The mitochondria-associated endoplasmic-reticulum subcompartment (MAM fraction) of rat liver contains highly active sphingolipid-specific glycosyltransferases

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Although most glycosphingolipids (GSLs) are thought to be located in the outer leaflet of the plasma membrane, recent evidence indicates that GSLs and their precursor, ceramide, are also associated with intracellular organelles and, particularly, mitochondria. GSL biosynthesis starts with the formation of ceramide in the endoplasmic reticulum (ER), which is transported by controversial mechanisms to the Golgi apparatus, where stepwise addition of monosaccharides on to ceramides takes place. We now report the presence of GSL-biosynthetic enzymes in a subcompartment of the ER previously characterized and termed 'mitochondria-associated membrane' (MAM). MAM is a membrane bridge between the ER and mitochondria that is involved in the biosynthesis and trafficking of phospholipids between the two organelles. Using exogenous acceptors coated on silica gel, we demonstrate the presence of ceramide glucosyltransferase (Cer-Glc-T), glucosylceramide galactosyltransferase and sialyltransferase (SAT) activities in the MAM. Estimation of the marker-enzyme activities showed that glycosyltransferase activities could not be ascribed to cross-contamination of MAM by Golgi membranes. Cer-Glc-T was found to have a marked preference for ceramide bearing phytosphingosine as sphingoid base. SAT activities in MAM led to the synthesis of G_{M3} ganglioside and small amounts of G_{D3}. G_{M1} was also synthesized along with G_{M3} upon incubation of the fraction with exogenous unlabelled G_{M3}, underlying the presence of other sphingolipid-specific glycosyltransferases in MAM. On the basis of our results, we propose MAM as a privileged compartment in providing GSLs for mitochondria.

Key words: biosynthesis, ganglioside, glycosyltransferase, Golgi, mitochondria-associated membrane (MAM), sphingolipid.

INTRODUCTION

Glycosphingolipids (GSLs) are ubiquitous membrane components of essentially all eukaryotic cells that have generally been assumed to be localized primarily at the cell surface [1a,2]. Although plasma membranes contain 76 % of the total ganglioside-bound sialic acid [3], Keenan et al. [4] and others [3,5,6] have observed gangliosides or neutral glycolipids associated wih internal membrane components such as the endoplasmic reticulum (ER) and mitochondria. Recently, we presented evidence for a mitochondrial content of 0.62 nmol/mg of protein and 0.1 nmol/mg of protein for glucosylceramide (Glc-Cer) and lactosylceramide (Lac-Cer) respectively, these compounds being mostly present in the outer mitochondrial membrane [7]. The lipid moiety of GSLs, ceramide, is produced on the ER [8,9] and then carried to the Golgi apparatus, where stepwise glycosylation reactions of ceramide occur (see [10] for a review). Thus the question of ceramide transport from the ER to Golgi arises. Transport via vesicular membrane flow [11,12] as well as non-vesicular transport [13,14] are described in the literature without being unambiguously clarified. Apart from the transport of complex GSLs to the cell surface, which seems to occur via transport vesicles, trafficking of neutral glycolipids or gangliosides between the Golgi apparatus and other subcellular compartments is as yet undefined. To explain the presence of neutral glycolipids [3,7] and gangliosides [3,6] within mitochondria, an alternative hypothesis to the undefined transport mechanism evoked above would be that the mitochondria-associated membrane (MAM) fraction, a functional ER-like compartment [15,16] possesses glycolipid glycosyltransferases involved in the synthesis of these molecules, thereby simplifying the acquisition of glycolipids by mitochondria. Glycosyltransferases involved in the biosynthesis of glycosylphosphatidylinositols have been already reported to be confined to the MAM fraction [17]. In addition, MAM has been hypothesized to be involved in the trafficking of an inner-mitochondrial-membrane glycoprotein synthesized in the ER [18] as well as in the trafficking of ceramides between the ER and mitochondria [19]. The purpose of the present study was to investigate the presence of glycolipid glycosyltransferases in the MAM fraction which could represent an alternative pathway for the synthesis of neutral glycolipids and gangliosides aimed to mitochondria.

MATERIALS AND METHODS

Materials

Leupeptin, pepstatin, PMSF, bicinchoninic acid, ceramides, Glc-Cers and Lac-Cers were from Sigma (L'Isle d'Abeau,

Abbreviations used: MAM, mitochondria-associated membrane; GSL, glycosphingolipid; ER, endoplasmic reticulum; Cer-Glc-T, ceramide glucosyltransferase; Gal-T_1 glucosylceramide (Glc-Cer) galactosyltransferase; HPTLC, high-performance TLC; Lac-Cer, lactosylceramide; Glc-Cer, glucosylceramide; Gal-T_2, Lac-Cer synthase; SAT, sialyltransferase; the nomenclature of Svennerholm [1] is used for gangliosides.

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France). Supelclean™ LC-NH_2 cartridges were from Supelco (L'Isle d'Abeau, France). Analytical-grade solvents and high-performance TLC (HPTLC) silica-gel-60 plates were from Merck (Nogent, France). Percoll was from Amersham Pharmacia Biotech (Paris, France). UDP-[^{14}C]glucose (303 mCi/mmol), UDP-[^{14}C]galactose (278 mCi/mmol), UDP-[^{3}H]galactose (5–15 Ci/mmol), CMP-[^{14}C]sialic acid (249 mCi/mmol) and [^{14}C]palmitoyl-CoA (56 mCi/mmol) were purchased from NEN PerkinElmer (Paris, France), and BioMax MR films were from Kodak (Paris, France).

Methods

Isolation and characterization of the MAM and other subcellular fractions from rat liver

Crude mitochondria were isolated from the livers of Sprague–Dawley rats as previously reported in [20]. Briefly, livers were immersed in ice-cold medium consisting of 250 mM sucrose in 10 mM Hepes buffer, pH 7.4, with 1 mM EDTA. Crude mitochondria were prepared from the cellular homogenate by differential centrifugations. The MAM fraction and purified mitochondria were separated on a self-forming 30 % Percoll gradient as described by Vance [15] (the stock solution of Percoll, after a 1:3 dilution in PBS before use to obtain the working solution, gives a continuous gradient upon centrifugation). Golgi membranes were isolated according to the procedure of Croze and Morré [21]. Subcellular fractions were stored in the presence of 1 μM leupeptin, 1 μM pepstatin and 100 μM PMSF before use. The following marker enzymes were measured to assess the cross-contamination of subcellular fractions: NADPH:cytochrome *c* reductase for whole microsomes [15], UDP-galactose:*N*-acetylglucosamine galactosyltransferase for the Golgi apparatus [22], glucose-6-phosphate phosphatase [23] for the MAM fraction and cytochrome *c* oxidase for mitochondria [24].

GSL glycosyltransferase assays

All enzymes were assayed using the same amount of proteins (150 μg) of each subcellular fraction. The sphingolipid acceptors were adsorbed on to silica-gel particles as described by Matsuo et al. [25]: after dissolving the acceptors in a small volume of chloroform, 1 mg of silica gel was added to the solutions, then the solvent was evaporated under a stream of nitrogen before use, resulting in the adsorption of the sphingolipids on silica-gel particles. For each glycosyltransferase, the incubation time was selected from the linear portions of the curves obtained in preliminary experiments performed to determine the kinetics, which were found to be very similar for each enzyme in all subcellular fractions (results not shown).

UDP-glucose:ceramide (β1-1)-glucosyltransferase (Cer-Glc-T) was assayed using ceramides adsorbed on silica gel in a final volume of 200 μl of 0.05 M Mes buffer, pH 6.5, containing 5 mM $MnCl_2$, 5 mM $MgCl_2$, 0.5 % CHAPS, 20 μM donor UDP-[^{14}C]glucose and 150 μg of proteins of each subcellular fraction. Incubation was carried out for 30 min at 37 °C. The reaction was terminated by the addition of 2 ml of chloroform/methanol (2:1, v/v).

UDP-galactose:Glc-Cer (β1-4)-galactosyltransferase (Gal-T_2; Lac-Cer synthase) was assayed using Glc-Cer adsorbed on silica gel as an acceptor. Galactosyltransferase was assayed for 1 h at 37 °C using 150 μg of protein in 200 μl of 0.1 M Hepes buffer, pH 7.4, containing 5 mM $MnCl_2$, 5 mM $MgCl_2$ and 20 μM of donor UDP-[^{14}C]galactose. The reaction was terminated by the addition of 2 ml of chloroform/methanol (2:1, v/v) mixture.

CMP-*N*-acetylneuraminate:GSL sialyltransferases (SATs) were assayed using Lac-Cer or G_{M3} ganglioside as acceptors adsorbed on silica gel. The reaction mixture contained 150 μg of protein in 100 μl of 0.2 M sodium cacodylate buffer, pH 6.5, containing 10 mM $MnCl_2$, 1 % Triton CF 54 and 50 μM CMP-[^{14}C]sialic acid. Incubation was carried out for 2 h at 37 °C, then 1 ml of chloroform/methanol (2:1, v/v) was added.

Characterization of the reaction products

For neutral-glycolipid biosynthesis, the reaction product formed on the surface of the solid support was extracted with chloroform/methanol (2:1, v/v), concentrated under nitrogen and spotted on to silica-gel HPTLC plates, run in the appropriate solvent systems (see the Results section) and the products visualized by autoradiography using BioMax MR films. Glc-Cer and galactosylceramide were separated on HPTLC plates that had been impregnated by spraying with methanolic 1 % (w/v) sodium borate and dried at 100 °C prior to use. Solvent system I was chloroform/methanol/water/28 % ammonia (40:10:0.9:0.15, by volume) [26]. Alternatively the lower phase of partition was evaporated to dryness under reduced pressure, taken up in chloroform and further fractionated using solid-phase extraction on LC-NH_2 cartridges as previously described [27]. The fractions were allowed to migrate in solvent system II, which was chloroform/methanol/water (65:25:4, by vol.). Standard GSLs co-migrating with the radioactive spots were visualized by spraying with orcinol/sulphuric reagent and heating for 1 min at 150 °C.

The reaction products of the SAT assays were extracted with chloroform/methanol (1:1, v/v), filtered, evaporated to dryness and the gangliosides were freed of contaminants as described by Popa et al. [28]. The dry residue was taken up in 2 ml of methanol/PBS, pH 7.4 (1/1, v/v) and the material applied to a 3 ml styrene/divinylbenzene column equilibrated in the same solvent. After washing with 10 ml of water, elution was performed with 5 ml of methanol and then 5 ml of chloroform/methanol (1:1, v/v). The organic phases were pooled and concentrated under nitrogen, spotted on silica-gel-60 HPTLC plates and run in solvent system III (chloroform/methanol/aq. 0.22 % $CaCl_2$, 60:35:8, by vol.) before visualization by autoradiography. Standard gangliosides co-migrating with the radioactive spots were visualized by spraying with resorcinol/HCl, covering the plate with a glass plate and heating 5 min at 120 °C.

Other methods

[^{14}C]Palmitoylsphingosine was synthesized using diethylcyanophosphonate (Fluka; called 'diethylphosphoryl cyanide' in [29]) as a coupling agent in the presence of triethylamine [29] then purifying the labelled ceramide on an LC-NH_2 column [27]. The protein concentration was determined using bicinchoninic acid as described by Smith et al. [30].

RESULTS

Characterization of the isolated subcellular fractions

We investigated the synthesis of Glc-Cer in the ER-like membrane fraction (MAM) which was co-isolated with crude mitochondria as previously described [15]. This investigation was performed in comparison with pure Golgi membranes, microsomes and mitochondria isolated from rat liver. Cross-contamination of each fraction was estimated by measuring the content of marker

Table 1 Specific marker-enzyme activities in subcellular fractions from rat liver

The units for cytochrome *c* oxidase activity are nmol of cytochrome *c* oxidized/min per mg of protein; those for galactosyltransferase are nmol of [^{3}H]galactose incorporated/h per mg of protein; those for NADPH:cytochrome *c* reductase are nmol of cytochrome *c* reduced/min per mg of protein; and those for glucose-6-phosphate phosphatase are μmol of P_i formed/h per mg of protein. Abbreviation: ND, not determined.

	Enzyme activity			
Marker enzyme . . .	Cytochrome *c* oxidase	Galactosyltransferase	NADPH:cytochrome *c* reductase	Glucose-6-phosphate phosphatase
Fraction / Subcellular fraction . . .	Mitochondria	Golgi	Microsomes	MAM
Homogenate	28.4 ± 4.1 ($n = 3$)	3.2 ± 0.5 ($n = 3$)	11.8 ± 1.9 ($n = 3$)	39.4 ± 6.1 ($n = 3$)
Microsomes	43.3 ± 5.5 ($n = 3$)	19.5 ± 3.0 ($n = 3$)	35.2 ± 8.2 ($n = 3$)	139.2 ± 33.0 ($n = 3$)
Golgi	25.6 ± 4.0 ($n = 3$)	195.0 ± 29.0 ($n = 4$)	3.1 ± 0.8 ($n = 3$)	26.0 ± 1.2 ($n = 4$)
MAM	17.3 ± 2.5 ($n = 5$)	14.6 ± 3.7 ($n = 4$)	14.1 ± 2.8 ($n = 3$)	298.5 ± 76.2 ($n = 4$)
Mitochondria	427.0 ± 23.0 ($n = 4$)	ND	0.7 ± 0.1 ($n = 3$)	23.8 ± 2.7 ($n = 3$)

enzymes respectively characteristic of microsomes (NADPH: cytochrome *c* reductase), Golgi (*N*-acetylglucosamine galactosyltransferase), mitochondria (cytochrome *c* oxidase) and MAM fraction (glucose-6-phosphate phosphatase) as previously described [15,16]. As shown in Table 1, the Golgi marker enzyme in the MAM had a specific activity not exceeding 7.5 % of that found in the Golgi fraction. For the mitochondrial marker enzyme, cytochrome *c* oxidase activity was low in MAM as compared with purified mitochondria. Moreover, Golgi membranes were only slightly contaminated by the MAM fraction (up to 8.7 %; see Table 1). Since the level of contamination of MAM by Golgi membranes was low in the present study, these subcellular fractions, and particularly MAM, were considered to be suitable for a comparative study of glycolipid-specific glycosyltransferase activities.

Synthesis of Glc-Cer in the MAM

For the measurement of Cer-Glc-T activity, silica-gel 60 was used as a solid support, since it was previously demonstrated that allowing the ceramide substrate to adsorb on to this material resulted in a higher incorporation of radiolabelled glucose into the ceramide [25] when compared with micellar systems. In addition, 0.5 % CHAPS was included in the incubation mixture, since it was reported that release of ceramide from the solid support during the assay was negligible and does not affect the result. After a 30 min incubation time as described in the Materials and methods section, lipid extracts were resolved by TLC using developing solvent systems I and II in the presence of authentic standards. Figure 1 shows the presence of a major radioactive spot, after partitioning in the organic phase, which co-migrated with authentic Glc-Cer (Figure 1) with previous impregnation of the silica-gel plate with sodium borate. This radioactive spot was scraped off the plate, eluted, and further fractionated on LC-NH_2 columns as described in [27]. More than 98 % of the radioactivity was recovered in fraction 4 – the fraction in which the neutral glycolipids are eluted. A similar result was obtained when [*palmitoyl*-^{14}C]ceramide was used as the acceptor and unlabelled UDP-glucose as the sugar donor under the same conditions as above (results not shown). A comparative experiment was then performed with the MAM fraction along with microsomes, Golgi membranes and mitochondria. After a 30 min incubation time in the presence of exogenous unlabelled ceramide and UDP-[^{14}C]glucose, the lipids were extracted and resolved by TLC. As depicted in Figure 2, [^{14}C]Glc-Cer was mainly synthesized in Golgi membranes (lane 3) and MAM (lane 2) and, to a lesser extent, in microsomes

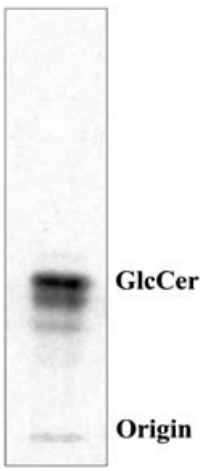

Figure 1 Autoradiography of lipids from the MAM incubated with UDP-[^{14}C]glucose and exogenous ceramides

Extracted lipids were resolved by TLC in solvent system I on silica-gel plates previously sprayed with methanolic 1 % sodium borate and dried at 100 °C before use. Identification was performed by co-migration with unlabelled commercial standards. The major radioactive band was Glc-Cer ('GlcCer').

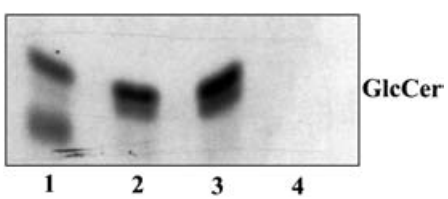

Figure 2 Autoradiography of lipids from rat liver subcellular fractions incubated with UDP-[^{14}C]glucose and exogenous ceramides

Extracted lipids from subcellular fractions (150 μg of protein/incubation) were resolved by TLC using solvent system II. The lipids were isolated from: whole microsomes (lane 1), MAM fraction (lane 2), Golgi fraction (lane 3) and purified mitochondria (lane 4). Identification of the labelled compounds was performed by co-migration with commercially available Glc-Cer ('GlcCer').

(lane 1), whereas purified mitochondria were clearly devoid of Cer-Glc-T activity under our experimental conditions. The specific activity of Cer-Glc-T was estimated in each subcellular fraction by counting the radioactivity of the spot co-migrating with authentic Glc-Cer. The apparent specific activities for Glc-Cer synthesis (c.p.m./min per mg of protein) were respectively

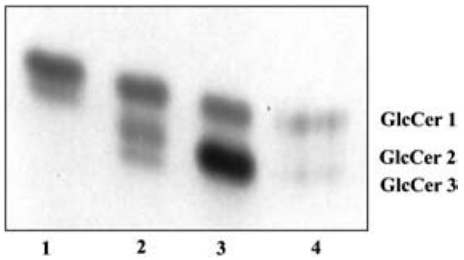

Figure 3 Autoradiography of lipids from the MAM incubated with UDP-[^{14}C]glucose in the presence of various exogenous ceramides

Lipids extracted from the MAM fraction (150 μg of protein/incubation) were resolved by TLC using solvent system I. Incubations were performed in the presence of: ceramides type III (Sigma) (lane 1), ceramides type IV (Sigma) (lane 2), phytosphingosine-containing ceramides (lane 3) and endogenous acceptors (lane 4). Migration of standard compounds was visualized with orcinol/sulphuric acid spray reagent. GlcCer 1, Glc-Cer with sphingosine and normal fatty acids (Sigma); GlcCer 2, phytosphingosine-containing Glc-Cer from human thyroid [26]; GlcCer 3, Glc-Cer with sphingosine and α-hydroxy fatty acids from human thyroid [26].

1460, 2260 and 750 for MAM, Golgi and whole microsomes (mean values for three independent experiments with variations $< 5\,\%$) compared with 74 c.p.m./min per mg of protein for the whole homogenate. These results show an enrichment of Cer-Glc-T activity of 30-fold in the Golgi fraction and of 20-fold in the MAM fraction relative to the homogenate. The substrate specificity of Glc-Cer synthase in the MAM fraction was further assessed by coating silica-gel particles with ceramides containing either sphingosine and non-hydroxy fatty acids (ceramides type III; Sigma), sphingosine and hydroxy fatty acids (ceramides type IV; Sigma) or ceramides with phytosphingosine and non-hydroxy fatty acids, as exogenous substrates. After a 30 min incubation time in the presence of UDP-[^{14}C]glucose, the lipid extracts were resolved by TLC as described above. Although Glc-Cer synthase was detected using endogenous substrates (Figure 3, lane 4), the results depicted in Figure 3 show that exogenous ceramides bearing phytosphingosine (4-hydroxysphinganine) were the best acceptors for Glc-Cer synthase as compared with standard ceramides (Sigma) type III containing normal fatty acids (lane 1) and type IV containing α-hydroxy fatty acids (lane 2). The radioactive spot in lane 3 migrating below standard sphingosine-containing Glc-Cer corresponds to phytosphingosine-containing Glc-Cer, as assessed by co-migration with the known standard purified from human thyroid [26] on borate-impregnated silica-gel plates. However, phytosphingosine-containing Glc-Cers could not be seen in lane 4 of Figure 3, which shows the Glc-Cers of MAM using endogenous substrates. The lower spot migrated like the standard of Glc-Cer that contains sphingosine and α-hydroxy fatty acids and which was purified from human thyroid [26]. The apparent specific activity for Cer-Glc-T in the MAM fraction was determined for each exogenous acceptor after scraping the radioactive spots from the silica-gel plate and counting radioactivity by liquid-scintillation spectrometry. The activities of Glc-Cer synthase were estimated as 4730, 4860 and 24 800 c.p.m./min per mg of protein (mean values for three independent experiments, with variations of less than 5 %) for ceramides type III, ceramides type IV and ceramides bearing phytosphingosine respectively, as compared with the control value of 1330 c.p.m./min per mg of protein for Glc-Cer synthase using endogenous substrates. All these results clearly demonstrate that phytosphingosine-containing ceramides are preferential substrates for Glc-Cer synthase in the MAM.

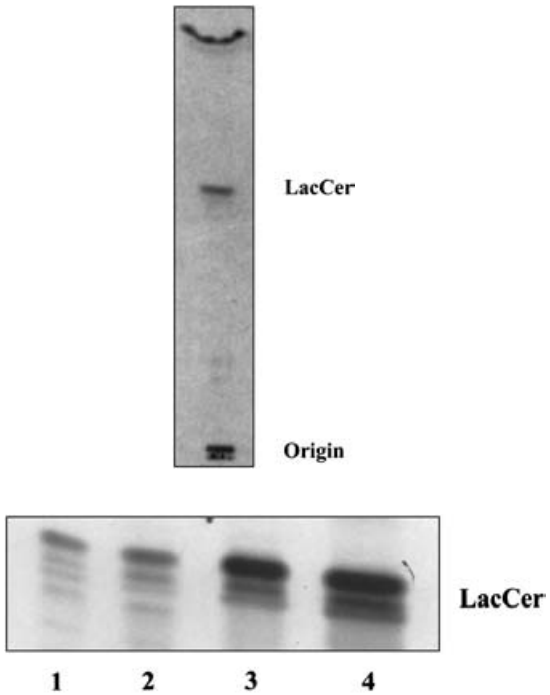

Figure 4 Autoradiography of lipids from the MAM and Golgi incubated with UDP-[^{14}C]galactose on endogenous acceptors and exogenous Glc-Cer

Upper panel: extracted lipids from the MAM fraction (150 μg of protein/incubation) were resolved by TLC using solvent system II. Identification was performed using commercial Lac-Cer ('LacCer') as standard. Lower panel: extracted lipids from rat liver subcellular fractions (150 μg of protein/incubation): MAM fraction with endogenous acceptors (lane 1), Golgi fraction with endogenous acceptors (lane 2), MAM with exogenous Glc-Cer (lane 3), and Golgi with exogenous Glc-Cer (lane 4). The solvent system was as in the legend to the upper panel.

Synthesis of Lac-Cer in the MAM

The second step in the synthesis of GSLs is the addition of a galactose residue from UDP-galactose on to Glc-Cer, yielding Lac-Cer. Although this enzyme has been previously localized to the Golgi apparatus [31,32], we investigated the presence of galactosyltransferase activity in the MAM fraction. As described for Glc-Cer synthase, the activity of the Glc-Cer galactosyltransferase (Gal-T_1; Lac-Cer synthase) was assayed using endogenous acceptors and exogenous Glc-Cer adsorbed on silica-gel 60. After a 30 min incubation time of MAM in the presence of UDP-[^{14}C]galactose, the lipids were extracted and resolved by TLC in solvent II. As a representative experiment, Figure 4, upper panel, shows the presence in the MAM of a radioactive spot which co-migrates with authentic Lac-Cer. The Lac-Cer synthase in MAM was further compared with that in Golgi using endogenous or exogenous acceptors (Figure 4, lower panel). After scraping material from the plate and counting for radioactivity the spot which co-migrated with authentic Lac-Cer, the apparent specific activity of Lac-Cer synthase (mean values for three independent experiments with variations of less than $< 5\,\%$) was 1570 c.p.m./min per mg of protein in the MAM using exogenous Glc-Cer as against 220 c.p.m./min per mg of protein with endogenous acceptors. This activity was 2110 c.p.m./min per mg for Golgi using exogenous Glc-Cer (lane 4) and 360 c.p.m./min per mg with endogenous acceptors (lane 2). These results demonstrate the presence of Lac-Cer synthase activity in the MAM fraction.

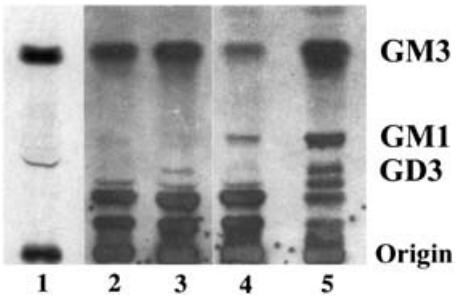

Figure 5 Autoradiography of lipids from rat liver subcellular fractions incubated with CMP-[^{14}C]sialic acid with and without exogenous acceptors

Reaction products obtained with 150 μg of protein of each fraction with or without exogenous substrates were resolved by TLC using solvent system III. Identification of the labelled products was performed by co-migration with gangliosides purified from melanoma tumours [49]. Lane 1, MAM fraction with endogenous acceptors; lane 2, MAM with exogenous Lac-Cer; lane 3, Golgi fraction with exogenous Lac-Cer; lane 4, MAM with exogenous G_{M3}; lane 5, Golgi with exogenous G_{M3}. Radioactive spots below G_{D3} in lanes 2–5 are unidentified.

Synthesis of gangliosides in the MAM

The sequential addition of further sialic acid residues and monosaccharides yielding GM_3 and more complex gangliosides is catalysed by glycosyltransferases which have been previously restricted to the luminal face of the Golgi membranes [10,33]. The presence of Glc-Cer synthase and Lac-Cer synthase in the MAM prompted us to investigate the presence of SAT activities in the MAM. Again, the assay was performed using Lac-Cer adsorbed on to silica gel as an exogenous acceptor incubated for 2 h in the presence of CMP-[^{14}C]*N*-acetylneuraminate. After purification of the gangliosides with our recently published new procedure [28], the gangliosides were resolved by TLC using solvent III. Figure 5 shows the results obtained in a representative experiment. In lane 1, a major radioactive spot co-migrated with authentic G_{M3}ganglioside and a minor one co-migrated with authentic G_{D3}, strongly suggesting the presence of SATs in the MAM. When Lac-Cer was used as an exogenous substrate, as depicted in lanes 2 and 3, the SAT activity was markedly enhanced, but many slow-migrating unidentified radioactive spots also appeared that could not be removed by reverse-phase chromatography on C_{18}-bonded silca gel. By scraping off and counting for radioactivity the radioactive spots co-migrating with ganglioside standards, the G_{M3} synthase activity recovered in the MAM (lane 2) was 80 % of that found in the Golgi fraction (lane 3): 325 c.p.m./min per mg of protein as against 406 c.p.m./min per mg of protein (mean values for three independent experiments with variations of less than 10 %). In both fractions, trace amounts of G_{M1} and G_{D3} were detectable. In a second set of experiments, MAM and Golgi were incubated with CMP-[^{14}C]sialic acid and exogenous G_{M3}. As seen in lanes 4 and 5, the presence of exogenous unlabelled G_{M3} led to a significant modification of the ganglioside profile revealed by TLC. In the Golgi fraction (lane 5), the synthesis of G_{M3} gangliosides was surprisingly stimulated in the presence of exogenous unlabelled G_{M3} as compared with lane 3, whereas the synthesis of G_{D3} and, most of all, G_{M1}, were also enhanced. In the MAM fraction, addition of exogenous G_{M3} instead of Lac-Cer in the incubation mixture resulted mostly in an enhanced synthesis of GM1 (lane 4), whereas the syntheses of G_{M3} and G_{D3} were apparently not modified. These results suggest that MAM contains also other glycosyltransferases such as Gal-T_2 (Lac-Cer synthase) and *N*-acetylgalactosaminyltransferases involved in the glycosylation of sphingolipids. At this stage of our study we can conclude that, under our experimental conditions, MAM has the capacity for synthesizing neutral GSLs that can be further sialylated and glycosylated in the same compartment.

DISCUSSION

The results described in the present paper point to a novel localization of glycolipid biosynthesis in a subcompartment of the ER. This ER subcompartment has been previously identified on the basis of biochemical [15,16] and morphological data [7] as a region of ER termed mitochondria-associated membrane or MAM [16] and tightly associated with mitochondria. Cell homogenization apparently disrupts the connection between the MAM and the bulk of the ER, resulting in its isolation together with mitochondria in a 10 000 ***g*** pellet [15]. Moreover, a physical linkage between MAM and mitochondria has been suggested [34,35] that dissociates during Percoll centrifugation. Since MAM is enriched in lipid-biosynthetic enzymes, it has been demonstrated by many groups [20,34,36,37] that the MAM is a specialized domain of the ER that links this organelle to the mitochondrion and mediates the transfer of newly synthesized phospholipids between these two subcellular compartments. More recently, the involvement of MAM has also been suggested for the transfer of signalling ceramides to mitochondria [19]. In the MAM, the levels of activities of the glycosyltransferases involved in the biosynthesis of neutral glycolipids and gangliosides were compared with those usually described in the Golgi fraction. Although MAM contains mitochondrial marker proteins (a likely consequence of the close association between the MAM and mitochondria), the level of contamination of MAM by Golgi assessed by the marker proteins was very low (an average of 7.5 % of cross-contamination of MAM by Golgi membranes) in the present study (see Table 1). Considering the biosynthesis of neutral GSLs, Cer-Glc-T and Gal-T_1 activities were found to represent respectively 65 and 74 % of those recovered in the Golgi subfraction. Thus the glycosyltransferase activities found in the MAM cannot be merely ascribed to the contamination by Golgi membranes that could occur during the procedure of subcellular fractionation. Although MAM cannot be considered as a major cellular site for neutral-glycolipid synthesis, the enzymic activities found in this compartment are high enough to suggest that the presence of neutral glycolipids in rat liver mitochondria [7] can be accounted for by a transfer from MAM. Cer-Glc-T activity showed phytosphingosine to be a preferential exogenous substrate in the MAM (Figure 3), as well as in Golgi membranes (results not shown). This finding is surprising, since previous studies [38,39] reported a preference of Cer-Glc-T for short-chain ceramides containing sphingosine as a substrate in the Golgi apparatus. Although phytosphingosine is the primary long-chain base found in yeast ceramides [40], it has been found so far only in low amounts in some animal sphingolipids [41]. Nevertheless, phytosphingosine has already been reported as a major sphingoid base of Glc-Cer in rat intestinal cells [42] and human thyrocytes [43]. In addition, a recent paper [44] described the first mammalian identified alkaline ceramidase localized in both Golgi and ER as being specific for the hydrolysis of phytosphingosine.

Gal-T_1 was also shown in the MAM fraction, a prerequisite for a further synthesis of gangliosides. At this stage of our investigations, the presence of ceramide galactosyltransferase activity, which is specifically localized in the ER, was not addressed in MAM. The next step of our study was to determine whether ganglioside synthesis can occur in the MAM. We firstly restricted our investigations to SAT activities, since previous studies [3] reported G_{M3} as the major ganglioside recovered in mitochondria together with small amounts of G_{D3}. More recently,

radioactive G_{M3} was also detected in purified mitochondria of HL-60 cells that were incubated with exogenous ^{14}C-labelled ceramides [45]. The results presented here show, for the first time, that SAT activities are present in a subcellular compartment different from the Golgi apparatus. Such activities in the MAM yield mainly G_{M3} and trace amounts of G_{D3}. Moreover, addition of exogenous G_{M3} in the incubation medium resulted in the synthesis of G_{M1}, thereby suggesting the possible presence of Gal-T_1 and *N*-acetylgalactosaminyltransferase activities in the MAM. Thus MAM is able to synthesize molecular species which are those actually described in mitochondria.

MAM has been very recently reported by Merrill [46] to have the capacity to synthesize ceramides. Since we have demonstrated here that MAM possess glycosyltransferase activities involved in the biosynthesis of neutral glycolipids and gangliosides, we suggest that mitochondrial GSLs can originate from MAM, thus avoiding the elaborate machinery that would be necessary to transport GSLs from the Golgi apparatus to mitochondria. As MAM has already been considered as a pre-Golgi compartment of the secretory route [16], the presence of such glycosyltransferases in MAM is consistent with that notion. For the synthesis of complex sphingolipids, the transport of the precursor ceramide from the ER to the Golgi was reported to occur via vesicular and non-vesicular pathways ([47]; see [19] for a review). The non-vesicular pathway involves stable membrane contacts between the ER and Golgi [47], a close apposition which was reported in a high-voltage electron-microscopic tomographic study [48]. Such contacts have been suggested to provide ceramide directly for *de novo* synthesis of sphingolipids [47]. In the light of our results, further studies are needed to determine whether the two ER subdomains, i.e. MAM and that involved in the non-vesicular trafficking of ceramide between ER and Golgi, have structural and/or functional similarities.

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