

Transport of sphingomyelin to the cell surface is inhibited by brefeldin A and in mitosis, where C₆-NBD-sphingomyelin is translocated across the plasma membrane by a multidrug transporter activity

Ardy van Helvoort¹, Maria Luisa Giudici², Marion Thielemans¹ and Gerrit van Meer^{1,*}

¹Department of Cell Biology, Medical School AZU H02.314, Institute of Biomembranes, University of Utrecht, 3584 CX Utrecht, The Netherlands

²Department of Biomedical Sciences and Biotechnology, University of Brescia, Brescia, Italy

*Author for correspondence

SUMMARY

Sphingomyelin is a major lipid of the mammalian cell surface. The view that sphingomyelin, after synthesis in the Golgi lumen, reaches the outer leaflet of the plasma membrane on the inside of carrier vesicles has been challenged by inconsistencies in the results of transport studies. To investigate whether an alternative pathway to the cell surface exists for sphingomyelin, brefeldin A and mitotic cells were used to block vesicular traffic between the Golgi complex and the plasma membrane. Exogenous sphingomyelinase was applied in the cold to assay for the presence of sphingomyelin on the surface of CHO cells. Newly synthesized radiolabeled sphingomyelin was found to equilibrate with cell surface sphingomyelin within 1.5 hours at 37°C. Brefeldin A and mitosis inhibited this transport but, surprisingly, not the surface appearance of

the short-chain sphingomyelin analog *N*-6[7-nitro-2,1,3-benzoxadiazol-4-yl]aminohexanoyl(C₆-NBD)-sphingomyelin as assayed by depletion of this lipid in the medium by the scavenger albumin. Transport of C₆-NBD-sphingomyelin in the presence of brefeldin A was blocked by cyclosporin A and PSC 833, inhibitors of the multidrug resistance P-glycoprotein. The same was observed in HepG2 and HeLa cells, and for short-chain glucosylceramide, which demonstrates the general nature of the transporter-dependent sphingolipid translocation across the plasma membrane.

Key words: Sphingomyelin, Glucosylceramide, Glycosphingolipid, MDR, P-glycoprotein, Flippase

INTRODUCTION

Sphingomyelin (SM) is a major lipid in the plasma membrane of mammalian cells. Besides a structural function as a bilayer-stabilizing lipid, it fulfils an important role as the precursor for the lipid second messenger ceramide in the so-called 'sphingomyelin cycle' (Hannun and Obeid, 1995; Kolesnick and Fuks, 1995). Concerning the cellular organization of SM, the prevailing view over the last decade has been that SM is essentially limited to the exoplasmic leaflet of the Golgi, the plasma membrane and endosomes, that it cannot translocate towards the cytosolic leaflet, and that it can therefore only be transported between organelles on the inside of carrier vesicles (Koval and Pagano, 1991). Indeed, data on endocytosis and recycling of SM analogs suggest that exogenously inserted SM remains exclusively oriented towards the lumen of the endocytotic organelles (Kok et al., 1991; Koval and Pagano, 1991; Mayor et al., 1993; Presley et al., 1993). However, from studying the properties of the pool of SM that is hydrolyzed during signal transduction it has been proposed that a significant fraction of the plasma membrane SM is organized in its cytosolic leaflet (Linardic and Hannun, 1994; Andrieu et al., 1996).

SM is synthesized by the enzyme phosphatidylcholine:ceramide cholinephosphotransferase (SM synthase) on the luminal side of the Golgi apparatus (Futerman et al., 1990; Jeckel et al., 1990). Although the original studies could not exclude the presence of a fraction of the enzyme in endosomes (Allan and Kallen, 1994) we have found no evidence for this in a study where we discriminated endosomes from Golgi membranes (A. van Helvoort, unpublished). In contrast, part of the SM synthase activity has been located on the cell surface (Futerman et al., 1990; van Helvoort et al., 1994). Since ceramide is synthesized in the ER, the Golgi SM synthase seems responsible for the synthesis of new SM while the activity on the cell surface may regulate the relative concentrations of the lipid second messengers ceramide and diacylglycerol during signal transduction processes.

Essentially no translocation of newly synthesized SM has been observed from the luminal to the cytosolic leaflet of the Golgi membrane (Helms et al., 1990; Karrenbauer et al., 1990; Jeckel et al., 1992). Although SM translocation has been measured in the ER (Herrmann et al., 1990; Buton et al., 1996), the low molar percentage of SM in the ER and in the ER-Golgi intermediate compartment (Schweizer et al., 1994) seems to suggest that little SM is transported along the retrograde

pathway from Golgi to ER. Anterograde transport of SM from the Golgi to the plasma membrane appears to be mediated by transport vesicles from the following observations: (i) SM analogs have been found on the inside of transport vesicles (Kobayashi et al., 1992; Babia et al., 1994). (ii) Transport of the SM analog C₆-NBD-SM to the cell surface of epithelial cells was inhibited by the microtubule-depolymerizing drug nocodazole to the same extent as transport of proteins (van Meer and van 't Hof, 1993). (iii) In mitotic cells, where vesicular traffic from the Golgi is blocked (Warren et al., 1995), the SM analog C₆-NBD-SM no longer reached the outside of the plasma membrane (Kobayashi and Pagano, 1989). (iv) In the presence of brefeldin A (BFA), where no vesicular traffic between Golgi and plasma membrane occurs (Klausner et al., 1992), SM transport was to a large extent inhibited as measured by cell fractionation (Warnock et al., 1994), by a sphingomyelinase (SMase) assay (Kallen et al., 1993), and by the reduced release of a water-soluble SM analog (Brüning et al., 1992). Remarkably, in contrast with these findings, BFA did not inhibit transport to the cell surface of [³H]choline-labeled SM in primary hepatocytes (Shiao and Vance, 1993) nor of C₆-NBD-SM in HepG2 cells (van Meer and van 't Hof, 1993).

In the present study we show that transport of SM to the surface of CHO cells is inhibited by BFA and in mitosis, whereas transport of C₆-NBD-SM continues. The latter process is sensitive to inhibitors of the multidrug transporter P-glycoproteins. The results reveal an unexpected transport pathway for short-chain SM analogs involving a multidrug transporter activity.

MATERIALS AND METHODS

Materials

Nocodazole, BFA, Hoechst 33258, thymidine, SMase (*Staphylococcus aureus*), UDP-glucose, verapamil, progesterone and BSA fraction V were purchased from Sigma (St Louis, MO). Okadaic acid was from LC Services Corp. (Woburn, MA), cyclosporin A from Sandoz (Nürnberg, Germany) and PSC 833 was a kind gift from Dr D. Cohen, Sandoz (New Jersey, NJ). C₆-NBD-ceramide was from Molecular Probes (Eugene, OR). [1-¹⁴C]C₆-ceramide (0.15 MBq/μmol) was synthesized as before (van der Bijl et al., 1996). L-[3-³H]serine (0.8 GBq/μmol) and [methyl-³H]choline chloride (3.0 GBq/μmol) were from NEN (Boston, MA). Cell culture plastics were obtained from Costar (Cambridge, MA) and cell culture media were from Gibco (Paisley, UK). Chemicals and solvents were of analytical grade and obtained from Riedel-de Haën (Seelze, FRG).

Cell culture

Cell monolayers were grown on plastic dishes under 5% CO₂. Wild-type Chinese hamster ovary (CHO) cells (Dr P. Rottier, Utrecht, Netherlands) were grown on 75 cm² plastic flasks in αMEM supplemented with ribo- and deoxyribonucleosides and 10% FCS; HepG2 cells in MEM with 10% FCS; HeLa cells (Dr G. Warren, London, UK) in MEM with 5% FCS. All cells used were free of mycoplasma contamination.

Preparation of prometaphase cells and G₁ cells

Mitotic CHO cells were isolated from monolayers in 850 cm² plastic roller bottles. After a debris-spin prometaphase cells were accumulated for 2 hours in fresh growth medium containing nocodazole (40

ng/ml; Zieve et al., 1980). Prometaphase cells were then isolated by shaking (Klevecz, 1975). To obtain mitotic HeLa cells a 16 hour thymidine block was followed by 9 hours without thymidine. After a debris-spin, prometaphase cells were accumulated for 4 hours in medium containing nocodazole and isolated as CHO cells. Mitotic cell populations were >95% in prometaphase as determined by chromosome structure. For this, cells were fixed in 1.5% paraformaldehyde + 1% Triton X-100 + Hoechst (0.5 μg/ml) for 0.5 hour on ice and viewed by fluorescence microscopy.

To obtain G₁ cells, prometaphase cells were washed, resuspended in growth medium and incubated for 1 hour at 37°C to allow completion of mitosis. After this procedure cells were >90% in G₁, as determined by the presence of interphase nuclei under a fluorescence microscope.

Drug incubations

Cells were preincubated with 1 μg BFA/ml Hanks' balanced salt solution without bicarbonate, 10 mM Hepes, pH 7.35 (HBSS; 1:1,000 dilution of an ethanolic stock) for 30 minutes at 37°C. The media of subsequent incubations contained BFA. Cells were incubated with 1 μM okadaic acid (1:1,000 from DMSO) for 30 minutes at 37°C. Preincubation with inhibitors of multidrug resistance (MDR) P-glycoproteins was for 10 minutes at 37°C. Subsequent 37°C media contained the MDR inhibitor. Progesterone (20 μM), PSC 833 (10 μM), and verapamil (20 μM) were diluted from ethanolic stocks. Cyclosporin A (10 μM) was diluted from a 42 mM solution in poly(oxyethylene)-40-castor oil, 33% ethanol. Control incubations were performed in 0.1% ethanol.

Cell fractionation and enzyme assays

Crude ER/Golgi membranes were prepared (Burger et al., 1996), and cell fractionation was carried out on a linear sucrose gradient (Strous et al., 1993). Fractions (1 ml) were collected and stored at -20°C. Sphingolipid synthesis was assayed in each gradient fraction by incubating 250 μl for 1 hour at 10°C with 250 μl 250 mM sucrose, 10 mM Hepes, 2 mM MnCl₂, 2 mM MgCl₂, 2 mM UDP-Glc, 1 mM EDTA, 10 μM C₆-NBD-ceramide, pH 7.2. Lipid products were extracted and analyzed by TLC (see below).

Sphingolipid transport assay

For kinetic transport studies, delivery of short-chain lipid analogs to the cell surface was assayed as by van Meer and van 't Hof (1993). Short-chain ceramide ([¹⁴C]C₆- or C₆-NBD-ceramide) was incorporated into the cell for 1 hour at 10°C from BSA-complexes (5-10 μM short-chain ceramide in 1 ml HBSS+BSA; 1% w/v) to allow accumulation of short-chain glucosylceramide (GlcCer) and SM. This was followed by 2 hours at 10°C in HBSS+BSA to wash out residual ceramide and to remove any short-chain SM that had been synthesized on the cell surface. Subsequent delivery of the newly synthesized short-chain lipids to the outer leaflet of the plasma membrane at 37°C was routinely assayed up to 1 hour by continuous depletion by HBSS+BSA. Each transport incubation was followed by a 30 minute 10°C wash with HBSS+BSA. In other transport studies cells were incubated with 5 μM C₆-NBD-ceramide in 1 ml HBSS+BSA for 1 hour at 37°C, followed by a 30 minute 10°C wash with HBSS+BSA.

Delivery of natural SM to the cell surface was assayed according to the method of Shiao and Vance (1993). Cellular SM was labeled at 37°C for 1-4 hours with 75 KBq [³H]serine/ml HBSS or 370 KBq [³H]choline/ml HBSS, during which period transport of the newly synthesized radiolabeled lipids can occur. Subsequently, the cells were incubated for 1 hour at 15°C with or without 1 U exogenous SMase/ml HBSS. The percentage of [³H]SM that is hydrolyzable, 100×(1-[[³H]SM_{SMase-treated}]/[³H]SM_{mock-treated}]) is used as a measure of [³H]SM transport to the cell surface. The distribution of unlabeled SM was determined using a phosphate determination (Rouser et al., 1970).

Lipid analysis

Lipids were extracted from media and cells as before (van der Bijl et al., 1996). After two-dimensional separation (I: chloroform/methanol/25% ammonia {65:35:5 v/v}; II: chloroform/acetone/methanol/acetic acid/water {50:20:10:10:5 v/v}) on silica-60 TLC plates, lipid spots were visualized by I₂ vapor for unlabeled lipids, UV light for C₆-NBD-lipids, or by autoradiography for radiolabeled lipids. Lipid spots were scraped and quantitated by phosphate determination, fluorescence spectrophotometry (Kontron, Switzerland), or liquid scintillation counting (Packard, IL) as described by van der Bijl et al. (1996).

RESULTS

SM-synthase and ceramide glucosyltransferase are Golgi enzymes in CHO cells

The bulk of both the SM-synthase and the ceramide glucosyltransferase (CGlcT) have been found to cofractionate with Golgi markers in rat liver and human liver-derived HepG2 cells (Futerman et al., 1990; Jeckel et al., 1990, 1992; Futerman and Pagano, 1991). When a postnuclear supernatant of CHO cells was fractionated on a linear 0.7-1.5 M sucrose gradient, the activities of both enzymes colocalized (Fig. 1) at the density of the protein galactosyltransferase (not shown). The localization in the Golgi was confirmed by pretreating the cells with

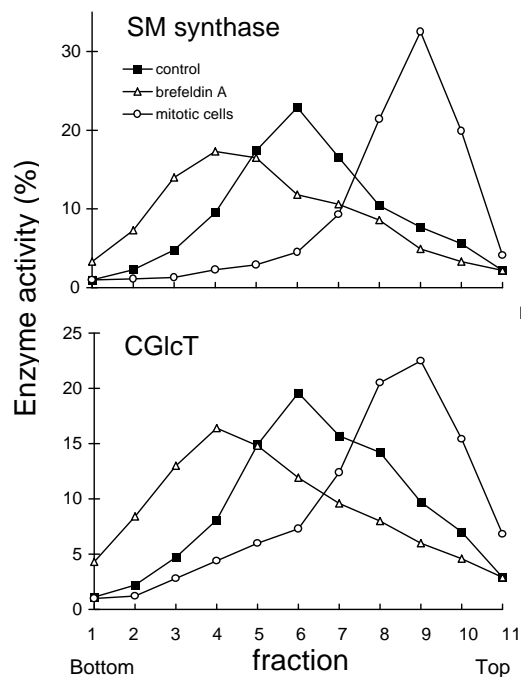


Fig. 1. Localization of SM synthase and ceramide glucosyltransferase (CGlcT) activities on sucrose gradients. A post nuclear supernatant from control, BFA-treated and mitotic CHO cells was fractionated on a linear 0.7-1.5 M sucrose density gradient. Fractions collected from the bottom (fraction 1) to the top (fraction 11) were assayed for the enzyme activities as in Materials and Methods. Data are the average of 3 experiments and are expressed as a percentage of total enzyme activity in the gradient. The absolute SM synthase activity in the homogenate of the BFA-treated cells was 1.9 ± 0.3 times that of the control homogenate and in the mitotic homogenate 0.7 ± 0.1 times control. CGlcT activity was equal between the three.

BFA. In most cells this treatment leads to fusion of the Golgi with the ER (Klausner et al., 1992) shifting Golgi enzymes to the density of the ER (Strous et al., 1993). Indeed, BFA redistributed both enzyme activities to higher density (Fig. 1). Complete redistribution was observed after 5 minutes incubation with BFA. The redistribution of the Golgi enzymes to the ER was readily reversible: 30 minutes after removal of the drug at 37°C both enzymes had resumed the profiles of control cells (not shown).

BFA treatment increased the activity of SM synthase, but not that of CGlcT, roughly twofold (Fig. 1, legend), which has been attributed to a higher concentration of the substrate phosphatidylcholine in a merged ER-Golgi compartment (Brüning et al., 1992; Hatch and Vance, 1992; Kallen et al., 1993). To address fusion of the SM-synthase compartment with the ER directly, we tested whether after BFA treatment the sphingolipid precursor ceramide, synthesized in the ER (Mandon et al., 1992), could reach the SM synthase by diffusion through a continuous membrane. When control cells were incubated with [³H]serine at 10°C where no vesicular traffic occurs, no [³H]SM was formed from [³H]ceramide (Fig. 2, left panel). However, after treatment of the cells with BFA [³H]ceramide was efficiently converted to [³H]SM (Fig. 2, right panel), implying free lipid diffusion and, therefore, membrane continuity between ER and Golgi.

In the transport experiments (below) mitotic cells were used as a physiological control for the effects of BFA on SM transport. During mitosis, the Golgi apparatus is fragmented and disperses over the cytosol (Warren et al., 1995). On sucrose gradients, a remarkable shift to the top of the gradient was observed for both sphingolipid synthesizing enzymes in prometaphase cells (Fig. 1). A similar shift was found after treatment of CHO, HepG2 and HeLa cells for 0.5 hour at 37°C with 1 μM okadaic acid (not shown), which mimics mitotic

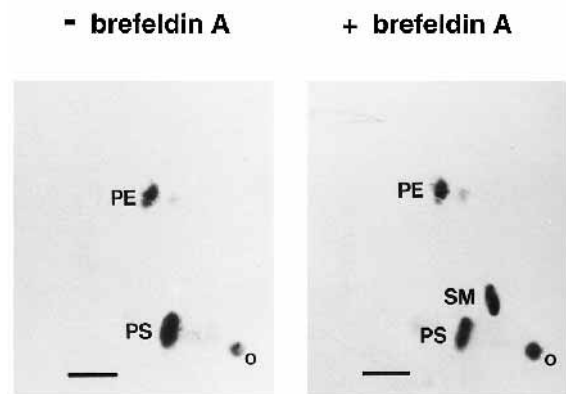


Fig. 2. After brefeldin A-treatment [³H]ceramide reaches the SM synthase at 10°C. CHO cells were preincubated for 30 minutes at 37°C in the presence or absence of BFA (1 μg/ml). Subsequently, the cells were incubated with 75 KBq [³H]serine/ml for 4 hours at 10°C. Lipids were extracted and separated on 10 cm² TLC plates as described in Materials and Methods. One typical experiment out of 4 is shown. Synthesis of [³H]SM was observed after BFA treatment only. [³H]phosphatidylserine (PS) synthesis was reduced 2-fold but [³H]phosphatidylethanolamine (PE) synthesis did not change. Virtually no label was found in ceramide at the solvent front (not shown; <1% of total label on either plate). Radioactivity at origin (O) represents free [³H]serine. Bar, 1 cm.

Golgi disassembly (Lucocq, 1992). Various ER markers remained in the lower part of the gradient. This implies that no membrane continuity between Golgi and ER was induced in mitosis or by okadaic acid. In line with this, synthesis of SM from exogenous precursors was not enhanced in either case and no increase in [^3H]SM labeling from [^3H]serine at 10°C was observed in mitotic cells (not shown).

Transport of natural SM from the Golgi to the exoplasmic leaflet of the plasma membrane is inhibited in the presence of brefeldin A and in mitotic cells

To determine the contribution of the secretory pathway to the transport of SM from its site of synthesis to the outer leaflet of the plasma membrane, SM was radiolabeled biosynthetically and its transport to the cell surface was measured under conditions where vesicular traffic from the Golgi to the plasma membrane is blocked. For this we chose treatment of the cells with BFA (Klausner et al., 1992) and mitotic cells (Warren, 1993). The fraction of radiolabeled SM at the cell surface was determined after various time-intervals by hydrolysis using exogenous SMase (Verkleij et al., 1973), but at 15°C to prevent lipid traffic during the SMase treatment (Shiao and Vance, 1993).

Fig. 3 shows that the relative amount of SM at the cell surface was not altered by BFA: in both control and BFA-treated cells just over 60% of the cellular SM was hydrolyzed. To assay the transport of newly synthesized SM to the exoplasmic leaflet of the plasma membrane, cells were labeled with [^3H]serine, which is incorporated in the sphingosine of SM's ceramide backbone, or with [^3H]choline to label the headgroup. In control cells, [^3H]serine-labeled SM reached the cell surface at 37°C, whereas transport of [^3H]choline-labeled SM lagged behind. The latter was probably due to an increasing rate of

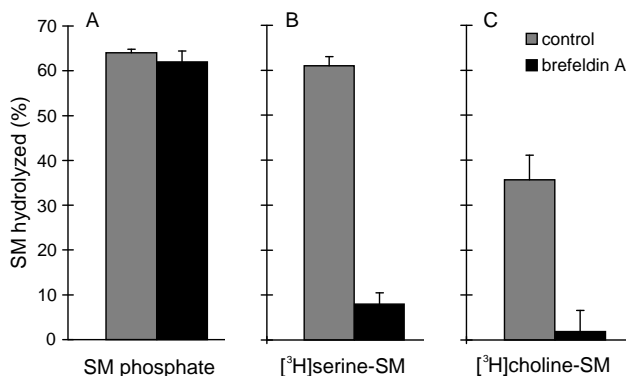


Fig. 3. Transport of natural [^3H]SM to the surface of brefeldin A-treated CHO cells. SM at the surface of control cells (hatched bars) and BFA-treated cells (solid bars) was quantitated with an exogenous SMase treatment at 15°C as in Materials and Methods.

(A) Hydrolyzable SM at the cell surface as determined by SM-phosphate. (B) The hydrolyzable pool of newly synthesized SM was determined after a 1.5 hour labeling with [^3H]serine (75 KBq/ml) at 37°C. [^3H]SM synthesis was 170 Bq in control cells and 2,500 Bq after BFA treatment. (C) [^3H]SM hydrolysis after a 3 hour labeling with [^3H]choline (370 KBq/ml). [^3H]SM synthesis was 170 Bq in control cells and 680 Bq after BFA treatment. Average of 3 independent experiments (\pm s.d.; $n=3$).

[^3H]choline-SM synthesis during the incubation caused by the large phosphatidyl-[^3H]choline precursor pool (Kallen et al., 1993). In both cases, a strong inhibition of [^3H]SM transport to the cell surface was observed. A clear inhibition of [^3H]SM transport was also observed in mitotic cells (Fig. 4). As a control, mitotic cells were further incubated in the absence of nocodazole for 1 hour to reach G₁. In G₁ cells 60% of the [^3H]SM was hydrolyzed, like in interphase cells. The addition of nocodazole (40 ng/ml) to interphase cells did not affect vesicular transport nor synthesis (not shown). The results suggest that the bulk of the newly synthesized SM follows the vesicular secretory pathway from the Golgi to the cell surface.

Transport of short-chain SM analogs from the Golgi to the exoplasmic leaflet of the plasma membrane continues in the presence of brefeldin A and in mitosis

Analogues of sphingolipids carrying a short acyl chain have been used extensively to study the synthesis and transport of sphingolipids (reviewed by Koval and Pagano, 1991; van der Bijl et al., 1996). In this type of approach, a short-chain ceramide is presented to the outside of the cell. First, it partitions into the plasma membrane, across which it can translocate because of the absence of a polar headgroup. From there, it can exchange between membranes as a monomer due to its short chain. Upon reaching the SM synthase and CGlcT in the Golgi it is converted to short-chain SM and glucosylceramide (GlcCer). So far, the procedure can be carried out at 10°C. Upon warming to 37°C, transport to the cell surface can be monitored by trapping the short-chain products as soon as they appear on the surface using BSA as a short-chain lipid scavenger in the extracellular medium.

Transport of [^{14}C]C₆-SM to the cell surface was inhibited significantly in the presence of BFA and in mitotic cells (Fig. 5A,B). Transport of C₆-NBD-SM in mitotic cells (Fig. 6B) was 50% reduced as compared to control interphase cells (Fig. 6A). In contrast, C₆-NBD-SM transport was not inhibited by BFA, and transport of short-chain GlcCer was hardly affected under any condition. If BFA inhibits all vesicular traffic from the

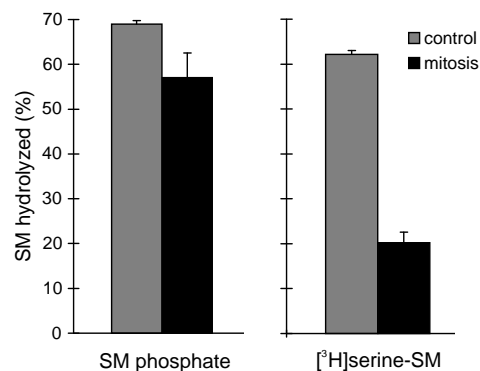


Fig. 4. Transport of natural [^3H]SM to the surface of CHO cells in G₁ and in mitosis. The hydrolyzable pool of unlabeled SM and of newly synthesized SM was quantitated in control cells (G₁-phase) and mitotic cells as in Fig. 3 after labeling with [^3H]serine (75 KBq/ml) for 2 hours at 37°C. [^3H]SM synthesis was 750 Bq in G₁ cells and 330 Bq in mitotic cells. Data are from 2 independent experiments (\pm s.d.; $n=5$).

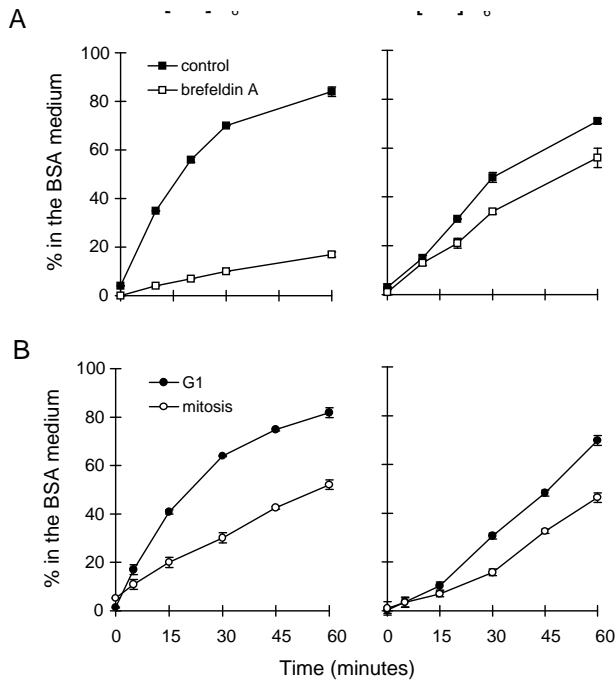


Fig. 5. Transport of [^{14}C]C₆-SM and [^{14}C]C₆-GlcCer to the CHO cell surface in the presence of brefeldin A and in mitosis. After pretreatment with or without BFA (A) and preparation of cells in mitosis and G₁ (B), the cells were incubated with [^{14}C]C₆-ceramide for 1 hour at 10°C followed by 2 hours at 10°C in HBSS+BSA. Arrival of the short-chain lipid products [^{14}C]C₆-SM and -GlcCer at the cell surface was assayed by BSA depletion into the medium during a 37°C incubation in the presence or absence of BFA (A) or nocodazole (B). Lipids were extracted from cells and media and quantitatively analyzed, all as in Materials and Methods. Data are the mean of 2 different experiments in duplicate (\pm s.d.). Synthesis per 10⁷ cells of [^{14}C]C₆-GlcCer was 55 pmol for all 4 conditions whereas synthesis of [^{14}C]C₆-SM was 50 pmol in control and G₁ cells, 225 pmol after BFA-treatment, and 25 pmol during mitosis.

merged ER-Golgi compartment to the cell surface (Klausner et al., 1992) and if no vesicular traffic occurs from the mitotic Golgi to the plasma membrane (Warren, 1993), the short-chain analogs that reached the cell surface under these conditions followed a non-vesicular pathway.

To exclude the possibility that the different transport properties of C₆-NBD-SM and endogenous [^3H]SM in the presence of BFA were due to a different site of synthesis, we performed a competition experiment to show that C₆-NBD-ceramide reached the site of [^3H]SM synthesis. Labeling of cellular SM with [^3H]serine decreased with increasing amounts of C₆-NBD-ceramide (Fig. 7). This was not due to inactivation of the SM synthase by C₆-NBD-ceramide as synthesis of C₆-NBD-SM, which saturated at 25 μM in the control and at 5 μM in the BFA-treated cells, was constant up to 75 μM ceramide. The inhibition implies that C₆-NBD-ceramide efficiently reached the site of [^3H]ceramide and [^3H]SM synthesis, in BFA-treated cells the ER-Golgi compartment, where at least a fraction must have been converted to C₆-NBD-SM. Since in BFA-treated cells all C₆-NBD-SM becomes available for depletion by BSA in the medium in prolonged incubations (not shown), C₆-NBD-

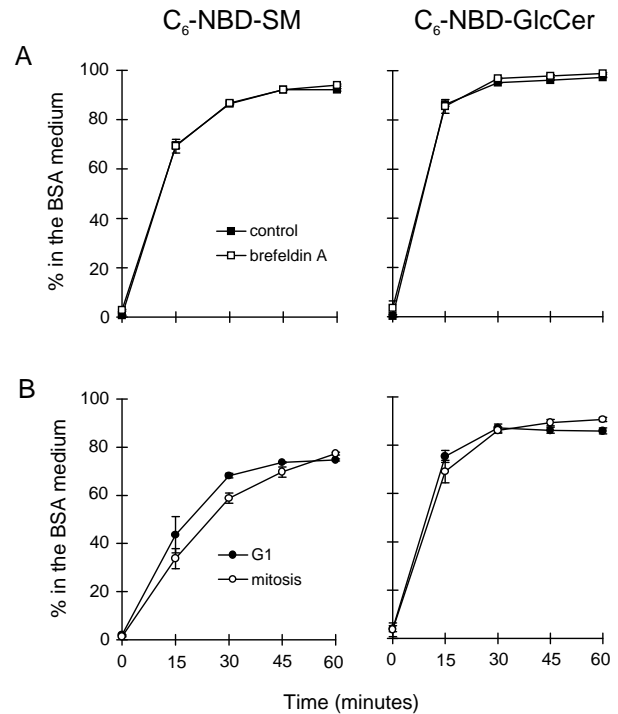


Fig. 6. Transport of C₆-NBD-SM and C₆-NBD-GlcCer to the CHO cell surface in the presence of brefeldin A and in mitosis. Cells were incubated with C₆-NBD-ceramide at 10°C as described for [^{14}C]C₆-ceramide in Fig. 5, after which arrival of C₆-NBD-SM and -GlcCer at the cell surface was assayed by BSA depletion at 37°C. Data are the mean of 2 different experiments in duplicate (\pm s.d.). Synthesis per 10⁷ cells of C₆-NBD-GlcCer was 40 pmol for all 4 conditions whereas synthesis of C₆-NBD-SM was 25 pmol in control cells, 85 pmol in BFA-treated cells, 20 pmol in G₁ cells and 14 pmol in mitotic cells.

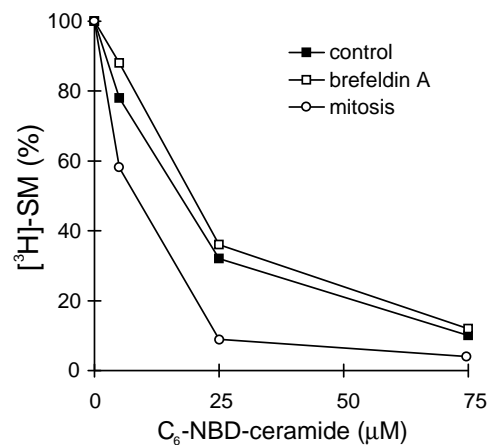


Fig. 7. Inhibition of endogenous SM synthesis by C₆-NBD-ceramide in CHO cells. Control cells, BFA-treated cells and mitotic cells were labeled with 75 KBq [^3H]serine for 1 hour at 37°C in the presence of increasing concentrations of C₆-NBD-ceramide in HBSS+BSA. Values were obtained in 2 experiments and are expressed as a percentage of the incorporation in the absence of C₆-NBD-ceramide: control, 150 Bq; BFA, 850 Bq; mitotic cells, 50 Bq.

SM can be transported to the cell surface in the absence of vesicular traffic.

Translocation of short-chain SM across the plasma membrane in the presence of brefeldin A and in mitosis

In the absence of vesicular traffic, the only possible pathway for intracellular C₆-NBD-lipids to reach the outer leaflet of the plasma membrane would be translocation across the plasma membrane. The family of the multidrug resistance P-glycoproteins has been found to translocate amphipathic substances to the outside of the plasma membrane. One of these, the mouse *mdr2* or human MDR3 P-glycoprotein, has been implicated in translocation of phosphatidylcholine across the bile canalicular membrane of the hepatocyte (reviewed by Menon, 1995). Therefore, we tested the influence of a series of known inhibitors of the multidrug transporting MDR1 P-glycoprotein on the transport of C₆-NBD-SM and -GlcCer to the cell surface. Table 1 shows that indeed transport of C₆-NBD-SM and -GlcCer in the presence of BFA was virtually blocked by cyclosporin A and its analog PSC 833 at concentrations where these agents inhibit drug transport by the MDR1 P-glycoprotein (Boesch et al., 1991; Cardarelli et al., 1995). Verapamil and progesterone, known to be partial inhibitors of P-glycoprotein activity at 20 µM (Tsuruo et al., 1981; Ueda et al., 1992), inhibited transport of both lipids by about 50%. At the same time, C₆-NBD-SM transport to the surface in the absence of BFA was virtually unaffected by cyclosporin A (Table 1), indicating that cyclosporin A did not interfere with the vesicular pathway to the plasma membrane. Transport of C₆-NBD-GlcCer was inhibited to nearly the same extent in the absence and presence of BFA (Table 1). These findings suggest that an MDR activity in the CHO plasma membrane is responsible for the translocation of C₆-NBD-SM and -GlcCer over the plasma membrane in BFA-treated cells, and that this activity accounts for most transport of C₆-NBD-GlcCer to the surface of control cells.

Similarly, the transport of C₆-NBD-GlcCer in mitotic and G₁ cells (Fig. 6B) was virtually blocked by cyclosporin A, and affected by PSC 833 and verapamil to the same extent as in BFA-treated and control cells, respectively (not shown). The inhibitors reduced SM transport in mitotic cells, but less than in BFA-treated cells where transport was reduced by up to 85%

Table 1. Transport of C₆-NBD-SM and C₆-NBD-GlcCer to the cell surface in the presence of inhibitors of the multidrug resistance P-glycoprotein

Addition	- Brefeldin A		+ Brefeldin A	
	C ₆ -NBD-SM	C ₆ -NBD-GlcCer	C ₆ -NBD-SM	C ₆ -NBD-GlcCer
	(percentage of each lipid in BSA-medium)			
None	73±8	86±2	70±3	86±3
Cyclosporin A	62±3	12±2	10±1	8±3
PSC 833	59±7	29±1	14±1	21±1
Progesterone	53±1	47±2	32±0	40±1
Verapamil	66±2	49±1	34±1	44±1

Before ceramide addition, cells were incubated for 10 minutes at 37°C in HBSS+BSA with or without 1 µg BFA/ml followed by 10 minutes at 37°C in the absence or presence of MDR inhibitors cyclosporin A (10 µM), PSC 833 (10 µM), progesterone (20 µM), and verapamil (20 µM). After addition of the ceramide analog synthesis and transport were quantitatively analyzed after 1 hour at 37°C by measuring the extracted lipid products from the cells and the BSA-containing medium as in Materials and Methods. Data are the average of 2 experiments in duplicate (± s.d.; n=4).

(Table 1). The inhibition of SM transport was underestimated as the transport values in these experiments were not corrected for SM synthesis on the cell surface (in contrast to the experiments in Figs 5 and 6 where synthesis and transport were studied independently). While intracellular SM synthesis was stimulated by BFA (4-fold, see legends to Figs 5 and 6), it was reduced in mitotic cells (2-fold) without effect on cell surface synthesis (not shown). As a consequence, cell surface synthesis was negligible in BFA-treated cells (≤5% of total synthesis as measured at 10°C; van Helvoort et al., 1994, and not shown) but constituted an appreciable fraction of SM synthesis in mitotic cells (± 20%). The data indicate that in mitotic cells transport of C₆-NBD-GlcCer, and partly that of C₆-NBD-SM, occurred by an MDR-activity.

Lipid translocation across the plasma membrane is not limited to CHO cells

Also in the human liver-derived HepG2 cell line, transport of C₆-NBD-SM and -GlcCer continued in the presence of BFA. As in CHO cells (Table 1), the transport of C₆-NBD-SM in the presence of BFA and of C₆-NBD-GlcCer in the presence and absence of BFA was sensitive to verapamil (Fig. 8). In control cells, verapamil inhibited transport of C₆-NBD-GlcCer by half with only a minor effect on transport of C₆-NBD-SM. In the presence of BFA, verapamil reduced transport of both lipids by more than half. This suggests that HepG2 cells possess a translocator in the plasma membrane similar to CHO cells.

Also in mitotic HeLa cells, prepared in parallel to the mitotic HeLa cells that were used to demonstrate a block in lipid transport in the early Golgi (Collins and Warren, 1992), transport of C₆-NBD-lipids to the cell surface continued (not

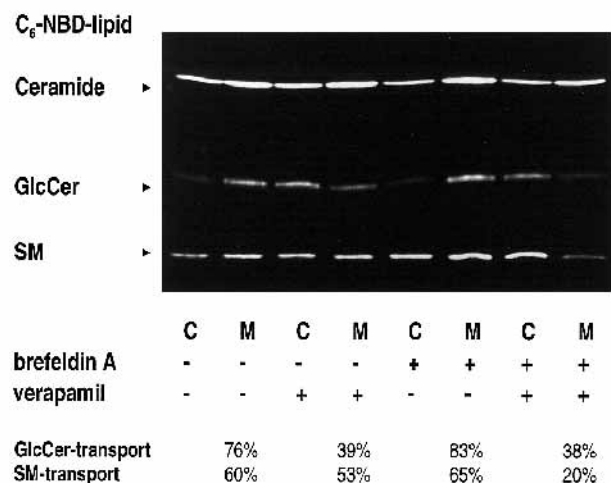


Fig. 8. Inhibition of C₆-NBD-SM and C₆-NBD-GlcCer transport to the surface of HepG2 cells by MDR inhibitors. Before ceramide addition, cells were preincubated for 10 minutes at 37°C in HBSS+BSA with or without 1 µg BFA/ml followed by a second preincubation for 10 minutes at 37°C in the absence or presence of verapamil (20 µM) and BFA. After addition of C₆-NBD-ceramide, synthesis and transport were analyzed after 1 hour at 37°C by measuring the extracted lipid products from the cells and the BSA-medium as in Materials and Methods. The picture represents a one-dimensional separation on a 10 cm × 20 cm TLC plate (solvent II) of the C₆-NBD-lipids extracted from cells (C) and media (M) of the different conditions.

shown). This suggests that all events involved in C₆-NBD-lipid transport in CHO cells also occur in HeLa cells, including translocation across the plasma membrane.

DISCUSSION

Newly synthesized proteins and lipids reach the outside of the cell via the vesicular secretory pathway. In addition, lipids can in principle be delivered to the surface after translocation from the cytosolic leaflet of the plasma membrane to the exoplasmic leaflet (van Helvoort and van Meer, 1995). Here, we have inhibited the vesicular transport pathway in two ways and show that transport of some lipid analogs to the cell surface continued. This process was sensitive to inhibitors of the multidrug resistance P-glycoprotein which suggests that this or a related protein is responsible for translocating the analogs across the plasma membrane.

Brefeldin A and SM transport to the cell surface

BFA induces fusion between cisternae of the Golgi complex and the ER in a number of cell types (Klausner et al., 1992). For the present study on SM transport it was important to define the location of the SM synthase in the presence of BFA. In CHO cells the SM synthase redistributed to the density of ER on a sucrose density gradient (Fig. 1). More convincingly, ceramide produced in the ER at 10°C was efficiently converted to SM after BFA but not in control cells (Fig. 2) implying that in BFA-treated cells SM synthase resided in a membrane continuous with the ER. In the presence of BFA newly synthesized endogenous SM remained virtually inaccessible to exogenous SMase at 37°C (Fig. 3). This confirms a similar observation with backbone-labeled SM in BFA-treated BHK cells (Kallen et al., 1993) and a more modest reduction in SM transport to the CHO plasma membrane as measured by cell fractionation (Warnock et al., 1994). Because BFA efficiently blocks vesicular protein transport to the surface (Klausner et al., 1992; Strous et al., 1993; Warnock et al., 1994) delivery of natural SM to the cell surface appears to require a vesicular transport step.

Our complementary observation that also choline-labeled SM transport to the surface was inhibited by BFA as monitored by SMase (Fig. 3) is in contrast with a study on hepatocytes (Shiao and Vance, 1993). Interestingly, in liver-derived HepG2 cells SM synthase did not shift to the ER density after BFA (van Meer and van 't Hof, 1993) while other Golgi enzymes did (Strous et al., 1993), and no SM was formed from newly synthesized ceramide at 10°C (not shown). Further studies will have to elucidate whether these differences with fibroblasts reflect a different localization of the SM synthase in hepatocytes.

Like endogenous long-chain SM, SM carrying a C₆-chain reached the cell surface much more slowly in the presence of BFA (Fig. 5A). In contrast, transport of the more water-soluble C₆-NBD-SM to the CHO cell surface was not inhibited by BFA (Fig. 6A). The fact that MDR inhibitors prevented arrival of C₆-NBD-SM on the cell surface in the presence of BFA (Table 1), suggests that the final step in the transport was translocation across the plasma membrane by an MDR-like protein. In a direct study, we have demonstrated that the human MDR1 and mouse *mdr1a* P-glycoprotein can translocate C₆-NBD-SM

to the cell surface (van Helvoort et al., 1996). Apparently, CHO cells (HepG2 and HeLa cells) express sufficient MDR activity to translocate C₆-NBD-SM. If natural SM is a substrate for the MDR activity, the presence of this activity would conflict with the proposed occurrence of a signaling pool of SM on the inside of the plasma membrane.

To reach the translocator in the plasma membrane C₆-NBD-SM must first have translocated from the lumen of the BFA-induced ER-Golgi compartment to the cytosolic leaflet, after which equilibration with the cytosolic leaflet of the plasma membrane should be fast because of the short hydrophilic side-chain. Indeed, rapid translocation of SM analogs across the ER membrane has been reported (Herrmann et al., 1990; Buton et al., 1996). The small contribution of the translocation pathway to delivery of C₆-NBD-SM to the surface of control cells (Table 1) implies that very little SM translocated to the cytosolic side of the Golgi (cf. Helms et al., 1990; Jeckel et al., 1992; Burger et al., 1996) and that little SM was transported back to the ER as compared to the SM flux into the anterograde vesicular pathway.

Transport of GlcCer to the cell surface

After synthesis on the cytosolic surface of the Golgi, C₆-NBD-GlcCer can translocate towards the Golgi lumen (Lannert et al., 1994; Burger et al., 1996), from where it can follow the secretory pathway to the cell surface. This translocation across the Golgi membrane is insensitive to MDR inhibitors (K. N. J. Burger, personal communication). Thus the sensitivity of C₆-NBD-GlcCer transport to the MDR inhibitors (Table 1) suggests that most of the newly synthesized C₆-NBD-GlcCer equilibrated with the inner leaflet of the plasma membrane and subsequently translocated to the exoplasmic leaflet. Therefore, most C₆-NBD-GlcCer bypassed the Golgi lumen on its route to the cell surface which may explain why only 1% is converted to higher glycolipids (data not shown; van der Bijl et al., 1996). This is different for galactosylceramide which is synthesized in the lumen of the ER. Up to one-third of short-chain GalCer received an additional galactose or sulfate before reaching the cell surface (van der Bijl et al., 1996). Like C₆-NBD-SM, C₆-NBD-GlcCer and [¹⁴C]C₆-GlcCer were found to be a substrate for P-glycoproteins of the MDR1 type (van Helvoort et al., 1996). Whether endogenous GlcCer can be translocated by P-glycoproteins and, if so, what fraction follows the MDR pathway as compared to translocation towards the Golgi lumen is unclear at present.

Endogenous GlcCer may have access to the cytosolic leaflet of the plasma membrane via the cytosolic leaflet of transport vesicles or via cytosolic transfer proteins (Sasaki, 1990). The first mechanism may explain the faster rates of transport of C₆-GlcCer to the cell surface of control cells vs. BFA-treated and mitotic cells (Fig. 5). Most likely, cytosolic transfer is responsible for the transport of endogenous GlcCer to the plasma membrane that was observed by cell fractionation in the presence of BFA and at 15°C (Warnock et al., 1994), because BFA blocks the Golgi pathway for glycosphingolipids (van Echten et al., 1990; Young et al., 1990) and SM (Kallen et al., 1993; Fig. 3) and because vesicular lipid transport to the cell surface was not observed at 15°C (Helms et al., 1990). The appearance of phosphatidylethanolamine on the cell surface in the presence of BFA (Vance et al., 1991) can also be explained by cytosolic transfer and translocation across the plasma

membrane. Obviously, as an alternative for the MDR activity one can never exclude the possibility that a specialized, non-Golgi vesicular pathway to the plasma membrane persists in the presence of BFA (Urbani and Simoni, 1990; van Leyen and Wieland, 1994).

Spingolipid transport to the mitotic cell surface

Most importantly, as was observed with BFA, transport of C₆-NBD-GlcCer to the cell surface was unchanged in mitosis. Again, it was fully sensitive to MDR inhibitors supporting the proposed presence of an MDR activity at the plasma membrane. Transport of endogenous SM to the cell surface was reduced in mitosis as compared to interphase cells (Fig. 4) but to a lesser extent than in the presence of BFA. The appearance of newly synthesized C₆-SM and C₆-NBD-SM on the cell surface was reduced by 50% as compared to control interphase cells (Figs 5, 6). All in all, the data suggest a strong reduction in vesicular traffic of endogenous SM, C₆-SM, and C₆-NBD-SM in mitotic cells, in line with the reported block in vesicular transport through the Golgi (Collins and Warren, 1992; Warren et al., 1995). The remaining appearance of C₆-NBD-SM on the mitotic cell surface was partly sensitive to MDR-inhibitors, while another part was synthesized on the cell surface (Results). The discrepancies between the present results and a previous study where no newly synthesized C₆-NBD-GlcCer and -SM appeared on the surface of mitotic CHO cells (Kobayashi and Pagano, 1989) would suggest that no MDR activity and no SM synthase were present on the surface of those CHO cells.

Lipid translocation across the plasma membrane by an MDR activity

We have observed an MDR inhibitor-sensitive translocation of C₆-NBD-GlcCer in all cell types tested: CHO, HepG2, HeLa, Caco-2, LLC-PK1, and MDCK cells. In the latter three cell types the activity is limited to the apical plasma membrane domain (K. N. J. Burger et al., unpublished observations) and it has indeed been reported that transport of C₆-NBD-SM continued in BFA-treated Caco-2 cells, but that it now appeared on the apical rather than the basolateral surface (van Meer and van 't Hof, 1993). Most epithelial cells express the MDR1 P-glycoprotein at their apical surface and also many tumor cells express this drug transporter. The human MDR1 P-glycoprotein in transfected cells is capable of translocating C₆-NBD-lipids (van Helvoort et al., 1996). This suggests the MDR1 P-glycoprotein as a candidate for the translocator activity observed in the present study.

The finding that C₆-NBD-GlcCer can be translocated across the apical membrane of epithelial cells forces us to reconsider the interpretation of previous transport data and broadens our views on the mechanism of epithelial lipid sorting (Simons and van Meer, 1988). The enrichment of GlcCer over SM on the apical surface could be generated by selective apical translocation of GlcCer vs vesicular traffic of SM to both cell surfaces. However, one essential question remaining is whether endogenous GlcCer can be translocated by the MDR1 P-glycoprotein. Further questions concern the substrate specificity of the lipid translocator and, last but not least, its physiological function.

We are grateful to Ruth Collins and Graham Warren who helped

us in the first experiments on mitotic cells. We thank Ger Strous for critical reading of the manuscript. The present work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO) to A.v.H., and EC contract B102-CT93-0348 to G.v.M.

REFERENCES

- Allan, D. and Kallen, K.-J. (1994). Is plasma membrane lipid composition defined in the exocytic or the endocytic pathway? *Trends Cell Biol.* **4**, 350-353.
- Andrieu, N., Salvayre, R. and Levade, T. (1996). Comparative study of the metabolic pools of sphingomyelin and phosphatidylcholine sensitive to tumor necrosis factor. *Eur. J. Biochem.* **236**, 738-745.
- Babia, T., Kok, J. W., van der Haar, M., Kalicharan, R. and Hoekstra, D. (1994). Transport of biosynthetic sphingolipids from Golgi to plasma membrane in HT29 cells: involvement of different carrier vesicle populations. *Eur. J. Cell Biol.* **63**, 172-181.
- Boesch, D., Gavériaux, C., Jachez, B., Pourtier-Manzanedo, A., Bollinger, P. and Loor, F. (1991). *In vivo* circumvention of P-glycoprotein-mediated multidrug resistance of tumor cells with SDZ PSC 833. *Cancer Res.* **51**, 4226-4233.
- Brüning, A., Karrenbauer, A., Schnabel, E. and Wieland, F. T. (1992). Brefeldin A-induced increase of sphingomyelin synthesis. *J. Biol. Chem.* **267**, 5052-5055.
- Burger, K. N. J., van der Bijl, P. and van Meer, G. (1996). Topology of sphingolipid galactosyltransferases in ER and Golgi: transbilayer movement of monohexosyl sphingolipids is required for higher glycosphingolipid biosynthesis. *J. Cell Biol.* **133**, 15-28.
- Buton, X., Morrot, G., Fellmann, P. and Seigneuret, M. (1996). Ultrafast glycerophospholipid-selective transbilayer motion mediated by a protein in the endoplasmic reticulum membrane. *J. Biol. Chem.* **271**, 6651-6657.
- Cardarelli, C. O., Aksentijevich, I., Pastan, I. and Gottesman, M. M. (1995). Differential effects of P-glycoprotein inhibitors on NIH3T3 cells transfected with wild-type (G185) or mutant (V185) multidrug transporters. *Cancer Res.* **55**, 1086-1091.
- Collins, R. N. and Warren, G. (1992). Sphingolipid transport in mitotic HeLa cells. *J. Biol. Chem.* **267**, 24906-24911.
- Futerman, A. H. and Pagano, R. E. (1991). Determination of the intracellular sites and topology of glucosylceramide synthesis in rat liver. *Biochem. J.* **280**, 295-302.
- Futerman, A. H., Stieger, B., Hubbard, A. L. and Pagano, R. E. (1990). Sphingomyelin synthesis in rat liver occurs predominantly at the *cis* and *medial* cisternae of the Golgi apparatus. *J. Biol. Chem.* **265**, 8650-8657.
- Hannun, Y. A. and Obeid, L. M. (1995). Ceramide: an intracellular signal for apoptosis. *Trends Biochem. Sci.* **20**, 73-77.
- Hatch, G. M. and Vance, D. E. (1992). Stimulation of sphingomyelin biosynthesis by brefeldin A and sphingomyelin breakdown by okadaic acid treatment of rat hepatocytes. *J. Biol. Chem.* **267**, 12443-12451.
- Helms, J. B., Karrenbauer, A., Wirtz, K. W. A., Rothman, J. E. and Wieland, F. T. (1990). Reconstitution of steps in the constitutive secretory pathway in permeabilized cells. *J. Biol. Chem.* **265**, 20027-20032.
- Herrmann, A., Zachowski, A. and Devaux, P. F. (1990). Protein-mediated phospholipid translocation in the endoplasmic reticulum with a low lipid specificity. *Biochemistry* **29**, 2023-2027.
- Jeckel, D., Karrenbauer, A., Birk, R., Schmidt, R. R. and Wieland, F. (1990). Sphingomyelin is synthesized in the *cis* Golgi. *FEBS Lett.* **261**, 155-157.
- Jeckel, D., Karrenbauer, A., Burger, K. N. J., van Meer, G. and Wieland, F. (1992). Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions. *J. Cell Biol.* **117**, 259-267.
- Kallen, K.-J., Quinn, P. and Allan, D. (1993). Effects of brefeldin A on sphingomyelin transport and lipid synthesis in BHK21 cells. *Biochem. J.* **289**, 307-312.
- Karrenbauer, A., Jeckel, D., Just, W., Birk, R., Schmidt, R. R., Rothman, J. E. and Wieland, F. T. (1990). The rate of bulk flow from the Golgi to the plasma membrane. *Cell* **63**, 259-267.
- Klausner, R. D., Donaldson, J. G. and Lippincott-Schwartz, J. (1992). Brefeldin A: Insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* **116**, 1071-1080.

- Klevecz, R. R.** (1975). Automated cell cycle analysis. *Meth. Cell Biol.* **10**, 157-173.
- Kobayashi, T. and Pagano, R. E.** (1989). Lipid transport during mitosis. Alternative pathways for delivery of newly synthesized lipids to the cell surface. *J. Biol. Chem.* **264**, 5966-5973.
- Kobayashi, T., Pimplikar, S. W., Parton, R. G., Bhakdi, S. and Simons, K.** (1992). Sphingolipid transport from the trans-Golgi network to the apical surface in permeabilized MDCK cells. *FEBS Lett.* **300**, 227-231.
- Kok, J. W., Babia, T. and Hoekstra, D.** (1991). Sorting of sphingolipids in the endocytic pathway of HT29 cells. *J. Cell Biol.* **114**, 231-239.
- Kolesnick, R. and Fuks, Z.** (1995). Ceramide: A signal for apoptosis or mitogenesis? *J. Exp. Med.* **181**, 1949-1952.
- Koval, M. and Pagano, R. E.** (1991). Intracellular transport and metabolism of sphingomyelin. *Biochim. Biophys. Acta* **1082**, 113-125.
- Lannert, H., Bünning, C., Jeckel, D. and Wieland, F. T.** (1994). Lactosylceramide is synthesized in the lumen of the Golgi apparatus. *FEBS Lett.* **342**, 91-96.
- Linardic, C. M. and Hannun, Y. A.** (1994). Identification of a distinct pool of sphingomyelin involved in the sphingomyelin cycle. *J. Biol. Chem.* **269**, 23530-23537.
- Lucocq, J.** (1992). Mimicking mitotic Golgi disassembly using okadaic acid. *J. Cell Sci.* **103**, 875-880.
- Mandon, E. C., Ehses, I., Rother, J., van Echten, G. and Sandhoff, K.** (1992). Subcellular localization and membrane topology of serine palmitoyltransferase, 3-dehydroshinganine reductase, and shinganine *N*-acyltransferase in mouse liver. *J. Biol. Chem.* **267**, 11144-11148.
- Mayor, S., Presley, J. F. and Maxfield, F. R.** (1993). Sorting of membrane components from endosomes and subsequent recycling to the cell surface occurs by a bulk flow process. *J. Cell Biol.* **121**, 1257-1269.
- Menon, A. K.** (1995). Flippases. *Trends Cell Biol.* **5**, 355-360.
- Presley, J. F., Mayor, S., Dunn, K. W., Johnson, L. S., McGraw, T. E. and Maxfield, F. R.** (1993). The *End2* mutation in CHO cells slows the exit of transferrin receptors from the recycling compartment but bulk membrane recycling is unaffected. *J. Cell Biol.* **122**, 1231-1241.
- Rouser, G., Fleischer, S. and Yamamoto, A.** (1970). Two-dimensional thin-layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5**, 494-496.
- Sasaki, T.** (1990). Glycolipid transfer protein and intracellular traffic of glucosylceramide. *Experientia* **46**, 611-616.
- Schweizer, A., Clausen, H., van Meer, G. and Hauri, H.-P.** (1994). Localization of *O*-glycan initiation, sphingomyelin synthesis, and glucosylceramide synthesis in Vero cells with respect to the endoplasmic reticulum-Golgi intermediate compartment. *J. Biol. Chem.* **269**, 4035-4041.
- Shiao, Y.-J. and Vance, J. E.** (1993). Sphingomyelin transport to the cell surface occurs independently of protein secretion in rat hepatocytes. *J. Biol. Chem.* **268**, 26085-26092.
- Simons, K. and van Meer, G.** (1988). Lipid sorting in epithelial cells. *Biochemistry* **27**, 6197-6202.
- Strous, G. J., van Kerkhof, P., van Meer, G., Rijnbout, S. and Stoorvogel, W.** (1993). Differential effects of brefeldin A on transport of secretory and lysosomal proteins. *J. Biol. Chem.* **268**, 2341-2347.
- Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, Y.** (1981). Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res.* **41**, 1967-1972.
- Ueda, K., Okamura, N., Hirai, M., Tanigawara, Y., Saeki, T., Kioka, N., Komano, T. and Hori, R.** (1992). Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. *J. Biol. Chem.* **267**, 24248-24252.
- Urbani, L. and Simoni, R. D.** (1990). Cholesterol and vesicular stomatitis virus G protein take separate routes from the endoplasmic reticulum to the plasma membrane. *J. Biol. Chem.* **265**, 1919-1923.
- van der Bijl, P., Lopes-Cardozo, M. and van Meer, G.** (1996). Sorting of newly synthesized galactosphingolipids to the two surface domains of epithelial cells. *J. Cell Biol.* **132**, 813-821.
- van Echten, G., Iber, H., Stotz, H., Takatsuki, A. and Sandhoff, K.** (1990). Uncoupling of ganglioside biosynthesis by Brefeldin A. *Eur. J. Cell Biol.* **51**, 135-139.
- van Helvoort, A., van 't Hof, W., Ritsema, T., Sandra, A. and van Meer, G.** (1994). Conversion of diacylglycerol to phosphatidylcholine on the basolateral surface of epithelial (Madin-Darby canine kidney) cells. Evidence for the reverse action of a sphingomyelin synthase. *J. Biol. Chem.* **269**, 1763-1769.
- van Helvoort, A. and van Meer, G.** (1995). Intracellular lipid heterogeneity caused by topology of synthesis and specificity in transport. Example: sphingolipids. *FEBS Lett.* **369**, 18-21.
- van Helvoort, A., Smith, A. J., Sprong, H., Fritzsche, I., Schinkel A. H., Borst, P., and van Meer, G.** (1996). MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell* (in press).
- van Leyen, K. and Wieland, F.** (1994). Complete Golgi passage of glycotriptides generated in the endoplasmic reticulum of mammalian cells. *FEBS Lett.* **352**, 211-215.
- van Meer, G. and van 't Hof, W.** (1993). Epithelial sphingolipid sorting is insensitive to reorganization of the Golgi by nocodazole, but is abolished by monensin in MDCK cells and by brefeldin A in Caco-2 cells. *J. Cell Sci.* **104**, 833-842.
- Vance, J. E., Aasman, E. J. and Szarka, R.** (1991). Brefeldin A does not inhibit the movement of phosphatidylethanolamine from its sites of synthesis to the cell surface. *J. Biol. Chem.* **266**, 8241-8247.
- Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D. and van Deenen, L. L. M.** (1973). The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. *Biochim. Biophys. Acta* **323**, 178-193.
- Warnock, D. E., Lutz, M. S., Blackburn, W. A., Young, W. W. Jr and Baenziger, J. U.** (1994). Transport of newly synthesized glucosylceramide to the plasma membrane by a non-Golgi pathway. *Proc. Nat. Acad. Sci. USA* **91**, 2708-2712.
- Warren, G.** (1993). Membrane partitioning during cell division. *Annu. Rev. Biochem.* **62**, 323-348.
- Warren, G., Levine, T. and Misteli, T.** (1995). Mitotic disassembly of the mammalian Golgi apparatus. *Trends Cell Biol.* **5**, 413-416.
- Young, W. W. Jr, Lutz, M. S., Mills, S. E. and Lechler-Osborn, S.** (1990). Use of brefeldin A to define sites of glycosphingolipid synthesis: GA2/GM2/GD2 synthase is trans to the brefeldin A block. *Proc. Nat. Acad. Sci. USA* **87**, 6838-6842.
- Zieve, G. W., Turnbull, D., Mullins, J. M. and McIntosh, J. R.** (1980). Production of large numbers of mitotic mammalian cells by use of the reversible microtubule inhibitor nocodazole. *Exp. Cell Res.* **126**, 397-405.

(Received 15 July 1996 - Accepted 10 October 1996)