Conversion of dihydroceramide to ceramide occurs at the cytosolic face of the endoplasmic reticulum

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Abstract Dihydroceramide desaturase is responsible for the introduction of the 4,5-*trans* double bond into ceramide. Here, we describe the localization of this enzyme in the endoplasmic reticulum (ER) using ER- and Golgi-enriched fractions from rat liver. Furthermore, enzyme topology was studied. Mild proteolysis of ER-derived vesicles under conditions which assure membrane integrity (latency of mannose 6-phosphatase was at least 91%) resulted in an up to 90% inactivation of dihydroceramide desaturase activity. This indicates a cytosolic orientation of dihydroceramide desaturase activity in the ER membrane.

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Key words: Dihydroceramide desaturase; Ceramide; Biosynthesis; Localization; Topology

1. Introduction

Ceramide anchors glycosphingolipids and sphingomyelin in the outer leaflet of the plasma membrane. Its physiological role is, however, not restricted to this structural function. Over the last few years it has become clear that ceramide plays important roles in cellular metabolism. It is involved in what has become known as the sphingomyelin cycle [1]. It also serves as a mediator of cellular apoptosis, differentiation [2], and senescence [3], and is involved in the coordination of cellular responses to stress situations [4].

It was observed that not only ceramide derived from the breakdown of sphingomyelin, but also that from de novo synthesis is involved in the cellular responses to inducers of apoptosis [5] and differentiation [6]. Taking into consideration that dihydroceramide does not mimic the effects of ceramide [7,8], it seems possible that dihydroceramide desaturase, the enzyme which converts dihydroceramide into ceramide, plays an important role in the tight regulation of the turnover of ceramide used as a lipid second messenger.

The de novo synthesis of ceramide starts with the condensation of L-serine and palmitoyl-CoA catalyzed by the pyridoxal phosphate dependent serine palmitoyltransferase. Its product, 3-dehydrosphinganine, is immediately reduced by the NADPH dependent 3-dehydrosphinganine reductase yielding D-*erythro*-sphinganine [9]. The sequence of the last two steps leading to ceramide remained unclear for some time, although experimental results strongly favored the formation of dihydroceramide as a biosynthetic intermediate [9–12]. The discovery of fumonisin B_1 as a potent inhibitor of the *N*-acylation of sphingoid bases [12,13] helped us and others to demonstrate that this is, indeed, the case in many cell types [14,15]. Thus, D-*erythro*-sphinganine is first acylated by the sphinganine *N*-acyltransferase and ceramide is subsequently formed by introduction of a 4,5-*trans* double bond by the NADH dependent dihydroceramide desaturase.

The first three enzymes involved in sphingolipid biosynthesis are well characterized and were found to be localized in the endoplasmic reticulum (ER) membrane with a cytosolic topology [16,17]. We have recently established an in vitro assay for dihydroceramide desaturase activity using rat liver microsomes as an enzyme source [18]. The aim of the present study was to clarify the localization and topology of this enzyme, thus filling the 'gap' in the pathway of the de novo synthesis of ceramide.

2. Materials and methods

2.1. Materials

Buffers, salts and sucrose were purchased from Merck (Darmstadt, Germany). ^{14}C-Labeled fatty acids, detergents, NADH, proteases, protease inhibitors, sugar phosphates and other biochemicals were of analytical grade and were supplied by Sigma (Munich, Germany). X-OMAT Scientific Imaging Film was purchased from Kodak. Male Wistar rats (250–300 g) were supplied by Charles River Wiga GmbH (Sulzfeld, Germany). ^{14}C-Labeled CMP-*N*-acetyl neuraminic acid was purchased from Amersham (Braunschweig, Germany). The diagnostic kit for quantitative determination of inorganic phosphate was supplied by Serva (Heidelberg, Germany). GM1a was a kind gift of Fidia Research Laboratories (Abano Terme, Italy).

2.2. Isolation of ER and Golgi vesicles

Golgi vesicles were isolated from rat liver according to Sandberg et al. [19] as described in detail by Yusuf et al. [20,21]. Briefly, rat liver was homogenized in 0.3 M sucrose and centrifuged twice at 7500×*g*. To the combined supernatants the same volume of 2 M sucrose was added. The solution was carefully overlaid with 1.05 M and 0.3 M sucrose, respectively, and then centrifuged at 150 000×*g* for 2 h. The Golgi fraction was recovered from the interface between the 1.05 M and 0.3 M sucrose layers. The ER fraction was recovered in the pellet, which was resuspended in MOPS-KOH buffer (100 mM, pH 8.0), and centrifuged at 105 000×*g* for 60 min. The pellet was resuspended in MOPS-KOH buffer (100 mM, pH 7.4). Aliquots were frozen in liquid nitrogen and stored at −80°C. The Golgi fraction was concentrated by centrifugation at 150 000×*g* for 45 min on a 1.05 M sucrose layer. The band at the interface was carefully saved. Aliquots were frozen in liquid nitrogen and stored at −80°C.

2.3. Protease treatment of ER-derived vesicles

ER-derived vesicles containing 1 mg of protein were incubated with various amounts of either trypsin or pronase (from *Streptomyces griseus*) for 20 min at 30°C in a final volume of 1 ml of MOPS-KOH buffer (100 mM, pH 7.4). Disruption of ER-derived vesicles was performed by incubation at 4°C for 15 min in the presence of CHAPS in a final concentration of 0.5% (w/v) prior to protease treatment. Pronase digestion also included 0.5 mM $CaCl_2$. The reactions were terminated by diluting the samples 9-fold with ice-cold MOPS-KOH buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 5 mM EDTA. After centrifugation at 105 000×*g* for 60

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Table 1
Localization of glucose 6-phosphatase, GD1a synthase (sialyltransferase IV) and dihydroceramide desaturase in ER- and Golgi-derived vesicles

		ER fraction	Golgi fraction
	Protein per fraction (mg)	106 ± 10	2.5 ± 0.8
Glucose 6-phosphatase	Specific activity (nmol/min/mg)	17.2	9.6
	Total activity (nmol/min)	1823	24
	Relative total activity (%)	98.7	1.3
GD1a synthase (sialyltransferase IV)	Specific activity (pmol/min/mg)	1.1	74.1
	Total activity (pmol/min)	116.6	244.5
	Relative total activity (%)	32.3	67.7
Dihydroceramide desaturase	Specific activity (pmol/min/mg)	885	252
	Total activity (nmol/min)	93.8	0.6
	Relative total activity (%)	99.3	0.7

Rat liver was fractionated into vesicles derived from ER and Golgi according to the procedure described in Section 2. Total activities were calculated by multiplying the specific enzymatic activity of each fraction by the milligrams of protein recovered from each fraction. The latency of mannose 6-phosphatase of ER-derived vesicles was at least 93%. The vesicles were enriched 6.8-fold over homogenate in glucose 6-phosphatase activity and 1-fold in GD1a synthase activity. Golgi vesicles were enriched 1.2-fold over homogenate in glucose 6-phosphatase activity and 34.5-fold in GD1a synthase activity.

2.4. Assays of enzymatic activities

Dihydroceramide desaturase activity was determined as described recently [18]. Briefly, 100 μg of protein, 15 nmol *N*-[1-^{14}C]octanoyl-D-*erythro*-sphinganine (as complex with 2 equivalents of bovine serum albumin) and 1 mM NADH were incubated in 330 μl MOPS-KOH buffer (100 mM, pH 7.4) at 37°C for 60 min. The reaction was terminated by addition of chloroform/methanol. After extraction, the lipids were separated by TLC, visualized by autoradiography and quantitated by densitometry.

Glucose 6-phosphatase activity (marker enzyme for ER fraction) [22] and GD1a synthase activity (sialyltransferase IV; marker enzyme for Golgi fraction) [23] were measured according to previously described methods.

The integrity of ER vesicles was examined by measuring the latency of mannose 6-phosphatase [22].

Contamination of ER- and Golgi-derived vesicles with other cellular membranes was determined by measuring 5′-nucleotidase activity (marker enzyme for plasma membrane fraction) and acid phosphatase activity (marker enzyme for lysosomal fraction) as previously described [22].

2.5. Miscellaneous procedures

N-[1-^{14}C]Octanoyl-D-*erythro*-sphinganine was prepared as described [18]. Protein was determined by the method of Bradford [24] using bovine serum albumin as standard protein.

2.6. Presentation of data

All data presented are means of three separate experiments. At least double determinations were performed in each experiment. All individual values were in the range of ±12% of the mean.

3. Results and discussion

3.1. Subcellular distribution of dihydroceramide desaturase

The fraction of ER-derived vesicles used in this study was enriched 6.8-fold over homogenate in glucose 6-phosphatase activity and 1-fold in GD1a synthase activity. Golgi vesicles were enriched 1.2-fold over homogenate in glucose 6-phosphatase activity and 34.5-fold in GD1a synthase activity. This method of separation of the Golgi compartment from ER membranes yielded good results, as can be concluded from the specific and total activities of both marker enzymes (Table 1). About 70% of total GD1a synthase activity was found in the Golgi fraction, whereas the glucose 6-phosphatase activity was almost quantitatively recovered in the ER fraction.

Contamination with plasma membrane and lysosomes was assessed by measuring the enrichment of 5′-nucleotidase and acid phosphatase activity over the respective homogenate activity in the fractions. ER-derived membranes showed a 3.3-fold 5′-nucleotidase and a 1.9-fold acid phosphatase enrichment. For Golgi vesicles, the enrichment was found to be 2.7-fold and 1-fold, respectively.

Based on the latency of glucose 6-phosphatase towards mannose 6-phosphate [22], a substrate which cannot be transported across the liver ER membrane [25], at least 93% of the

Table 2
Effects of protease treatment on dihydroceramide desaturase activity in intact and disrupted ER-derived vesicles from rat liver

Protease	(μg/mg protein)	Dihydroceramide desaturase activity			
		In disrupted vesicles		In intact vesicles	
		specific (nmol/h/mg)	relative (%)	specific (nmol/h/mg)	relative (%)
Trypsin	0	18.6	100	22.5	100
	50	9.5	51.1	10.7	47.6
	100	5.5	29.6	7.8	34.7
	300	3.9	21.0	5.5	24.4
Pronase	0	20.5	100	25.0	100
	25	6.7	32.7	8.7	34.8
	50	3.7	18.0	5.3	21.2
	100	1.2	5.9	2.1	8.4

ER-derived vesicles were incubated with either trypsin or pronase as described in Section 2. The latency of mannose 6-phosphatase of intact ER-derived vesicles remained unchanged after proteolysis (92 ± 2%), but was susceptible to both proteases in disrupted vesicles. Disruption of vesicles was achieved by treatment with 0.5% CHAPS (w/v) as described in Section 2. Addition of the dihydroceramide desaturase assay components to intact vesicles did not alter the membrane integrity (mannose 6-phosphatase latency was 91 ± 2%).

ER-derived vesicles were sealed and of the correct membrane topology ('right side out') after the preparation procedure.

As shown in Table 1, dihydroceramide desaturase activity was almost quantitatively (>99%) recovered in the ER-derived vesicles. Furthermore, its distribution among the two membrane fractions was paralleled by that of the ER marker enzyme, glucose 6-phosphatase. Therefore, our results strongly suggest that dihydroceramide desaturase is located in the endoplasmic reticulum.

3.2. Topology of dihydroceramide desaturase

Mild proteolysis of ER-derived vesicles with various amounts of trypsin or pronase in either the presence or absence of detergent (CHAPS) led to a concentration dependent reduction of dihydroceramide desaturase activity (Table 2). This result was not due to disturbance of membrane integrity, since in the absence of detergent the latency of mannose 6-phosphatase remained unaltered after treatment with proteases (92% latency). Subsequent addition of all dihydroceramide desaturase assay components (bovine serum albumin, *N*-[1-^{14}C]octanoyl-D-*erythro*-sphinganine and NADH) did not affect the latency of mannose 6-phosphatase either (91% latency). Only in the presence of CHAPS was mannose 6-phosphatase susceptible to proteolysis, as was shown before [26]. These findings strongly suggest a cytosolic orientation of the dihydroceramide desaturase activity.

The antiluminal topology of this enzyme is not unexpected, since we showed recently that dihydroceramide desaturase appears to be part of the ER resident cytochrome b_5 electron transport system [18], which is also localized in the cytosolic leaflet. Moreover, all known fatty acyl CoA desaturases receive the required electrons from this system and share the same cytosolic topology in the ER [27]. Furthermore, dihydroceramide, the substrate for dihydroceramide desaturase, is likewise synthesized on the cytosolic face of the ER [16] and would, therefore, be directly accessible to the active site of this enzyme.

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