Trans-Golgi Network and Subapical Compartment of HepG2 Cells Display Different Properties in Sorting and Exiting of Sphingolipids

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In HepG2 cells, the subapical compartment (SAC) is involved in the biogenesis of membrane polarity. By contrast, direct, apical transport originating from the trans-Golgi network (TGN), which may contribute to polarity establishment, has been poorly defined in these cells. Thus, although newly synthesized sphingolipids can be directly transported from the TGN to the apical membrane, numerous apical resident proteins are traveling via the transcytotic route. Here, we developed an in vitro transport assay and compared the molecular sorting of 6-[N-(7-nitrobenz-2-oxa-1,3 diazol-4-yl)amino] hexanoyl-sphingomyelin (C6NBD-SM) and C6NBD-glucosylceramide (C6NBD-GlcCer) in TGN and SAC. SM is released from both TGN and SAC in the luminal leaflet of transport vesicles. This holds also for GlcCer released from SAC but not for a substantial fraction that departed from the Golgi. Distinct transport vesicles, enriched in either SM or GlcCer are released from SAC, consistent with their rigid sorting in this compartment. Different vesicle populations could not be recovered from TGN, although in situ experiments reveal that GlcCer is preferentially transported to the apical membrane, reflecting different transport mechanisms. The results indicate that in HepG2 cells sphingolipids are mainly sorted in the SAC membrane and that the release of SM from SAC and TGN is differentially regulated.

Polarized cells have distinct plasma membrane domains, i.e. the apical and basolateral membranes each displaying a specific protein and lipid composition. These differences are generated and maintained, despite a continuous membrane flux between these domains and intracellular organelles, sorting and vesicular transport being instrumental in these events (1). Two major pathways have been implicated in polarized sorting. De novo synthesized proteins and lipids are sorted to either plasma membrane in the trans-Golgi network (TGN)* (2, 3). In addition, molecular redistribution can also be mediated by the endosomal sorting machinery (4). Thus, proteins and lipids internalized from the basolateral and apical membrane meet in an apically localized common endosome (5), defined as the subapical compartment (SAC) in HepG2 cells (6). Here, molecules are sorted and either recycled to the original membrane or transcytosed to the opposite membrane domain (7), thereby securing and maintaining a polarized distribution. The existence of two such pathways raise intriguing questions concerning the relative contributions of SAC and TGN to polarity generation and maintenance and the molecular nature of the sorting machinery involved in either pathway.

Detergent-insoluble membrane domains, called rafts (8), have been implicated in sorting (9), although raft formation per se is probably not sufficient for apical targeting (10). (Glyco-) sphingolipids, together with cholesterol, are the main lipid constituents of rafts and the finding that inhibition of sphingolipid synthesis, but not cholesterol depletion, interferes with the apical sorting of detergent-insoluble GPI-anchored proteins in Fischer rat thyroids cells (11) indicates that sphingolipids are especially important for the sorting of apical cargo. In many epithelial cells newly synthesized proteins are sorted in the TGN and directly transported to either the basolateral or apical membrane (3). In contrast, hepatocytes transport most (12, 13), but not all (14, 15) newly synthesized apical proteins first to the basolateral membrane, prior to reaching the apical (bile canalicular (BC)) membrane via transcytosis. Yet, in these cells, newly synthesized analogs of SM and GlcCer can be transported directly from the TGN to the BC membrane (16), although it has not been determined whether, similar to MDCK and Caco-2 cells (17, 18), sphingolipids are actually sorted in the TGN. In the latter studies, it was shown that de novo synthesized C6NBD-labeled sphingolipid analogs can be sorted in the TGN, directing C6NBD-GlcCer preferentially to the apical membrane, whereas SM distributes about equally over apical and basolateral domains.

In MDCK cells, it has been demonstrated that the common recycling endosome is enriched in raft lipids (19). This could imply that by analogy the formation of sphingolipid-enriched membrane domains may be important for polarized sorting in the SAC as well. In this context, we have recently demonstrated that the SAC is intimately involved in regulating the distribution of both of these lipids during transcytotic transport (20, 21). At steady state, SM is transported predominantly from the SAC to the basolateral membrane, whereas GlcCer is recycling between the SAC and the BC membrane (20). Circumstantial evidence suggests that the exiting of both lipid analogs from SAC is vesicle-mediated (20). However, de novo synthesized GlcCer analogs (22, 23) as well as the natural counterpart (24) may at least in part be transported as monomers, likely from an early Golgi compartment, where its synthesis occurs at
the cytosolic surface (25, 26). Following arrival at the inner leaflet of the plasma membrane, transmembrane translocation of the GlcCer analogs has been shown to occur via ABC transporter proteins (23, 27).

To understand the mechanisms that govern these various sorting processes, the regulation of sphingolipid transport in the biosynthetic and endocytic pathways, and how vesicular transport derived from SAC and TGN contribute to the biogenesis and maintenance of polarity, the isolation of the putative transport vesicles and the reconstitution of vesicular transport of both proteins and lipids in permeabilized cells are desirable. Thus far, most studies focusing on these issues have been restricted to the analysis of vesicular protein transport (28–30), whereas only a few in vitro studies have been developed to characterize the vesicular transport of lipids (31, 32). Here, we developed an in vitro assay using permeabilized HepG2 cells to study the exit of fluorescently tagged SM and GlcCer from the SAC and TGN. The purpose was to reveal the occurrence and nature of sorting in either compartment, thereby improving our knowledge of where and how membrane domains enriched in sphingolipids may contribute to the sorting of apical and basolateral proteins. We demonstrate that both sphingolipids can exit SAC and TGN in transport vesicles. However, whereas SAC-derived trafficking is exclusively vesicle-mediated, a fraction of the newly synthesized GlcCer exits from Golgi by a mechanism that does not involve its packaging into the lumenal side of transport vesicles. Moreover, evidence is presented that SM and GlcCer are released in distinct vesicles from the apical transport compartment, thus highlighting the prominent role of this compartment as a sorting station in the transcytotic pathway.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium and Hank’s balanced salt solution were from Invitrogen, and trifluoperazine (TFP) was from Calbiochem (La Jolla, CA). C6NBD was obtained from Molecular Probes (Eugene, OR), and cyclosporin A (CSA) was from Alexis (Laülfingen, Switzerland). High performance-TLC plates and sodium dithionite were purchased from Merck, and PDMP was from Matreya (Pleasant Gap, PA). BSA (fraction V), Dowex 1X8-D-glucosylsphingosine, and sphingosylphosphorylcholine from Sigma. MK571 was kindly provided by E. Vellenga (University of Groningen). All other materials were of analytical grade.

Cell Culture—HepG2 cells were grown in 25-cm² cell culture flasks (Costar) in Dulbecco’s modified Eagle’s medium containing 4500 mg of glucose/liter, supplemented with 10% fetal calf serum and 2 mM l-glutamine. For the experiments, the cells were used 3 days after plating when they were maximally polarized (33).

Synthesis of C6NBD-labeled Sphingolipids—C6NBD-ceramide, C6NBD-GlcCer, and C6NBD-SM were synthesized from C6NBD-1-palmitoyl-glucosylsphingosine, and sphingosylphosphorylcholine, respectively, as described elsewhere (34). Sphingolipids were stored at −20 °C and routinely checked for purity.

Labeling of HepG2 Cells with C6NBD-Sphingolipids—To analyze sphingolipid transport of intact HepG2 cells, they were labeled with 4 μM of C6NBD-sphingolipids. For in vitro studies 5 μM of sphingolipids were used. To label the TGN, the cells were incubated with C6NBD-ceramide in Hanks’ balanced salt solution for 90 min at 18 °C. Noninternalized ceramide was removed from the plasma membrane by back-exchange with 5% BSA at 4 °C in Hanks’ balanced salt solution (2 × 30 min). The cells were incubated for an additional 90 min at 18 °C in presence of 5% BSA. To label the SAC, the cells were incubated with C6NBD-SM and -GlcCer in Hanks’ balanced salt solution for 30 min at 37 °C. Noninternalized sphingolipids were removed by back-exchange, and the sphingolipids were chased into the SAC for 2 h at 18 °C in presence of BSA. Sphingolipids that had been transported to the BC membrane were quenched for 10 min with 30 mM sodium dithionite at 4 °C. At these conditions, the agent does not permeate the membrane and exclusively abolishes NBD fluorescence that is localized in the external leaflet of the BC (32).

Transport of Sphingolipids in Intact Cells—To study basolateral transport after labeling of the TGN, HepG2 cells were incubated for various time intervals at 37 °C in presence of 5% BSA, acting as a scavenger for basolaterally arrived lipid. The incubation medium and the cells were collected separately, and the lipids of both fractions were extracted and analyzed by TLC as described previously (24). To study apical transport the cells were grown on coverslips, and the TGN was labeled as described above. The cells were incubated for various time intervals at 37 °C in back-exchange medium. Transport to the BC membrane was analyzed by fluorescence microscopy. BC structures were identified in phase contrast and characterized as C6NBD-positive structures with NBD fluorescence, as has been described in detail elsewhere (33). Images were scanned and processed with Paint Shop Pro.

In vitro Budding Assay—After labeling either TGN or SAC of HepG2 cells with C6NBD-sphingolipids, the cells were scraped from the culture dish into 1 ml of transport buffer (140 mM KCl, 0.5 mM KH2PO4, 20 mM Hepes-KOH, pH 7.3). The cells were permeabilized by pressing them four times through a 25-gauge syringe, and cell debris was removed by a brief centrifugation step (10 min, 2000 × g). The permeabilized cells were treated for 30 min at 4 °C with 5% BSA, washed once in transport buffer, and incubated at 37 °C in the presence of cytosol (2 mg/ml), ATP, and an ATP-regenerating system, and 1% BSA. After the incubation, the cells were removed by centrifugation at 2000 × g, and the supernatant was centrifuged for 2 h at 100,000 × g to recover membrane-associated sphingolipids, which were protected from BSA. The lipids of all fractions were extracted and analyzed by TLC. To calculate the release of C6NBD-sphingolipids in the luminal leaflet of vesicles, the amount recovered in the 100,000 × g pellet was correlated to the total amount of sphingolipid prior to the incubation (2000 × g pellet + 100,000 × g supernatant + 100,000 × g pellet). The amount of sphingolipids that became accessible to BSA (100,000 × g supernatant) was determined similarly.

Fractionation of Vesicles by Gradient Centrifugation—The vesicular supernatant obtained from the in vitro budding assay was immediately loaded on a continuous gradient of 20–50% sucrose (w/w) in transport buffer. The gradient was centrifuged for 16 h at 100,000 × g in an SW50 rotor (Beckman) and fractionated. Immediately before lipid extraction the obtained gradient fractions were treated for 5 min with 30 mM sodium dithionite at 4 °C to quench any NBD fluorescence associated with sphingolipids that were extracted by BSA and/or exposed in the outer leaflet of the membranes.

Preparation of Cytosol—HepG2 cells were grown for 3 days on 165-cm² dishes, washed and scraped into 0.5 ml of transport buffer. The cells of 5–10 dishes were collected and homogenized with a tight Dounce homogenizer. The homogenate was centrifuged for 10 min at 1000 × g to remove cell debris and nuclei, and the post nuclear supernatant was centrifuged two times at 100,000 × g. The final supernatant was used in the in vitro experiments.

Transmission Electron Microscopy—Gradient fractions containing vesicles enriched in C6NBD-sphingolipids were collected and adjusted with transport buffer to a sucrose concentration of 10% (w/w). The vesicles were pelleted for 3 h at 100,000 × g. After fixation for 15 min with 0.5% glutaraldehyde in the transport buffer containing 10% sucrose, the pellets were preincubated for 15 min in the dark with 0.15% (w/w) dianisobenzidine (DAB). For photoconversion of DAB by the NBD-labeled sphingolipids, the pellets were illuminated for 30 min at a wavelength of 460 nm (for experimental details see Ref. 36). Thereafter the pellets were incubated for 60 min at room temperature with 1% OsO4 in cacodylate buffer (pH 7.4) and embedded in Epon. After sectioning, the structures containing osmiophilic precipitates were analyzed using a Philips CM100 electron microscope at 80 kV. The micrographs were scanned and processed with Paint Shop Pro.

Determination of Galactosyltransferase Activity—To assay for galactosyltransferase (GTase) activity, the samples were incubated for 1 h at 37 °C in a containing (final concentrations) 100 mM sodium cacodylate (pH 7.3), 20 mM N-acetylglucosamine, 20 mM MgCl2, 4 mM ATP, 0.5% Triton X-100, and 0.7 mM UDP-galactose (including 100,000 dpm [14C]UDP-galactose). The incubation was stopped by the addition of 450 μl of cold H2O, and [14C]N-acetyllactosamine was separated from [14C]UDP-galactose by a Dowex 1X8–200 column. The columns were washed in the presence of N-acetylglucosamine were corrected for control values (containing H2O), and the specific activity (dpm/μg protein) was determined. Finally the amount of GTase in each sample was determined by multiplying the specific activity with the protein content.

RESULTS

Both Golgi-derived transport of de novo synthesized sphingolipids and SAC-derived glycosphingolipid transport contribute to the biogenesis and maintenance of polarized membrane...
domains in HepG2 cells. Although direct sphingolipid transport from Golgi to the BC membrane can occur in liver cells (16), the mechanism of transport has not been determined, and it is also unknown whether sorting of the lipid species occurs as observed in other epithelial cells (17, 18). Accordingly, we first determined the mechanism by which newly synthesized GlcCer and SM reach the basolateral and apical membrane in optimally polarized HepG2 cells. This flux was then compared with the transport of both these lipids from the SAC, involved in membrane dynamics related to endocytotic events at either membrane surface, to appreciate the relative contribution of either pathway to membrane polarity development in liver cells.

**Direct Transport of Newly Synthesized C_{6}NBD-SM and -GlcCer from TGN to BC Membrane**—To analyze the exit of newly synthesized sphingolipids from the TGN in HepG2 cells, the cells were labeled for 90 min with C_{6}NBD-ceramide at 18°C. After a wash, the sphingolipids were chased for an additional 90 min at 18°C in the presence of 5% BSA. The latter will preclude the re-entry of basolaterally arrived lipids into the cell. After the chase, the perinuclear region of the cells was brightly labeled (Fig. 1, A and B), which is typical for a Golgi localization in these cells (16). 50% of the ceramide was metabolized to C_{6}NBD-SM and -GlcCer (in a molar ratio of 3:1, Fig. 1C), the only products that could be detected. The accumulation of the newly synthesized lipids in the Golgi is reflected by the observation that only approximately 10% of both sphingolipids were transported from the Golgi, presumably exiting from the TGN, to the basolateral plasma membrane during the incubation at 18°C (Fig. 1D).

During the incubation at 18°C, no significant labeling of the BC could be detected (Fig. 1B), implying that sphingolipid transport from Golgi to BC was impeded, whereas evidently C_{6}NBD-Cer did not acquire access to the BC. However, when the temperature was subsequently raised to 37°C in the presence of 5% BSA, 50–60% of the BCs were C_{6}NBD-positive after a 30-min incubation period (Fig. 2, A–C). Treatment with sodium dithionite after the incubation reduced the amount of labeled BCs to control levels (Fig. 2C), providing evidence that the C_{6}NBD-labeled sphingolipids were transported directly to the exoplasmic leaflet of the BC membrane. After longer incubation times (30–60 min) the amount of positive BCs decreased again, indicating that both sphingolipids are transcytosed from the apical to the basolateral membrane (33), where they are captured by BSA (Fig. 2C).

Because the BC membrane is not accessible to BSA, the direct quantification of the apically transported sphingolipid analogs is not possible. To discriminate between the fate of either newly synthesized C_{6}NBD-GlcCer or -SM, the cells were therefore incubated with 10 μM PDMP, which completely inhibited the synthesis of C_{6}NBD-GlcCer but did not affect synthesis of SM (data not shown). At these conditions, the fraction of the total pool of SM that was transported over a 30-min time interval to the basolateral membrane was not affected (Fig. 2D), but the number of labeled BCs after 30 min of transport was reduced by approximately 40% (Fig. 2D). Because only 25% of the total newly synthesized sphingolipid fraction (C_{6}NBD-GlcCer plus C_{6}NBD-SM) at control conditions consists of GlcCer, this result indicates that, in relative amounts, C_{6}NBD-GlcCer is the major sphingolipid that is transported to the BC membrane. Yet, evidently, a fraction of C_{6}NBD-SM is also transported from the Golgi to the BC membrane.

**Mechanism of Sphingolipid Transport from TGN to Basolateral and Apical Membranes**—Interestingly, C_{6}NBD-GlcCer transport from the Golgi to the basolateral membrane was much faster than that of C_{6}NBD-SM. After 15 min at 37°C, 32% of the pool of C_{6}NBD-GlcCer and 18% of that of C_{6}NBD-SM had reached the basolateral membrane (Fig. 3, A and B). These different transport kinetics to the basolateral membrane of HepG2 cells are reminiscent of the fast transport of natural GlcCer compared with other sphingolipids in Chinese hamster ovary cells (24). With increasing incubation times the difference between SM and GlcCer with regard to the fraction of the total lipid pools that reached the basolateral membrane diminished, amounting to 75–80% of the pools of both sphingolipids after 60 min. As shown above (Fig. 2C) the percentage of labeled BCs decreased concomitantly, emphasizing the integration of the newly synthesized sphingolipids in a dynamic transport process between apical and basolateral membrane. This is not the case after short term incubations (30 min; Fig. 2C). Accordingly, this would therefore suggest that the rapid transfer of GlcCer from the Golgi to basolateral membrane after 15 min involves a direct transport step and, given its pool size compared with that of SM, may be related to a mechanism that differs from that of SM.

GlcCer is synthesized at the cytoplasmic leaflet of the cis-Golgi (25) and its transport from the Golgi surface to the cytoplasmic leaflet of the plasma membrane, either by monomeric flow or in the cytoplasmic leaflet of transport vesicles,
has been described (24). In contrast to its natural counterpart, the short chain fluorescent GlcCer analog can be translocated to the exoplasmic leaflet by the basolaterally localized multi-drug resistance protein MRP1 to become accessible for BSA (23, 37). Indeed, incubation with the MRP1 inhibitor MK571 significantly reduced the amount of C6NBD-GlcCer that became accessible to BSA but had no effect on the basolateral transport of C6NBD-SM (Fig. 3, A and B). The effect of MK571 on GlcCer transport was time-dependent, showing 60% inhibition of lipid translocation after 15 min, which diminished to approximately 35% after 30 min, whereas the effect had largely disappeared after 60 min. These data thus indicate that early (i.e. up to 15–30 min) after the onset of sphingolipid biosynthesis in HepG2 cells, the transport mechanism of SM and GlcCer from Golgi to the basolateral membrane at least partly differs. However, after longer incubation times, an increasing pool of the C6NBD-GlcCer is transported directly to the exoplasmic leaflet of the plasma membrane via a (slower) MRP-independent pathway that originates from an intracellular site that differs from the cytoplasmic surface of the Golgi. In contrast to trafficking of de novo synthesized GlcCer from Golgi to the basolateral membrane, GlcCer (and SM) exiting from SAC, after specific accumulation in this compartment following endocytic internalization (see “Experimental Procedures”), reaches the outer leaflet of the (apical) plasma membrane, entirely independent of multidrug resistance protein 1 (MDR1) activity (20).

To analyze how newly synthesized C6NBD-GlcCer and -SM...
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are transported from the Golgi to the apical membrane, we examined the effect of CSA, an inhibitor of the MDR1 protein that is localized in the BC membrane of HepG2 cells (37). Incubation with 5 μM CSA, which completely abolished the translocation of rhodamine 123 into the BCs (data not shown) neither affected the number of labeled BCs nor the accessibility of the C₆NBD-labeled sphingolipids to sodium dithionite (Fig. 3C). In conjunction with the observation that upon inhibition of GlcCer biosynthesis BC labeling is reduced by almost 50% (see above), the results imply that the major part of the C₆NBD-GlcCer (and -SM) is transported directly to the exoplasmic leaflet of the BC membrane, i.e. the lipid must have been localized in the lumenal leaflet of transport vesicles. To obtain direct evidence for this notion, we subsequently developed an assay to isolate the putative transport vesicles, released from TGN and SAC.

C₆NBD-SM and -GlcCer Can Be Recovered in the Lumenal Leaflet of Transport Vesicles Released from the TGN and the SAC—HepG2 cells were mechanically permeabilized to induce holes in the cell surface that are large enough for the release of transport vesicles. Because of cell detachment, the filter stripping method described previously (28, 32) was not suitable for HepG2 cells, and permeabilization was therefore carried out by pressing the cells through a 25-gauge syringe. After this treatment 80–90% of the cells were permeable for trypan blue and had largely lost their plasma membrane as revealed by phase contrast microscopy (not shown). Using this protocol, 15–20% of the total cell associated C₆NBD-sphingolipids (ceramide, SM, and GlcCer) were released from the cells following the Golgi labeling procedure, whereas 25–30% of the total pool of C₆NBD-SM and -GlcCer were released from SAC-labeled cells (Fig. 4). To remove any sphingolipids that remained exposed on the cytoplasmic leaflet of intracellular membranes following permeabilization, the cell pellet was washed with BSA. 30% of both C₆NBD-ceramide and -GlcCer was localized in the cytoplasmic leaflet of the Golgi membrane (Fig. 4A). In contrast, only 10% of the newly synthesized C₆NBD-SM was accessible to BSA. After labeling the SAC of HepG2 cells with C₆NBD-SM and -GlcCer, 10–15% of both sphingolipids were accessible to BSA after permeabilization (Fig. 4B).

After these treatments, the remaining cell-associated fractions of C₆NBD-Cer and newly synthesized GlcCer and SM, associated with the Golgi, as well as the association of both sphingolipids with SAC, displayed a largely localized appearance, as reflected by bright fluorescent spots within the cellular lumen (not shown). Under these conditions, C₆NBD-GlcCer and -SM should be localized solely in the lumenal leaflet of the SAC or the TGN/Golgi (Ref. 33; see also “Discussion”). Accordingly, if transport vesicles exclusively mediate subsequent transport and assuming that the topology is maintained, the lipids should now be recovered in the lumenal leaflet of such vesicles after their release and thus must be inaccessible to BSA and pelletable at high speed. After an incubation for 30 min at 37 °C in presence of ATP and cytosolic proteins, 8% of either newly synthesized sphingolipid was recovered in the high speed pellet. In contrast, at 4 °C or in the absence of ATP and cytosol only 2–4% of C₆NBD-GlcCer and -SM were recovered (Fig. 5A). Comparably low vesicle budding was obtained when ATP or cytosol alone was omitted from the assay (data not shown). Essentially the same requirements applied for the release of C₆NBD-SM and -GlcCer from the SAC. Again 2–3% of C₆NBD-SM and -GlcCer were released at 4 °C or at 37 °C in the absence of ATP and/or cytosol. However, in presence of ATP and cytosol the vesicular release of both sphingolipids increased 2–3-fold, amounting to 7–8% of the initial cell-associ-
ence demonstrates that the sphingolipids were released from cells that were left intact during the preparation. Although newly synthesized C₆NBD-SM became accessible to BSA in significant amounts only after incubation at 37 °C, this was not the case for C₆NBD-ceramide and C₆NBD-GlcCer. Even at 4 °C 20% of the C₆NBD-GlcCer and 10% of the C₆NBD-ceramide became accessible to BSA (Fig. 5C). Incubation at 37 °C did not markedly change the relative amount of released C₆NBD-ceramide and -GlcCer.

**Distinct C₆NBD-SM and -GlcCer-containing Vesicles Are Released from the SAC but Not from the TGN—**To analyze whether C₆NBD-SM and -GlcCer are sorted in the SAC- and/or TGN membrane, which might be reflected by distinct vesicle populations enriched in either lipid, we fractionated the vesicle-containing supernatants by sucrose gradient centrifugation. The fractions were treated on ice for 5 min with sodium dithionite to ensure that only C₆NBD-sphingolipids in the luminal leaflet of transport vesicles were analyzed. In SAC-derived vesicles, C₆NBD-SM was clearly enriched over C₆NBD-GlcCer in vesicles obtained at a density corresponding to 30–33% sucrose (w/w), whereas C₆NBD-GlcCer was enriched at a sucrose density between 40 and 44% (Fig. 6A). Although neither lipid was exclusively retrieved in a particular vesicle fraction, it is evident that distinct “preferential” transport vesicles from the SAC for either sphingolipid can be identified.

To further characterize the potentially different nature of the carriers responsible for the exit of C₆NBD-SM and -GlcCer from the SAC, we took advantage of the ability of NBD to photoconvert DAB into an osmiophilic precipitate that can be visualized by electron microscopy (36). Prior to incubation with DAB and processing for electron microscopy, the gradient fractions corresponding to 30–33 and 40–44% sucrose, respectively, were adjusted to 10% sucrose to avoid possible morphological alterations caused by osmotic effects. The 30–33% fraction contained labeled and unlabeled spherical vesicles of different diameters. Especially small vesicles of approximately 50 nm in diameter were heavily labeled with DAB and are most likely those containing C₆NBD-SM (arrows in Fig. 7, A and B).

Such vesicles were only occasionally observed in the 40–44% fraction, in which the main DAB-positive structures consisted of larger, cup-like vesicles with a diameter of 80–100 nm (arrowsheads in Fig. 7B). No DAB-positive, osmiophilic structures were obtained when the pellets were kept in the dark, although faint vesicular structures could be identified (Fig. 7, C and D). The analysis of TGN-derived vesicles was hindered by the different amounts of the sphingolipids, because HepG2 cells synthesize much more C₆NBD-SM than C₆NBD-GlcCer. As observed for the SAC-derived vesicles, C₆NBD-SM was found predominantly at a sucrose concentration of 30–33% (Fig. 6B). C₆NBD-GlcCer was recovered at slightly lower sucrose concentrations, in contrast to its location at a higher density seen for SAC-derived vesicles. However, the difference in localization between SM and GlcCer, present in the TGN-derived vesicles, was usually not large enough to support the view that sorting

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**Table I**

| Fraction | Protein content | Specific activity | GTase Amount |
|----------|-----------------|------------------|-------------|
| Cell lysate | 100 | 0.99 ± 0.25 | 17.0 ± 5.14 |
| S1 | 17.1 ± 0.96 | 0.91 ± 0.20 | 3.37 ± 0.58 |
| S2 | 3.7 ± 0.25 | 1.08 ± 0.25 | 1.73 ± 0.52 |
| S3 | 1.6 ± 0.40 | 1.02 ± 0.06 | 3.83 ± 1.70 |
| Cells at 4 °C | 37.6 ± 0.54 | 1.05 ± 0.08 | 37.5 ± 2.65 |
| Cells at 37 °C | 35.7 ± 0.58 | 0.75 ± 0.07 | 0.91 ± 0.23 |
| Vesicles at 4 °C | 1.2 ± 0.19 | 0.44 ± 0.10 | 1.41 ± 0.34 |
| Vesicles at 37 °C | 3.2 ± 0.15 | 0.25 ± 0.11 | 0.50 ± 0.19 |

* The values given are calculated from those of the vesicles at 37 °C corrected for those of the vesicles at 4 °C.
higher concentrations of TFP (50 μM) was inhibited by 70%, and a slight effect on the amount of C6NBD-SM (sphingolipids were extracted and analyzed by TLC. In fractions were treated with sodium dithionite for 5 min on ice, and the fractionated. Fraction 1 corresponds to the bottom of the gradient. The buffer. The gradient was centrifuged for 16 h at 100,000 × g. To calculate the enrichment of one sphingolipid over the other at a particular sucrose concentration. In B the distribution of C6NBD-SM and -GlcCer over the whole gradient is shown. For both TGN- and SAC-derived vesicles a representative TLC plate as well as the mean and extreme values of two gradients are shown. Of the two sphingolipids into distinct transport vesicles occurred.

The Release of C6NBD-SM-containing Vesicles from the SAC and the TGN Is Differently Affected by the Calmodulin Antagonist TFP.—We have previously shown that C6NBD-SM and -GlcCer are located in different domains in the SAC. Among others, the evidence relied on the selective regulation of their exiting to the apical and basolateral domain upon treatment of the cells with the calmodulin antagonist TFP (38). The preferential localization of SM and GlcCer in distinct SAC-derived transport vesicles (Fig. 6A) is therefore fully consistent with this notion. To further corroborate these observations, the present assay system provides the possibility to investigate in a direct manner whether TFP modulates vesicular exiting of the sphingolipids.

In intact cells, TFP inhibited the transport of C6NBD-SM and -GlcCer from the SAC to the basolateral membrane, whereas it had no effect on the recycling of C6NBD-ceramide (B) or C6NBD-ceramide (B). After permeabilization and BSA treatment the cells were incubated at 37 °C in the presence of BSA, ATP, and cytosol. The cells were removed, and the supernatant was layered on top of a sucrose (w/w) in transport buffer. The gradient was centrifuged for 16 h at 100,000 × g and fractionated. Fraction 1 corresponds to the bottom of the gradient. The fractions were treated with sodium dithionite for 5 min on ice, and the sphingolipids were extracted and analyzed by TLC. In A the relative amount of C6NBD-SM (■) and C6NBD-GlcCer (○) in each fraction is calculated to demonstrate the enrichment of one sphingolipid over the other at a particular sucrose concentration. In B the distribution of C6NBD-SM and -GlcCer over the whole gradient is shown. For both TGN- and SAC-derived vesicles a representative TLC plate as well as the mean and extreme values of two gradients are shown.

Surprisingly, incubation with 20 μM TFP increased the vesicular release of C6NBD-SM from the TGN by 30%, whereas it had no effect on the release of C6NBD-GlcCer (Fig. 8B). It is unlikely that this increased release of C6NBD-SM was due to nonspecific fragmentation of the TGN, because higher concentrations of TFP (100 μM) slightly inhibited the release of C6NBD-SM and abolished the release of C6NBD-GlcCer (Fig. 8B). Interestingly, the data thus suggest that distinct mechanistic features are involved in mediating transport vesicle assembly and/or release from SAC on the one hand and Golgi on the other.

**DISCUSSION**

The present study demonstrates that the mode of sorting of SM and GlcCer during their exit from Golgi and SAC, the major sorting compartments in the biogenesis and maintenance of polarized membrane domains, differs. This is reflected by the recovery of transport vesicles, distinctly enriched in these sphingolipids. In addition, sphingolipid sorting in HepG2 cells is mainly associated with the SAC, highlighting its prominent role in polarity development of these cells.

**In Vitro Budding System and Sphingolipid Release from SAC and TGN**—To appreciate the involvement of SAC and TGN, including their hierarchy, in the polarized transport itinerary of hepatocytes, the isolation and characterization of the respective transport vesicles will be imperative. In fact, a variety of in vitro systems have been developed to analyze vesicular transport of proteins from the TGN to the plasma membrane (28–30). Also in hepatocytes distinct constitutive pathways from the TGN to the basolateral plasma membrane have been described (39, 40). However, although lipids, especially sphingolipids, play an important role in the polarized sorting of apical and basolateral proteins in epithelial cells (11), only very few in vitro systems have been developed to study the polarized sorting of de novo synthesized sphingolipids in the context of the assembly of transport vesicles (31, 32). Therefore,


FIG. 8. TFP treatment affects the release of C₆NBD-SM from the SAC and TGN differently. HepG2 cells were incubated with a mixture of C₆NBD-SM and -GlcCer (A) or C₆NBD-ceramide (B). After permeabilization and BSA treatment the cells were incubated at 4 °C or at 37 °C in presence of BSA, ATP, and cytochalasin D. During BSA treatment and vesicle formation the cells were incubated with (lane 2 and hatched bars in A and B) or without 20 μM TFP (lane 1 and white bars in A and B). In some experiments cells labeled with C₆NBD-ceramide were incubated with 100 μM TFP (lane 3 and black bars in B). The cells were removed, and the supernatant was centrifuged at 100,000 × g. The sphingolipids in the vesicle pellet were extracted and analyzed by TLC.

To calculate the temperature-dependent release of sphingolipids, the values obtained at 4 °C were subtracted from those obtained at 37 °C, and the resulting values for the control cells were set as 100. Representative TLC plates, showing the release at 37 °C, and the mean values and standard deviations of at least three independent experiments are shown.

As a first approach, an in vitro assay was developed to characterize transport and sorting of sphingolipids from either compartment. To this end, we used mechanically permeabilized HepG2 cells that had been labeled with C₆NBD-tagged sphingolipid analogs. Our data reveal that the release of C₆NBD-SM- and -GlcCer-containing vesicles from both compartments was temperature-dependent and required the addition of ATP and cytosolic proteins. A significant contribution of nonspecific fragmentation of the organelles to the observed sphingolipid transport is excluded, because no significant release of the Golgi marker GTase occurred during vesicle formation, whereas the TGN-derived vesicles (Fig. 5A) did not contain significant amounts of the Golgi-localized C₆NBD-ceramide.

From an experimental point of view it is relevant to emphasize that, although after internalization at the plasma membrane a fraction of the fluorescent sphingolipid analogs may be transported to the Golgi, as occurs in undifferentiated cells (35, 41, 42), in polarized HepG2 cells both C₆NBD-GlcCer and -SM are specifically accumulated in the SAC. As shown previously (20, 33), localization in this compartment can be readily distinguished from the Golgi apparatus, e.g. in its sensitivity to monensin and nocodazole. Indeed, the present results demonstrate that, prior to their vesicular release, newly synthesized and internalized sphingolipid analogs are located in the intended “target” compartments, i.e. Golgi and SAC. This is, for example, apparent from the distinct effects of TFP on the release of SM (Fig. 8), present in either compartment, or the differences in accessibility of GlcCer to BSA at 4 °C (Fig. 5, compare C and D).

In the context of the latter observation, it is interesting that only de novo synthesized C₆NBD-GlcCer, after its translocation to the lumenal leaflet in the Golgi, became accessible to BSA at 4 °C. Because all accessible sphingolipid analogs had been removed from the cytoplasmic leaflet of internal membranes prior to the incubation, this implies that a temperature-independent (and energy-independent) activity exists in the Golgi membranes that translocates C₆NBD-GlcCer but not C₆NBD-SM from the luminal to the cytoplasmic leaflet. Because such a translocation was not observed in the SAC-labeled membranes, the data reveal that the compartments display different properties concerning C₆NBD-GlcCer translocation and emphasize Golgi specificity rather than a nonspecific translocation of the lipid caused by the C₆NBD group.

Finally, although these analogs resemble the properties of natural sphingolipids in many aspects (42, 43), a direct comparison would be preferable. However, to specifically label SAC with sphingolipids or to accumulate newly synthesized sphingolipids in Golgi, the use of exchangeable or modifiable (dithioflavin quenching) sphingolipids is essential. In this manner, “contamination” of SAC- or Golgi-derived transport vesicle fractions with (natural) sphingolipid-containing vesicle fractions of similar density, but derived from different sources (plasma membrane, endocytic pathway), is avoided. Besides, a lack of suitable markers currently precludes further purification of the recovered vesicle fractions. It is most relevant, however, that the analogs used are sorted in SAC in a manner that depends on the progress of polarity (20, 21). This emphasizes their validity as markers for apically and basolaterally directed transport from SAC (or TGN) and for characterization of the respective transport vesicles (see also below).

**Sorting of SM and GlcCer in SAC and TGN Prior to Vesicle Release**—The present data reveal that despite the transcytotic processing of apical proteins (12), the Golgi of hepatic cells does display (direct) sphingolipid sorting capacity. This fact gains considerable significance by recent observations that several hepatic apical proteins may reach the apical membrane by direct transport (14, 15, 44), which might link sphingolipid sorting to raft assembly. In this regard, questions have been raised concerning the ability of NBD lipids to partition into rafts, triggered in artificial liposomal systems, given their relative water solubility (45). However, in biological systems it has been shown that exogenous addition of C₆NBD-SM to...
veloping neurons triggered raft domain formation and the apical
sorting of the Thy-1 receptor to axonal domains (46). Other,
but similar, short chain fluorescent (BODIPY) sphingolipid
analogs are sorted in a cholesterol-dependent, i.e. a potentially
raft-mediated mechanism, in fibroblasts derived from patients
suffering from various sphingolipid storage diseases (47).
Nevertheless, further work will be needed to directly clarify these
issues.

The present data are schematically summarized in Fig. 9.
During the C6NBD-ceramide labeling at 18 °C (Fig. 9A, steps 1
and 2), not only C6NBD-SM but also C6NBD-GlcCer accumu-
lated in the Golgi. This differs from LLC-Pk1 cells (23) and
indicates that in liver cells, C6NBD-GlcCer release from the
TGN is also largely vesicular. Inhibition of MRPI activity con-
ﬁrmed previous ﬁndings (23) that transport of C6NBD-SM to
the plasma membrane is exclusively in the luminal leaflet of
transport vesicles (Fig. 9A, step 3a). Because C6NBD-GlcCer is
partly transported to the cytoplasmic leaflet of the basolateral
membrane, prior to its translocation to the exoplasmic leaflet
(Fig. 9A, step 4), the temperature dependence of this process
(>18 °C) may suggest involvement of transport vesicles (Fig.
9A, step 3b). Nevertheless, monomeric transport, possibly
mediated by a glycosphingolipid transfer protein (48), cannot
yet be excluded (Fig. 9A, step 3c). Interestingly, even in presence
of MK571, the majority of the C6NBD-GlcCer became accessible
to BSA after prolonged incubation. These data indicate that
only in an early phase (15–30 min) after de novo synthesis of
GlcCer, may vesicle-independent transport occur. At later
stages, when the lipid has acquired its correct topological
transmembrane distribution, transport of GlcCer is predomi-
nantly of a vesicular nature.

Interestingly, inhibition of the apically localized MDR1 had
no effect on the number of labeled BCs and the accessibility of
the lipids to sodium dithionite, indicating that the sphingolipid
analogs were transported directly to the exoplasmic leaflet of
the BC membrane. Furthermore, inhibition of GlcCer synthesis
reduced the number of labeled BCs, in spite of the fact that the
pool of C6NBD-SM, synthesized in control cells, is more than
3-fold in excess of that of GlcCer. Together, these results imply
that (i) C6NBD-GlcCer is, in relative amounts, the major sphin-
golipid that is transported to the BC membrane, whereas
C6NBD-SM is predominantly transported to the basolateral
plasma membrane (indicated in Fig. 9A, steps 3a and 3d) and
(ii) the majority of apically transported C6NBD-GlcCer is local-
ized in the luminal leaflet of transport vesicles. This is in
contrast to studies on LLC-Pk1 cells, where MDR1 activity was
required for the transport of C6NBD-GlcCer to the outer leaflet
of the apical membrane (23, 27), thus indicating cell type-de-
pendent differences in sphingolipid sorting in polarized cells.

By using the in vitro assay, direct evidence demonstrated
that C6NBD-GlcCer and -SM, after their accumulation in the
SAC, are released in distinct transport vesicles (Fig. 9B). Thus,
TFF, which blocks the exit of C6NBD-SM but not that of
C6NBD-GlcCer from the SAC in intact cells (38), inhibited only
the release of SM-containing vesicles but had no effect on the
release of C6NBD-GlcCer (Fig. 8). This shows that the vesicular
release of C6NBD-SM and -GlcCer is diﬀerentially regulated and
indicates that both sphingolipids are localized in distinct
membrane domains of the SAC, presumably accomplished by
lateral sorting in the luminal leaflet (38). Intriguingly the sucrase fractions enriched in the apically sorted C6NBD-GlcCer
contained DAB-positive cup-shaped vesicles. These are remi-
niscent of the vesicles derived from the common recycling en-
dosome of MDCK cells that contain predominantly the apically
directed polymeric immunoglobulin receptor (7). Because the
fractions of the 30–33% sucrase contain virtually no C6NBD-
GlcCer, we assume that the small DAB-positive vesicles found
there contain exclusively C6NBD-SM, which is predominantly
transported to the basolateral membrane under our experi-
mental conditions. Similarly, in MDCK cells the transferrin
receptor is released from recycling endosomes predominantly
in small vesicles of 60-nm diameter (7). Further work should
clarify whether the C6NBD-SM identiﬁed in the heavier gra-
dient fractions is localized only in the small vesicles that were
occasionally seen or whether it is also localized in the larger
cup-shaped vesicles. If so, another issue would be whether
C6NBD-GlcCer and -SM are present in the same vesicles or
whether they are sorted into distinct but morphologically sim-
ilar vesicles.

Using diﬀerent epithelial cells, it has been shown that de
novo synthesized sphingolipid analogs can be sorted into dis-
tinct transport vesicles at the Golgi (31, 32). However, a more
pronounced sorting capacity of these lipids in the SAC of
HepG2 cells is in line with the fact that in hepatocytes most
newly synthesized apical proteins are transported to the baso-
lateral membrane, prior to their transport to the apical mem-
brane via transcytosis. In WIF-B cells, transcytosis via an
endosomal subapical compartment has been demonstrated
(49), implying that a heavy burden may be placed on the
sorting capacity of the SAC. Cell type-dependent differences in
the relative contribution of distinct sorting sites (SAC versus
TGN) may also explain that sorting of sphingolipid analogs was
not observed in the transcytotic pathway in MDCK cells (50).
Indeed the initial sorting observed for de novo synthesized
sphingolipids was abolished after prolonged incubation times,
indicating that the SAC equivalent in these cells (i.e. the com-
mon endosome) has a lower sorting capacity than in HepG2
cells and underlining the importance to investigate vesicular
transport in diﬀerent cell types to fully understand polarity
establishment. The current approach may provide an excellent
opportunity to analyze the mechanisms that mediate the sort-
ing of sphingolipids and proteins in the SAC (and the TGN), by
careful analysis of the molecular mechanisms that underlie
the formation of the transport vesicles and the regulation of their
release.

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