

Glucosylceramide Is Synthesized at the Cytosolic Surface of Various Golgi Subfractions

Dieter Jeckel,* Achim Karrenbauer,* Koert N. J. Burger,† Gerrit van Meer,† and Felix Wieland*

*Institut für Biochemie I der Universität Heidelberg, Germany; and †Department of Cell Biology, Medical School, University of Utrecht, The Netherlands

Abstract. In our attempt to assess the topology of glucosylceramide biosynthesis, we have employed a truncated ceramide analogue that permeates cell membranes and is converted into water soluble sphingolipid analogues both in living and in fractionated cells. Truncated sphingomyelin is synthesized in the lumen of the Golgi, whereas glucosylceramide is synthesized at the cytosolic surface of the Golgi as shown by (a) the insensitivity of truncated sphingomyelin synthesis and the sensitivity of truncated glucosylceramide synthesis in intact Golgi membranes from rabbit liver to treatment with protease or the chemical reagent DIDS; and (b) sensitivity of truncated sphingomyelin export and insensitivity of truncated glucosylceramide export to decreased temperature and the presence of GTP- γ -S

in semiintact CHO cells. Moreover, subfractionation of rat liver Golgi demonstrated that the sphingomyelin synthase activity was restricted to fractions containing marker enzymes for the proximal Golgi, whereas the capacity to synthesize truncated glucosylceramide was also found in fractions containing distal Golgi markers. A similar distribution of glucosylceramide synthesizing activity was observed in the Golgi of the human liver derived HepG2 cells. The cytosolic orientation of the reaction in HepG2 cells was confirmed by complete extractability of newly formed NBD-glucosylceramide from isolated Golgi membranes or semiintact cells by serum albumin, whereas NBD-sphingomyelin remained protected against such extraction.

THE Golgi apparatus is the site of modification of glycoproteins as well as of sphingolipid synthesis. Glycoproteins and sphingolipids are believed to be transported in vesicles through the Golgi apparatus and to the plasma membrane (reviewed in 24, 34). For our understanding of the molecular mechanisms that underlie biosynthesis and transport of these compounds it is crucial to know the topology of the enzymes involved. A quite detailed concept has evolved as to the distribution of trimming and processing enzymes that act on glycoproteins during their transport from the proximal via medial to the distal Golgi cisternae (19), and recently a corresponding concept has been proposed for the synthesis of glycolipids (28, 36). This concept involves a distribution within the Golgi of glycosyltransferases corresponding to their function in the course of glycosphingolipid biosynthesis: "early" glycosyltransferases, i.e., GlcCer synthase or lactosylceramide synthase, are thought to be restricted to the "early" (proximal) Golgi, whereas "late" reactions (i.e., sialyltransferases) are located in the "late" (distal) Golgi. In the literature there has been a report that suggested the key enzyme of glycosphingolipid synthesis, UDP-glucose-ceramide: glucosyl transferase, to be located at the cytosolic side of the Golgi apparatus (11). However, this suggestion has not been widely accepted probably due to problems intrinsic to the experiments carried out in the study. In particular, the investigation of the topology

of the GlcCer product in the intact membranes was hampered by the limited accessibility of the sphingolipid to externally added glucosylceramidase (<50% after 3 h at 37°C, even in disrupted membranes). Two approaches have been developed to circumvent this problem. First, a substrate for sphingomyelin and glycosphingolipid synthesis has been devised that due to a short fluorescent fatty acyl chain can be "back-exchanged" from a membrane surface to serum albumin or liposomes (20). Second, we have developed a ceramide the products of which intercalate into the lipid layer even less firmly. They are strongly amphiphilic and readily exchange between membrane and aqueous phase in the absence of any acceptor. With this compound, a ceramide that is truncated to a length of eight carbon atoms in both hydrophobic chains (t-Cer)¹, we have characterized the site of sphingomyelin biosynthesis as being the cis-Golgi (15). Predominant localization of this activity to the cis and medial cisternae was independently demonstrated by Futerman and colleagues (12). Moreover, we have used the t-Cer in living CHO cells to generate t-Sph as a luminal marker for the cis Golgi, which has allowed us to access the rate of vesicular flow from this organelle to the plasma membrane (16). Fi-

1. *Abbreviations used in this paper:* t-Cer, (truncated) C8, C8-Ceramide; t-Sph, (truncated) C8, C8-sphingomyelin; t-GlcCer, (truncated) C8, C8-glucosylceramide; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; SL-O, Streptolysin O.

nally, in isolated Golgi membranes t-Cer appeared to be a potent acceptor for glucose from UDP-glucose and for phosphorylcholine from phosphatidylcholine (15, 16) and the resulting truncated sphingolipid analogues were recovered in the aqueous medium.

In the current study, both our assay systems confirm earlier suggestions (11) that the synthesis of glucosyl ceramide occurs at the cytosolic side of the Golgi. In addition, evidence is presented that GlcCer synthesis takes place in the Golgi, where in contrast to Sph-synthesis it is not limited to cisternae containing proximal Golgi markers.

Materials and Methods

Materials

^3H -UDP-galactose (specific activity 47.5 Ci/mmol) and ^3H -UDP-N-acetylglucosamine (specific activity 8.3 Ci/mmol) were purchased from NEN-research products. UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, and FBS from Boehringer (Mannheim, Germany) and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and BSA, fraction V from Sigma Chemical Co. (St. Louis, MO). Culture media and solutions were obtained from Gibco (Glasgow, UK), and culture plastics were from Costar (Cambridge, MA). Streptolysin-O was from Wellcome Diagnostics (Dartford, UK). C8, C8-Ceramide was prepared according to (16). The N-6-[7-nitro-2,1,3-benzoxadiazol-4-yl]aminocaproyl-sphingolipid analogue NBD-Cer was synthesized as described (35).

Fractionation of Golgi

Isolation and Subfractionation of Golgi from Liver. Intact Golgi membranes from rabbit liver were isolated according to (31). Partial separation of Golgi cisternae from rat liver was performed according to (32). Marker enzyme assays were conducted according to the literature: esterase and galactosyltransferase as described by (4) and N-acetylglucosaminylphosphotransferase as reported by (25). Galactosyltransferase activity was 35-fold enriched compared with the starting postnuclear supernatant, t-Sph-synthase activity was 30-fold enriched, and t-GlcCer synthase activity 39-fold, whereas Esterase activity was decreased to one fourth. The overall yield of galactosyltransferase in the Golgi enriched fraction was 25%, of t-Sph-synthase 21%, of t-GlcCer-synthase 27%, and of Esterase <0.2%.

Protein was determined with the Bicinchoninic acid method according to Zhang and Halling (37) after precipitation with TCA (final volume 1 ml, containing 150 μg deoxycholate as a carrier and 80 mg TCA), with BSA as a standard.

Subfractionation of Golgi from HepG2 Cells. The human hepatoma cell line HepG2 (clone a16) was cultured as monolayers on plastic dishes in MEM containing 10% deplemented FBS (17, 27). For experiments monolayers were 80% confluent. Fresh culture medium was added 1 d before the experiment. HepG2 cells were washed with ice-cold homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Hepes, pH 7.4) and scraped into 750 μl of this buffer with a rubber policeman. Subsequently, they were passed 15 times through two 30-gauge needles (30G1/2) connected by 100 mm of polythene tubing of 0.28-mm inner diameter. A post-nuclear supernatant was prepared by a 10-min spin at 375 g_{max} , and the volume was made 1 ml with homogenization buffer. The homogenate was loaded on top of a 10.3 ml 0.7 M-1.5 M linear sucrose gradient (containing 1 mM EDTA and 10 mM Hepes, pH 7.4). Gradients were spun for 3 h at 38,000 rpm (250,000 g_{max}) in a SW41 rotor (Beckman, Palo Alto, CA), after which they were harvested into 11 fractions of 1 ml on ice. Fractions were assayed for enzyme activities immediately, or extracted for lipid analysis.

Treatment of Golgi with Pronase or DIDS

Pronase treatment of membranes (250 μg protein) (protein/protease = 5:1) was performed in 50 mM NaCl, 10 mM EDTA in 10 mM Tris/maleate, pH 7.4, in a total volume of 70 μl at 37°C for different time intervals. After incubation the samples were diluted threefold with ice-cold buffer, overlaid on 1 ml 10 mM Tris/maleate, pH 7.4, containing 500 mM sucrose and 2 mg BSA/ml, and the membranes pelleted by centrifugation (30 min at 170,000 g and 4°C). The pellets were resuspended in 250 mM sucrose in

10 mM Tris/maleate, pH 7.4, and aliquots used for determination of t-Sph- and t-GlcCer-synthesis.

DIDS-treatment as described by (30), with the following modifications: Golgi membranes (50 μg protein) were preincubated with various concentrations of DIDS at 25°C for 10 min in 15 μl of 50 mM NaCl, 10 mM EDTA in 10 mM Tris/maleate, pH 7.4, before addition of ^3H -t-Cer and UDP-glucose. t-Sph and t-GlcCer-synthesis was followed as described above.

Determination of Sphingomyelin and Glucosylceramide Synthesis

A standard assay to follow t-Sph and t-GlcCer synthesis in Golgi membranes contained in a total volume of 20 μl 10–50 μg intact Golgi membranes, 100 μM ^3H -t-Cer (specific activity 150 $\mu\text{Ci}/\mu\text{mol}$), 50 mM NaCl, 10 mM EDTA, 4 mM UDP-glucose in 10 mM Tris/maleate, pH 7.4. This mixture was incubated under vigorous shaking at 37°C for 40 min. Reaction was stopped by addition of 1 vol i-propanol, centrifuged (10,000 g , 2 min) and an aliquot of the mixture analyzed by TLC on Whatman silica gel plates LK 6 in butanone-2/acetone/water/formic acid = 30/3/5/0.04. The chromatograms were evaluated by an automatic TLC-2D-analyzer (digital autoradiograph, Berthold, Wildbad, FRG). The yield of the radioactivity determination in the TLC scanner was calculated by correlation to the ^3H counts found by liquid scintillation counting: a spot of 45,000 counts (counts per hour) is equivalent to 55,000 cpm and to 12.5 pmol of t-sphingolipid. An assay mixture without UDP-glucose led to synthesis of t-Sph exclusively.

Fractions from sucrose density gradients were similarly assayed except that the final volume of the reaction mixture was 12.5 μl and the concentration of t-Cer was 50 μM . With 3.3×10^5 dpm ^3H -t-Cer per assay, fractions with maximal activity incorporated $\sim 3 \times 10^4$ dpm into t-Sph and (in the presence of UDP-Glc) $\sim 4 \times 10^4$ dpm into t-GlcCer.

The synthesis of NBD-Sph and NBD-GlcCer was determined in cell homogenates or gradient fractions from HepG2 cells by the use of the fluorescent precursor NBD-Cer (20), in an assay similar to those described by Constantino-Ceccarini et al. (10) and Futerman et al. (12). To a 250- μl aliquot from each gradient fraction was added 200 μl of NBD-Cer containing liposomes (13 nmol C6-NBD-Cer, 13 mol% in unilamellar phosphatidylcholine liposomes [35]) and 50 μl UDP-glucose (final concentration 500 μM). The mixture was incubated for 15 min at 37°C, and the reaction stopped by the addition of 1.6 ml of ice-cold methanol/chloroform (2.2:1, vol/vol). The fluorescent lipids were extracted according to Bligh and Digher (8). All aqueous solutions were acidified to 10 mM acetic acid. Fluorescent lipids were analyzed by thin layer chromatography in chloroform/acetone/methanol/acetic acid/water, 50:20:10:10:5 (vol/vol). Fluorescence was quantitated as described before (33). A nonfluorescent cell extract gave a background value for the GlcCer or Sph spots equivalent to 1 pmol NBD-Sph. In the enzyme assay using NBD-Cer, the total background of the assay mixture without sample was 3 pmol for Sph and 5 pmol for GlcCer. This amounted to 10 and 20% of the maximal activity measured on gradients, respectively ($\pm 5\%$; $n = 11$).

Assay for Sidedness of NBD-Sph and NBD-GlcCer in Organelles

Incorporation of NBD-lipids into Intact Cells. HepG2 monolayers were washed twice with HBSS without bicarbonate, 10 mM Hepes (4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid), pH 7.4 (HBSS). NBD-Cer was applied to the cells (13 mol% in unilamellar phosphatidylcholine liposomes, 100 nmol NBD-Cer in 4 ml for 9-cm dishes; (35)) for 1 h at 10°C. Under these conditions, the lipid partitions into the cellular membranes and reaches the site where it is metabolically converted to NBD-GlcCer and NBD-Sph. After removal of the liposomes and two washes with HBSS, cells were incubated for 3 h in the presence of 1% (wt/vol) BSA at either 0°, 10°, 15° or 20°C (identical results). The figures represent the mean of 4–10 experiments. After removal of the BSA-containing medium and two washes with HBSS, the cells were homogenized as described above.

Assay for Sidedness

To test for the fraction of the NBD-lipids that was lumenally located in the organelles (see Results) each sample was split into two 500- μl fractions. To the control fraction was added 250 μl of homogenization buffer, and to the test fraction 250 μl of a 20% (wt/vol) BSA solution in the same buffer. The samples were left on ice for 30 min, after which they were fractionated on sucrose gradients as described above.

Permeabilized Cells

Synthesis of *t*-Sph and *t*-GlcCer in and Secretion from Permeabilized CHO-15B Cells. Semiintact CHO-15B cells were prepared by the swelling-scraping method according to Beckers et al. (6). Incubation of semiintact cells at low temperature or in the presence of GTP- γ -S was carried out as described (14). For the determination of *t*-GlcCer synthesis 500 μ M UDP-glucose was added.

Sidedness of NBD-Sph and NBD-GlcCer in Permeabilized HepG2 Cells. After washing HepG2 monolayers three times with ice-cold PBS without Ca^{2+} and Mg^{2+} streptolysin-O (SL-O) was bound for 10 min at 0°C (0.5 U in 0.5 ml per 3-cm dish) (2). Excess SL-O was removed by two washes with PBS without Ca^{2+} and Mg^{2+} , followed by two additional washes with transport buffer (80 mM K^+ -glutamate, 15 mM KCl, 5 mM NaCl, 0.8 mM CaCl_2 , 2 mM MgCl_2 , 1.6 mM EGTA, 20 mM HEPES/KOH, pH 7.2). Permeabilization was carried out by raising the temperature to 37°C for 10 min. After this procedure 100% of the cells were stained by trypan-blue, a stain that cannot permeate intact membranes.

After four washes with transport buffer at 0°C, permeabilized cells were incubated in 1 ml of the buffer containing 1 mM UDP-glucose, an energy-regenerating system (0.5 mM ATP, 2 mM creatine phosphate, and 114 μ g/ml creatine kinase), and NBD-Cer (concentration as above) for 3 h at 12°C. Under these conditions synthesis of NBD-lipids in permeabilized cells was comparable to that in intact cells. Subsequently, the medium was replaced by 1 ml of 3% (wt/vol) BSA in transport buffer and the depletion of cellular NBD-lipids into the BSA-medium was followed in time at 12°C. A control experiment showed that during depletion for 3.5 h, only little additional NBD-lipid synthesis had occurred: 4% for GlcCer, 13% for Sph.

Results

t-GlcCer Synthase Is Accessible to Protease

A widely applied method to study the topology of membrane proteins is to assay the sensitivity to digestion with proteases of the protein under study. We have investigated the influence of protease treatment on the *t*-GlcCer synthesizing activity of

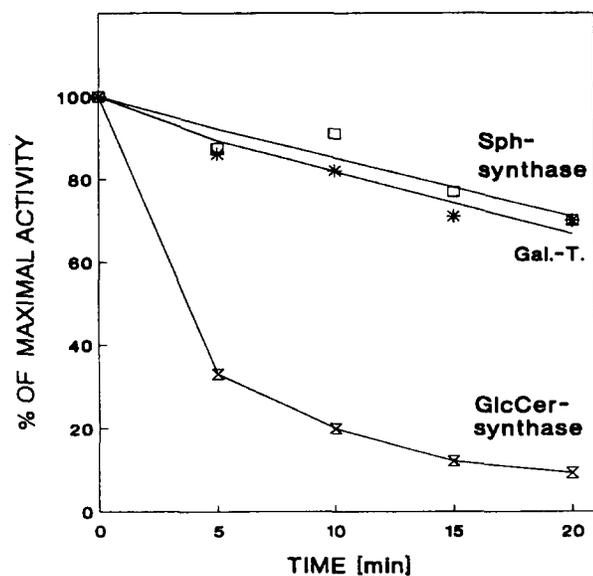


Figure 1. GlcCer synthase in intact membranes is accessible to protease. Intact Golgi membranes were incubated with pronase E at 37°C, and at the time points indicated samples were diluted with ice-cold buffer (containing an excess of BSA) and centrifuged through a layer of buffered sucrose solution containing 2 mg BSA/ml. The pellets were resuspended and aliquots used for the determination of *t*-Sph-synthase activity (\square — \square); galactosyltransferase activity ($*$ — $*$); and *t*-GlcCer synthase activity (\times — \times). For experimental details see Materials and Methods.

rabbit liver Golgi membranes. The effects of pronase E treatment on the *t*-GlcCer synthase and the *t*-Sph synthase activities (and, as a control, luminal galactosyltransferase) were followed in time. The results are shown in Fig. 1. After 20 min, *t*-GlcCer synthase is depressed to <10% of its original activity, whereas *t*-Sph synthase and the activity of the luminal enzyme galactosyltransferase are only slightly affected.

GlcCer Synthase Is Inhibited by a Nonmembrane-permeable Drug

DIDS has been used to block various membrane channels and proteins (11, 30). The drug covalently binds to basic amino acid residues and thereby inactivates enzymes in a quite unspecific manner. It has been shown that the compound is unable to penetrate membranes. If inhibition of an enzyme is observed after addition of the drug to isolated intact membranes it is likely that the active center of this enzyme is localized at the accessible (cytosolic) side of the membrane. We have incubated intact isolated Golgi membranes with increasing concentrations of DIDS and subsequently measured their *t*-GlcCer as well as *t*-Sph synthase activities. The results are shown in Fig. 2. Sphingomyelin synthase is not affected up to concentrations of 200 μ M of the drug. In contrast, GlcCer synthase activity is decreased to 50% at a DIDS concentration of \sim 60 μ M.

t-GlcCer Is Released from Semiintact Cells under Conditions Where *t*-Sph Is Retained

Permeabilized (semiintact) (29) cells have been shown to retain the ability to expedite vesicular biosynthetic transport from the ER to the Golgi (6) and to the plasma membrane (14). *t*-Sph has served as a luminal marker for monitoring the flow of vesicles from the proximal Golgi to the plasma

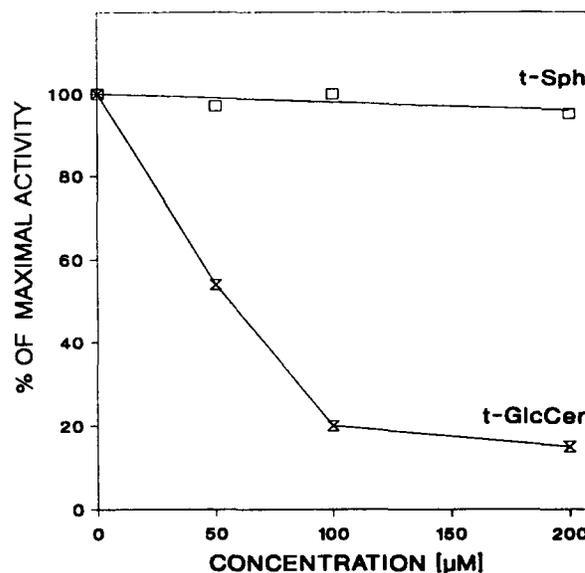


Figure 2. Decrease of GlcCer synthase activity by DIDS, a protein modifying reagent that cannot pass membranes. Golgi membranes were incubated with increasing concentration of DIDS for 10 min at 25°C. Thereafter, the activities of *t*-GlcCer synthesis (\times — \times) and of *t*-Sph synthesis (\square — \square) were determined as described in Materials and Methods.

membrane in permeabilized as well as in intact CHO cells (14). It has been shown that vesicular transport is inhibited by lowering the temperature (21) as well as by the addition of the nonhydrolyzable GTP analogue GTP- γ -S (22, 23, 36). t-Sph-transport in semiintact cells is vesicular, because it is inhibited by lowering the temperature to 15°C or by the presence of GTP- γ -S (14). We have investigated the secretion of t-GlcCer under these conditions. Both markers are secreted at 37°C (14), whereas at 15°C t-Sph is efficiently retained (Fig. 3 A). In contrast, at this temperature t-GlcCer is released into the medium to an extent indistinguishable from the 37°C control experiment (Fig. 3 B).

In nonpermeabilized, living cells at 15°C both truncated sphingolipids, t-Sph and t-GlcCer are retained to about the same extent (Fig. 3, C and D).

As shown in Fig. 4, in the presence of GTP- γ -S, t-Sph ex-

port is clearly reduced in permeabilized cells at 37°C (Fig. 4 A). In contrast, t-GlcCer secretion is insensitive to this nucleotide (Fig. 4 B).

Distribution of t-GlcCer Synthase within the Golgi Rat Liver Apparatus

The ease with which t-GlcCer synthase can be determined in isolated intact membranes allowed us to localize the activity on sucrose density gradients. To this end, rat liver Golgi was subjected to sucrose gradient fractionation (32). In Fig. 5, a distribution of enzyme activities is shown that reside in the ER (3), the proximal Golgi (13), and the distal Golgi (26). Although no baseline separation was achieved, the marker enzyme activities reproducibly appear in distinct peaks (Fig. 5 B), shifted from each other. As shown earlier

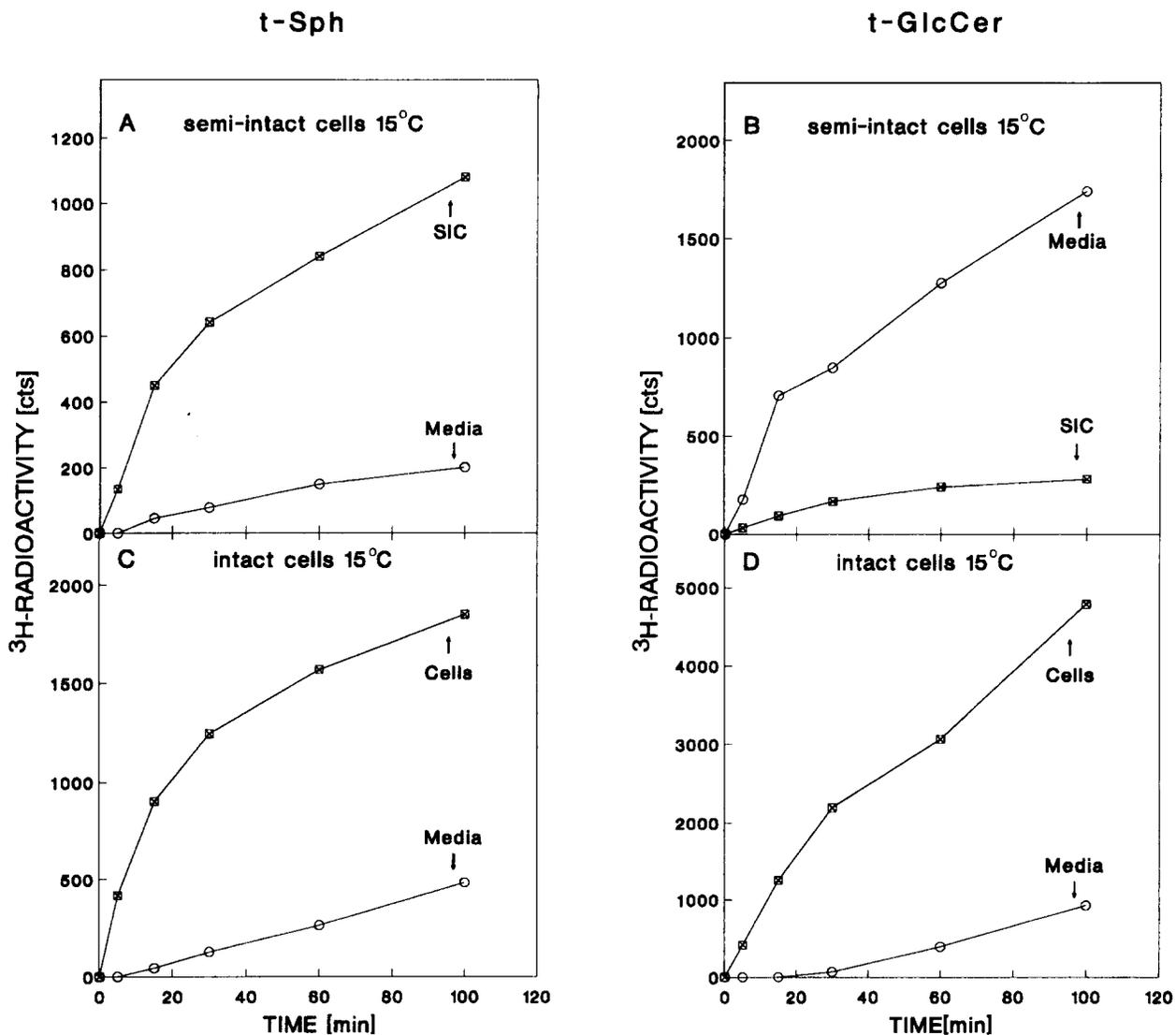


Figure 3. t-GlcCer is not retained in semiintact cells at 15°C. CHO 15B cells were permeabilized according to Beckers et al. (6) and aliquots of the resuspended semiintact cells were incubated with ^3H -t-Cer at the temperatures and for the times indicated. ^3H -t-Sph and ^3H -t-GlcCer were assayed as described in Materials and Methods. \circ - \circ shows the content of the ^3H -t-sphingolipid in the media, and \square - \square shows that in the semiintact cells (SIC) (A and B). As a control, intact CHO 15B cells were incubated with ^3H -t-Cer at 15°C, and ^3H -t-sphingolipids determined in the cells (\square - \square) and media (\circ - \circ) (C and D).

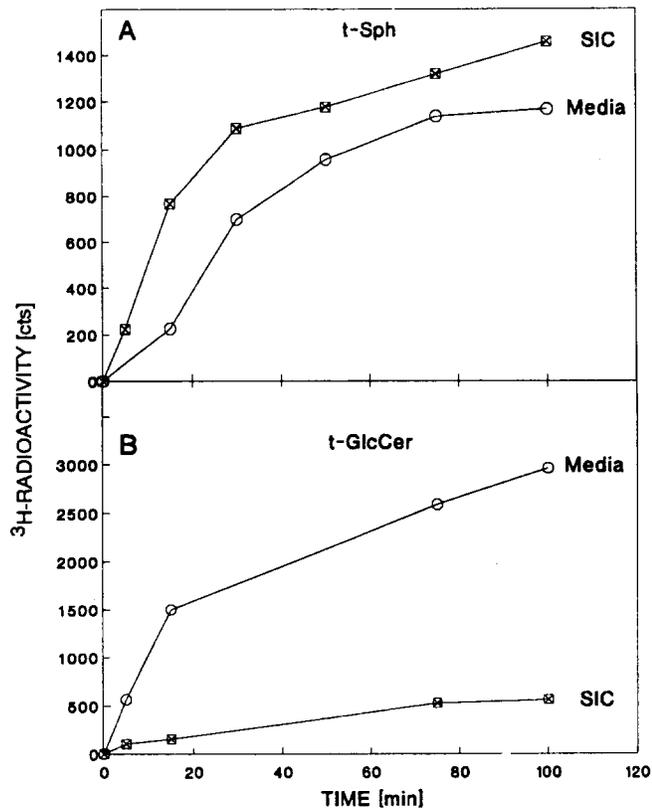


Figure 4. GTP- γ -S does not inhibit secretion of t-GlcCer in semi-intact cells at 37°C. CHO 15B cells were permeabilized according to Beckers et al. (6). The semiintact cells were incubated in the presence of 100 μ M GTP- γ -S as described (14), and the individual 3 H-t-sphingolipids quantitated in aliquots taken at the time points indicated. (○—○) media; (□—□) semiintact cells.

(15), t-Sph synthesis follows the profile of the proximal Golgi marker enzyme N-acetyl-glucosaminylphosphotransferase, while in studies by Futerman et al. (12) cofractionation of Sph-synthase was observed with the proximal Golgi marker mannosidase II. Although t-GlcCer synthase activity is observed along the phosphotransferase peak as well, a second peak of t-GlcCer synthase is obtained that coincides with that of galactosyltransferase, a distal Golgi marker (Fig. 5 A). The protein profile of the gradient is shown in Fig. 5 C. In four experiments the separation achieved was as shown in Fig. 5 A, whereas in eight experiments only one instead of two fractions was present between the proximal and distal activity peaks. The assays of t-Sph-synthase and t-GlcCer synthase activities were performed under conditions where the substrate t-Cer was not limiting as determined by the amount of t-Cer left in each incubation after the assay, which was in excess and therefore about the same in each fraction.

To exclude that these findings are specific to the cells and the assay system used, we have extended this study by the application of an established marker for sphingolipid synthesis, NBD-Ceramide (20), to another established liver cell system, the human hepatoma HepG2. HepG2 cells were incubated with NBD-Cer, and after breaking the cells the distribution of the NBD-lipid-derivatives along a sucrose density gradient was determined in the presence or absence of scavenger BSA in the homogenization buffer. Lipids in the

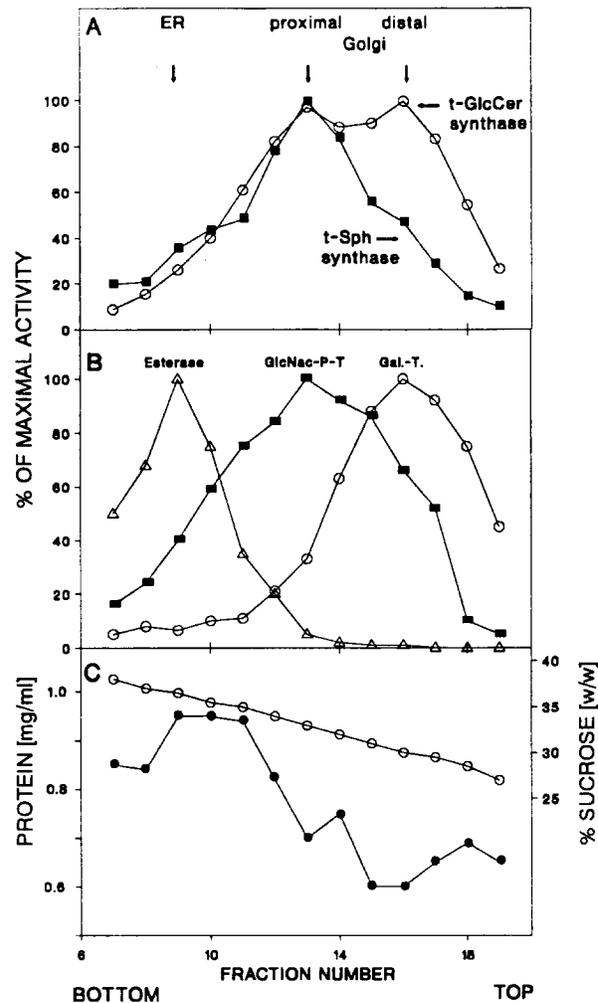


Figure 5. Partial separation of t-GlcCer- and t-Sph synthase activities in rat liver Golgi. Rat liver Golgi membranes were submitted to sucrose density gradient centrifugation as described (32), and 3 H-t-Sph (■—■) and 3 H-t-GlcCer synthase (○—○) activities were assayed (A). The gradient was characterized by measuring the activities of marker enzymes for the ER (Esterase; ref. 4) (Δ — Δ) for the proximal Golgi (GlcNAc-phosphotransferase; ref. 25) (■—■) and for the distal Golgi (galactosyltransferase; ref. 4) (○—○) (B). In C the protein content (●—●) and the sucrose concentration of the fractions (○—○) are shown.

outer leaflet of an organelle that come into contact with BSA will bind to the protein and therefore stay with the soluble protein fraction on top of the gradient, whereas lipids in the inner leaflet of a membrane are protected against depletion by the BSA and will migrate with the membrane fraction. Fig. 6 shows the gradient profiles obtained. In Fig. 6 A the distribution of free NBD-Cer is given in the presence or absence of BSA. In the absence of the scavenger protein, the bulk amount of NBD-Cer migrates to the position where most of the membranes are, i.e., about two thirds down the gradient. In contrast, in the presence of BSA most of the NBD-Cer is found on top of the gradient. Fig. 6, B and C shows the distribution of the GlcCer- and Sph-derivatives, respectively. Most of the GlcCer analogue runs with the bulk of the membranes and is accessible to BSA, whereas the

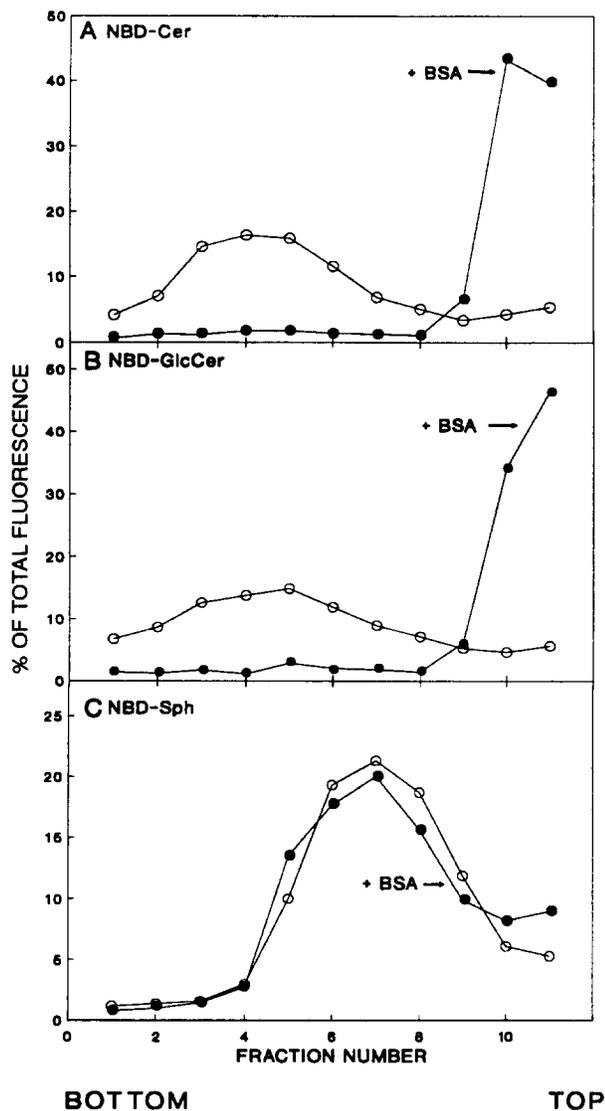


Figure 6. Sidedness of NBD-sphingolipids in HepG2 cells. HepG2 cells were incubated with NBD-Cer and homogenized. The fraction of the newly synthesized NBD-sphingolipids present on the cytoplasmic surface of the Golgi was quantitated by assaying its accessibility to the scavenger protein BSA. The postnuclear supernatant was incubated in the presence or absence of BSA for 30 min at 0°C, and then fractionated on a sucrose gradient. The individual NBD-lipids in each fraction were quantitated. (○—○) No scavenger protein added; (●—●) BSA added to the homogenization buffer.

main fraction of the Sph-derivative migrates with the membranes to the Golgi area of the gradient both in the presence and absence of BSA. These findings indicate that the NBD-Sph is located at the inner, luminal leaflet and NBD-GlcCer at the outer, cytosolic leaflet of the membranes, in full accordance with the results obtained in CHO cells with the truncated sphingolipid analogues. The sidedness of GlcCer versus Sph was assayed in an additional experiment in permeabilized HepG2 cells, as shown in Fig. 7. After treatment with streptolysin-O, HepG2 cells were incubated with NBD-Cer at 12°C for 3 h. Thereafter, the ceramide-containing

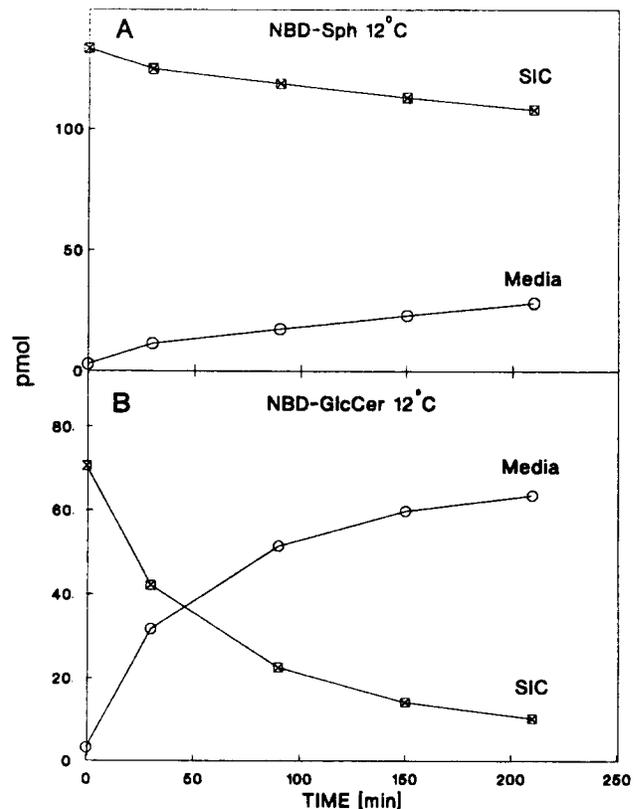


Figure 7. NBD-Cer is not retained in semiintact cells at 12°C. HepG2 cells were permeabilized with streptolysin O, and incubated with NBD-Cer for 3 h at 12°C to allow for NBD-sphingolipid synthesis. Starting at $t = 0$, at each time point the medium was replaced by a buffer containing 3% BSA. NBD-lipids in media and cells were assayed as described under Materials and Methods. (○—○) shows the content of NBD-lipids in the media; (□—□) shows that in the semiintact cells (SIC). Each curve represents the mean of two experiments, with deviations less than the symbol size.

medium was replaced by a medium containing BSA as a scavenger. More than 85% of the NBD-GlcCer was recovered in the BSA-containing medium, whereas only 20% of the NBD-Sph was accessible to the scavenger.

The distribution of sphingomyelin synthase and UDP-glucose/ceramide glucosyltransferase activities (as assayed with NBD-Cer) along a sucrose density gradient after application of a total postnuclear supernatant is shown in Fig. 8. Both sphingolipid activities are clearly separated from the maximum of the ER marker and, like in subfractionated rat liver Golgi (see Fig. 5 A), GlcCer synthase shows two maxima whereas Sph synthase runs essentially in a single peak.

Discussion

Both the protease digestion and the protein modification experiments shown here confirm earlier findings by Coste et al. (11), and suggest a cytosolic orientation of the GlcCer synthase enzyme. However, neither type of experiment excludes the possibility that the active site of the enzyme is lu-

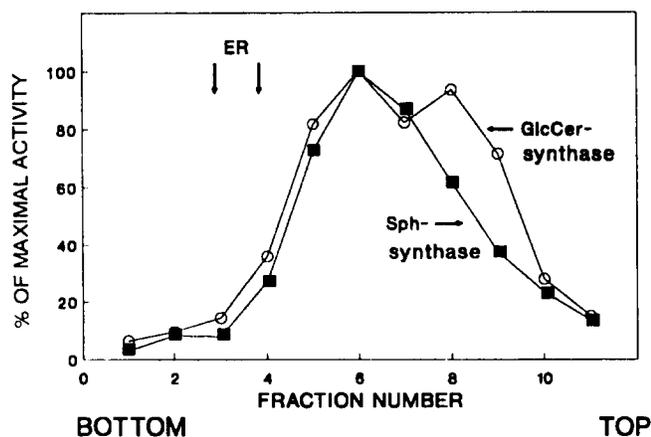


Figure 8. NBD-Sph- and NBD-GlcCer synthase activities in Golgi membrane fractions after separation by sucrose density gradient centrifugation. HepG2-postnuclear supernatant was subjected to sucrose density gradient centrifugation as described in Materials and Methods. Esterase activity was determined as an ER-marker. The fractions with maximal esterase activities are indicated with arrows. Sph- (■—■) and GlcCer synthase (○—○) activities were determined by an NBD-Cer assay as described in Materials and Methods.

minal, and that the enzyme is inhibited by nicks or covalent modifications introduced into its cytosolic moiety by protease or protein modification reagents, respectively. Therefore, we have investigated the topology of sphingolipid biosynthesis in experiments that do not aim to modify the enzymes involved but rather to quantitate the amount of sphingolipids produced at either side of the Golgi membrane system. This could be achieved on the one hand by combining semiintact CHO cells with the truncated ceramide that gives rise to water soluble truncated sphingomyelin and glucosyl ceramide, and on the other hand by the use of permeabilized or homogenized HepG2 cells combined with short chain NBD-sphingolipids that can be "back-exchanged" from membrane surfaces by BSA. In previous work we have shown that the truncated Sph and GlcCer analogues do not permeate membranes but, rather, remain trapped in membrane vesicles (14, 16). The same is true for the NBD-sphingolipid analogues (7). In addition, neither of these lipids is released from intact cells during mitosis (18), nor when they are synthesized at a temperature where vesicular transport to the plasma membrane is blocked (15°C). Although at this temperature vesicular transport is blocked in semiintact cells as well, the t-GlcCer analogue and NBD-GlcCer in the presence of BSA diffuse out of the cell and are recovered in the medium. In contrast, the Sph analogues remain in the Golgi. This indicates that the latter analogues are synthesized in the Golgi lumen, and GlcCer on the cytosolic side of the organelle.

GlcCer serves as the precursor for virtually all complex glycosphingolipids including the gangliosides. First, a galactosyl moiety is added to GlcCer to yield LacCer. This and the subsequent reactions in the assembly are likely to be localized in the lumen of the Golgi: cells with a mutation in the Golgi UDP-Gal carrier can only synthesize GlcCer (9).

To make GlcCer available for the galactosyl transferase a carrier must be postulated that would translocate newly synthesized GlcCer from the cytosolic leaflet to the luminal leaflet of the organelle (11). In addition, translocation of GlcCer may be required for its transport to and expression on the cell surface. It has been observed previously that part of the t-GlcCer and NBD-GlcCer was Golgi associated under conditions not very different from the present ones (16, 33). We are directing our present efforts at defining the requirements for translocation to the Golgi lumen and the exact location of this event. It is unlikely that the translocator is also present in the plasma membrane. In that case, t-GlcCer and NBD-GlcCer should have been able to leave the cell even in the absence of vesicular transport at low temperature or in mitotic cells, which is not observed (see above). After synthesis, endogenous "normal" long chain GlcCer cannot escape the Golgi membrane because its long hydrophobic chains are tightly embedded into the bilayer. It can therefore efficiently reach, and be translocated by, the carrier. In contrast, the t-GlcCer derivative is not restricted to the bilayer but in equilibrium with the cytosol and might thus escape the translocation event. Similarly, NBD-GlcCer will not be limited to the cytosolic surface of the Golgi but equilibrate with other intracellular membranes. This was indeed observed after cell fractionation (Fig. 6B) where NBD-GlcCer was located in other membranes than the synthesizing activity (Fig. 8). Nevertheless, translocation of both GlcCer analogues is efficient since they are rapidly transported to the surface of intact cells (16, 20, 33, 35). This may be explained by the fact that the volume of the cytosol is relatively small, which would guarantee a significant concentration of t-GlcCer in the Golgi membrane. A similar argument applies to the NBD-GlcCer. In semiintact cells, however, the cytosol can exchange freely with the surrounding medium. Under this condition t-GlcCer is diluted several hundredfold while NBD-GlcCer will equilibrate with the excess of scavenger BSA in the medium. The analogues will thus have a correspondingly smaller chance to be translocated into the lumen of the Golgi. Consequently they will diffuse out of semiintact cells even at low temperature.

A variety of Golgi enzymes have been ascribed to different subsets of the Golgi apparatus. For the analysis of Golgi subsites we have fractionated the Golgi on sucrose gradients and followed the activities of N-acetylglucosaminyl-phosphotransferase (25) and of galactosyltransferase (26), established markers of the proximal and the distal Golgi, respectively. Although baseline separation of these marker enzyme activities was not achieved, the activity peaks are clearly and reproducibly shifted from each other. The maximal activities of t-Sph and t-GlcCer synthesis coincide with the proximal Golgi. For Sph this confirms earlier work (12, 15). A second peak of glucosylceramide synthesis activity is clearly and reproducibly separated and appears at a buoyant density similar to that of membranes enriched in galactosyltransferase, a "late" Golgi enzyme.

Our finding that part of the GlcCer may be synthesized on the distal Golgi would contradict the straight forward hypothesis that the sequence of biosynthetic events correlates with the localization of the enzymes involved, i.e., "early" enzymes located to the "early" proximal Golgi, and "late" enzymes to the "late" distal Golgi because the possibility exists

that the late Golgi compartment displaying GlcCer synthesis may be distal to the galactosyl-transferase compartment. On the one hand, GlcCer synthesized there may be accessible for the postulated carrier protein and translocated to the luminal side, after which it would be transported as GlcCer to the cell surface. This mechanism would easily explain the fact that an appreciable part of the glycolipid escapes further sugar transfer reactions and is expressed at the cell surface as the monohexosylceramide. On the other hand, it may be subject to cytosolic transfer by specific proteins (1) and thus be delivered to those cisternae containing the galactosyl-transferase. In this way the glucolipid would be available to higher glycolipid synthesizing compartments according to need because with a GlcCer carrier the cytoplasmic leaflet of these membranes would become depleted of the glucolipid at the same pace galactosyl transfer occurs in the lumen to yield lactosylceramide.

The results obtained with CHO cells and both rat and rabbit liver Golgi using t-Cer have been reproduced with human liver derived HepG2 cells and NBD-ceramide, indicating that our findings are not restricted to a special assay or cell system. Earlier data (11) and the data presented here together present strong evidence that glucosylceramide is in fact synthesized at the cytosolic surface of the Golgi apparatus. Localization studies by sucrose density gradient centrifugation have revealed different patterns of sphingomyelin and glucosylceramide synthase activities with glucosylceramide synthase found in both a fraction containing a proximal Golgi marker and a fraction colocalizing with a distal Golgi marker. These findings raise a number of questions concerning the intracellular distribution of GlcCer, the exact location of its synthesis and of the translocation event. Purification of the proteins involved and the use of immunoelectron microscopy will be required for their precise localization. Unfortunately, we are not aware of the existence of antibodies against the synthases. Apart from the fact that GlcCer is the most abundant glycosphingolipid in most cells and that its distribution may be important for the physicochemical properties of specific membranes or membrane leaflets, it is the precursor for most higher glycosphingolipids including all gangliosides. There is good hope that further work on the synthesis, translocation, and transport of glucosylceramide will lead to a better understanding of the organization and regulation of glycosphingolipid expression on the cell surface.

Independent of the present work, similar conclusions on the sidedness of GlcCer synthesis have been reached by Futerman and Pagano (Futerman, A. H., and R. E. Pagano. 1991. *Biochem. J.* 280:295-302) and by Trinchera and colleagues (Trinchera, M., M. Fabbri, and R. Ghidoni. 1991. *J. Biol. Chem.* 266:20907-20912). Futerman and Pagano furthermore observed that GlcCer synthesis was more widely distributed than the cis/medial Golgi cisternae and suggested significant synthesis in a pre- or early Golgi compartment. Trinchera et al. (1991) reported that besides GlcCer synthesis also the subsequent transfer of galactose to yield lactosyl ceramide occurred on the cytosolic surface of the Golgi apparatus.

We thank S. Adrian, I. Meißner, and G. Weiß for their expert technical assistance, and B. Schröter and I. Speckard for their help with the manuscript.

The work was supported by the Deutsche Forschungsgemeinschaft (SFB 352).

Received for publication 30 September 1991 and in revised form 2 December 1991.

References

1. Abe, A., and T. Sasaki. 1985. Purification and some properties of the glycolipid transfer protein from pig brain. *J. Biol. Chem.* 260:11231-11239.
2. Ahnert-Hilger, G., W. Mach, K. J. Fohr, and M. Gratzl. 1989. Poration by alpha toxin and streptolysin O: an approach to analyze intracellular processes. *Methods Cell Biol.* 31:63-90.
3. Beaufay, H., A. Amar-Costesec, D. Thinh-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. *J. Cell Biol.* 61:213-231.
4. Beaufay, H., A. Amar-Costesec, E. Feytmans, D. Thinh-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. *J. Cell Biol.* 61:188-200.
5. Beckers, C. J. M., and W. E. Balch. 1989. Calcium and GTP: Essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. *J. Cell Biol.* 108:1245-1256.
6. Beckers, C. J. M., D. S. Keller, and W. E. Balch. 1987. Semi-intact cells permeable to macromolecules: Use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell.* 50:523-534.
7. Bennett, M. K., A. Wandinger-Ness, and K. Simons. 1988. Release of putative exocytic transport vesicles from perforated MDCK cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:4075-4085.
8. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911-917.
9. Brändli, A. W., G. C. Hansson, E. Rodriguez-Boulan, and K. Simons. 1988. A polarized epithelial cell mutant deficient in translocation of UDP-galactose into the Golgi complex. *J. Biol. Chem.* 263:16283-16290.
10. Costantino-Ceccarini, E., and A. Cestelli. 1981. A novel assay method for the biosynthesis of galactosyl- and glucosylceramides. *Methods Enzymol.* 72:384-391.
11. Coste, H., M. B. Martel, and R. Got. 1986. Topology of glucosylceramide synthesis in Golgi membranes from porcine submaxillary glands. *Biochim. Biophys. Acta.* 858:6-12.
12. Futerman, A. H., B. Stieger, A. L. Hubbard, and R. E. Pagano. 1990. Sphingomyelin synthesis in rat liver occurs predominantly at the cis and medial cisternae of the Golgi apparatus. *J. Biol. Chem.* 265:8650-8657.
13. Goldberg, D. E., and S. Kornfeld. 1983. Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. *J. Biol. Chem.* 258:3159-3165.
14. Helms, J. B., A. Karrenbauer, K. W. A. Wirtz, J. E. Rothman, and F. T. Wieland. 1990. Reconstitution of steps in the constitutive secretory pathway in permeabilized cells. *J. Biol. Chem.* 265:20027-20032.
15. Jeckel, D. A., A. Karrenbauer, R. Birk, R. R. Schmidt, and F. T. Wieland. 1990. Sphingomyelin is synthesized in the cis Golgi. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 261:155-157.
16. Karrenbauer, A., D. Jeckel, W. Just, R. Birk, R. R. Schmidt, J. E. Rothman, and F. T. Wieland. 1990. The rate of bulk flow from the Golgi to the plasma membrane. *Cell.* 63:259-267.
17. Knowles, B. B., C. C. Howe, and D. P. Aden. 1980. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science (Wash. DC).* 209:497-499.
18. Kobayashi, T., and R. E. Pagano. 1989. Lipid transport during mitosis. Alternative pathways for delivery of newly synthesized lipids to the cell surface. *J. Biol. Chem.* 264:5966-5973.
19. Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631-664.
20. Lipsky, N. G., and R. E. Pagano. 1985. Intracellular translocation of fluorescent sphingolipids in cultured fibroblasts: endogenously synthesized sphingomyelin and glucocerebroside analogues pass through the Golgi apparatus en route to the plasma membrane. *J. Cell Biol.* 100:27-34.
21. Matlin, K. S., and K. Simons. 1983. Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell.* 34:233-243.
22. Melancon, P., B. S. Glick, V. Malhotra, P. J. Weidman, T. Serafini, M. L. Gleason, L. Orci, and J. E. Rothman. 1987. Involvement of GTP-binding "G" proteins in transport through the Golgi stack. *Cell.* 51:1053-1062.
23. Miller, S. G., and H.-P. H. Moore. 1991. Reconstitution of constitutive secretion using semi-intact cells: regulation by GTP but not calcium. *J. Cell Biol.* 112:39-54.
24. Pfeffer, S. R., and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56:829-852.
25. Reitman, M. L., and S. Kornfeld. 1981. Lysosomal enzyme targeting. *J. Biol. Chem.* 256:11977-11980.
26. Roth, J., and E. G. Berger. 1982. Immunocytochemical localization of galactosyl-transferase in HeLa cells: codistribution with thiamine pyrophosphatase in trans-Golgi cisternae. *J. Cell Biol.* 93:223-229.
27. Schwartz, A. L., S. E. Fridovich, B. B. Knowles, and H. F. Lodish. 1981. Characterization of the asialoglycoprotein receptor in a continuous hepatoma line. *J. Biol. Chem.* 256:8878-8881.

28. Schwarzmann, G., and K. Sandhoff. 1990. Metabolism and intracellular transport of glycosphingolipids. *Biochemistry*. 29:10865-10871.
29. Simons, K., and H. Virta. 1987. Perforated MDCK cells support intracellular transport. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2241-2247.
30. Spiro, M. J., and R. G. Spiro. 1985. Effect of anionspecific inhibitors on the utilization of sugar nucleotides for N-linked carbohydrate unit assembly by thyroid endoplasmic reticulum vesicles. *J. Biol. Chem.* 260: 5808-5815.
31. Tabas, I., and S. Kornfeld. 1979. Purification and characterization of a rat liver Golgi α -mannosidase capable of processing asparagine-linked oligosaccharides. *J. Biol. Chem.* 254:11655-11663.
32. Trinchera, M., and R. Ghidoni. 1989. Two glycosphingolipid sialyltransferases are localized in different sub-Golgi compartments in rat liver. *J. Biol. Chem.* 264:15766-15769.
33. van't Hof, W., and G. van Meer. 1990. Generation of lipid polarity in intestinal epithelial (Caco-2) cells: Sphingolipid synthesis in the Golgi complex and sorting before vesicular traffic to the plasma membrane. *J. Cell Biol.* 111:977-986.
34. van Meer, G. 1989. Lipid traffic in animal cells. *Annu. Rev. Cell Biol.* 5:247-275.
35. van Meer, G., E. H. K. Stelzer, R. W. Wijnaendts-van-Resandt, and K. Simons. 1987. Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. *J. Cell Biol.* 105:1623-1635.
36. Wattenberg, B. W. 1990. Glycolipid and glycoprotein transport through the Golgi complex are similar biochemically and kinetically. Reconstitution of glycolipid transport in a cell free system. *J. Cell Biol.* 111:421-428.
37. Zhang, J. X., and P. Halling. 1990. pH and buffering in the bicinchoninic acid (4,4'-dicarboxy-2,2'-biquinoline) protein assay. *Anal. Biochem.* 188:9-10.