kinetics in Figure 2 and Figure 4A (supernatant), respectively, and are shown on a logarithmic plot. The initial rate of SL-LacCer inward redistribution in liposomes (not shown) was very low (0.007%/min) as deduced from a linear fit of data in Figure 2. The gray bar in (B) corresponds to cytosolic−lumenal movement of SL-GalCer in fresh prepared Golgi membranes (*). The initial rate of lumenal−cytosolic movement is similar to that of cytosolic−lumenal movement (shown for SL-GlcCer, coarse bar; see Figure 6). The initial rate of cytosolic−lumenal movement of spin-labeled phospholipid (SL-PC) and phosphosphingolipid (SL-SM) in Golgi membrane was taken from fitting of respective kinetics (not shown). Experiments were carried out as described in the legend of Figure 4; SL-PC or SL-SM (2% mol fraction of total phospholipids) was initially incorporated in the cytosolic leaflet of the Golgi membrane. The membranes were preincubated with 4 mM diisopropyl fluorophosphate in experiments using SL-PC to prevent PC metabolism. Latency of UDP-galactose galactosyltransferase in the Golgi was identical before and after the incubation, 89% and 86%, respectively. Experiments were performed four times with similar results. The final transbilayer distribution was similar to that observed for SL-glycosphingolipids (see Figure 4A). The standard error of fitting is given. The correlation coefficient of fits was not smaller than 0.94.

from the cytosolic leaflet, leaving the membranes labeled only in the lumenal leaflet. If, during further incubation, SL-GlcCer translocates from the lumenal leaflet to the cytosolic leaflet, it will be extracted by BSA. In the Golgi we found a rapid outward translocation of SL-GlcCer with a half-time of ≈2.5 min (Figure 6), indicating that the transbilayer movement of SL-GlcCer occurs in both directions and with the same efficiency as confirmed by the very similar initial rates (Figure 5). Furthermore, an identical outward transverse diffusion of SL-GlcCer was found with membrane that had been incubated for 3 h with the spin-label before the assay (data not shown). This confirms that ceramide production is negligible and has no effect on the flip-flop data.

To determine whether the transbilayer movement of SL-GlcCer in the Golgi is a saturable process, the effect of substrate concentration on the initial rate of translocation was investigated (Figure 7). Experiments were carried out for mole fractions ranging from 0 to 0.1. Higher values were not used since a loss of vesicle integrity was observed, as indicated by a reduced latency of UDP-galactose galactosyltransferase for mole fractions above 0.1. Translocation of SL-GlcCer appears to undergo a progressive saturation

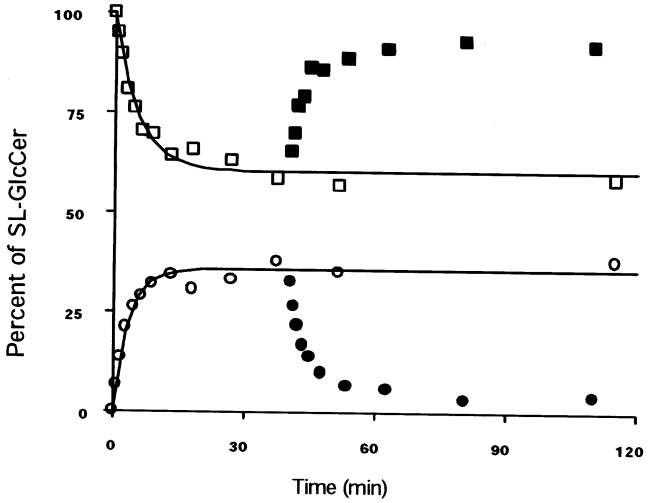

FIGURE 6: Lumenal−cytosolic translocation of SL-GlcCer in the Golgi membranes from rat liver. Golgi membranes were preincubated in the presence of 0.26 mM castanospermin (20 min at 20 °C), labeled with SL-GlcCer (2 mol % of total phospholipids), and assayed for SL-GlcCer in the cytosolic (□) and lumenal (○) leaflet at 20 °C (as in Figure 2). After 40 min, the labeled membranes were mixed with 20% (w/v) BSA and further incubated at 20 °C. At indicated times, aliquots were assayed for SL-GlcCer transmembrane distribution: cytosolic (■); lumenal (●) leaflet. Experiments were performed four times; data points corresponding to two of them are shown.

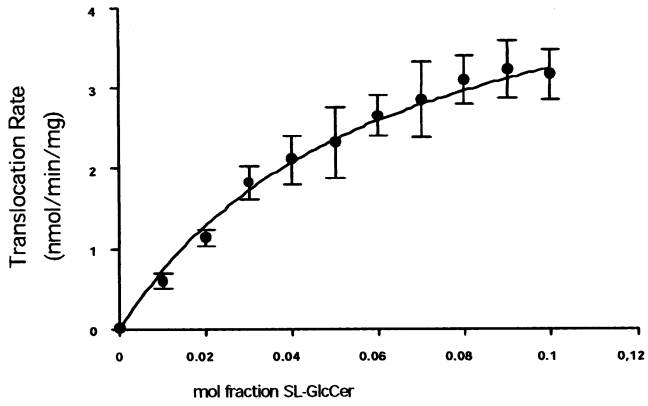

FIGURE 7: Concentration dependence of SL-GlcCer translocation in the Golgi membranes from rat liver. Golgi membranes were incubated at 20 °C in the presence of increasing amounts of SL-GlcCer (●). Initial rates were calculated from the amount of SL-GlcCer translocated in 1.5 min (average of BSA and SDS extraction values; each data point is presented as the mean ± standard deviation; the curve was fitted using the Michaelis equation). The latency of UDP-galactose galactosyltransferase was measured to be 84 ± 2% after labeling with 0.1 mol fraction of SL-GlcCer and 85% without labeling. It was checked by ESR that, in each condition, all of the probe was incorporated in the membrane.

with an apparent K_m corresponding to a mole fraction of 0.06 and a calculated apparent maximum velocity of (5.2 ± 0.1) nmol min^{-1} (mg of protein)$^{-1}$ for SL-GlcCer.

SM, like monohexosylsphingolipids, is synthesized from ceramide in the Golgi apparatus. Usually, a very slow spontaneous diffusion of SL-SM through membranes has been observed (*29*), except for the ER where translocation is fast (*24*−*26*). We have measured the cytosolic−lumenal movement of SL-SM in Golgi membranes (not shown). Although translocation of SL-SM occurs in the Golgi apparatus, nevertheless as indicated by the initial rates (Figure 5) it is about 6.5 times slower than that found for SL-GlcCer

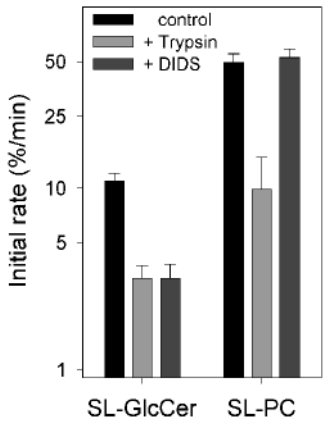

FIGURE 8: Effects of protease and DIDS treatment on the cytosolic−lumenal movement of SL-GlcCer and SL-PC in the Golgi membranes from rat liver. Transbilayer movement was assayed as in Figure 4. The initial rates were obtained from fitting of the kinetics of cytosolic−lumenal movement (supernatant, not shown) to a monoexponential function (see Figure 5) for SL-GlcCer and SL-PC with trypsin digestion (light gray bar) or DIDS treatment (gray bar). Black bars refer to the control (no digestion/treatment). The standard error of fitting is given. The correlation coefficient of the fits was not smaller than 0.94. The latency of UDP-galactose galactosyltransferase was assayed after treatment with trypsin, 87 ± 3%, or trypsin−soybean trypsin inhibitor, 85 ± 2%, and before and after preincubation with DIDS: 84 ± 4% and 85 ± 4%, respectively. The membranes were preincubated with 4 mM diisopropyl fluorophosphate in experiments using SL-PC to prevent PC metabolism. Experiments were performed three times.

or SL-GalCer. The final distribution of SL-SM was almost similar to that of SL-GlcCer or SL-GalCer.

Effects of Chemical and Enzymatic Treatments on the Transbilayer Movement of SL-GlcCer and SL-PC in the Golgi. The occurrence of a very rapid and saturable flip-flop of monohexosylsphingolipids in the Golgi and ER suggests the involvement of a specific protein in the transport process. To further examine the possibility of a protein-mediated glycosphingolipid translocation, we have investigated the effect of protein-modifying reagents. SL-PC flip-flop, which is protein mediated in the ER (*23*−*25*), was studied in parallel for comparison. The transbilayer movement of SL-GlcCer in Golgi membranes was found to be affected by trypsin pretreatment (Figure 8); 30% of translocation activity remained after trypsin incubation (based on initial rates of translocation). Trypsin also affected the transbilayer movement of SL-PC (Figure 8) but to a larger extent; i.e., about 20% of the translocation activity remained in the membrane after proteolysis. The final distribution of SL-GlcCer and SL-PC, respectively, was not affected by protease treatment. The trypsin digestion under the present experimental conditions did not modify the membrane integrity as judged from the latency of UDP-galactose galactosyltransferase activity which remained unchanged (see legend of Figure 8). More extensive trypsin treatments led to a decreased latency and loss of membrane integrity.

The effect of DIDS, a nonpenetrating membrane probe which reacts with amino and sulfhydryl groups, was also investigated. Golgi membranes pretreated with this reagent displayed a strongly reduced transbilayer movement of SL-GlcCer (Figure 8); i.e., only about 30% of the initial translocation activity remained. In contrast, DIDS treatment had no effect on the transbilayer movement of SL-PC in the Golgi (Figure 8). To assess for a possible effect of such

protein treatment on the lipid packing and dynamics, ESR spectra of SL-PC incorporated in intact and trypsin or DIDS-modified Golgi membranes were compared (not shown). No significant differences were found; i.e., empirical spin-label correlation times (*55*) were 1.92×10^{-9} s for intact membranes, 2.05×10^{-9} s for trypsin-treated membranes, and 1.87×10^{-9} s for DIDS-treated membranes.

Preincubation at 20 °C of Golgi (0.8 mg/mL) with *N*-ethylmaleimide (20 mM) was tested. The result of four experiments showed only a small decrease in the rate of the transbilayer movement for SL-GlcCer in the Golgi, corresponding to an inhibition of 5−10% (not shown).

DISCUSSION

The goal of this study was to investigate the transbilayer movement of glycosphingolipids in ER and Golgi membranes as well as plasma membranes. For this purpose, we have synthesized spin-labeled analogues of such lipids. It may be asked whether the spin-labels used are faithful reporters of natural glycosphingolipid movements. During the last two decades many studies on the transverse diffusion of phospholipids in liposomes or in biological membranes were carried out, in particular in this laboratory, using spin-labeled glycerophospholipids carrying a doxylpentanoyl chain. It was shown that the passive flip-flop of such doxylpentanoylphospholipids in liposomes is nondetectable for several hours as for natural phospholipids (*56*). Tilley et al. (*57*) used radioactive long-chain phospholipids to measure flip-flop rates in human erythrocytes. The technique they used for monitoring the probe orientation did not allow analysis of rapid kinetics; nevertheless, they found an overall behavior of long-chain glycerophospholipids similar to what has been observed in the same cells with doxylpentanoyl derivatives (*54*). More recently, we have found that the doxyl group had no effect upon protein-mediated phospholipid translocation in ER (*25*). On the other hand, a qualitative effect of the labeled chain on SL-glycosphingolipid flip-flop cannot be excluded since in other cases an effect of acyl chain composition on transverse diffusion has been shown (*58*).

Most previous studies devoted to the biosynthesis and trafficking of glycosphingolipids made use of fluorescent NBD-labeled analogues. While these analogues have many advantages for studies on intact cells where the probes can be visualized in various organelles, they are less suitable for quantitative investigations carried out on isolated membranes (for a comprehensive review see ref *59*). Indeed, in agreement with Thi Dao et al. (*60*), we have found that monohexosylsphingolipid (hexanoyl) NBD analogues are more difficult to extract with BSA (97% extracted in 10 min at 4 °C) than the spin-labeled (doxylpentanoyl)glycosphingolipids (98% extracted in 25 s at 4 °C), a feature which makes the former unsuitable to study fast translocation phenomena. Furthermore, the present study indicates that the passive transbilayer movement of NBD-GlcCer in LUVs is significantly slower than that of SL-GlcCer. Similarly in the case of glycerophospholipids, spin-labeled analogues are more appropriate for studies of passive and protein-mediated transverse diffusion than NBD analogues (*36*, *61*). The relatively slow transbilayer movement of NBD analogues probably originates from the polar character of the NBD group as pointed out already by Chattopadhyay and London (*62*).

In the present investigation, we have found that all spin-labeled monohexosylsphingolipid analogues undergo a slow but detectable transverse diffusion in egg PC liposomes with half-times in the order of a few hours. In contrast, the dihexosylsphingolipid LacCer has a barely detectable transverse diffusion in LUVs. Previous experiments using spin-labeled gangliosides also demonstrated that higher glycosphingolipids were unable to diffuse through the membrane of liposomes (*63*). The ability of monohexosylsphingolipids to flip-flop contrasts with the much lower capacity of phospholipids to undergo spontaneous transbilayer movement. Even the zwitterionic PC and SM are no exception. This indicates that the ability of a polar lipid to undergo passive flip-flop depends on the presence or absence of charged substituents on its polar headgroup and not only on the net charge of the polar headgroup. The size of the polar headgroup also appears to be important. Indeed, lipids with moderately sized polar headgroups bearing no charge, such as monohexosylsphingolipids, appear to be able to flip-flop relatively rapidly. Accordingly, the protonated forms of both phosphatidylglycerol (*64*) and phosphatidic acid (*65*) undergo a rapid transbilayer passive diffusion in liposomes.

The experiments performed on Golgi and ER membranes isolated from rat liver show that transbilayer movement of both monohexosylsphingolipids is much more rapid (30 times) than in liposomes. In contrast, the transbilayer movement of LacCer remains very slow. The fast transbilayer diffusion of SL-GlcCer in the Golgi apparatus is not due to the lipid composition of Golgi membrane since the transbilayer diffusion of SL-GlcCer in LUVs made from Golgi-extracted lipids is similar to that found in LUVs made from egg PC. We have performed several control experiments to make sure that this rapid flip-flop of GlcCer and GalCer is not due to degradation of the glycosphingolipids, e.g., to ceramide which undergoes a very rapid transbilayer diffusion.

The transbilayer movement of GlcCer appears to display a saturable behavior. However, it cannot be ruled out that the saturation regime shown in Figure 7 actually results from nonlytic modifications of membrane structure that produce indirect changes in transport activity, although, a priori, a prelytic regime would accelerate lipid flip-flop rather than inhibit it. Note that the low percentage of naturally occurring glycolipids rules out any important competition between spin-labeled and endogenous glycolipids. Finally, a strong argument in favor of the role of a protein in the transbilayer movement of GlcCer is the fact that it is sensitive to proteases and protein modification reagents. Such treatments, which are known to inhibit enzymatic activity, did not change membrane integrity as indicated by UDP-galactose galactosyltransferase latency.

Overall, our data suggest that the transbilayer movement of monohexosylsphingolipids is likely to involve one or several proteins of the Golgi or of the ER membranes. The most likely explanation is that there exists in both membranes a flippase that is specific for monohexosylsphingolipid transbilayer transport. The fact that GlcCer transverse diffusion occurs identically in both directions, leads to a symmetric lipid distribution, and is independent of ATP would indicate that this flippase is a facilitated nonvectorial transporter. Also, it appears that it is not coupled to any biosynthetic pathway since it does not involve the conversion of monohexosylceramides to higher order products.

Bishop and Bell (*23*) as well as ourselves (*24*−*26*) have previously characterized a protein activity that translocates glycerophospholipids in the ER. This protein is able to transport glycerophospholipids and sphingomyelin rapidly and nonvectorially; we now find that this protein or a similar one also exists in the Golgi membranes. Both this glycerophospholipid transporter and the monohexosylsphingolipid transporter suggested by the present study are sensitive to trypsin. However, only the putative monohexosylsphingolipid transporter is sensitive to DIDS. This suggests that the two transport activities correspond to distinct proteins. An alternative hypothesis is that a single protein is involved and that DIDS affects its interaction with glycosphingolipids and not with phospholipids.

The question of the specificity of the protein(s) responsible for fast lipid translocation will ultimately be resolved if a flippase or a family of flippases is purified and reconstituted. For the moment, we have unambiguously demonstrated only protein-mediated lipid translocation in the ER and Golgi membranes. Recently, Menon and co-workers (*27*) found by fractionating of detergent-solubilized rat liver ER and subsequent reconstitution of protein fractions phospholipid translocation-active proteoliposomes. Flippase activity was associated with a specific membrane protein fraction. Remarkably, this study raised the possibility that at least two different proteins are able to mediate fast lipid movement. Recently, evidence was provided that yeast *RFT1* which encodes an essential protein with multiple transmembrane domains that is localized in the ER is required for the translocation of lipid-linked oligosaccharides across the ER membrane (*66*). Homologues of *RFT1* are found essentially in all eukaryotic genomes.

Can one explain such rapid translocation by a general property of the lipid phase of these membranes that would be affected by trypsin and/or DIDS treatment of, for example, ion channels? It has been suggested some years ago that the ER membrane contains localized nonbilayer structures (*67*). However, the NMR line shape that gave rise to such hypothesis can be explained by the presence of smaller vesicles (*68*). Furthermore, for these localized nonbilayer structures to exist, the presence of a protein component able to destabilize the bilayer organization is required (*69*), indicating again a direct involvement of a protein. Finally, in a previous publication (*25*), we have shown that treatments of the ER membranes which eliminate this particular ^{31}P NMR component do not modify the rapid translocation of glycerophospholipids in ER membranes. Other explanations such as the putative role of a transmembrane potential do not seem to apply for these noncharged lipids.

Recent reports on the glycosphingolipid biosynthesis showed that GlcCer is synthesized at the cytosolic leaflet of the cis Golgi apparatus (*16*, *17*) and that LacCer synthesis occurs at the lumenal leaflet. The results presented here show that GlcCer, after its synthesis, is able to reach the lumenal leaflet where it can be converted into LacCer. Our data indicate that LacCer is most likely unable to cross the membrane and therefore remains in the lumenal leaflet where it may undergo subsequent modifications. In the same fashion, GalCer is able to cross the membrane and be converted to Ga_2Cer or sulfatide (SGalCer) in the lumenal leaflet of the Golgi (*19*). Both monohexosylsphingolipids are able to cross the membrane in both directions with the same efficiency, so this process must lead to a symmetric transmembrane distribution. At steady state, approximately 40% of the analogue was protected against BSA extraction. As the latency of UDP-galactose galactosyltransferase is 85% and as, due to vesicle size, there exists a surface area difference between the two leaflets of Golgi or ER membranes, it can be concluded that an almost symmetric distribution of monohexosylsphingolipids exists over the two leaflets. In contrast, all higher glycosphingolipids synthesized at the lumenal leaflet are expected to be unable to translocate. Discrepancies exist concerning the topology of LacCer biosynthesis: a study by Trinchera et al. (*70*) localized this activity at the cytosolic leaflet of the cis Golgi apparatus while more recent reports (*19*, *21*) conclude it takes place at the lumenal leaflet. Our results are consistent with the latter reports, as LacCer appears to be unable to translocate in Golgi membranes. If LacCer would be synthesized at the cytosolic leaflet, it would not be able to reach the lumenal leaflet where all enzymes involved in complex glycosphingolipid biosynthesis are located. This raises the interesting possibility that a monohexosylsphingolipid flippase may play a central role in the regulation of higher glycosphingolipid biosynthesis. For example, inhibition of this putative flippase activity would slow the equilibration of monohexosylsphingolipids between both leaflets and thus lead to a decrease of the synthesis of higher order glycosphingolipids. Nevertheless, recently it has been shown that the MDR1 P-glycoprotein (MDR1 Pgp, ABCB1) translocates both analogues of GlcCer (*71*) and natural GlcCer (see ref *12*) from the cytoplasmic to the exoplasmic leaflet of the plasma membrane of mammalian cells. It has been suggested (*12*) that MDR1 Pgp might also translocate GlcCer in the Golgi in parallel with the energy-independent flippase. Importantly, the MDR1 Pgp transport in the plasma membrane could only be detected by overexpression of the protein. Therefore, we can preclude that the slow decrease of the amount of spin-labeled glycosphingolipids in the exoplasmic leaflet of HepG2 and red blood cells is due to a fast inward movement and an even more rapid outward transport, the latter mediated by MDR1 Pgp. In those cells, MDR1 Pgp is not overexpressed, if it is present at all. The slow transbilayer movement of glycosphingolipid analogues in the plasma membrane is consistent with recent results of Pagano and co-workers. They have shown for human skin fibroblasts that fluorescent analogues of LacCer and globoside were internalized almost exclusively by clathrin-independent endocytosis (*72*).

Interestingly, we detected a similar rapid movement of monohexosylsphingolipids in the ER (Figure 4) possibly catalyzed by a similar flippase. In a recent report Burger and collaborators (*19*) studied the topology of galactosyltransferases and found that 2-hydroxy fatty acyl GalCer (HFA-GalCer) is synthesized in the lumenal leaflet of the ER. After its synthesis, HFA-GalCer appears to have rapid access to the cytosolic leaflet of the ER. Though there is a small difference in structure between HFA- and NFA-GalCer, it is possible that HFA-GalCer is also recognized by the monohexosylsphingolipid flippase described in the current study and translocated from the lumenal to the cytosolic leaflet of the ER (see also ref *20*). Indeed, Burger et al. (*19*) found that both NFA- and HFA-GalCer were translocated from the cytosolic to the lumenal leaflet of the Golgi apparatus.

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