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Reconstitution of Glucosylceramide Flip-Flop across Endoplasmic Reticulum

*IMPLICATIONS FOR MECHANISM OF GLYCOSPHINGOLIPID BIOSYNTHESIS**

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Background: Lipid flip-flop, a key feature of many glycolipid biosynthetic pathways, requires as yet unidentified flippase proteins.
Results: Glucosylceramide flips slowly across protein-free vesicles but rapidly across vesicles reconstituted with ER membrane proteins.
Conclusion: Glucosylceramide flipping is facilitated by ATP-independent ER phospholipid flippases.
Significance: Defining how glucosylceramide is transported across membranes is critical for understanding the glycosphingolipid biosynthetic pathway.

Most glycosphingolipids are synthesized by the sequential addition of monosaccharides to glucosylceramide (GlcCer) in the lumen of the Golgi apparatus. Because GlcCer is synthesized on the cytoplasmic face of Golgi membranes, it must be flipped to the non-cytoplasmic face by a lipid flippase in order to nucleate glycosphingolipid synthesis. Halter *et al.* (Halter, D., Neumann, S., van Dijk, S. M., Wolthoorn, J., de Mazière, A. M., Vieira, O. V., Mattjus, P., Klumperman, J., van Meer, G., and Sprong, H. (2007) Pre- and post-Golgi translocation of glucosylceramide in glycosphingolipid synthesis. *J. Cell Biol.* 179, 101–115) proposed that this essential flipping step is accomplished via a complex trafficking itinerary; GlcCer is moved from the cytoplasmic face of the Golgi to the endoplasmic reticulum (ER) by FAPP2, a cytoplasmic lipid transfer protein, flipped across the ER membrane, then delivered to the lumen of the Golgi complex by vesicular transport. We now report biochemical reconstitution studies to analyze GlcCer flipping at the ER. Using proteoliposomes reconstituted from Triton X-100-solubilized rat liver ER membrane proteins, we demonstrate rapid ($t_{1/2}$ < 20 s), ATP-independent flip-flop of *N*-(6-((7-nitro-2–1,3-benzoxadiazol-4-yl)amino)hexanoyl)-D-glucosyl-β1–1′-sphingosine, a fluorescent GlcCer analog. Further studies involving protein modification, biochemical fractionation, and analyses of flip-flop in proteoliposomes reconstituted with ER membrane proteins from yeast indicate that GlcCer translocation is facilitated by well characterized ER phospholipid flippases that remain to be identified at the molecular level. By reason of their abundance and membrane bending activity, we considered that the ER reticulons and the related Yop1 protein could function as phospholipid-GlcCer flippases. Direct tests showed that these proteins have no flippase activity.

Glycosphingolipids (GSLs)[2] are eukaryotic lipids composed of a hydrophobic ceramide moiety crowned with a sugar headgroup (1–3). Located principally in the outer leaflet of the plasma membrane, they have diverse functions in cellular and organismal biology. For example, they are involved in embryogenesis (4), endocytosis of microbial toxins, such as cholera toxin (5), and nanoscale organization of the plasma membrane (6). Defects in GSL metabolism and turnover cause a number of lysosomal storage diseases (7). A recent review by Lingwood (1) provides a comprehensive survey of GSL functions.

The simplest GSLs are the monohexosylsphingolipids glucosylceramide (GlcCer) and galactosylceramide (GalCer). Larger GSLs are typically derivatives of GlcCer that are formed by adding monosaccharides to the glucosyl headgroup (3). Whereas GlcCer is synthesized on the cytoplasmic face of the Golgi complex from ceramide and UDP-glucose (8, 9), higher order GSLs are synthesized in the Golgi lumen (10). This requires transport of cytoplasmically synthesized sugar nucleotides into the Golgi lumen as well as transbilayer movement of GlcCer. The import of nucleotide sugars into the lumen of the Golgi complex is mediated by specific transporters that have been identified (11). However, the mechanism by which GlcCer moves from the cytoplasmic to the lumenal face of the Golgi is unknown. Because the spontaneous movement of GlcCer across synthetic membranes is slow (12) a transport mechanism must exist to increase it to a physiologically appropriate rate. Using a spin-labeled derivative, Buton *et al.* (12) demonstrated protein-mediated translocation of GlcCer across rat liver ER and Golgi membranes, consistent with the existence of a GlcCer transporter or flippase.

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[2] The abbreviations used are: GSL, glycosphingolipid; ER, endoplasmic reticulum; GlcCer, glucosylceramide; GalCer, galactosylceramide; NBD-GlcCer, *N*-(6-((7-nitro-2–1,3-benzoxadiazol-4-yl)amino)hexanoyl)-D-glucosyl-β1–1′-sphingosine; NBD-PC, 1-myristoyl-2-(6-((7-nitro-2–1,3-benzoxadiazol-4-yl)amino)hexanoyl)-*sn*-glycero-3-phosphocholine; NBD-PE, 1-myristoyl-2-(6-((7-nitro-2–1,3-benzoxadiazol-4-yl)amino)hexanoyl)-*sn*-glycero-3-phosphoethanolamine; NEM, *N*-ethylmaleimide; PPR, protein/phospholipid ratio; TE, Triton extract; ER, endoplasmic reticulum; RER, rough ER; DEPC, diethylpyrocarbonate.

Halter *et al.* (13) recently proposed that newly synthesized GlcCer suffers two fates (Fig. 1*A*). A small fraction moves to the cytoplasmic face of the plasma membrane by a non-vesicular mechanism, whereupon it is translocated to the cell surface. This pool is unlikely to contribute significantly to GSL synthesis. The majority of GlcCer is transported to the ER by FAPP2 (phosphatidylinositol 4-phosphate adaptor protein) (Fig. 1*A*, *step 1*), a cytoplasmic protein with sequence homology to glycolipid transfer protein. At the ER, GlcCer is flipped across the membrane (Fig. 1*A*, *step 2*), ultimately reaching the lumen of the Golgi complex by vesicular transport (Fig. 1*A*, *step 3*), where it is converted to lactosylceramide and higher order GSLs (Fig. 1*A*, *step 4*). A key feature of this model is that GlcCer flipping for GSL synthesis occurs at the ER rather than at the Golgi complex. The low specificity of the ER phospholipid flippases suggests that these as yet unidentified transporters (14) may also be responsible for flipping GlcCer (13, 15). We now report biochemical reconstitution studies to test this proposal.

EXPERIMENTAL PROCEDURES

Materials—Egg phosphatidylcholine, egg phosphatidylglycerol, *N*-(6-((7-nitro-2–1,3-benzoxadiazol-4-yl)amino)hexanoyl)-D-glucosyl-β1–1′-sphingosine (NBD-GlcCer), 1-myristoyl-2-(6-((7-nitro-2–1,3-benzoxadiazol-4-yl)amino)hexanoyl)-*sn*-glycero-3-phosphoethanolamine (NBD-PE), and 1-myristoyl-2-(6-((7-nitro-2–1,3-benzoxadiazol-4-yl)amino)-hexanoyl)-*sn*-glycero-3-phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids. SM2 Bio-Beads were from Bio-Rad; 4,4′-diisothiocyanato-2,2′-stilbene disulfonate was from Invitrogen; Triton X-100 (Ultrol grade) was from Calbiochem; and diethylpyrocarbonate (DEPC), EZview Red anti-HA resin, HA peptide, anti-HA antibodies for immunoblotting, hydroxyapatite resin (HA-Ultrogel), and *N*-ethylmaleimide (NEM) were from Sigma.

Buffers—Buffer A contained 10 mM HEPES-NaOH, pH 7.4, 100 mM NaCl, 1% (w/v) Triton X-100, and Buffer B contained 10 mM HEPES-NaOH, pH 7.4, 100 mM NaCl.

Reconstitution of Rat Liver ER Membrane Proteins into Proteoliposomes—Rat liver ER membranes and Triton X-100-solubilized rat liver ER membrane proteins (Triton extract (TE)) were prepared as described previously (16). TE (typically ~1.5 mg/ml protein determined by the micro-BCA method) was either used directly or snap-frozen and stored at −80 °C. Proteoliposomes were reconstituted from a mixture of TE and Triton X-100-solubilized lipids as described (14, 16, 17). Briefly, chloroform stocks of egg phosphatidylcholine (3.7 μmol), egg phosphatidylglycerol (0.4 μmol), and NBD-lipid (0.012 μmol) were combined in a glass screw-cap tube, dried under nitrogen, and solubilized in Buffer A. The solution was then mixed with an indicated amount of TE in a final volume of 1 ml. Unilamellar vesicles were generated by staged treatment of the solution with a total of 300 mg of washed SM2 Bio-Beads. For all assays, protein-free liposomes were reconstituted in parallel. The protein/phospholipid ratio (PPR; mg of protein/mmol of phospholipid) of the vesicles was determined as described (16). There was no evidence of hydrolysis of the NBD-lipids during the reconstitution procedure as determined by thin layer chromatography of organic solvent extracts of liposomes or proteoliposomes (Fig. 3*B*). Chromatography was done using silica 60 plates and chloroform/methanol/water (65:25:4 by volume) as the solvent system. Fluorescence was visualized with a Typhoon scanner.

Vesicle Size Analysis—Reconstituted vesicles were analyzed by dynamic light scattering in a DynaPro DLS (Protein Solutions Inc.) instrument at 25 °C for 1 min at a 90° scattering angle. The vesicles were diluted ~10× to a final phospholipid concentration of 0.4 mM in Buffer B before dynamic light scattering measurements. The majority of the vesicles (both liposomes and proteoliposomes) had a diameter of 154.7 ± 5.6 nm. There was some evidence of heterogeneity in the vesicle population, reflected in minority populations with an average diameter of ~700 nm and ≥1 μm. These large apparent diameters suggest vesicle aggregates rather than individual large vesicles.

Flippase Assay—Proteoliposomes containing NBD-lipids were assayed for flippase activity as described previously (17–19). Briefly, 50 μl of proteoliposomes (~4 mM phospholipid) was diluted to 2 ml in Buffer B in a cuvette. The sample was stirred continuously at 23 °C, and its fluorescence intensity (excitation 470 nm, emission 530 nm) was recorded in a PTI spectrofluorimeter before and after the addition of sodium dithionite (added to a final concentration of 5 mM from a 1 M stock prepared in unbuffered Tris). Protein-free liposomes containing the appropriate NBD-lipid were analyzed in parallel. The assay is described in more detail under "Results."

Spontaneous Flipping of NBD-lipids—The spontaneous flipping of NBD-PE and NBD-GlcCer was determined at 23 °C using asymmetric liposomes in which the NBD-lipid was restricted to the inner leaflet. Asymmetric liposomes were prepared as follows. NBD-lipid-containing symmetric liposomes were prepared from Triton X-100-solubilized lipids as described above and then incubated with 10 mM dithionite for 15 min at 23 °C to reduce all of the NBD-lipid in the outer leaflet. The treated vesicles containing fluorescent NBD-lipid only in the inner leaflet were passed over a Biogel P6 column (1 ml of bed volume per 200-μl liposome sample) to remove excess dithionite and then transferred to a water bath at 23 °C and incubated for up to 30 h. Aliquots were removed at regular intervals and treated with dithionite to determine the extent to which NBD-lipids had moved from the inner to the outer leaflet.

Protein Modification—TE was treated with NEM and DEPC as described previously (18). Stock solutions of NEM (200 mM) and DEPC (200 mM) were prepared in Buffer B and added to TE at a final concentration of 40 mM. In some experiments, a combination of NEM and DEPC was used. The pH of TE was unaffected by adding the reagents. Samples were incubated at 23 °C for 45 min before being reconstituted into proteoliposomes. Mock-treated liposomes and proteoliposomes were analyzed in parallel.

Glycerol Gradient Centrifugation—TE was fractionated by velocity gradient sedimentation on a linear glycerol gradient as described previously (14, 19). Briefly, 300 μl of TE was loaded onto a 3.7-ml linear gradient of 10–35% (w/v) glycerol (prepared in Buffer A). A parallel gradient was loaded with a mixture of sedimentation standards (150 μg each of ovalbumin (3.6 S), bovine serum albumin (4.2 S), β-amylase (8.9 S), and catalase

(11 S)). The gradients were centrifuged at 4 °C for 18.5 h at 45,000 rpm (162,000 × g_{av}) in a swing out rotor (MLS 50, Beckman-Coulter). 14 fractions (300 μl each) were collected from the top and pooled pairwise after discarding the first and the last fractions. The gradient was characterized by determining the refractive index of individual fractions and migration of sedimentation standards. Fractions were subjected to buffer exchange on a Biogel P6 column (equilibrated in Buffer A) to remove glycerol. Pairwise pooled fractions were reconstituted into proteoliposomes and taken for NBD-PE and NBD-GlcCer flippase activity measurement.

Hydroxyapatite Chromatography—400 μl of TE (adjusted to 10 mM HEPES/NaOH, pH 7.4, 50 mM NaCl, 0.5% (w/v) Triton X-100) was incubated with ~200 μl of hydroxyapatite resin (pre-equilibrated with 10 volumes of 10 mM HEPES/NaOH, pH 7.4, 1 mM NaCl, 0.5% (w/v) Triton X-100) in a microcentrifuge tube. After 1.5 h at 4 °C with end-over-end mixing, the sample was centrifuged to pellet the resin and recover the supernatant (flow-through fraction). Resin-bound proteins were eluted first with 4 × 200 μl of 10 mM HEPES/NaOH, pH 7.4, 1 M NaCl, 0.5% (w/v) Triton X-100 and subsequently with 4 × 200 μl of 10 mM HEPES/NaOH, pH 7.4, 0.3 M NaH_2PO_4, 0.5% (w/v) Triton X-100. Each elution step was done for 20 min at 4 °C with end-over-end mixing. All fractions were adjusted to Buffer A by buffer exchange on a Biogel P6 column. Fractions were reconstituted into proteoliposomes at a PPR of 1 mg/mmol for the flippase activity assay.

Yeast TE—The yeast strains used in this study were BY4741 (*MATa his3Δ1 leu2Δ met15Δ ura3Δ*) and NDY257 (*BY4741 rtn1::kanMX4 rtn2::kanMX4 yop1::kanMX*). Yop1-HA, a C-terminally tagged version of Yop1 was expressed in both strains at endogenous levels by transforming the cells with pYop1-HA (a plasmid based on pYC2/CT (Invitrogen), a CEN-URA plasmid with the original *GAL1* promoter removed) (20). Yeast TE was prepared as follows. Yeast cultures were grown to an A_{600} of ~2. The cells were collected by centrifugation, washed, and resuspended in 3 volumes of disruption buffer (20 mM Hepes-NaOH, pH 7.5, 10 mM $MgCl_2$, 5% (w/v) glycerol, 1 mM dithiothreitol, protease inhibitor mixture) per volume of packed cells (1 g wet weight of cells is ~1 ml of packed cell volume). Prechilled glass beads (4 volumes) were added, and the sample was vortexed for 30 s at high speed. After a 30-s cooling period on ice, the sample was subjected to 11 further cycles of vortexing and cooling before being centrifuged to remove unbroken cells and glass beads. The resulting supernatant was centrifuged at 70,000 rpm (~200,000 × g_{av}) in a Beckman-Coulter TLA 100.3 rotor for 30 min at 4 °C to collect microsomes. The microsomes were resuspended in 0.5 volume of resuspension buffer (20 mM Hepes-NaOH, pH 7.5, 100 mM NaCl) and incubated with an equal volume of ice-cold extraction buffer (20 mM Hepes-NaOH, pH 7.5, 100 mM NaCl, 2% (w/v) Triton X-100, 0.5 mM PMSF, protease inhibitor mixture) for 30 min on ice with occasional gentle mixing. The sample was centrifuged at 200,000 × g_{av} as before, and the supernatant (TE) was removed, flash-frozen in liquid nitrogen, and stored at −80 °C. Approximately 1 ml of TE (~1 mg/ml) was obtained from 400 OD units of cell culture. We previously showed that ice-old Triton X-100 selectively solubilizes ER membrane proteins from crude yeast microsomes (21).

Yop1-HA was affinity-purified from TE (prepared from the NDY257+pYop1-HA strain) using anti-HA affinity resin. Briefly, 50–100 μl of the resin was equilibrated with Buffer A. TE (500 μl; 1 mg/ml) was incubated with the washed resin for 1.5 h at 4 °C with end-over-end mixing. The sample was centrifuged to pellet the resin, and the supernatant (flow-through fraction) was collected. The resin was washed before eluting Yop1-HA with HA peptide in three steps in a total volume of 500 μl. For each elution step, the resin was incubated with 150–200 μl of HA peptide (200 μg/ml in Buffer A at 4 °C for 30 min with occasional vortex mixing. The eluted material was pooled. SDS-PAGE and immunoblotting with anti-HA antibody indicated that (i) the flow-through fraction had no detectable Yop1-HA and (ii) 70–100% of the bound protein was recovered in the pooled eluate. Purity of the eluted Yop1 was verified by silver-stained SDS-PAGE.

Estimate of Number of Yeast ER Membrane Proteins—We estimate the number of ER membrane proteins in yeast as follows. The surface area of yeast ER is ~100 μm^2 (22). The cross-sectional area of a membrane protein depends on the number of transmembrane helices; here we use 20 nm^2/membrane protein (23). The cross-sectional area of a phospholipid is 0.6 nm^2 (24). If 50% of the surface area of the ER corresponds to protein molecules, then there are 2.5×10^6 proteins in the ER and 1.6×10^8 phospholipids. Assuming that the average molecular mass of ER membrane proteins is 50 kDa, the protein/phospholipid ratio of the ER is ~0.8 g/mmol, corresponding to a weight ratio of ~1:1 (g/g), as generally expected for cellular membranes (25).

RESULTS

Spontaneous Flipping of NBD-labeled Lipids Is Slow in Vesicles Reconstituted from Triton X-100-solubilized Phospholipids—A previous report indicated that NBD-GlcCer (Fig. 1) moves spontaneously across the membrane of large unilamellar vesicles with a half-time of ~5 h at 20 °C (12). The vesicles used in these measurements were prepared by hydrating a lipid film and subjecting the resulting suspension to freeze-thaw cycles followed by extrusion through a 0.2-μm filter (12). The present study makes use of a detergent-based vesicle reconstitution protocol in which Triton X-100-solubilized phospholipids and ER membrane proteins are incubated with detergent-adsorbing Bio-Beads. Although greater than 99% of the detergent is removed during this procedure (26) to generate large unilamellar vesicles, we considered whether residual detergent in the vesicles could affect the spontaneous flip-flop of lipids.

To address this point, we measured the rate at which NBD-GlcCer and NBD-PE (Fig. 1) move spontaneously across the membrane of protein-free liposomes prepared by our detergent-based reconstitution procedure. We reconstituted liposomes with trace quantities of NBD-GlcCer or NBD-PE and used sodium dithionite (27) to eliminate the fluorescence of all of the NBD-lipid located in the outer leaflet. The resulting asymmetric vesicles were passed over a gel filtration column to remove excess dithionite before being incubated for up to 30 h at 23 °C. Spontaneous flipping of NBD-lipids from the inner to

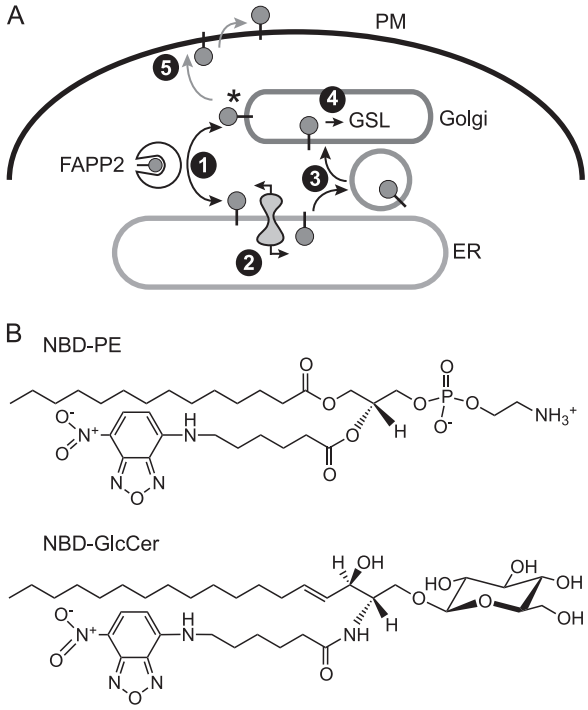

FIGURE 1. **Intracellular transport of glucosylceramide and structure of fluorescent lipids.** *A*, trafficking of glucosylceramide. GlcCer is synthesized on the cytoplasmic face of the Golgi complex (indicated by the *asterisk*) (*1*). The majority of GlcCer is moved to the cytoplasmic face of the ER by FAPP2, a cytoplasmic lipid transfer protein. This is probably a bidirectional process (*2*). GlcCer is flipped across the ER membrane by an ATP-independent flippase (*3*). Lumenal GlcCer enters ER-derived transport vesicles and thus enters the lumen of the Golgi complex on vesicle fusion (*4*). In the Golgi lumen, GlcCer is galactosylated to form lactosylceramide, which is then further elaborated to generate higher order GSLs (*5*). A minor fraction of GlcCer moves to the PM by a non-vesicular route and is flipped across the plasma membrane. The model is based the work of Halter *et al.* (13). *B*, NBD-PE and NBD-GlcCer.

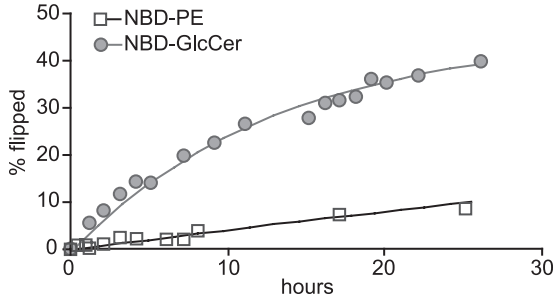

FIGURE 2. **Spontaneous flipping of NBD-GlcCer and NBD-PE.** Asymmetric liposomes with the NBD-labeled lipid confined to the inner leaflet at time zero were incubated at 23 °C. Transbilayer movement of the labeled lipid from the inner to the outer leaflet was monitored over time by determining the fraction of sample fluorescence that could be eliminated by dithionite, a membrane-impermeant reductant. The data were fit to a monoexponential equation of the form, % flipped = $50(1 - \exp(-kt))$. The half-time of flipping (equal to $0.69/k$) was ~80 h for NBD-PE and ~9 h for NBD-GlcCer.

the outer leaflet was assessed by removing aliquots at different times and determining the fraction of fluorescence that could be reduced by freshly added dithionite. Fig. 2 shows that the spontaneous flipping of NBD-PE is extremely slow, with a half-time estimated at ~80 h, whereas that of NBD-GlcCer is somewhat faster, with a half-time of ~9 h. These estimates are similar to those reported by Buton *et al.* (12). We conclude that the spontaneous movement of NBD-GlcCer and NBD-PE across membranes occurs on a time scale of tens of hours and is unaffected by residual detergent in our reconstituted vesicles. The absence of a formal charge in the glucosyl headgroup of NBD-GlcCer probably accounts for its faster spontaneous flipping rate compared with that of NBD-PE, which is zwitterionic at pH 7.4 (28).

NBD-GlcCer Flippase Activity in Proteoliposomes Reconstituted with Rat Liver ER Membrane Proteins—For our studies, we used highly enriched ER membrane preparations that were essentially devoid of Golgi membrane contamination. Starting with a rat liver homogenate, we used a classical procedure (29) to separate smooth membranes from rough ER (RER). The RER fraction was washed to remove peripheral proteins and permeabilized to eliminate lumenal content before being used to prepare TE (16). SDS-PAGE and immunoblotting confirmed that the RER fraction is highly enriched in the ER membrane protein ribophorin I (30) relative to plasma membrane and Golgi membrane markers (dipeptidylpeptidase IV (31) and α2,6-sialyltransferase (32), respectively), which are found mainly in smooth membranes and barely detectable in the RER fraction. The results are identical to those reported previously (14) and confirm the purity of the ER preparation.

We previously used a variety of assays to demonstrate ATP-independent phospholipid flippase activity in proteoliposomes reconstituted with ER membrane proteins (14, 18, 33–36). The most convenient of these assays (17, 18) exploits the ability of sodium dithionite to eliminate selectively the fluorescence of NBD-lipids located on the outer leaflet of sealed vesicles. Thus, when dithionite is added to large unilamellar vesicles containing NBD-lipids symmetrically distributed between the two leaflets of the membrane, fluorescence is expected to drop by 50% as NBD-lipids in the outer leaflet react. However, if vesicles contain a lipid flippase, NBD-lipids will be flipped from the inner to the dithionite-accessible outer leaflet, leading to a predicted 100% loss of fluorescence.

Fig. 3*C* demonstrates the principle of the flippase assay using NBD-PE as the reporter lipid (chromatographic analyses (Fig. 3*B*) confirm that NBD-lipids remain intact during reconstitution into both liposomes and protein-containing vesicles). Upon adding dithionite to NBD-PE-containing protein-free liposomes, fluorescence dropped rapidly by ~47%, consistent with quantitative reduction of NBD-PE molecules located in the outer leaflet and protection of the NBD-PE pool located in the inner leaflet (the addition of Triton X-100 to permeabilize the vesicles resulted in complete loss of fluorescence). For proteoliposomes containing ER membrane proteins, the extent of reduction was greater, consistent with transport of NBD-PE from the inner to the outer leaflet. Thus, for vesicles reconstituted at a PPR of 3.7 mg of protein per mmol of phospholipid (*sample P1*, Fig. 3*A*), ~77% of the fluorescence was reduced upon adding dithionite. For vesicles prepared at a higher PPR (Fig. 3*A*, *trace P2*; PPR ~37 mg/mmol), ~94% of the fluorescence was reduced. Because no metabolic energy source was added to the samples, the NBD-PE flippase activity is "ATP-independent." The rate of fluorescence loss was comparable for both liposomes and proteoliposomes (half-time ~20 s), indicating that NBD reduction rather than lipid flipping is the rate-limiting step in the assay (17, 18, 36). Thus, the assay yields end

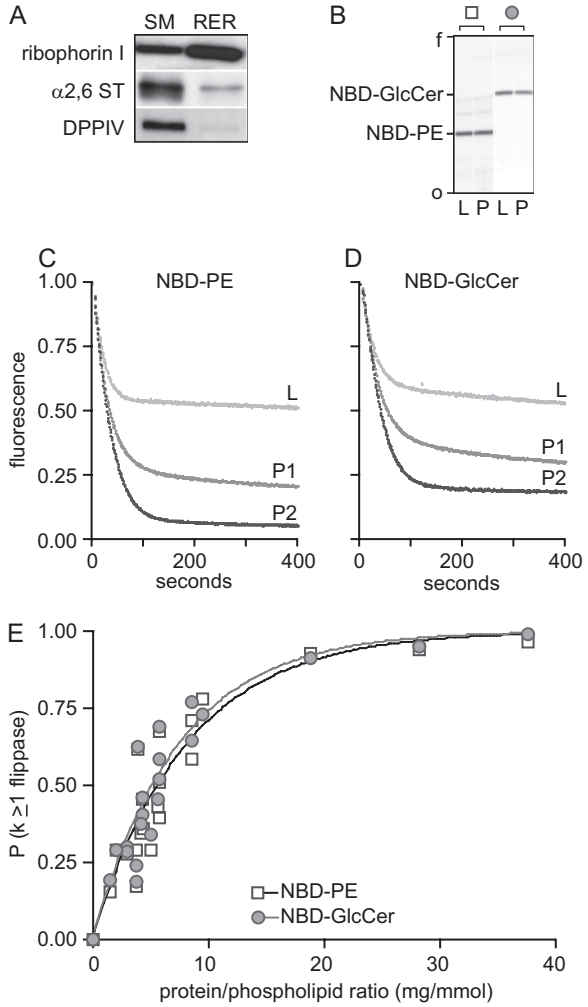

FIGURE 3. **Reconstitution of NBD-PE and NBD-GlcCer flipping in proteoliposomes.** Large unilamellar vesicles were prepared from Triton X-100-solubilized egg phosphatidylcholine, egg phosphatidylglycerol, and either NBD-PE or NBD-GlcCer. Different amounts of Triton X-100-solubilized rat liver ER membrane proteins were included in the reconstitution mixture to generate vesicles with different PPR values (mg of protein/mmol of phospholipid). *A*, rat liver homogenates were fractionated to yield smooth microsomes (*SM*) and RER. Smooth microsomes and RER samples were analyzed (equal protein loading) by SDS-PAGE and immunoblotting using antibodies to ribophorin I (~65 kDa), α2,6-sialyltransferase (*α2,6 ST*; ~50 kDa) and dipeptidylpeptidase IV (*DPPIV*; ~110 kDa). *B*, thin layer chromatograms of organic solvent extracts of liposomes and proteoliposomes reconstituted with NBD-PE or NBD-GlcCer. The migration position of NBD-lipid standards is indicated; *o*, origin; *f*, solvent front. *C*, liposomes (*L*) and proteoliposomes (*P1* and *P2*, with PPR of 3.7 and 37 mg/mmol) were reconstituted with NBD-PE. Dithionite was added at time 0, and the reduction in fluorescence was monitored over time at ambient temperature. *D*, as in *C*, except that vesicles were reconstituted with NBD-GlcCer. *E*, raw data obtained from experiments, such as those shown in *C* and *D*, indicate the percentage (*y*) of NBD-PE or NBD-GlcCer that can be reduced by dithionite as a function of the PPR of the reconstituted sample. The transformation $f(y) = (y - y_0)/(y_{max} - y_0)$, where y_0 is the percentage reduction obtained with liposomes and y_{max} is the maximum percentage reduction observed, yields a monoexponential graph of $p(\geq 1)$ *versus* PPR, where $p(\geq 1)$ is the probability that a particular vesicle in the sample has ≥1 flippase. The monoexponential fit constant for this graph is the PPR value at which the average number of flippases per vesicle is 1, and ~63% of the vesicles in the population possess ≥1 flippase (17).

point information indicating the proportion of flippase-containing vesicles within a given sample.

We generated a series of fluorescence traces similar to those shown in Fig. 3*C* by systematically varying the PPR at which vesicles were reconstituted. Transformation of the fluorescence data (see the legend to Fig. 3*E*) yielded a graph of the probability, as a function of the PPR, that an individual vesicle contains ≥1 flippase(s) (Fig. 3*E*). The graph is a monoexponential function whose fit constant is the PPR at which (i) the average number of flippases per vesicle is 1 and (ii) ~63% of the vesicles have one or more flippase molecules. The fit constant that we obtained is 8.1 mg/mmol. Because the average diameter of the vesicles in the sample is ~155 nm (obtained by dynamic light scattering measurements (see "Experimental Procedures")) and the average molecular mass of ER membrane proteins being reconstituted is 50 kDa (37), it can be calculated (17) that functional phospholipid flippases represent ~3% of protein molecules in the Triton X-100-solubilized mixture of ER membrane proteins used for reconstitution. Identical data were obtained when NBD-PC was used as the lipid reporter (data not shown).

To determine whether the proteoliposomes could translocate glucosylceramide, we used NBD-GlcCer. Upon adding dithionite to NBD-GlcCer-containing protein-free liposomes (*L* in Fig. 3*D*), fluorescence was rapidly reduced by ~45%. For proteoliposomes, the extent of fluorescence reduction was greater. Thus, for proteoliposomes with a PPR of 3.7 and 37 mg of protein per mmol of phospholipid (*samples P1* and *P2*, respectively, in Fig. 3*D*), ~68 and ~81% of the fluorescence was reduced upon adding dithionite. The extent of fluorescence reduction was not as great as that seen for corresponding samples reconstituted with NBD-PE. The reason for this is unclear, but it may be due to differences in sample heterogeneity, as suggested previously (17).

We systematically varied the PPR at which vesicles were reconstituted and determined the extent of dithionite-mediated reduction of NBD-GlcCer fluorescence. Transformation of the data yielded a plot of $p(\geq 1$ flippase) *versus* PPR that was very similar to that obtained for NBD-PE (Fig. 3*E*). The monoexponential fit constant for the NBD-GlcCer data set was ~7.4 mg/mmol, comparable with the value of 8.1 mg/mmol obtained for NBD-PE. This result suggests that either the same flippase(s) is responsible for translocating NBD-PE and NBD-GlcCer, or if each lipid has a dedicated flippase(s), then the flippase(s) must be comparably abundant in the mixture of ER proteins used for reconstitution.

Effect of Protein Modification Reagents on NBD-PE and NBD-GlcCer Flippase Activity—Phospholipid flippase activity in the ER is due to two distinct pools of flippases that can be distinguished by their sensitivity to the protein modification reagents NEM and DEPC (18, 19). Thus, as shown in Fig. 4*B* (*NBD-PE*), treatment of Triton X-100-solubilized rat liver ER membrane proteins (TE) with NEM or DEPC prior to reconstitution resulted in the loss of phospholipid flippase activity in ~35 or ~55% of flippase-containing vesicles, respectively. However, treatment of TE with a combination of NEM and DEPC resulted in an increased, roughly additive effect because activity was lost in ~75% of the vesicles.

We reasoned that if the same flippases are responsible for translocating both NBD-GlcCer and NBD-PE, then the effect of NEM and DEPC on NBD-GlcCer translocation in the reconstituted system should be the same as that observed for NBD-PE translocation. Accordingly, we measured NBD-GlcCer flippase

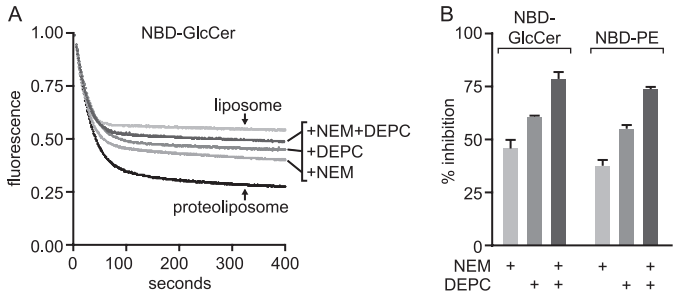

FIGURE 4. **Effect of protein modification reagents on translocation of NBD-GlcCer and NBD-PE.** Triton X-100-solubilized rat liver ER membrane proteins were treated with NEM (40 mM), DEPC (40 mM), or both for 45 min at ambient temperature before being taken for reconstitution. Mock-treated samples were used as controls. *A*, fluorescence traces generated on adding dithionite to NBD-GlcCer vesicles prepared without protein (liposome), with rat liver ER membrane proteins (proteoliposome), or with protein samples treated with NEM, DEPC, or NEM + DEPC as indicated. The PPR of all of the protein-containing samples was ~3.7 mg/mmol. *B*, combined data (means ± S.E. (*error bars*) from at least three independent experiments) showing the effect of protein modification reagents on the translocation of NBD-GlcCer and NBD-PE in vesicles reconstituted at a PPR of ~3.7 mg/mmol.

activity in proteoliposomes that had been reconstituted using TE treated with NEM, DEPC, or a combination of the two reagents.

Fig. 4*A* shows fluorescence traces from a representative experiment with NBD-GlcCer-containing vesicles. Upon adding dithionite, fluorescence dropped by ~46 and ~72% for liposomes and proteolipsomes (PPR ~3.7 mg/mmol), respectively. However, for proteoliposomes prepared from treated TE, the extent of fluorescence reduction was ~58% (+NEM), ~55% (+DEPC), and ~51% (+NEM+DEPC), corresponding to ~48, ~60, and ~78% inhibition, respectively. Fig. 4*B* presents data combined from several experiments. The results show clearly that translocation of both NBD-PE and NBD-GlcCer in reconstituted vesicles is similarly affected by NEM and DEPC, consistent with the conclusion that the same flippases are responsible for translocating both lipids.

NBD-PE and NBD-GlcCer Flippase Activities Fractionate Identically on Velocity Gradient—To test our conclusion that the same ER membrane proteins translocate NBD-PE and NBD-GlcCer, we fractionated TE by velocity sedimentation on a glycerol gradient. We loaded TE onto a linear 10–35% (w/v) glycerol gradient, centrifuged the sample at ~160,000 × g_{av} for 18.5 h at 4 °C, and collected 14 fractions from the top. The refractive index of each fraction was measured, confirming the linearity of the gradient (Fig. 5*A*). To facilitate further analyses, we pooled fractions pairwise, starting with fraction 2. TE protein was distributed throughout the gradient, with the most protein being recovered in the pool corresponding to fractions 6 and 7 (Fig. 5*A*). The pooled fractions were reconstituted and taken for flippase assays. Fig. 5*C* shows that NBD-PE and NBD-GlcCer flippase activity fractionated as the same broad peak, consistent with our conclusion that the same proteins are responsible for flipping both lipids. We note that the activity profile does not correlate with the protein profile (Fig. 5, compare *A* and *C*; the peak of the protein profile is indicated by a *gray bar* running through all *panels* of the figure), confirming previous conclusions that flippase activity in our system is not simply a result of reconstituting any membrane protein.

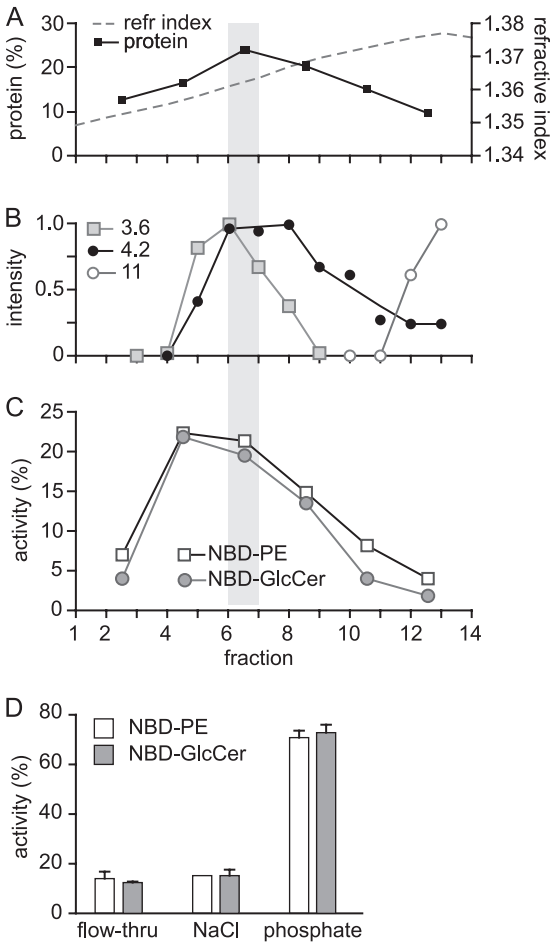

FIGURE 5. **Velocity gradient sedimentation analysis and hydroxyapatite chromatography of NBD-PE and NBD-GlcCer flippase activities.** *A–C*, velocity gradient sedimentation analysis of TE; *D*, hydroxyapatite chromatography of TE. *A*, refractive index of individual fractions and protein content of fractions pooled pairwise, starting with fraction 2. The *gray bar* running through all three *panels* indicates the peak of the protein recovery profile. *B*, sedimentation behavior of ovalbumin (3.6 S), bovine serum albumin (4.2 S), and catalase (11 S). Fractions were analyzed by SDS-PAGE, and the relative amount of a particular standard in each fraction was determined by densitometry of the Coomassie-stained gel. *C*, fractions were pooled pairwise, starting with fraction 2, subjected to buffer exchange, and then reconstituted at the same PPR value (~1 mg/mmol) to determine NBD-PE and NBD-GlcCer translocation activity. Unfractionated TE was reconstituted in parallel. A specific activity (*SA*) measure was obtained by determining the extent of fluorescence reduction (y_{frac} and y_{TE}), subtracting the extent of reduction obtained with protein-free liposomes (y_o), and normalizing to the protein amount (p) used for reconstitution. Thus, $SA_{frac} = (y_{frac} - y_o)/p$. Total flippase activity was calculated as the product of specific activity and the total protein amount in the sample. The *graph* shows the total activity in each pooled fraction normalized to that of the load (TE). *D*, TE was fractionated on hydroxyapatite to yield a flow-through fraction as well as fractions eluted from the resin with 1 M NaCl and 0.3 M phosphate. The *chart* shows GlcCer and PL flippase activity (calculated as described in the legend to *C*) recovered in each fraction (mean ± range (*error bars*) of two independent experiments).

The flippase activity profile was quite broad, with a full-width-half-maximum of ~6 fractions (Fig. 5*C*). To determine whether this was simply a consequence of the intrinsic sedimentation behavior of proteins, we analyzed a mixture of sedimentation standards (3.6, 4.2, and 11 S) in parallel. The standards also displayed profiles that were relatively broad (Fig. 5*B*) but not as broad as the flippase activity profile: the full-width-half-maximum of the 3.6 and 4.2 S standards was ~3 and ~5 fractions, respectively. The greater breadth of the phospholip-

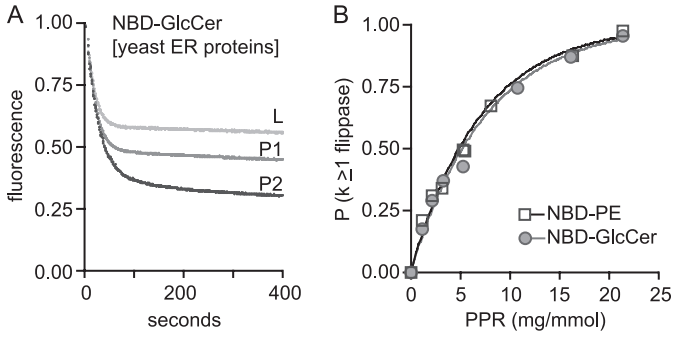

FIGURE 6. **NBD-GlcCer translocation in proteoliposomes containing yeast ER proteins.** *A*, liposomes (*L*) and proteoliposomes containing yeast ER membrane proteins (*P1* and *P2*, with PPR of 3.2 and 16 mg/mmol) were reconstituted with NBD-GlcCer. Dithionite was added at time 0, and the reduction in fluorescence was monitored over time at ambient temperature. *B*, raw data for NBD-GlcCer and NBD-PE flipping were obtained from experiments such as the one shown in *A*, using proteoliposomes prepared at a range of PPR values. The data were processed as described in the legend to Fig. 3*C* to generate a graph of $p(\geq 1)$ *versus* PPR. The *lines* through the two data sets are monoexponential fits with a fit constant (in each case) of ~7.5 mg/mmol.

id/GlcCer flippase activity profile compared with that of the standards suggests heterogeneity. It is possible that velocity gradient sedimentation incompletely separates two pools of flippase, probably the NEM- and DEPC-sensitive pools; one sediments slightly slower and the other slightly faster than ~4 S, resulting in a broad peak of activity. Future work will determine whether this is the case.

NBD-PE and NBD-GlcCer Flippase Activities Co-fractionate on Hydroxyapatite Chromatography—As a further test of our conclusion that the same proteins are responsible for flipping both NBD-PE and NBD-GlcCer, we fractionated TE on hydroxyapatite and analyzed flow-through, salt-eluted, and phosphate-eluted fractions for flippase activity. Fig. 5*D* shows that both NBD-PE and NBD-GlcCer flippase activity fractionated identically, with ~70% of the activity in each case being recovered in the pool of acidic proteins that is eluted from hydroxyapatite by 0.3 M phosphate buffer and ~10–15% in each case being recovered in the flow-through and salt-eluted fractions. The specific activity of the phosphate-eluted material was ~2.5 relative to TE. The identical behavior of both NBD-PE and NBD-GlcCer flippase activity on hydroxyapatite supports our conclusion that the same flippases are responsible for flipping both lipids.

NBD-GlcCer Flippase Activity in Yeast—Glucosylceramide is not found in *Saccharomyces cerevisiae* (38, 39), but phospholipid flippase activity has been demonstrated in yeast microsomes (19) as well as in proteoliposomes reconstituted with yeast ER membrane proteins (19, 21, 35). If the rat liver ER phospholipid flippases are able to translocate NBD-GlcCer, we reasoned that the same should be true in the yeast system. To test this, we assayed NBD-GlcCer flipping in proteoliposomes reconstituted with yeast ER membrane proteins. Fig. 6*A* shows fluorescence traces corresponding to dithionite reduction of NBD-GlcCer in liposomes and proteoliposomes (*P1* and *P2*, corresponding to PPR values of 3.2 and 16 mg/mmol). Similar to the data shown for rat liver ER proteins in Fig. 3*B*, these traces clearly indicate that proteoliposomes reconstituted with yeast ER proteins are capable of flipping NBD-GlcCer. Fig. 6*B* shows the corresponding protein dependence plot for NBD-GlcCer and NBD-PE flipping. As for the case of reconstituted rat liver ER membrane proteins, the protein dependence plots for NBD-PE and NBD-GlcCer flipping are identical, indicating that both lipids are probably translocated by the same yeast proteins. The fit constant for the protein dependence plot is ~7.5 mg/mmol; based on the assumptions described above, these data suggest that the phospholipid/GlcCer flippases represent ~3.2% by weight of proteins in yeast TE.

Role for Reticulons and Yop1 in Lipid Flipping?—A back-of-the-envelope calculation suggests that yeast ER has $\sim 2.5 \times 10^6$ membrane proteins (see "Experimental Procedures"). Because flippases represent ~3% of TE proteins, it can be deduced that there are ~75,000 phospholipid/GlcCer flippases per yeast cell, divided roughly equally between the NEM- and DEPC-sensitive pools. The reticulons (Rtn1 and Rtn2) and Yop1 are a family of membrane-active proteins implicated in the formation of ER tubules (20, 40–43). Found in most eukaryotes, these proteins are predicted to have the ability to insert into membranes by wedging into one leaflet, thereby promoting membrane curvature. Although non-essential, Rtn1 and Yop1 are very abundant, with $>10^4$ copies/yeast cell (40, 44), similar to the estimated size of the flippase pool. The abundance of Rtn1 and Yop1 and their predicted membrane activity suggest that they are good flippase candidates.

To examine this possibility, we assayed NBD-PE and NBD-GlcCer flipping in proteoliposomes reconstituted with TE from NDY257 cells lacking Rtn1, Rtn2, and Yop1. For comparison, we also prepared samples from the isogenic BY4741 wild-type strain. We anticipated that if Rtn1, Rtn2, and/or Yop1 contribute to the pool of functional phospholipid/GlcCer flippases, then this should be evident in a plot of the protein dependence of flippase activity; TE from the NDY257 strain should yield a shallower plot with a greater fit constant than TE from wild-type cells. As shown in Fig. 7*A*, essentially identical protein dependence plots were obtained for both NBD-PE and NBD-GlcCer using TE from either strain, indicating that Rtn1, Rtn2, and Yop1 do not contribute to NBD-PE and NBD-GlcCer flipping.

To verify this result, we prepared TE from a yeast strain (obtained from the yeast TAP-Fusion Library (Open Biosystems)) in which Yop1 is expressed from its chromosomal location with a C-terminal TAP tag (we verified expression of Yop1-TAP by SDS-PAGE and immunoblotting). We used IgG resin to quantitatively immunodeplete Yop1-TAP from the TE. Flippase activity assays showed that both mock-treated TE and Yop1-TAP-depleted TE were similarly active (data not shown), indicating that Yop1 does not contribute detectably to flippase activity.

We next tested purified Yop1. To do this, we expressed a HA-tagged variant of Yop1 at endogenous levels in NDY257 cells, affinity-purified the protein (Fig. 7*B*), and reconstituted it into vesicles. Association of Yop1-HA with vesicles was demonstrated by flotation of the reconstituted preparations on a discontinuous sucrose gradient (Fig. 7*C*). Reconstitutions were done using amounts of Yop1 up to 10-fold the normal amount found in TE but nevertheless considerably below the protein/phospholipid ratio that was previously shown to induce membrane tubulation (20). Proteoliposomes containing purified

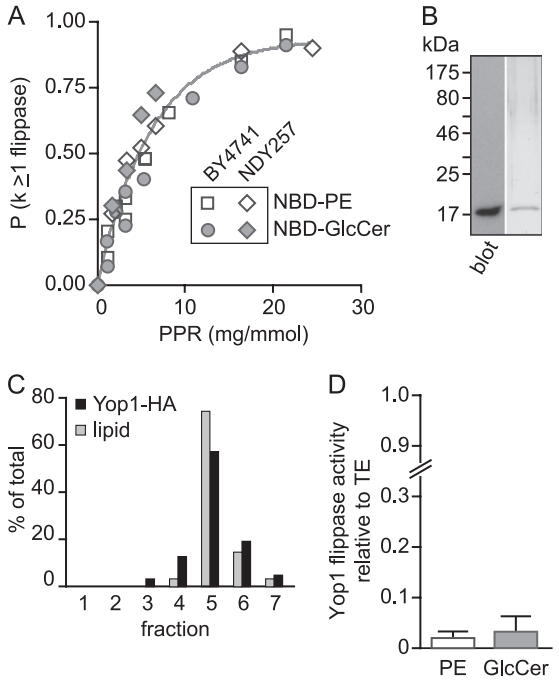

FIGURE 7. **A role for reticulons and Yop1 in lipid flipping?** *A*, proteoliposomes prepared at different protein/phospholipid ratio using TE from BY4741 wild-type cells as well as TE from NDY257 cells lacking Rtn1, Rtn2, and Yop1 were assayed for their ability to translocate NBD-PE and NBD-GlcCer. The *line* through the points is a monoexponential fit encompassing all four data sets. *B*, Yop1-HA was expressed in NDY257 cells and affinity-purified after preparing TE. The purified sample was analyzed by SDS-PAGE and immunoblotting using anti-HA antibodies or by silver-staining the gel. *C*, purified Yop1-HA was reconstituted into proteoliposomes. Membrane association of Yop1-HA was demonstrated by flotation in a discontinuous sucrose gradient, as described previously (21), using steps of 750, 750, 1000, 1000, and 750 μl of 0, 5, 10, 20, and 30% (w/v) sucrose, respectively. The vesicles floated as a sharp band at the interface between the 10 and 20% sucrose layers. Fractions (600, 600, 600, 400, 800, 600, and 650 μl, harvested from the top) were analyzed for phospholipid content and taken for SDS-PAGE immunoblotting to quantitate Yop1-HA. Fraction 5 includes the interface between the 10 and 20% sucrose layers. *D*, proteoliposomes containing purified Yop1-HA were tested for their ability to flip NBD-PE and NBD-GlcCer. Vesicles reconstituted with TE from Yop1-HA-expressing NDY257 cells were used for comparison. The concentration of Yop1-HA was adjusted to be the same as that of Yop1-HA in TE; this was verified by immunoblotting. We determined the extent of fluorescence reduction (y_{Yop1} and y_{TE}) obtained using different volumes (v) of sample for reconstitution, subtracted the extent of reduction obtained with protein-free liposomes (y_o), and divided by sample volume to get a specific activity measure (*i.e.* $SA_{Yop1} = (y_{Yop1} - y_o)/v$ and $SA_{TE} = (y_{TE} - y_o)/v$. The volumes of TE chosen were such that $(y_{TE} - y_o)$ was between 5 and 15% (*i.e.* on the rising portion of the protein dependence plot shown in *A*). The *bar chart* presents the average of the ratio SA_{Yop1}/SA_{TE}. The specific activity of the flow-through fraction obtained after quantitative depletion of Yop1-HA from TE was the same as that obtained for TE. *Error bars*, S.E.

Yop1-HA showed no flippase activity (Fig. 7*C*); in comparison, TE from NDY257 cells expressing Yop1-HA and Yop1-HA-depleted TE were both fully active. The cumulative data indicate that Yop1 and the reticulons do not contribute to phospholipid/GlcCer flippase activity in the ER.

DISCUSSION

The results presented in this paper show that transbilayer movement of NBD-GlcCer is slow ($t_{½}$ ~9 h) in protein-free vesicles, but fast ($t_{½}$ <20 s) in proteoliposomes containing rat liver or yeast ER membrane proteins. Fast flipping of NBD-GlcCer in proteoliposomes is due to specific proteins, representing ~3% of ER membrane proteins. The activity is partially sensitive to the protein modification reagents NEM and DEPC but can be largely eliminated when both reagents are used in combination, indicating two distinct flippase pools. Velocity sedimentation analysis indicates a broad profile of flippase activity with a maximum at ~4 S, and hydroxyapatite chromatography indicates that flippase activity resides in a pool of acidic proteins in the TE. The characteristics of the GlcCer flippase activity listed above are identical to those of the ER phospholipid flippases, indicating that these latter well characterized but as yet unidentified proteins (14, 18, 19, 34, 45, 46) probably flip both GlcCer and common phospholipids.

We recently showed that G-protein-coupled receptors, specifically opsin and the β1-adrenergic receptor, can facilitate the rapid flip-flop of phospholipids (17). Preliminary experiments indicate that opsin, reconstituted at a density of ~1 molecule/vesicle, is also able to flip NBD-GlcCer rapidly.[3] This result shows that an established phospholipid flippase can flip a monohexosyl GSL, and thus it is unsurprising that the ER phospholipid flippases would be able to do this as well despite the structural differences between the two classes of lipid.

Our studies use fluorescently tagged lipids to approximate the behavior of natural phospholipids whose transbilayer translocation is quite difficult to measure. The slight water solubility of these analogs gives them a higher off rate from membranes and allows them to exchange spontaneously between membranes on a time scale of tens of seconds. Whereas these characteristics are a potential concern in cellular studies, they do not impact the *in vitro* measurement of transbilayer transport except possibly to affect transport kinetics (47), a parameter that is not measured in our end point assays. For such assays, the use of tagged lipids is not in dispute (33, 48).

The original view of GSL biosynthesis placed the GlcCer translocation step in the Golgi complex (10, 49, 50). This was confirmed by studies of Buton *et al.* (12), who showed that spin-labeled GlcCer is able to flip across rat liver Golgi vesicles in an ATP-independent fashion. However, subsequent cell culture studies contradicted these results by noting that the short chain analogs of GlcCer are translocated across Golgi membranes by an ATP-binding cassette transporter that does not transport natural GlcCer (13). This matter remains to be resolved. Our focus in this paper is on the more likely scenario that GlcCer flips at the level of the ER.

Halter *et al.* (13) suggest a remarkable transport itinerary for GlcCer, from the Golgi complex to the ER via the cytoplasm, followed by a return to the Golgi in the lumenal aspect of secretory vesicles (Fig. 1*A*). The complexity of this transport route presents many possibilities for regulating GSL synthesis by controlling the availability of GlcCer in the Golgi lumen. Flip-flop of GlcCer at the ER membrane is unlikely to be a regulated step because it is facilitated by flippases that are known to function at well over the capacity needed for cell growth (33).

We previously reported that the ER phospholipid/GlcCer flippases represent ~0.6% of ER membrane proteins (14). We now present a refined analysis that indicates an abundance closer to ~3%, corresponding to ~37,000 molecules each in

[3] M. Chalat, unpublished result.

yeast, of the NEM- and DEPC-sensitive flippase pools. High throughput measurements indicate that ER membrane proteins, such as the translocon component Sec61 and the reticulon Rtn1, are present at levels that are compatible with our estimates: ~25,000 copies/yeast cell of Sec61 and ~37,000 copies/yeast cell of Rtn1 (44). Yop1, a membrane-bending molecule similar to Rtn1 (42), is also present at levels comparable with that of Rtn1 (40). Sec61 is not responsible for phospholipid flippase activity (19) and is therefore unlikely to be involved in flipping GlcCer; velocity sedimentation analyses indicate that the protein sediments rapidly (19) and is thus easily separated from the more slowly sedimenting peak of phospholipid/GlcCer flippase activity. We now report that the reticulons (Rtn1 and Rtn2) and Yop1 also lack phospholipid/GlcCer flippase activity.

The ER is a biogenic membrane that supports the biosynthesis of a number of different lipids, some of which are critically required for post-translational modification of proteins (46). Thus, glycerophospholipids, glycosylphosphatidylinositol, dolichol-PP-oligosaccharide, dolichol-P-monosaccharides, dolichyl phosphate, and glucosylceramide must all flip across the ER membrane to support cell growth and metabolism. We recently showed that glycerophospholipids, dolichol-PP-oligosaccharide, and dolichyl-phosphate-mannose are transported across the ER by ATP-independent flippases that can be biochemically resolved (21, 51). Because it is likely that dolichyl phosphate is flipped by the dolichol-P-monosaccharide flippases (51) and glycosylphosphatidylinositol is translocated by phospholipid/GlcCer flippases (52), we conclude that the ER has four different lipid flippases. Identification of these flippases remains an objective of future work.

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