The long-chain sphingoid base of sphingolipids is acylated at the cytosolic surface of the endoplasmic reticulum in rat liver

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Ceramide, a key intermediate in sphingolipid metabolism, is synthesized by acylation of sphinganine followed by dehydrogenation of dihydroceramide to ceramide. Using radioactive sphinganine, we have examined the site and topology of dihydroceramide synthesis in well-characterized subcellular fractions from rat liver. [4,5-^{3}H]Sphinganine was introduced as a complex with BSA and was metabolized to [4,5-^{3}H]dihydroceramide upon incubation of rat liver homogenates or microsomes with fatty acyl CoA. Conditions were established in a detergent-free system in which dihydroceramide synthesis was not limited by either substrate availability or by amounts of microsomal protein or reaction time. The distribution of dihydroceramide synthesis was found to exactly parallel that of an endoplasmic reticulum (ER) marker upon subfractionation of microsomes, and no endogenous activity was detected in either purified Golgi apparatus or plasma membrane fractions. Limited protease digestion demonstrated that sphinganine N-acyltransferase is localized at the cytosolic surface of intact ER-derived vesicles. These results are discussed with regard to the subsequent transport of (dihydro)-ceramide from the ER to sites of further metabolism in a pre-Golgi apparatus compartment and in the cis and medial cisternae of the Golgi apparatus.

INTRODUCTION

The secretory pathway has been the focus of considerable attention in recent years (Rothman and Orci, 1992). In particular, the molecular machinery by which proteins are transported from the endoplasmic reticulum (ER) to and through the Golgi apparatus has been studied in detail. Less information is available about the transport of lipids along the secretory pathway (Pagano, 1990; Wattenberg, 1990; Moreau et al., 1991; Puoti et al., 1991). As a prerequisite for studying lipid transport through the ER and Golgi apparatus, biochemical markers for each compartment are required. Thus, we have recently shown that two sphingolipids are synthesized at discrete locations along the secretory pathway. Glucosylceramide (GlcCer), the precursor of complex glycosphingolipids such as gangliosides, is synthesized by the glucosylation of ceramide at the cytosolic surface of a pre- or early Golgi apparatus compartment (Futerman and Pagano, 1991). Sphingomyelin (SM), a major component of plasma membranes, is synthesized by the transfer of phosphocholine from phosphatidylcholine to ceramide at the lumenal surface of the cis and medial Golgi apparatus (Futerman et al., 1990).

Surprisingly, the site of synthesis of ceramide, a key intermediate in sphingolipid metabolism, has never been rigorously determined. An early study examining the site of acylation of sphingoid bases using subcellular fractions from mouse brain (Morell and Radin, 1970) suggested that ceramide synthesis occurs in microsomes, but no attempts were made to distinguish between the various membranes that co-purify in a microsomal fraction. Another study using rat liver fractions (Walter et al., 1983) suggested that ceramide synthesis occurs in the ER, but the levels of synthesis in either the ER or other subcellular fractions were not quantified, and no evidence concerning the topology of ceramide synthesis was presented. In the current report, using well-characterized subcellular fractions from rat liver (Futerman et al., 1990; Futerman and Pagano, 1991), and using an assay of ceramide synthesis which does not require detergents either to disperse the substrates or incorporate them into membranes, we demonstrate that acylation of the sphingoid long-chain base occurs at the cytosolic surface of the ER, with no endogenous synthesis occurring in either a pre-Golgi apparatus compartment or in the Golgi apparatus. These results are discussed with regard to the transport of sphingolipids along the secretory pathway.

MATERIALS AND METHODS

Materials

Rats (Wistar) were obtained from the Weizmann Institute Breeding Centre. $NaB^{3}H_{4}$ (9–11 Ci/mmol) was from either Du Pont–New England Nuclear or from Amersham Corp. UDP-[4,5-^{3}H]Gal (34 Ci/mmol) was from Du Pont–New England Nuclear. Fumonisin B_1 was a generous gift from Dr. A. Merrill (Department of Biochemistry, Emory University School of Medicine, Atlanta, GA, U.S.A.). Silica gel 60 plates were from Merck. The following chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.): sphinganine (DL-*erythro*-2-amino-1,3-octadecanediol); sphingosine (*trans*-D-*erythro*-2-amino-4-octadecene-1,3-diol); *N*-palmitoyl-DL-dihydrosphingosine; ceramide (from bovine brain); *N*-palmitoyl-D-sphingosine; palmitoyl CoA; stearoyl CoA; arachidonyl CoA; fatty acids, even-carbon straight chains; defatted BSA; Pronase. Chymotrypsin (CDI grade) was from Worthington. Solvents (analytical grade) were from Bio-Lab Laboratories Ltd., Jerusalem, Israel.

Preparation of [4,5-^{3}H]sphinganine

The structures of the sphingolipids referred to in this study are shown in Figure 1. The long-chain sphingoid base, sphinganine, was labelled by reduction of D-*erythro*-sphingosine (Figure 1a) with $NaB^{3}H_{4}$ according to the method originally described by Schwarzmann (1978), in which lysogangliosides were hydro-

Abbreviations used: ER, endoplasmic reticulum; GlcCer, glucosylceramide; PM; plasma membrane; SM, sphingomyelin.
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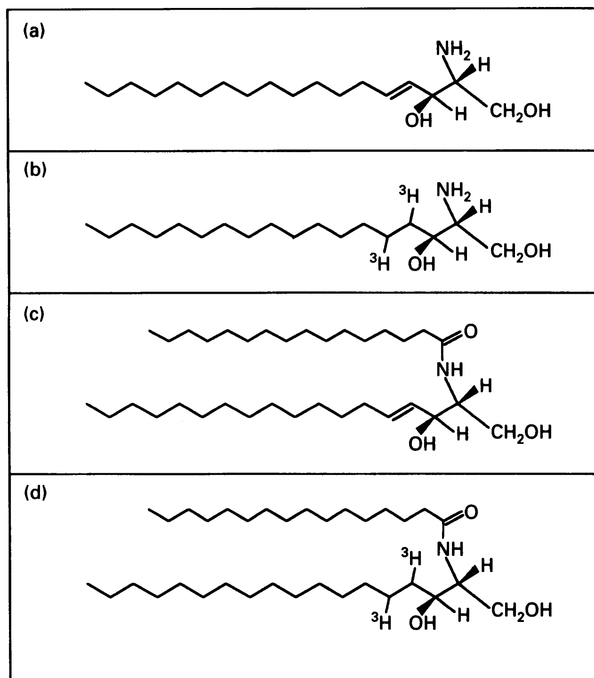

Figure 1 The structure of sphingoid bases and ceramides

Structures are as follows: (**a**) sphingosine, (**b**) [4,5-^{3}H]sphinganine ([4,5-^{3}H]dihydrosphingosine), (**c**) ceramide (*N*-palmitoylsphingosine), (**d**) [4,5-^{3}H]dihydroceramide (*N*-palmitoyl-[4,5-^{3}H]dihydrosphingosine).

genated at the sphingosine double bond with tritium gas generated *in situ* using Pd^{2+} as a catalyst. Briefly, 5–10 mg of sphingosine and 1.7 mg of $PdCl_2$ were dissolved in tetrahydrofuran in a screw-capped vial. NaB^3H_4 (100 mCi) was dissolved in diethyleneglycol dimethyl ether and added immediately to a screw-capped vial. After flushing with N_2, the reaction was allowed to proceed for 3–4 h at room temperature with stirring. Sphingolipids were extracted into diethyl ether, dried under N_2 and purified by t.l.c. with silica gel 60 plates using the following solvents: (1) $CHCl_3/CH_3OH/H_2O$ (65:25:4, by vol.); (2) $CHCl_3/CH_3OH$/15 mM $CaCl_2$ (60:35:8, by vol.); (3) $CHCl_3/CH_3OH$/2 M NH_4OH (40:10:1, by vol.). [4,5-^{3}H]Sphinganine (Figure 1b) was identified by comparison with authentic sphinganine. Following t.l.c., [4,5-^{3}H]sphinganine was extracted from the silica by the procedure of Bligh and Dyer (1959) using saline solutions of neutral pH.

Even after extensive purification by t.l.c., a minor radioactive product was observed which had an R_F value of 0.6 using $CHCl_3/CH_3OH$/2 M NH_4OH (40:10:1, by vol.) as the developing solvent. This product was a result of decomposition of [4,5-^{3}H]sphinganine on the silica surface of t.l.c. plates (see below). [4,5-^{3}H]Sphinganine was stored at −80 °C in toluene/ethanol (99:1, v/v) under an N_2 atmosphere to minimize decomposition during storage.

N-Palmitoyl-[4,5-^{3}H]dihydrosphingosine ([4,5-^{3}H]dihydroceramide; Figure 1d) was prepared by reducing *N*-palmitoyl-DL-sphingosine (ceramide; Figure 1c) as described above. [4,5-^{3}H]Dihydroceramide was identified by comparison with authentic dihydroceramide.

Subcellular fractionation

An intact Golgi apparatus fraction (Bergeron et al., 1982), a plasma membrane (PM) fraction (Hubbard et al., 1983), and microsomal subfractions (Ehrenreich et al., 1973) were prepared exactly as described previously (Futerman et al., 1990). To prepare the microsomal subfractions, microsomes were fractionated on a discontinuous sucrose gradient and the following fractions collected: light Golgi subfraction (fraction 1); intermediate Golgi subfraction (fraction 2); heavy Golgi subfraction (fraction 3); light smooth vesicles (fraction 4); heavy vesicles (fraction 5); rough microsomes (fraction 6) (Futerman and Pagano, 1991).

Measurement of dihydroceramide synthesis in liver fractions

Defatted-BSA–sphinganine–[4,5-^{3}H]sphinganine complexes were prepared as described for other lipids (Pagano, 1989), except that dialysis of the complex was not performed. An equimolar ratio of BSA and sphinganine was used. [4,5-^{3}H]Sphinganine ($1–3 \times 10^6$ c.p.m.) was added to the unlabelled sphinganine before preparing a complex with defatted BSA. Liver fractions were pre-incubated with various amounts of BSA–sphinganine–[4,5-^{3}H]sphinganine at 37 °C for 5 min before addition of fatty acids or fatty acyl CoAs (see the Results section for discussion of the molar ratios of the lipids and BSA). The reaction was terminated by addition of 2–3 ml of $CHCl_3/CH_3OH$ (1:2, v/v) and the samples were immediately placed on ice. Lipids were extracted (Bligh and Dyer, 1959) with all solutions at neutral pH. Lipid extracts were dried under a stream of N_2 and subsequently separated by t.l.c. using $CHCl_3/CH_3OH$/2 M NH_4OH (40:10:1, by vol.) as the developing solvent. [4,5-^{3}H]Dihydroceramide was identified after visualization of the radioactive lipids by fluorography using EN^{3}HANCE spray (Du Pont–New England Nuclear); the R_F values for [4,5-^{3}H]sphinganine and [4,5-^{3}H]dihydroceramide were 0.15 and 0.7 respectively. 4,5-^{3}H-labelled lipids were recovered from the plates by scraping, and radioactivities determined by liquid-scintillation counting in a Packard 1500 Tri-Carb scintillation counter using Lumax/toluene (1:3, v/v) as the scintillation fluid, after allowing the samples to stand at room temperature overnight.

Dihydroceramide synthesis was quantified after examining the following: (1) no statistically significant difference was observed in the recovery of [4,5-^{3}H]sphinganine and [4,5-^{3}H]dihydroceramide in the organic and aqueous phases of the extraction procedure (Bligh and Dyer, 1959). (2) Degradation of [4,5-^{3}H]sphinganine and [4,5-^{3}H]dihydroceramide. Previous studies (Wang et al., 1991) have demonstrated that long-chain bases decompose in acid and base, and that this decomposition is accelerated on glass surfaces (Merrill and Wang, 1992). In the current study, the amount of the decomposition product present varied between 5 and 20% of the initial [4,5-^{3}H]sphinganine added. No change in the amount of the decomposition product was seen during different times of incubation, but the amount of decomposition product was directly proportional to the amount of [4,5-^{3}H]sphinganine remaining after termination of incubations with liver fractions, confirming that [4,5-^{3}H]sphinganine decomposes on the silica/glass surface of t.l.c. plates. Thus, in calculating the percentage conversion of [4,5-^{3}H]sphinganine into [4,5-^{3}H]dihydroceramide, the radioactivity in the ^{3}H-labelled degradation product was added to that in [4,5-^{3}H]sphinganine. In contrast, [4,5-^{3}H]dihydroceramide did not decompose on glass surfaces. (3) Background radioactivity in the area corresponding to [4,5-^{3}H]dihydroceramide was subtracted using results from identical assays that omitted microsomal protein. This correction was particularly significant when large amounts of [4,5-^{3}H]sphinganine were applied to t.l.c. plates and small amounts of [4,5-^{3}H]dihydroceramide were formed. (4) The

efficiency of extraction and counting in Lumax/toluene (1:3, v/v) were identical for both radioactive lipids.

Protease treatment of microsomes

Microsomes (prepared as above except that 20 mM Hepes, pH 7.4, was included at all stages including the initial homogenization) were incubated with various amounts of either chymotrypsin or Pronase in a final volume of 280 μl containing 0.25 M sucrose, 20 mM Hepes, pH 7.4, and 0.1 mM $(NH_4)_6Mo_7O_{24},4H_2O$ (Van Veldhoven and Mannaerts, 1991), in either the presence or absence of Triton X-100 [final concentration 0.2% (v/v)] for 20 min at 30 °C. Reactions with Pronase also included 0.5 mM $CaCl_2$. The reactions were terminated by dilution of the samples 6-fold in ice-cold homogenization buffer that included 1 mM phenylmethanesulphonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml antipain and 100 k-i.u./ml aprotonin, and in the case of Pronase, 1.5 mM EGTA. Microsomal integrity after incubation with or without proteases was examined by measuring the latency of mannose-6-phosphatase (Van Veldhoven and Mannaerts, 1991), and dihydroceramide synthesis was measured as described above.

Miscellaneous procedures

Protein concentrations, and galactosyltransferase, alkaline phosphodiesterase and glucose-6-phosphatase activities were measured as described (Futerman et al., 1990).

RESULTS

Characterization of dihydroceramide synthesis in microsomes

Recent experiments (Merrill and Wang, 1986) supported earlier observations (Ong and Brady, 1973) that the 4-*trans* double bond of sphingolipids is added after acylation of the sphingoid base [reviewed in Merrill and Jones (1990)]. Thus, to determine the site of acylation of the sphingoid base of sphingolipids, we examined the metabolism of [4,5-^{3}H]sphinganine in rat liver microsomal fractions, since earlier reports suggested that ceramides are synthesized in liver and brain microsomes [reviewed in Kishimoto (1983)].

Microsomes were incubated with BSA–sphinganine (1 μM)–[4,5-^{3}H]sphinganine (1×10^6 c.p.m.) and a variety of fatty acids (30 μM), including hexanoic, octanoic, decanoic, lauric, myristic, palmitic, stearic, arachidic, behenic and lingoceric acids. No metabolism of [4,5-^{3}H]sphinganine was observed with any of the fatty acids tested. In contrast, incubation with either stearoyl CoA or palmitoyl CoA (30 or 75 μM) yielded significant amounts of a radioactive product that had an identical R_F value to dihydroceramide (Figure 2b). Addition of 2 μM fumonisin B_1 {previously shown to inhibit the conversion of [^{3}H]sphingosine into [^{3}H]ceramide, both in intact cells and *in vitro* (Wang et al., 1991)} inhibited the acylation of [4,5-^{3}H]sphinganine by more than 90% (Figure 2d).

Mild alkaline hydrolysis (1 M KOH/methanol, 70 °C, 18 h) (Figure 2e) of the acylation product yielded [4,5-^{3}H]sphinganine only and no [4,5-^{3}H]sphingosine, demonstrating that [4,5-^{3}H]dihydroceramide was formed *in vitro* and was not subsequently reduced to [^{3}H]ceramide during the reaction. This was confirmed by analysing the acylation products using a t.l.c. system ($CHCl_3$/acetic acid) (9:1, v/v) that separates dihydroceramide (*N*-palmitoyl-DL-dihydrosphingosine) and ceramide (*N*-palmitoyl-DL-sphingosine) (Morell and Radin, 1970); no [^{3}H]ceramide was observed (results not shown). [4,5-^{3}H]Dihydroceramide was not metabolized to either [^{3}H]SM or [^{3}H]GlcCer *in vitro*, although we have recently observed that [4,5-^{3}H]sphinganine is metabolized to a variety of complex sphingolipids (including [^{3}H]SM, [^{3}H]GlcCer and a variety of ^{3}H-labelled gangliosides) when added to cultures of hippocampal neurons (R. Harel and A. H. Futerman, unpublished work).

Using palmitoyl CoA as the fatty-acid donor, dihydroceramide synthesis was stimulated up to 1.5-fold by Mg^{2+}, with maximal stimulation at 1 mM (Figure 3). Mn^{2+} stimulated synthesis at low concentrations (0.5 mM), but higher concentrations inhibited synthesis (Figure 3); these results are similar to those obtained using chicken liver microsomes and Tween-20 to dissolve the lipid substrates (Sribney, 1966). All subsequent assays of dihydroceramide synthesis were performed in the presence of 2 mM $MgCl_2$.

The Mg^{2+}-stimulated hydrolysis of ceramide to sphingosine has been reported to occur at neutral pH values in rat liver PM (Slife et al., 1989) and other tissues (Spence et al., 1986), using an assay in which lipid substrates were solubilized by detergents. To exclude the possibility that newly synthesized [4,5-^{3}H]dihydroceramide was degraded in liver microsomes in the presence of Mg^{2+}, thus reducing the apparent synthesis of [4,5-^{3}H]dihydroceramide, the following experiment was performed: [4,5-

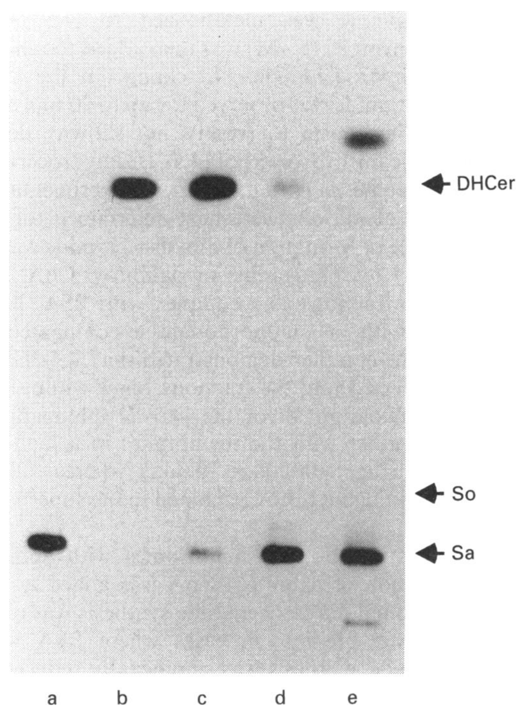

Figure 2 Chromatographic analysis of the acylation of [4,5-^{3}H]sphinganine in rat liver microsomes

Samples were as follows: lane a, [4,5-^{3}H]sphinganine; lane b, [4,5-^{3}H]dihydroceramide; lane c, microsomes (500 μg of protein) incubated for 60 min in a final volume of 1 ml of Hepes buffer (20 mM, pH 7.4) containing 2 mM $MgCl_2$, 3×10^6 c.p.m. of [4,5-^{3}H]sphinganine, 20 μM BSA and 75 μM palmitoyl CoA. The reaction was stopped by addition of 2–3 ml of $CHCl_3/CH_3OH$ (1:2, v/v) and lipids extracted (see the Materials and methods section) prior to t.l.c.; lane d, incubations were carried out as for the sample in lane c but in the presence of 2 μM Fumonisin B_1; lane e, [4,5-^{3}H]dihydroceramide formed by incubation of liver microsomes under identical conditions as in lane c was extracted from t.l.c. by the procedure of Bligh and Dyer (1959) and degraded by mild alkaline hydrolysis (1 M KOH/methanol, 70 °C, 18 h). The identity of the minor band with an R_F higher than [4,5-^{3}H]dihydroceramide is unknown. The following lipids were used as standards: sphinganine (Sa); sphingosine (So); *N*-palmitoyl-DL-dihydrosphingosine (DHCer).

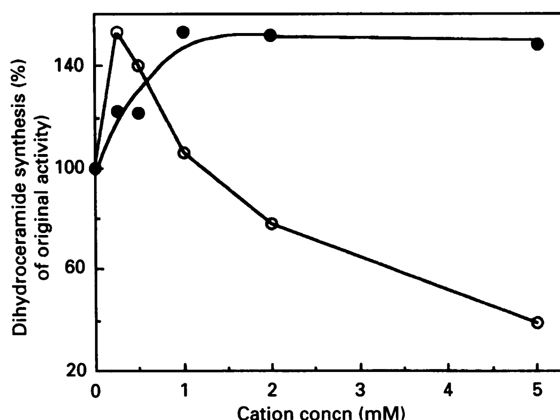

Figure 3 Cation dependence of dihydroceramide synthesis

Rat liver microsomes (250 μg of protein) were incubated with BSA (30 μM)–[4,5-^{3}H]sphinganine (1×10^{6} c.p.m.)–sphinganine (2 μM) and 30 μM palmitoyl CoA in a final volume of 500 μl of Hepes (20 μM, pH 7.4) for 20 min in the presence of various amounts of either $MgCl_2$ (●) or $MnCl_2$ (○). Dihydroceramide synthesis is expressed as a percentage of the original activity in the homogenate in the absence of cations. Results are means of two independent experiments.

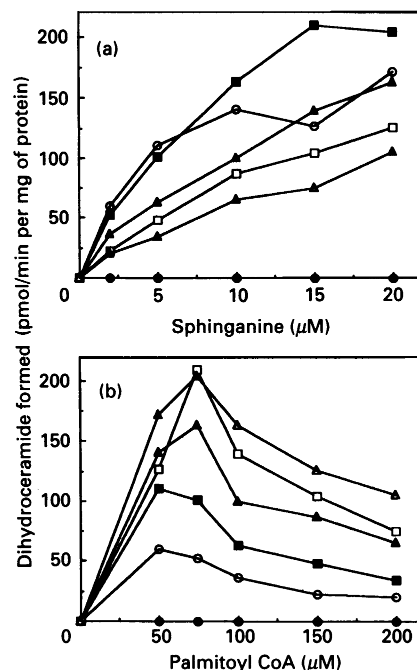

Figure 4 Effect of sphinganine and palmitoyl CoA on dihydroceramide synthesis

Liver microsomes (250 μg of protein) were incubated with [4,5-^{3}H]sphinganine (2×10^{6} c.p.m.) and various amounts of sphinganine and palmitoyl CoA, in the presence of 20 μM BSA for 20 min in a final volume of 500 μl of Hepes (20 mM, pH 7.4). Results are shown as the amount of dihydroceramide formed/min per mg of protein versus (**a**) increasing concentrations of sphinganine in the presence of various concentrations of palmitoyl CoA [0 μM (●), 50 μM (○); 75 μM (■); 100 μM (▲); 150 μM (□); 200 μM (△)] and (**b**) increasing concentrations of palmitoyl CoA in the presence of various concentrations of sphinganine (0 μM (●); 2 μM (○); 5 μM (■); 10 μM (▲); 15 μM (□); 20 μM (△)].

^{3}H]sphinganine (3×10^{6} c.p.m.) and palmitoyl CoA (7.5 μM) were incubated with microsomes until more than 90 % of the [4,5-^{3}H]sphinganine was metabolized to [4,5-^{3}H]dihydroceramide. Fumonisin B_1 (5 μM) was then added to inhibit synthesis of [4,5-^{3}H]dihydroceramide. No change in the level of [4,5-^{3}H]dihydroceramide was observed for up to 90 min at 37 °C after addition of Fumonisin B_1 (results not shown), demonstrating that no significant hydrolysis of [4,5-^{3}H]dihydroceramide occurs in liver microsomes *in vitro* under these experimental conditions.

Incubation conditions were next determined for microsomes so that the rate of formation of dihydroceramide was not limited by availability of sphinganine or palmitoyl CoA. Sphinganine was added to fractions as a complex with BSA. Incubation of membranes with a sulphorhodamine-conjugated BSA–[4,5-^{3}H]sphinganine complex demonstrated that [4,5-^{3}H]sphinganine rapidly transferred into the fractions, since within less than one minute of incubation all of the [4,5-^{3}H]sphinganine could be recovered together with the membranes in a high-speed pellet (Beckmann airfuge, 100000 ***g***, 10 min), whereas all the sulphorhodamine-conjugated BSA remained in the supernatant (results not shown).

After pre-incubation of microsomes with sphinganine, an aqueous solution of palmitoyl CoA was added to the reaction mixture. Maximal dihydroceramide synthesis was observed only when BSA was present; in cases where BSA was omitted, dihydroceramide synthesis was significantly reduced because of the detergent-like effects of palmitoyl CoA (Richards et al., 1990). Similar findings have been reported for other palmitoyl-CoA-utilizing enzymes, including dihydroxyacetonephosphate acyltransferase (Das et al., 1992). Indeed, incubation of microsomes with 150 μM palmitoyl CoA affected microsomal integrity since a significant reduction in the latency of mannose-6-phosphatase was observed, whereas addition of 20 μM BSA together with palmitoyl CoA prevented this effect. The optimum ratio of BSA to palmitoyl CoA was determined to be 1:3–4; higher ratios reduced dihydroceramide synthesis due to the high-affinity binding of palmitoyl CoA to BSA, which reduced the amount of available palmitoyl CoA (Pauly and McMillin, 1988; Richards et al., 1990). Maximal dihydroceramide synthesis was obtained by preparing a complex of 20 μM BSA with 20 μM sphinganine (Figure 4a), and adding 75 μM palmitoyl CoA (Figure 4b). At concentrations above 75 μM palmitoyl CoA, dihydroceramide synthesis was reduced (Figure 4b) due to the unbound excess palmitoyl CoA. With these concentrations of substrate and BSA, the synthesis of dihydroceramide was linear between 10 and 40 min and at levels up to 600 μg of protein (results not shown), and under these conditions, the apparent $V_{max.}$ of the reaction was about 200 pmol of dihydroceramide formed/min per mg of protein.

Distribution of dihydroceramide synthesis in rat liver membrane fractions

Using well-characterized microsomal subfractions (Ehrenreich et al., 1973; Futerman and Pagano, 1991), the distribution of recovered dihydroceramide synthesis exactly paralleled that of an ER marker, glucose-6-phosphatase, with 92 % of each activity obtained in fractions 5 and 6, which consist predominantly of smooth microsomes and rough microsomes respectively. Virtually no synthesis was observed in fractions 1–3 (enriched Golgi apparatus fractions) and the small amount of synthesis detected in fraction 4 (light smooth vesicles of density intermediate between the smooth ER and an early Golgi apparatus compartment) could be accounted for by contamination by ER membranes, since a similar amount of glucose-6-phosphatase was recovered in this fraction. These results are in marked contrast with the distributions of SM and GlcCer synthesis, for which 61 % and 48 % of the recovered activities were obtained in fractions 1–4 (Futerman et al., 1990; Futerman and Pagano,

Table 1 Distribution of protein and enzyme activities in microsomal subfractions

Dihydroceramide synthesis was measured by incubating 250 μg of protein from fractions 4–6, the microsomal suspension and the homogenate or 10–150 μg of protein from fractions 1–3, with 20 μM sphinganine–[4,5-^{3}H]sphinganine (3 × 10^6 c.p.m.)–20 μM BSA and 75 μM palmitoyl CoA at 37 °C for 20 min in 1 ml of 20 mM Hepes, pH 7.4, containing 2 mM $MgCl_2$. Results are expressed as means ± S.D. of four gradients. The organelle in which most of each marker enzyme activity is found is noted in parentheses. Distribution and recoveries of protein and enzyme activities were calculated as follows: (1) The recovery of protein and enzyme activities in the microsomal suspension were calculated as the percentage of the initial homogenate activity present in the microsomal suspension. Percentage recoveries in the microsomal fraction were: protein, 16.2 ± 1.0; dihydroceramide synthesis, 29.3 ± 5.2%; glucose-6-phosphatase, 29.6 ± 6.0; alkaline phosphodiesterase, 34.1 ± 3.0; galactosyltransferase, 58.4 ± 11.1. (2) The recovery of protein and enzyme activities in the gradients is given by the sum of the values obtained for fractions 1–6 (total recovered activity) divided by the amount measured for the microsomal suspension × 100. Percentage recoveries in the gradients were: protein, 100.1 ± 6.6%; dihydroceramide synthesis, 65.3 ± 6.8%; glucose-6-phosphatase, 87.9 ± 22.0; alkaline phosphodiesterase, 109.7 ± 16.8; galactosyltransferase, 117.6 ± 56.3. (3) The distribution of protein and enzyme activities in the gradients is calculated as the ratio of the activity in that fraction divided by the total recovered activity × 100.

Fraction	Protein	Dihydroceramide synthesis	Glucose-6-phosphatase (ER)	Alkaline phosphodiesterase (PM)	Galactosyltransferase (Golgi)
1–3*	1.8 ± 0.5	2.2 ± 0.7	1.6 ± 1.7	13.4 ± 1.9	42.0 ± 4.3
4	6.0 ± 1.1	5.9 ± 0.7	6.2 ± 4.0	8.9 ± 1.1	28.3 ± 0.6
5	40.2 ± 3.5	31.7 ± 9.3	35.9 ± 2.0	44.0 ± 4.2	25.1 ± 5.1
6	52.0 ± 2.9	60.3 ± 9.0	56.2 ± 7.0	33.7 ± 6.7	4.5 ± 1.7

* Distribution in fractions 1–3 is the sum of the distributions in individual subfractions 1, 2 and 3.

Table 2 Distribution of protein and enzyme activities in Golgi apparatus and PM fractions

Dihydroceramide synthesis was measured as in Table 1. Results are means of three and two preparations for the Golgi apparatus and PM respectively. The organelle in which most of each marker enzyme activity is found is noted in parentheses. Distribution of protein and enzyme activities is given as the percentage of the initial homogenate activity present in the final membrane fraction after correcting for recoveries.

Fraction	Protein	Dihydroceramide synthesis	Alkaline phosphodiesterase (PM)	Galactosyltransferase (Golgi)	Glucose-6-phosphatase (ER)
PM	0.6	1.5	32.3	1.5	3.7
Golgi	1.0	1.0	3.7	47.0	2.5

1991) which contain 50–60% of the Golgi apparatus marker, galactosyltransferase (Table 1).

Since fractions 5 and 6 also contained significant amounts of the PM marker, alkaline phosphodiesterase, dihydroceramide synthesis was examined in an isolated PM-sheet preparation (Hubbard et al., 1983). Of the recovered alkaline phosphodiesterase 32% was obtained in the PM preparation but it only contained 1.5% of dihydroceramide synthesis (Table 2). Moreover, all of the dihydroceramide synthesis in the PM preparation could be accounted for as contamination by ER membranes, since 3.7% of recovered glucose-6-phosphatase was in this fraction. A similar lack of dihydroceramide synthesis by rat liver PM fractions has been reported (Slife et al., 1989).

Finally, dihydroceramide synthesis was assayed in an intact Golgi apparatus preparation (Bergeron et al., 1982). No enrichment in distribution or specific activity was found in this fraction (Table 2) confirming that no dihydroceramide synthesis occurs in the Golgi apparatus.

Topology of dihydroceramide synthesis in microsomes

The topology of dihydroceramide synthesis was examined by limited proteolytic digestion of intact and disrupted microsomes. Conditions were established so that microsomes were not disrupted by proteases, with microsomal integrity remaining at 85–95% after treatment with either Pronase (Figure 5) or chymotrypsin (results not shown). However, trypsin caused significant disruption of microsomes since mannose-6-phosphatase latency was reduced to 50% even at a low ratio of trypsin

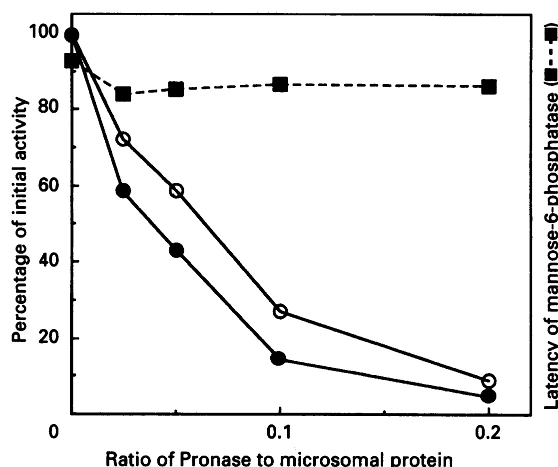

Figure 5 Topology of dihydroceramide synthesis in microsomes

Liver microsomes were incubated with Pronase in the presence or absence of Triton X-100 as described in the Materials and methods section. The susceptibility of dihydroceramide synthesis to Pronase treatment is expressed as a percentage of the activity in microsomes incubated without Pronase in either the presence (○) or absence (●) of Triton-X 100. The latency of mannose-6-phosphatase at the various ratios of Pronase to microsomal protein is calculated as follows (Van Veldhoven and Mannaerts, 1991): % latency = 100 × 1 − (activity without detergent/activity with detergent)]. Results are the means of two independent experiments.

to microsomal protein (results not shown). Pronase completely destroyed dihydroceramide synthesis in intact microsomes, and incubation with Pronase in the presence of Triton X-100 [final

concentration of 0.2 %, (v/v)] did not increase the susceptibility of dihydroceramide synthesis to digestion (Figure 5); similar results were obtained using chymotrypsin (results not shown). We therefore conclude that acyl CoA: sphinganine N-acyltransferase (EC 2.3.1.24) is located on the cytosolic surface of the ER.

DISCUSSION

Based on observations that ceramide is synthesized in microsomes (Morell and Radin, 1970), it has been assumed that the ER is the site of synthesis of ceramides (see for example Bruning et al., 1992; Kendler and Dawson, 1992), although there has been no direct evidence to date that synthesis occurs in the ER and not the Golgi apparatus. Therefore, in the present study we have determined the site and topology of dihydroceramide synthesis using well-characterized subcellular fractions in which the ER and Golgi apparatus can be well-separated, and have demonstrated that dihydroceramide is synthesized at the cytosolic surface of the ER in rat liver, with no synthesis occurring in the Golgi apparatus. Similar conclusions concerning the site and topology of dihydroceramide synthesis in mouse liver were reported recently (Mandon et al., 1992), although no attempts were made to work at saturating concentrations of substrates (see below).

[4,5-^{3}H]Dihydroceramide synthesis *in vitro*

In order to analyse dihydroceramide synthesis *in vitro*, we characterized an assay in which sphinganine was added to membrane fractions by spontaneous transfer from a BSA complex. This is in contrast with various other assays of ceramide synthesis in which the long-chain bases were dissolved in detergents (Sribney, 1966; Mandon et al., 1991) and/or dispersed by sonication (Mandon et al., 1991, 1992), or coated on Celite (Morell and Radin, 1970; Singh, 1983). These methods are often problematic since the activity of many integral membrane proteins is affected by detergents or other treatments that disrupt membrane integrity.

To compare enzyme activities in different subcellular fractions, it is essential that the substrates are present at saturating concentrations so that the rate of the reaction is directly proportional to the amount of enzyme present in the fractions. We therefore determined conditions in which the rate of formation of dihydroceramide was not limited by availability of either sphinganine or palmitoyl CoA. The rate of the reaction was found to be critically dependent on the ratio of palmitoyl CoA and BSA in the reaction mixture. At low ratios, fatty-acyl CoA bound tightly to BSA and was unavailable for the enzyme reaction (Pauly and McMillin, 1988) thus reducing the apparent concentration of palmitoyl CoA, and at high ratios, palmitoyl CoA acted as a detergent and severely reduced dihydroceramide synthesis, presumably by disrupting membrane integrity or enzyme structure. Thus, although BSA acts as a useful substrate depot for the delivery of acyl units to lipid-metabolizing enzymes *in vitro*, care must be taken that saturating conditions of fatty-acyl CoAs are present (Pauly and McMillin, 1988). Analysis of sphinganine N-acyltransferase in the absence of BSA using high concentrations of palmitoyl CoA [i.e. 200 μM; Wang et al. (1991)] may lead to an underestimation of enzyme activity.

Metabolic consequences of dihydroceramide synthesis in the ER

Sphingolipids contain four kinds of fatty acids, namely saturated long-chain fatty acids, very-long-chain fatty acids, monoenoic fatty acids and α-hydroxy-very-long-chain fatty acids (reviewed in Kishimoto, 1983; Merrill and Jones, 1990); the fatty-acyl specificity of sphinganine N-acyltransferase(s) will be determined only when the enzyme has been purified and reconstituted. Once sphinganine has been acylated at the cytosolic surface of the ER (Figure 6), dihydroceramide is presumably dehydrogenated to form ceramide, since the predominant form of the long-chain base in sphingolipids is sphingosine, not sphinganine. This putative dehydrogenase has never been observed either *in vitro* or *in vivo*. However, it has been suggested that the double bond of sphingolipids may be added in a different subcellular compartment from that in which dihydroceramide is synthesized (Merrill and Wang, 1986). An important unresolved issue in sphingolipid biochemistry concerns the identification and subcellular localization of the dehydrogenase (Figure 6).

Ceramide is metabolized to GlcCer at the cytosolic surface of a pre- or early Golgi apparatus compartment (Figure 6) (Futerman and Pagano, 1991); similar conclusions about the topology of GlcCer synthesis have been reached by others although Trinchera et al. (1991) reported that GlcCer synthesis occurs only in the Golgi apparatus, and Jeckel et al. (1992) reported that significant amounts of GlcCer are synthesized in a distal Golgi apparatus compartment. The latter conclusion is based on sucrose-gradient fractionation in which two major peaks of activity of GlcCer synthesis were observed, namely in proximal and distal Golgi apparatus fractions. It should be noted that the putative proximal Golgi apparatus marker used in this study, N-acetylglucosaminylphosphotransferase, has never been unequivocally demonstrated to be located in the proximal Golgi apparatus by immunocytochemical methods.

Ceramide is metabolized to SM at the lumenal surface of the cis and medial Golgi apparatus (Figure 6) (Futerman et al., 1990) or the cis Golgi (Jeckel et al., 1990). A rate-limiting step in sphingolipid synthesis may be delivery of ceramide from the ER to sites of subsequent metabolism. Support for this idea comes from recent experiments in which brefeldin A led to an increase of SM synthesis in Chinese hamster ovary cells. Brefeldin A causes redistribution of Golgi-associated enzymes to the ER (Klausner et al., 1992), and the increase of SM synthesis in the presence of brefeldin A was suggested to be due to translocation of SM synthase to the ER, resulting in an increase of the availability of ceramide to SM synthase since the pool of ceramide in the mixed ER–Golgi organelle may be larger than that in the Golgi apparatus alone (Bruning et al., 1992).

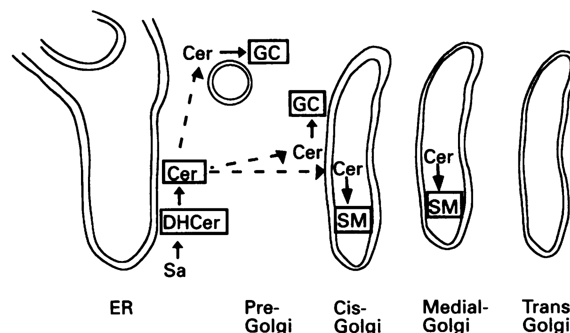

Figure 6 Topology and sites of synthesis of sphingolipids in the ER and Golgi apparatus

The sites and topology of dihydroceramide, GlcCer and SM synthesis are shown. Lipid products are shown in squares, enzyme reactions by continuous lines and putative transport pathways of lipids by broken lines. Note that the site of conversion of dihydroceramide into ceramide is not known. Abbreviations: Sa, sphinganine; DHCer, dihydroceramide; GC, GlcCer; Cer, ceramide.

Two potential mechanisms exist for transporting (dihydro)-ceramide out of the ER. Dihydroceramide might be transported by the same vesicles that transport proteins out of the ER and along the secretory pathway. Evidence that may support this possibility has been provided by studies in *Saccharomyces cerevisiae* in which the synthesis of mannosylated ceramide derivatives in the Golgi apparatus was dependent upon genes controlling the flow of secretory vesicles from the ER to Golgi apparatus (Puoti et al., 1991); however, no data was presented in these studies to definitively locate the enzymes of sphingolipid synthesis in *Saccharomyces* sp. Alternatively, ceramide may be transported as lipid monomers through the cytosol by protein-facilitated transport. Interestingly, all four stereoisomers of sphinganine are acylated *in vivo*, but only the D-*erythro* and to some extent the L-*threo* isomers are subsequently converted into SM and GlcCer (Stoffel and Bister, 1973), perhaps implying that D-*erythro*-ceramide is transported out of the ER by a protein-facilitated mechanism that is stereospecific towards the long-chain base. Evidence supporting this idea comes from observations that SM synthase is able to metabolize all four ceramide stereoisomers and GlcCer synthase is able to metabolize both D-*erythro*- and L-*erythro*-ceramide (Pagano and Martin, 1988). Resolution of the mechanism of transport of (dihydro)ceramide out of the ER awaits isolation of the vesicles responsible for transporting material from the ER to the Golgi apparatus; initial attempts to isolate these transition vesicles indicated that cerebrosides were not significantly enriched (Moreau et al., 1991). Alternatively, a cell-free assay of lipid flow from the ER to the Golgi apparatus could be established. Our demonstration that the early steps of sphingolipid synthesis are compartmentalized within the ER–Golgi complex, and our characterization of a detergent-free assay for ceramide synthesis that does not effect microsomal integrity, provide the biochemical tools for the establishment of such an assay.

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