Ceramide transport from endoplasmic reticulum to Golgi apparatus is not vesicle-mediated

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Ceramide (Cer) transfer from the endoplasmic reticulum (ER) to the Golgi apparatus was measured under conditions that block vesicle-mediated protein transfer. This was done either in intact cells by reducing the incubation temperature to 15 °C, or in streptolysin O-permeabilized cells by manipulating the intracellular environment. In both cases, Cer transfer was not inhibited, as demonstrated by the biosynthesis of ceramide monohexosides and sphingomyelin (SM) *de novo* from met-

abolically (with [¹⁴C]serine) labelled Cer. This assay is based on the knowledge that Cer is synthesized, starting from serine and palmitoyl-CoA, at the ER, whereas glycosphingolipids and SM are synthesized in the (early) Golgi apparatus. Formation of [¹⁴C]glycosphingolipids and [¹⁴C]SM was observed under conditions that block vesicle-mediated vesicular stomatitis virus glycoprotein transport. These results indicate that [¹⁴C]Cer is transferred from ER to Golgi by a non-vesicular mechanism.

INTRODUCTION

Protein transport between the endoplasmic reticulum (ER) and the Golgi apparatus has been extensively studied and reviewed [1,2]. Research in this field is on the verge of establishing the molecular machinery required for this vesicular transport process [3]. However, lipid transport between these two organelles has only recently started to receive attention. Obviously, lipids are an inherent part of the vesicle carriers involved in transport between ER and Golgi. Indeed, the formation of these transport vesicles might well be in concert with the synthesis of phospholipids [4]. However, not all types of lipid need necessarily be included in this process of vesicle formation or require such vesicles for interorganellar transport. Of particular interest in this respect is ceramide (Cer). Its synthesis starts with the condensation of serine and palmitoyl-CoA by serine C-palmitoyltransferase to form 3-oxosphinganine, which is subsequently reduced to sphinganine. The addition of an amide-linked fatty acid by ceramide synthase yields dihydroceramide. These steps have been shown to take place at the cytosolic surface of the ER [5,6]. Dihydroceramide is the precursor for Cer synthesis, involving introduction of the 4-*trans* double bond (desaturation [7,8]), which is likely to occur at the ER as well [7]. Alternatively, dihydroceramide can be converted into the dihydro forms of glucosylceramide (GlcCer) and sphingomyelin (SM) [7]. The biosynthesis of glycosphingolipids and SM from (dihydro)Cer, however, occurs in the (early) Golgi apparatus. This has been shown for SM [7,9,10] and GlcCer [7,9,11,12], the latter being a precursor for many other glycosphingolipids. Interestingly, GlcCer is synthesized at the cytosolic surface of the Golgi membrane [11,13,14]. Thus one can envision that Cer, after its synthesis at the cytosolic surface of the ER, is transferred through the cytosol to reach the cytosolic surface of the Golgi, where it can be used as a substrate for GlcCer synthesis. In addition, a fraction of the Cer must be translocated at the Golgi to reach the luminal leaflet where it can be used as a substrate for SM biosynthesis [15].

Some papers have described the transport mechanism of Cer between ER and Golgi [16–20]. Certain of the authors have reached the conclusion that Cer transport is non-vesicular, on the basis of observations in mitotic cells or cell-free systems [16,17], whereas others have obtained evidence for the opposite conclusion, showing ATP and temperature-dependence of ceramide conversion to (glyco)sphingolipids [18,19] or Cer enrichment in ER-derived transport vesicles [20]. This lack of consensus prompted the present work, in which we directly compared Cer and vesicular stomatitis virus glycoprotein (VSV-G protein) transport between ER and Golgi, in a cell physiological setting. This allows us to draw firm conclusions about the involvement of vesicular protein carriers in Cer transport, without the interference of possible artifacts associated with assays *in vitro* and the integration of modified (short-chain) sphingolipids into biological membranes. Results are presented showing that the transport of Cer is not mediated by the vesicular protein transport machinery. Intact and permeabilized cells were incubated with [¹⁴C]serine and conditions were imposed that block protein transfer from ER to Golgi, such as lowering the incubation temperature to 15 °C or the manipulation of intracellular ATP, Ca²⁺ or cytosol levels. Although protein trafficking was blocked under such conditions, as shown by the lack of VSV-G protein modification, the biosynthesis of [¹⁴C]glycosphingolipids and [¹⁴C]SM proceeded, demonstrating that newly synthesized [¹⁴C]Cer had reached the Golgi apparatus by a vesicle-independent mechanism.

EXPERIMENTAL

Materials

Reduced streptolysin O was purchased from Sanofi Diagnostics Pasteur (Marnes-la-coquette, France). L-[U-¹⁴C]serine was from Amersham International (Little Chalfont, Bucks., U.K.). Tran³⁵S-label was from ICN Pharmaceuticals (Irvine, CA,

Abbreviations used: BFA, brefeldin A; Cer, ceramide; CMH, ceramide monohexoside; C₆-NBD, 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl or 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoic acid; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; GlcCer, galactosylceramide; GlcCer, glucosylceramide; GTP[S], guanosine 5'-[γ-thio]triphosphate; HPTLC, high-performance thin-layer chromatography; NEM, N-ethylmaleimide; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; SM, sphingomyelin; UDPGlu, uridine 5'-diphosphoglucose; VSV-G protein, vesicular stomatitis virus glycoprotein.

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U.S.A.). ATP, phosphocreatine, creatine phosphokinase, BSA, EGTA, uridine 5'-diphosphoglucose (UDPGlu), apyrase, sphingomyelinase (from *Staphylococcus aureus*), D-erythro-sphingosine, brefeldin A (BFA) and mouse monoclonal anti-(VSV-G protein) were purchased from Sigma (St. Louis, MO, U.S.A.). Rabbit anti-(mouse IgG) was from Dako (Glostrup, Denmark). Protein A/G agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Endoglycosidase H (endo H), dithiothreitol and guanosine 5'-[γ -thio]triphosphate (GTP[S]) were from Boehringer (Mannheim, Germany) and MnCl_2 was from Merck (Darmstadt, Germany). The succinimidyl ester of 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoic acid (C_6 -NBD) was from Molecular Probes (Eugene, OR, U.S.A.). C_6 -NBD-Cer was synthesized from D-erythro-sphingosine and the succinimidyl ester of C_6 -NBD, by the method of Kok et al. [21].

Cell culture

Monocultures of HT29 G+ cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, supplemented with 10% (v/v) decompented (56 °C, 30 min) fetal calf serum, in a water-saturated atmosphere of air/ CO_2 (19:1). Cells were routinely seeded at a density of 6.6×10^4 cells/ cm^2 . During the exponential phase of growth, the culture medium was changed every 48 h. Under these conditions cell confluency was reached after 7 days of culture.

Preparation of subcellular fractions

Cytosol was prepared from HT29 G+ cells as described previously [22]. Rat liver Golgi membranes were isolated as described before [9].

Procedure for permeabilization with streptolysin O

HT29 G+ cells were permeabilized with streptolysin O by the procedure of Tan et al. [23], with concentrated streptolysin O binding buffer [1.37 M NaCl/27 mM KCl/20 mM EGTA/10 mM CaCl_2 /20 mM MgCl_2 /0.2 M Pipes (pH 7.3)] and permeabilization buffer [0.9 M KCl/16 mM EGTA/8 mM CaCl_2 /20 mM MgCl_2 /0.2 M Hepes (pH 7.3)], diluted with water. In brief, cells were washed three times with Hanks buffer (at 2 °C) then incubated with streptolysin O (1.3 units/ml in binding buffer/water [1:9, v/v] for 10 min at 2 °C). After two washes with binding buffer/water [1.3:8.7, v/v], the cells were transferred to 37 °C by adding warm (hypotonic) permeabilization buffer/water [0.8:9.2, v/v]. After 5 min at 37 °C (during which permeabilization occurred), an equal volume of permeabilization buffer/water [2.8:7.2, v/v] was added to restore the osmolarity of the medium, and allowed to equilibrate for 5 min at 37 °C. For the transport assay (see below), the medium was removed and replaced by transport buffer [140 mM KCl/0.5 mM KH_2PO_4 /20 mM Hepes (pH 7.3)].

The streptolysin O permeabilization procedure was optimized so that a high efficiency of permeabilization was obtained. With 1.3 units/ml streptolysin O in binding buffer/water [1:9, v/v], the procedure resulted in the permeabilization of more than 95% of the cells. This was established by permeabilization of cells grown to confluency in 45 mm Petri dishes, followed by incubation with transport buffer containing propidium iodide (0.2–1 $\mu\text{g}/\text{ml}$) for 10 min at 37 °C, and counting of the fraction of propidium iodide-stained cells.

The cytosol was efficiently washed out of the permeabilized cells. This was checked by incubating permeabilized cells with C_6 -NBD-Cer (results not shown). In intact cells, this fluorescent

sphingolipid precursor reaches all organelles by diffusion, including the Golgi apparatus, where it is converted into C_6 -NBD-GlcCer and C_6 -NBD-SM [24]. In permeabilized cells, however, C_6 -NBD-GlcCer synthesis was completely abolished. The latter could be restored by adding the substrate UDPGlu, which is normally present in cytosol supporting the biosynthesis of GlcCer on the cytosolic leaflet of the Golgi membrane. Thus in permeabilized cells UDPGlu was washed out with the cytosol.

The Golgi apparatus itself, however, remains intact in permeabilized cells. This is a prerequisite for the transport assay, because it is the target organelle for Cer. Morphological integrity of the Golgi apparatus was checked microscopically (results not shown) (see [22]), by co-labelling of permeabilized cells with propidium iodide as a permeabilization marker and C_6 -NBD-Cer as a vital stain for the Golgi [25]. In these experiments intact cells were incubated with C_6 -NBD-Cer for 30 min at 37 °C, followed by permeabilization as described above and staining with propidium iodide.

Cer transport assay

To study the temperature dependence of Cer transfer from ER to Golgi, intact HT29 G+ cells were pulse-loaded for 5 min at 37 °C with [^{14}C]serine (20 $\mu\text{Ci}/\text{ml}$) in minimal essential medium (without serine and fetal calf serum), after an initial incubation for 1 h at 37 °C in minimal essential medium (without serine and fetal calf serum). Thereafter the cells were incubated in DMEM (without fetal calf serum) at the desired incubation temperatures (2, 15 or 37 °C) for 2 h. Control cells were subjected to extraction (see below) immediately after the [^{14}C]serine pulse. For BFA treatment, cells were preincubated for 1 h with the drug (in minimal essential medium without serine and fetal calf serum), and it was added to all subsequent incubations.

Streptolysin O-permeabilized HT29 G+ cells were incubated with [^{14}C]serine (0.5 $\mu\text{Ci}/\text{ml}$) in transport buffer for 2 h at 37 °C, occasionally in the presence of (1) a GlcCer biosynthesis-supporting system (10 mM UDPGlu/10 mM MnCl_2), (2) an ATP-regenerating system [7 mM ATP/35 mM phosphocreatine/25 units/ml creatine phosphokinase/0.4 mM dithiothreitol/0.1% (w/v) BSA], (3) 1 mg/ml cytosol, (4) 100 μM GTP[S], (5) 50 mM EGTA, (6) 3 mM ZnCl_2 , (7) 30 units/ml apyrase or (8) 3 mM N-ethylmaleimide (NEM). Control cells were not permeabilized and were incubated with [^{14}C]serine (0.5 $\mu\text{Ci}/\text{ml}$) in DMEM (without fetal calf serum) for 2 h at 37 °C.

Lipid analysis and calculations

After the incubations as indicated above, equal numbers of cells for each condition were subjected to lipid extraction by the procedure of Bligh and Dyer [26]. Extracted lipids were separated by two-dimensional high-performance thin-layer chromatography (HPTLC), employing solvent system A [$\text{CH}_3\text{Cl}/\text{CH}_3\text{OH}/20\%$ (w/v) NH_4OH (70:30:5, by vol.)] in the first dimension and solvent system B [$\text{CH}_3\text{Cl}/\text{CH}_3\text{OH}/0.2\%$ CaCl_2 in water (50:42:11, by vol.)] in the second, followed by autoradiography. This procedure allowed the separation of radioactively labelled Cer, ceramide monohexoside (CMH), SM, phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtn). To characterize the sphingolipids, individual spots were scraped from the plate, then the lipids were eluted from the silica by washing with 10 vol. of $\text{CH}_3\text{Cl}/\text{CH}_3\text{OH}$ (1:1, by vol.) and 10 vol. of CH_3OH . (1) The presumed Cer spot was either run directly on HPTLC, with the solvent system C [$\text{CH}_3\text{Cl}/\text{CH}_3\text{OH}/\text{acetic acid}$ (90:2:8, by vol.)] or subjected to acid

hydrolysis. To the lipid was added 1 ml of CH_3OH and 1 ml of 1 M HCl, followed by an incubation at 70 °C for 18 h under argon. After neutralization with 1 M KOH the sample was desalted on a Sep-pak C_{18} cartridge. The eluate was applied to HPTLC and developed in solvent system D [$\text{CH}_3\text{Cl}/\text{CH}_3\text{OH}/3.75\%$ (w/v) NH_4OH (40:10:1, by vol.)] to separate Cer from sphingoid bases [6]. (2) The presumed CMH spots were run on HPTLC plates, which were impregnated with borate [27] by being sprayed with methanol containing boric acid (1.25 g/50 ml). These plates were developed in solvent system E [$\text{CH}_3\text{Cl}/\text{CH}_3\text{OH}/25\%$ (w/v) NH_4OH (65:35:5, by vol.)]. Glycolipids were identified by the orcinol test [28]. (3) The presumed SM spot was either run directly on HPTLC, employing solvent system F [$\text{CH}_3\text{Cl}/\text{CH}_3\text{OH}/\text{acetic acid}/\text{water}$ (25:15:4:2, by vol.)] or subjected to hydrolysis by sphingomyelinase, by the method of Gatt et al. [29], before HPTLC analysis in solvent system F.

For quantification, individual spots were scraped from the plate and counted in a liquid-scintillation counter. The radioactive counts were used to calculate the percentages of each lipid with respect to the total radioactivity incorporated into the lipid pool (set to 100%). Alternatively, radioactive counts were used to calculate the ratio of CMH to Cer or SM to Cer.

For analysis of the temperature dependence of sphingolipid biosynthesis, 25 μg of rat liver Golgi membranes were incubated for 2 h at different temperatures with 2 μM $\text{C}_6\text{-NBD-Cer}$ and 10 mM UDPGlu, followed by lipid extraction [26] and HPTLC separation, employing solvent system A. Individual $\text{C}_6\text{-NBD-sphingolipid}$ spots were scraped, followed by elution of the lipid from the silica by vigorous shaking in 3 ml of 1% (v/v) Triton X-100. After centrifugation of the silica particles, the supernatant was measured in a fluorimeter at 530 nm (excitation at 465 nm). Individual $\text{C}_6\text{-NBD-labelled}$ lipids were quantified and expressed as a percentage of the total $\text{C}_6\text{-NBD-labelled}$ lipid.

VSV-G protein transport assay

HT29 G+ monolayers were infected at 25 p.f.u./cell with the VSV temperature-sensitive mutant ts045 virus in DMEM (without fetal calf serum) for 1 h at 32 °C. After 4 h of infection in DMEM containing 10% (v/v) fetal calf serum, the cells were treated with trypsin. In transport studies in permeabilized cells, permeabilization with streptolysin O, as described above, was performed before trypsinization. Subsequently the cells were incubated for 15 min in methionine-free DMEM at 40 °C and pulse-labelled in suspension with 200 $\mu\text{Ci}/\text{ml}$ Tran ^{35}S -label for 10 min at 40 °C. Cells were washed with ice-cold PBS and chased for 2 h at various temperatures with various additions to permeabilized cells (see the Cer transport assay section). After the chase, cells were washed twice with ice-cold PBS and lysed for 15 min on ice with lysis buffer [1% (v/v) Triton-X-100/50 mM Tris/HCl (pH 8.0)/62.5 mM EDTA]. Subsequently the cells were centrifuged at 11000 g for 15 min. To supernatants, 200 μl of detergent solution [0.4% deoxycholic acid/1% (v/v) NP40/50 mM Tris/HCl (pH 8.0)/62.5 mM EDTA], 8 μl of 10% (w/v) SDS and 2 μl of mouse monoclonal anti-(VSV-G protein) antibody were added and incubated overnight at 4 °C. Thereafter the mixture was incubated with rabbit anti-(mouse IgG) for 1 h at 4 °C, then 20 μl of protein A/G agarose was added and incubated for 45 min at room temperature. The protein A/G agarose beads were centrifuged and washed three times with RIPA buffer [0.1% SDS/1% (w/v) deoxycholic acid/1% (v/v) NP40/10 mM Tris/HCl (pH 7.4)/0.15 M NaCl], three times with TENEN high-salt buffer [0.1% SDS/0.5% (v/v) NP40/10 mM Tris/HCl (pH 7.2)/0.5 M NaCl/1 mM EDTA] and once

with PBS. Finally the protein A/G agarose beads were re-suspended in 10 μl of BH1 buffer [0.1% (v/v) Triton X-100/1% (w/v) SDS/0.1 M sodium acetate (pH 5.5)] and proteins were eluted from the beads by being heated at 95 °C for 5 min. The supernatant was recovered after centrifugation and 30 μl of BH2 buffer [0.1 M sodium acetate (pH 5.5)/1 mM PMSF/5 $\mu\text{g}/\text{ml}$ aprotinin/1 mM benzamide] was added. Subsequently 0.25 m-unit of endo H was added and incubated overnight at 37 °C. Samples were analysed under reducing conditions by SDS/PAGE [7.5% (w/v) gel]; gels were subjected to fluorography.

RESULTS AND DISCUSSION

Identification of lipid products

Figure 1 shows an autoradiograph of a two-dimensional HPTLC plate on which a lipid extract was run from HT29 G+ cells incubated for 2 h at 37 °C with [^{14}C]serine. Individual lipid spots were clearly separated, the CMH consisting of several spots. The presumed Cer, CMH and SM spots were scraped from the HPTLC plate, followed by elution of the lipids for characterization and positive identification. The presumed Cer (spot 1 in Figure 1) co-migrated with Cer standard in solvent system C and resulted in the formation of sphingoid bases on acid hydrolysis (results not shown). The CMH spots (Figure 1, spots 2) were all stained by orcinol and could be further separated in solvent system E after borate impregnation. The CMHs were separated into four spots, two of which were identified as GlcCer and the other two as galactosylceramide (GalCer). The presumed SM (spot 5 in Figure 1) co-migrated with SM standard in solvent system F and was converted to Cer on hydrolysis by sphingomyelinase.

Transport of Cer from ER to Golgi in permeabilized cells

The streptolysin O permeabilization procedure resulted in the formation of stable pores in the plasma membrane, removal of the cytosol, and maintenance of the morphological integrity of the Golgi apparatus (see the Experimental section). Consequently this system could be used for studying the transport mechanism

Figure 1 Two-dimensional HPTLC profile of ^{14}C -labelled lipids

HT29 G+ cells were incubated with [^{14}C]serine (0.5 $\mu\text{Ci}/\text{ml}$) for 2 h at 37 °C. Thereafter, lipids were extracted and separated in two dimensions on HPTLC, as described in the Experimental section. Shown here is an autoradiograph of an HPTLC, exposed for 1 week. By using reference lipid markers and biochemical analyses, individual lipid spots were identified as Cer (1), CMHs (2), PtdEtn (3), PtdSer (4) and SM (5). 0 represents the origin of lipid extract application. CMHs consist of several spots, which can be further separated in solvent system E (results not shown; see the text for details).

of Cer from the ER to the Golgi and the potential involvement of cytosolic factors. Vesicular transport of proteins from ER to Golgi has been shown to require ATP [30–32], Ca^{2+} [30,33] and cytosolic (GTP-binding) proteins [30,32–34]. These factors can be removed from or supplied to the permeabilized cells, to study their effect on Cer transfer. In this way one can determine whether Cer transport requires similar cofactors to those needed to sustain protein transport by vesicular carriers. The Cer transfer assay is based on the knowledge that Cer is synthesized at the ER [5,6] and incorporates [^{14}C]serine supplied to the cells, whereas CMH and SM are synthesized in the (early) Golgi apparatus [10–12]. Therefore radioactive CMH and SM will only be detected when transfer of [^{14}C]Cer from the ER to the Golgi has occurred. Biosynthesis of SM has also been shown to occur in the plasma membrane of cells [9,10]. However, this synthase activity contributes a minor portion to total SM biosynthesis and evidently also implicates the transport of newly synthesized Cer out of the ER.

Figure 2 shows the results of this approach. In permeabilized HT29 G+ cells, some SM synthesis still occurred (s1 in Figure 2B), but CMH synthesis was abolished (s1 in Figure 2A). This result is consistent with that obtained by using $\text{C}_6\text{-NBD-Cer}$ as a sphingolipid precursor and can be attributed (as outlined in the Experimental section) to a lack of the substrate UDPGlu, which is effectively washed out. CMH synthesis was recovered by supplying the cells with UDPGlu and Mn^{2+} (s2), resulting in a relatively high rate of synthesis (Figure 2A). However, also Cer and SM synthesis were relatively high under these conditions (s2 in Figures 2B and 2C). Therefore it can be assumed that the increase in the [^{14}C]Cer pool results in an increase in the pools of both [^{14}C]CMH and [^{14}C]SM. On the addition of an ATP-regenerating system (s3), the relative synthesis of all sphingolipids (Cer, CMH and SM) decreased compared with the incubation without ATP (s2 in Figures 2A–C), whereas the absolute synthesis of all lipids increased markedly (Figure 2, legend). Thus an increase in the [^{14}C]Cer pool is reflected in a similar increase in the radioactive CMH and SM pools. ATP therefore enhances Cer biosynthesis but is not a necessary component for the Cer transport system. This is further shown by supplying permeabilized cells with apyrase (s8), which abolishes all ATP. Under these conditions CMH and SM synthesis still occurred in accordance with Cer synthesis. Furthermore additions of cytosol or GTP[S] to permeabilized, ATP-supplied cells had little or no effect on CMH and SM synthesis (s4 and s5 in Figures 2A–2C), whereas the addition of EGTA resulted in a slight decrease in the relative synthesis of all sphingolipids (s6 in Figures 2A–2C). Control experiments involved the analysis of VSV-G protein carbohydrate modification (acquisition of resistance to endoH) as an indication of its vesicular transfer from the ER to the Golgi compartment. Results showed that, under all incubation conditions, vesicular transport from ER to Golgi was abolished in permeabilized cells but not in intact control cells (Figure 3; compare s1–s8 with 37 °C). Nevertheless Cer transfer continued under conditions that blocked vesicular protein transport from ER to Golgi. In addition the inclusion of Zn^{2+} (s7), which has been reported to block the formation of transport vesicles completely at the ER [4], did not result in a decrease in the formation of CMH or SM (s7 in Figures 2A and 2B). In the same report [4] NEM was shown to inhibit transport from ER to Golgi. In our assay, NEM completely blocked CMH synthesis, whereas SM and total lipid synthesis were largely decreased (results not shown). However, NEM also completely blocked the synthesis of $\text{C}_6\text{-NBD-GlcCer}$ from $\text{C}_6\text{-NBD-Cer}$, and therefore inhibits the GlcCer synthase, interfering with the principle of our transport assay.

Considering the overall resemblance of the relative Cer synthesis on the one hand (Figure 2C), and especially the CMH, but also the SM synthesis on the other hand (Figures 2A and 2B), one can conclude that CMH and SM synthesis are not limited by Cer transport but depend only on the rate of biosynthesis of Cer. This is further shown in Figure 2(E), where the ratio of CMH/Cer biosynthesis is equal under all tested conditions in permeabilized cells supplied with UDPGlu and Mn^{2+} . With SM (Figure 2F) an effect of ATP is discerned (compare s3 with s2), which causes an increase of SM biosynthesis relative to that of Cer.

In conclusion, none of the factors known to interfere strongly with vesicular protein transport between ER and Golgi affect the relative synthesis of CMH. The only requirement is the presence of UDPGlu. Therefore Cer seems to reach the target organelle (Golgi) for CMH synthesis without the need for vesicular carriers. For SM these factors are not necessary either, although ATP has a stimulatory effect on synthesis (see below).

It is surprising that Cer formation continued in permeabilized cells, i.e. without an apparent requirement for palmitoyl-CoA and NADPH, because the initial steps of Cer biosynthesis have been shown to occur at the cytosolic leaflet of the ER [5,6]. However, the apparent dependence on these factors for Cer biosynthesis, as observed *in vitro* with isolated membranes, cannot be extrapolated to the situation in permeabilized cells. (1) The washing of isolated membranes is more rigorous than that of organelle membranes in permeabilized cells. Therefore it is likely that small molecules are not completely washed out from permeabilized cells. This has also been observed for NAD^+ by others (see, for example, [35]). Moreover palmitoyl-CoA might be even more refractory to complete wash-out owing to its partly hydrophobic character, supporting attachment to membranes. (2) In contrast with isolated ER/Golgi membranes, permeabilized cells contain intact (i.e. not perforated by streptolysin O) organelles, including mitochondria. These organelles contain a large fraction of the cellular pyridine nucleotides and might function as a source of NADPH in permeabilized cells. Small quantities of cofactors might be sufficient to sustain Cer biosynthesis. In this context NADPH oxidase has been shown to have a significantly higher substrate affinity for NADPH in permeabilized cells than in isolated membranes [36], indicating that the properties of enzymes, as observed *in vitro*, can differ from those in (permeabilized) cells.

Transport of Cer from ER to Golgi at lower temperature

Another established way of interfering with vesicular protein traffic between ER and Golgi is by lowering the incubation temperature. At temperatures of 15 °C and below, protein transport to the Golgi is known to be inhibited [31,32,37,38]. As shown in Figure 3, transfer of VSV-G protein from the ER to the Golgi was indeed abolished in HT29 G+ cells at 15 °C or below, because the acquisition of resistance to endo H did not occur at these temperatures in contrast with 37 °C. This temperature block can similarly be applied to investigate whether Cer transfer between ER and Golgi is blocked. Figure 4 shows the result of decreasing the incubation temperature to either 2 or 15 °C on Cer transfer and consecutive CMH/SM synthesis. The cells were pulsed with [^{14}C]serine for 5 min at 37 °C to provide an intracellular pool of the radioactive precursor (this was necessary because the cellular uptake of [^{14}C]serine was drastically decreased at 15 °C or below). During this pulse some [^{14}C]CMH and [^{14}C]SM synthesis occurred (Figures 4A and 4B). During the subsequent 2 h incubation at 2 °C the percentage of biosynthesis of these sphingolipids did not increase. However, at 15 °C an increase was observed, most prominently for CMH. At 37 °C the

Figure 3 Transport of VSV-G protein from ER to Golgi in permeabilized cells or at lower temperature

HT29 G+ cell monolayers were infected with VSV ts045 mutant virus, permeabilized with streptolysin O (only s1–s8), treated with trypsin, pulse-labelled with Tran³⁵S-label, then chased at different temperatures or, for permeabilized cells, with different additions (see below). Thereafter, cells were lysed and the VSV-G protein was immunoprecipitated. For each condition, half of the immunoprecipitate was subjected to treatment with endo H (+). Samples were analysed by SDS/PAGE and subjected to fluorography. Shown here is a typical result taken from three independent experiments. Additions to streptolysin O permeabilized cells were as described in the legend to Figure 2.

biosynthesis of both CMH and SM increased. Although this suggests that Cer transfer might have increased with temperature, this turned out not to be so. It is important to realize that the assay to monitor Cer transport involves a two-step process: (1) the transfer of [¹⁴C]Cer from ER to Golgi, and (2) the subsequent biosynthesis of [¹⁴C]CMH and [¹⁴C]SM. The latter process is probably temperature-dependent and hence might lead to aberrant conclusions on the temperature dependence of Cer transport if the proper controls are omitted. Therefore the temperature dependence of biosynthesis was tested in a system *in vitro*, utilizing isolated Golgi membranes from rat liver and C₆-NBD-Cer as a substrate. In this assay the delivery of the substrate to the biosynthetic enzymes is not a limiting factor and the temperature dependence of sphingolipid product formation can be measured. The profile of C₆-NBD-GlcCer biosynthesis in the Golgi at three different temperatures (Figure 5E) closely resembled that of [¹⁴C]CMH biosynthesis in intact HT29 G+ cells (Figure 4A). In addition, a second type of control experiment was performed, with the use of BFA to merge ER and Golgi membranes. Also in this experimental setup, intermembrane Cer transport is not required for CMH and SM biosynthesis because the synthases are now in the same membrane as the enzymes

Figure 2 Transport of Cer from ER to Golgi in permeabilized cells

HT29 G+ cells were permeabilized with streptolysin O as described in the Experimental section. Thereafter, the cells were incubated with transport buffer with various additions (see below). Control cells (c) were not permeabilized. Under each condition, [¹⁴C]serine (0.5 μCi/ml) was added to the incubation mixture and the cells were incubated for 2 h at 37 °C. Thereafter, the cells were harvested; this was followed by lipid extraction, HPTLC and scintillation counting of the individual spots on HPTLC, corresponding to Cer, CMH, SM, PtdEtn and PtdSer (see Figure 1). From the resulting radioactive counts the percentages of CMH (A), SM (B), Cer (C) and PtdSer + PtdEtn (D) of the total lipid pool was calculated and is depicted as a bar. (E, F) The fractional radioactive counts of CMH (E) and SM (F) divided by the radioactive count of Cer. PL represents the phospholipids (PtdSer + PtdEtn). Results are means ± S.D. for triplicate measurements. Additions to streptolysin O-permeabilized cells were as follows: s1, none (31.4 d.p.s./mg); s2, 10 mM UDPGlu and 10 mM MnCl₂ (38.2 d.p.s./mg); s3, UDPGlu/MnCl₂ and ATP + regenerating system (see the Experimental section) (349 d.p.s./mg); s4, UDPGlu/MnCl₂, ATP + regenerating system and cytosol (1 mg/ml of protein) (281 d.p.s./mg); s5, UDPGlu/MnCl₂, ATP + regenerating system, cytosol and 100 μM GTP[S] (GTPγS) (257 d.p.s./mg); s6, UDPGlu/MnCl₂, ATP + regenerating system, cytosol and 50 mM EGTA (316 d.p.s./mg); s7, UDPGlu/MnCl₂, ATP + regenerating system, cytosol and 3 mM ZnCl₂ (116 d.p.s./mg); s8, UDPGlu/MnCl₂, apyrase (30 units/ml) (29.7 d.p.s./mg). In parentheses are given the average (n = 3) incorporations of [¹⁴C]serine into the total lipid pool (d.p.s./mg of cellular protein). In control cells the incorporation was 241 d.p.s./mg.

Figure 4 Transport of Cer from ER to Golgi at lower temperature

HT29 G+ cells were loaded with [14 C]serine (20 μ Ci/ml) for 5 min at 37 °C after an initial incubation for 1 h at 37 °C in minimal essential medium (without serine and fetal calf serum). Thereafter, the cells were incubated for a further 2 h at the respective temperatures. Control cells (—) were subjected to lipid extraction immediately after the 5 min loading. After the incubations the cells were harvested; this was followed by lipid extraction, HPTLC and scintillation counting of the individual lipid spots on HPTLC, corresponding to CMH (A), SM (B), Cer (C) and PtdSer + PtdEtn (D; see Figure 1). From the radioactive counts the percentage of each lipid in the total lipid pool was calculated and is depicted as a bar. PL represents the phospholipids (PtdSer + PtdEtn). The average ($n = 3$) incorporation of [14 C]serine (d.p.s./mg of protein) into the total lipid pool was 694 (loading), 1131 (2 °C chase), 1149 (15 °C chase) and 1644 (37 °C chase). Results are means $\pm$ S.D. for triplicate measurements. The significance of differences between two means was determined by Student's t test. $P < 0.05$ was considered significant. Asterisks indicate results that were significantly different from all other conditions.

responsible for Cer generation. Thus non-transport components of the assay system were explicitly tested in this way. As shown in Figures 5(A) to 5(D), the profile of [14 C]CMH biosynthesis at three different temperatures closely resembled that in cells that had not been treated with BFA, in terms of the relative increases with temperature. Absolute biosynthesis of [14 C]CMH was higher at all temperatures in BFA-treated cells, as expected in view of earlier results [7,39]. Thus variation in [14 C]CMH formation from [14 C]Cer with temperature in intact cells results from temperature-dependent CMH synthase activity, and is not a consequence of limited Cer availability at lower temperature. This indicates that Cer transfer from ER to Golgi did occur at 15 °C. For [14 C]SM, the lack of substantial increase of its formation from [14 C]Cer in intact cells at 15 °C cannot be explained by an effect of lower temperature on SM synthase capacity only (compare Figure 4B with Figures 5B and 5F). Consequently another factor should be responsible for the large increase in SM biosynthesis observed at 37 °C in intact HT29 G+ cells. This result is consistent with the ATP-stimulated biosynthesis of SM in permeabilized cells (see above), indicating that an energy- and temperature-dependent process is involved. Several alternative explanations for this phenomenon can be conceived. (1) SM biosynthesis can occur only when Cer is available in the luminal leaflet of the Golgi membrane and thus might require temperature-dependent, ATP-stimulated translocation of Cer from the cytosolic leaflet, after its delivery to the Golgi through the cytosol. However, the assumption has often been made that natural Cer undergoes spontaneous flip-flop with a $t_{1/2}$ of only seconds [40]. By analogy with diacylglycerol, which

Figure 5 Temperature dependence of CMH and SM synthase activities

(A–D) HT29 G+ cells were loaded with [14 C]serine (20 μ Ci/ml) for 5 min at 37 °C, after an initial incubation for 1 h at 37 °C in minimal essential medium (without serine and fetal calf serum) with 5 μ g/ml BFA. Thereafter, the cells were incubated for a further 2 h at the respective temperatures, in the presence of BFA. Control cells (—) were subjected to lipid extraction immediately after the 5 min loading. After the incubations the cells were harvested; this was followed by lipid extraction, HPTLC and scintillation counting of the individual lipid spots on HPTLC, corresponding to CMH (A), SM (B), Cer (C) and PtdSer + PtdEtn (D; see Figure 1). From the radioactive counts the percentage of each lipid in the total lipid pool was calculated and is depicted as a bar. PL represents the phospholipids (PtdSer + PtdEtn). The average ($n = 3$) incorporation of [14 C]serine (d.p.s./mg of protein) into the total lipid pool was 418 (loading), 432 (2 °C chase), 349 (15 °C chase) and 559 (37 °C chase). (E, F) Golgi membranes (25 μ g) were incubated for 2 h at different temperatures with 1 nmol of C₆-NBD-Cer and 10 mM UDPGlu; this was followed by lipid extraction, HPTLC, scraping and measurement of the C₆-NBD-Cer/GlcCer/SM spots. The percentages of C₆-NBD-GlcCer (E) and C₆-NBD-SM (F) of the total pool of C₆-NBD-labelled lipid were calculated and are depicted as bars. With the Golgi incubations, pulse loading of the substrate was not necessary (—). Results are means $\pm$ S.D. for triplicate measurements. The significance of differences between two means was determined by Student's t test. $P < 0.05$ was considered significant. Asterisks indicate results that were significantly different from all other conditions. In (F) only the results of the 2 °C and 37 °C measurements were significantly different.

to some extent shows a structural similarity to Cer, the absence of a polar headgroup is thought to facilitate the transversion of Cer across the hydrophobic bilayer interior [41]. In addition, the fact that Cer is uncharged might also ease the translocation process. However, no experimental results concerning flip-flop characteristics of natural Cer are available. Moreover it is not immediately apparent that observations that C₆-NBD-Cer [7,9,25] and truncated (C₈-C₈-)Cer [42] readily permeate intact membranes without energy input can be extrapolated to the fate of endogenous Cer. Rather, we expect that movement across the bilayer depends delicately on the complete structure of the Cer molecule. Furthermore the exogenous insertion of short-chain

sphingolipid analogues into biological membranes might by itself cause local disturbances in membrane organization, which in turn might enhance movement across the bilayer. Finally, definite answers on the contribution of movement across the bilayer to the stimulation of SM biosynthesis by temperature and ATP must await the development of assays that can be applied to endogenous Cer in intact cells. (2) SM biosynthesis might (partly) occur in a sub-Golgi compartment distal to that which is involved in GlcCer synthesis. In that case, SM biosynthesis requires not only the transport of Cer from ER to Golgi but also intra-Golgi transport. The latter process is likely to be temperature- and ATP-dependent (compare [43]). The SM synthase activity has not been localized with high precision in a specific sub-Golgi compartment. However, experimental evidence is available that supports the conclusion on a distinction in the relative positioning of GlcCer and SM synthase in the Golgi [39]. (3) It cannot be excluded that the stimulation of SM biosynthesis by temperature and ATP is due to the contribution of the plasma membrane SM synthase, which is active in HT29 cells and responsible for 15–25% of total SM synthesis [9]. However, we consider this possibility unlikely because it cannot explain the difference in temperature sensitivity of SM synthesis between BFA-treated and untreated cells, as BFA does not cause a redistribution of the plasma membrane SM synthase to the ER.

Perspective

Taken together the results presented above indicate that Cer transfer from ER to Golgi, as reflected by the relative biosyntheses of CMH and SM, is not dependent on vesicular carriers that mediate protein transport. Under conditions in which we show that vesicular protein transport was blocked, i.e. by imposing an energy block or by lowering the temperature, Cer transfer still occurred. These results corroborate the studies of Moreau et al. [17], who used an entirely different experimental approach: in isolated ER–Golgi transition vesicles ceramide was found to be present in much smaller amounts than in ER and Golgi membranes, whereas in a cell-free system [^3H]Cer transport was independent of ATP or cytosol and was not inhibited by NEM [17]. A slight disadvantage of this method is that exogenous [^3H]Cer has to be incorporated into the ER to permit its transport to the Golgi to be followed. The question can thus be raised as to whether these labelled Cer molecules are incorporated in the membrane in a fashion different from their natural counterparts, which in turn might affect their transport mechanism. These potential problems are overcome in the present approach, involving the endogenous radiolabelling of Cer molecules, which are naturally incorporated into the membrane. Furthermore our results are in good agreement with those of Collins and Warren [16], who observed that Cer transport between ER and Golgi continues in intact mitotic cells. Although they did not perform control experiments on the inhibition of protein transport in their system, it is generally accepted that this process is blocked in mitotic cells.

In contrast, Kendler and Dawson [18] reached a different conclusion, based on a similar approach to ours, using [^3H]palmitate as a radioactive precursor instead of [^{14}C]serine. They showed that the transport of non-hydroxy fatty acid (NFA)[^3H]Cer from the ER to the Golgi and the concomitant synthesis of NFA[^3H]GalCer are ATP-dependent processes in oligodendrocytes. Depletion of ATP results in an increased NFA[^3H]Cer pool and a decreased NFA[^3H]GalCer pool. As a possible explanation, oligodendrocytes might dispose of a unique Cer transport and GalCer synthesis mechanism, related to the bulk synthesis of this glycolipid that occurs specifically in these

cells. In addition, results obtained by van Helvoort et al. [19] showing a dependence of SM biosynthesis on BFA treatment at 10 °C seem to be at odds with those reported in the present work. However, both these results can be reconciled by taking into account that the precise temperature at which the experiments are conducted is critical. At 15 °C, CMH and SM synthase activities are less decreased than at 10 °C, whereas protein transport is blocked, as shown in this study. Furthermore non-vesicular Cer transport by itself might exhibit a threshold temperature below which inhibition occurs. We also observe increased biosynthesis of SM in BFA-treated cells at 15 °C (compare Figures 5B and 4B), but without BFA its synthesis is still enhanced at 15 °C compared with that at 2 °C and the pulse. It is conceivable that also in our experimental setup differences between 2 and 10 °C are too small to be discerned. Moreover, van Helvoort et al. [19] do not report on CMH biosynthesis, which in our studies provides the most compelling evidence for non-vesicular Cer transport.

Taken together, our conclusion that Cer transport from ER to Golgi is non-vesicular is based on studies that, for the first time, directly compare Cer and protein transport in one and the same cell system in a physiological setting. As discussed, most results so far reported in the literature can be reconciled with this view. Transport of Cer between ER and Golgi might occur simply by diffusion or might involve lipid-transfer proteins. A distinction between these two alternatives requires further study.

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