Biochimica et Biophysica Acta 858 (1986) 6–12
Elsevier

BBA 73098

Topology of glucosylceramide synthesis in Golgi membranes from porcine submaxillary glands

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(Received February 18th, 1986)

Key words: UDPglucose; Ceramide glucosyltransferase; Glycolipid; Golgi membrane; Membrane topology; (Porcine submaxillary gland)

The topology of ceramide glucosyltransferase and de novo synthesized glucosylceramide was studied in sealed and 'right-side-out' vesicles of porcine submaxillary glands derived from Golgi apparatus. Pronase treatment which did not cause any breakdown of the luminal glycoprotein galactosyltransferase activity, inhibited the ceramide glucosyltransferase to more than 50% at a ratio proteinase to Golgi protein 1:100. Trypsin at the same concentration, while producing no inactivation of luminal galactosyltransferase, caused a complete loss of ceramide glucosyltransferase activity. The membrane-impermeable compound, DIDS, which did not cause any inhibition of the galactosyltransferase, inhibited the ceramide glucosyltransferase (70% reduction at 80 μM DIDS). Thus, the enzyme ceramide glucosyltransferase is accessible from the cytoplasmic side of the Golgi vesicles. The orientation of the newly synthesized glucosylceramide is studied by the ability of the enzyme glucosylceramidase to hydrolyse this compound both on intact and on disrupted vesicles. The same percentage (respectively, 36 and 30%) of hydrolysis was obtained during an incubation of 3 h, showing that glucosylceramide is not at all protected from external hydrolysis. Pronase-treated vesicles revealed an increase in glucosylceramidase hydrolysis (up to 45%), which indicates that glucosylceramide may be cryptic. All these results indicate that the ceramide glucosyltransferase, as well as related glucosylceramide, are cytoplasmically oriented in Golgi vesicles from porcine submaxillary glands.

Introduction

The initial step in the assembly of the oligosaccharide moiety of some glycolipids involves the synthesis of glucosylceramide from UDPglucose. Previous investigations in this laboratory showed that UDPglucose-ceramide glucosyltransferase from porcine submaxillary glands is associated with Golgi apparatus [1].

It was shown [2–7] that glycosyltransferases responsible for glycoprotein biosynthesis are membrane-bound enzymes and reside on the luminal side of the Golgi vesicles. This spatial arrangement poses a problem in logistic, because sugar nucleotides are synthesized in the cytosol and cannot normally penetrate the Golgi membranes. To solve this problem, a specific uptake mechanism appears to function in the production of an intra-luminal pool of CMP-NeuNAc [5,8] or GDPfucose [9]. The existence of similar carrier systems has also been shown for UDPgalactose,

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid.

UDP-GalNAc [10–12], UDPglucose [13] and UDP-GlcNAc [14]. Recently, Yusuf et al. [15] suggest a similar mecanism for gangliosides G_{M2} and G_{M1} synthesis.

For the synthesis of glucosylceramide, another possibility could be considered: synthesis on the cytoplasmic side of the Golgi membranes and then translocation to the luminal side. A similar model has been proposed for oligosaccharide-lipid synthesis into the endoplasmic reticulum [16].

In this report, studies are presented on the topological orientation of glucosylceramide and of the enzyme involved in its synthesis. These studies indicate that in sealed and 'right-side-out' Golgi vesicles: (1) ceramide glucosyltransferase can be inactivated by external proteolysis, (2) it is possible to inhibit the ceramide glucosyltransferase but not the galactosyltransferase by treatment of vesicles with membrane-impermeable compounds such as DIDS [17], (3) enzymatic glucosylceramide hydrolysis can be obtained.

Materials and Methods

Materials. Porcine submaxillary glands were obtained from a local slaughterhouse. UDP[U-^{14}C]galactose (309 Ci/mol) and UDP[U-^{14}C]glucose (294 Ci/mol) were purchased from Amersham International. DIDS, trypsin, Mops and CHAPS were obtained from Sigma; pronase (70 PUK) from Merck; 2,3-dimercaptopropanol, also called British Anti-Lewisite, was from Serva; ceramides from bovine brain were purchased from Serdary Research Laboratories, Canada.

Isolation and topology of Golgi vesicles. Golgi vesicles were isolated from porcine submaxillary glands according to the procedure of Leelavathi et al. [18] as previously described [1]. The vesicles were enriched 13-fold over the postnuclear supernatant in galactosyltransferase activity (25% yield), 18-fold in ceramide glucosyltransferase activity (30% yield) and showed low activity for NADPH-cytochrome *c* reductase.

The vesicles' integrity and their topologic orientation were determined by measuring the latency of galactosyltransferase when exogenous glycoproteins as ovomucoid were used as substrates [6]. Moreover, susceptibility of ovomucoid-β-D-galactosyltransferase to pronase and trypsin (ratio proteinase to membrane protein 1 : 10) was 0% on intact vesicles and, respectively, 95 and 97% when vesicles were disrupted by sonication (Branson sonicator at +4°C for 10 min at 20 W) prior to proteinase treatments. Thus, about 90–95% of the vesicles were sealed and 'right-side-out'.

Isolation of glucosylceramidase. A partially purified glucosylceramidase was prepared from human placenta essentially as described by Furbish et al. [19] up to the step of *n*-butanol extraction.

Enzyme assays. The following standard assay procedure was used to measure the enzymatic activities:

Galactosyltransferase was assayed on endogenous protein acceptors in a total volume of 80 μl comprising: 10 μM UDP[U-^{14}C]galactose (309 Ci/mol)/50 mM Mops buffer (pH 6.5)/5 mM $MnCl_2$/2.5 mM $MgCl_2$/0.25 M sucrose/1 mM NADH/5 mM 2,3-dimercaptopropanol/600–800 μg protein. After incubation for 2–10 min at 37°C, the reaction was stopped by addition of 8 ml of 50 mM Mops buffer (pH 6.5)/0.25 M sucrose at 4°C. Vesicles were isolated by centrifugation in a Beckman-50 rotor at 180000 × *g* for 45 min. After resuspension and washing in the same conditions, the resulting pellet was extracted by addition of 2 ml chloroform/methanol (1 : 1, v/v) 30 min at room temperature. After centrifugation for 30 min at 2500 × *g*, the pellet was washed again with 1 ml of chloroform/methanol (1 : 1, v/v). Proteins were dissolved in 500 μl of soluene 350 (Packard Instruments) and incorporation of [^{14}C]galactose was determined by liquid-scintillation counting.

For the exogenous activity, ovomucoid was used as acceptor according to Fitzgerald et al. [20]. The reaction mixture comprised in a final volume of 260 μl: 55 μM UDP[U-^{14}C]galactose (4.5 Ci/mol)/30 mM Mops buffer (pH 6.5)/9 mM $MnCl_2$/2.5 mM AMP/0.2% Triton X-100/500 μg ovomucoid/30–200 μg protein. Incubation was carried out for 30 min at 32°C and the reaction was stopped by the addition of 2 ml phosphotungstic acid 5% in 2 M HCl. Proteins were collected on glass microfiber filters Whatman (GF/B, 2.5 cm) and incorporation of [^{14}C]galactose was determined by liquid-scintillation counting.

The synthesis of glucosylceramide was assayed on endogenous acceptor in a total volume of 110 μl comprising: 50 μM UDP[U-^{14}C]glucose (70 Ci/mol)/50 mM Mops buffer (pH 6.5)/5 mM $MnCl_2$/2.5 mM $MgCl_2$/1 mM NADH/5 mM dimercaptopropanol/ 0.2–0.5 mg protein. For synthesis on exogenous ceramides, the method is adapted from Nescovic et al. [21]. Before incubation, detergent (CHAPS, 500 μg) and ceramides (75 μg) were dissolved in chloroform/methanol (2 : 1, v/v) and mixed in the incubation tubes. The organic solvent was evaporated under a gentle stream of nitrogen. The same buffer and cofactors as described for endogenous incorporation were added to the dry lipid film as well as the enzymatic fraction and vortexed twice for 30 s. The reaction was then initiated by the addition of 50 μM UDP[U-^{14}C]glucose. Reactions were carried out for 5–10 min at 37°C and were stopped by addition of 2 ml chloroform/methanol (2 : 1, v/v) 1 h at room temperature. The lipid extract was partitioned according to Folch et al. [22]. The lower phase was washed twice with the theoretical upper phase of Folch et al. [22] and dried.

Radiolabeled glucosylceramide was separated on silica-gel 60 thin-layer plates (Merck) in the solvent system chloroform/ methanol/ water (60 : 35 : 8, v/v), scraped off the plates and counted by liquid scintillation.

Proteinase treatment of Golgi vesicles. Proteinase treatment of membranes was performed in 10 mM Tris-HCl buffer (pH 7.5)/50 mM KCl/0.25 M sucrose at 37°C for 30 min. Different concentrations of pronase and trypsin were added in a total volume of 100 μl and a Golgi protein concentration of 8 mg/ml. At the end of the incubation, the membranes were diluted with 35 ml of 50 mM Mops buffer (pH 6.5)/0.25 M sucrose and vesicles were isolated by centrifugation for 45 min at 180 000 × *g*. The resulting pellets were resuspended in the original volume of ice-cold buffer, 70 mM Mops (pH 6.5)/0.25 M sucrose/8 mM $MnCl_2$/4 mM $MgCl_2$, and assayed immediately.

In the case of trypsin treatment, soybean trypsin inhibitor (Sigma) was added at a concentration of 50 μg for 1 μg of trypsin.

DIDS treatment of Golgi vesicles. Golgi vesicles (1 mg of protein) were incubated for 20 min at 25°C in 2 ml of 50 mM Mops buffer (pH 6.5)/0.25 M sucrose/20–80 μM DIDS. The suspension was then diluted to 35 ml of the same buffer at 4°C, and bovine serum albumin was added (to adsorb DIDS) to a final concentration of 0.5%. Following centrifugation at 180 000 × *g* for 45 min, the supernatant solution was discarded. The pellet was resuspended in 100 μl of 70 mM Mops buffer (pH 6.5)/0.25 M sucrose/8 mM $MnCl_2$/4 mM $MgCl_2$ and assayed immediately.

Glucosylceramidase treatment of Golgi vesicles. Before these treatments, [U-^{14}C]glucosylceramide was synthesized on endogenous ceramide as described in enzymatic assays, except that the specific activity of UDP[U-^{14}C]glucose was 294 Ci/mol. Vesicles were then diluted with 35 ml of 50 mM Mops buffer (pH 6.5)/0.25 M sucrose and centrifuged for 45 min at 180 000 × *g*.

Before glucosylceramidase treatment, pronase-treated vesicles were obtained as described before. Sonication-disrupted vesicles were obtained with Branson sonicator at +4°C for 10 min (50% duty cycle) at 20 W.

Glucosylceramidase treatment was performed on labeled vesicles (400 μg proteins) in 100 μl of (0.2 M/0.4 M) citrate phosphate buffer (pH 5.3)/ 1.25 M sucrose with 400 μl of the purified enzyme (8 μg proteins) as obtained elsewhere. Incubation was carried out for 1–3 h at 37°C and stopped by addition of 2 ml chloroform/methanol (2 : 1, v/v) for 1 h at room temperature. The lower phase of Folch et al. [22] was washed and labeled glucosylceramide was counted by liquid scintillation. An assay was carried out under the same conditions, without glucosylceramidase in order to determine an eventual endogenous hydrolysis of [U-^{14}C]glucosylceramide.

Results

Effect of proteinases on glucosylceramide synthetase

Incubation of intact vesicles derived from Golgi apparatus with UDP[U-^{14}C]glucose led to a rapid incorporation of radioactivity into lower-phase lipid. This phase contained essentially (more than 95%) a radiolabeled compound which was identified as glucosylceramide on thin-layer chromatography [1]. If the glucosylceramide is formed on the cytoplasmic face of the membrane, the enzyme involved in its synthesis must have proteinase-sen-

TABLE I

EFFECT OF PROTEINASE TREATMENT ON CERAMIDE GLUCOSYLTRANSFERASE AND GLYCOPROTEIN GALACTOSYLTRANSFERASE IN INTACT GOLGI VESICLES FROM PORCINE SUBMAXILLARY GLANDS

Golgi vesicles were preincubated with pronase or trypsin as described under Materials and Methods. After these treatments, the vesicles were reisolated by centrifugation and enzyme assays were carried out without detergent for endogenous acceptors or with detergent for exogenous acceptors. The ratios of proteinase-to-membrane protein are given in the table. The latency of ovomucoid β-D-galactosyltransferase remained unchanged (90%) after proteolytic treatments. The average results of four experiments are shown. Incorporation of radioactivity from UDPglucose in control incubations, designated as 100, was: 11200 cpm/5 min per mg protein on the endogenous acceptor and 37500 cpm/5 min per mg protein on the exogenous acceptor. Incorporation of radioactivity from UDPgalactose in control incubations, designated as 100, was: 40000 cpm/10 min per mg protein for endogenous transfer and 56800 cpm/10 min per mg protein on the exogenous acceptor.

Proteinase	Ratio to membrane protein	% lost protein from the Golgi fraction	% Enzyme inactivation			
			glucosyl ceramide transferase		glycoprotein galactosyltransferase	
			endogenous acceptor	exogenous acceptor	endogenous transfer	exogenous acceptor
Pronase	1:20	27	100	100	50	0
	1:50	19	90	100	25	0
	1:100	12	25	50	0	0
	1:200	5	10	20	0	0
	1:300	2	0	0	0	0
Trypsin	1:10	14	100	100	0	0
	1:50	4	90	95	0	0
	1:100	2	65	85	0	0

sitive sites on the side of the membrane facing the medium. This conclusion is based on the findings that the vesicles derived from the Golgi apparatus are sealed with a unique orientation 'right-side-out' (have the same membrane topology as in vivo) [4,6]. We have approached this problem by examining the degree to which the activities of various glycosyltransferases are affected by external proteolysis of Golgi vesicles.

As shown in Table I, glucosylceramide synthesis on endogenous ceramides, as well as on exogenous ceramides, could be inhibited by nearly 100% following treatment of Golgi vesicles with proteinases. At the same time, the galactosyltransferase activity, assayed with ovomucoid as the exogenous protein acceptor, was not affected, suggesting that the enzyme was protected from proteinase action.

Disruption of the Golgi vesicles by sonication and subsequent treatment with proteinases resulted in total inhibition of ovomucoid-β-D-galactosyltransferase (respectively, 95 and 97% for pronase and trypsin at a proteinase-to-membrane protein ratio of 1:10). This inhibition ruled out the possibility of a specific insensibility of galactosyltransferase to proteinases. Therefore, the lack of inhibition of galactosyltransferase by proteinases unless the vesicles had been disrupted was due to its specific orientation in the lumen of the Golgi.

The galactosyltransferase on endogenous acceptors (endogenous transfer) was inactivated to a lesser extent, 25–50%, when treated with pronase at a high ratio to membrane protein. This resulted from the inactivation of UDP galactose carrier protein which has a cytoplasmic domain in Golgi membranes [12]. This carrier protein is insensible to trypsin action.

The fact that glucosylceramide transferase activity on endogenous as well as exogenous ceramides was markedly inhibited suggests that the enzyme (or at least its active site) is accessible from the cytoplasmic side of Golgi vesicles.

Effect of anion-specific inhibitor on glucosylceramide biosynthesis

DIDS, a non-penetrating reagent, was shown to interact with the cytoplasmically oriented anion-binding sites of glycosyltransferases [23]. Synthesis of glucosylceramide was found to be affected by the inclusion of DIDS in the incubation medium (Table II).

With increasing amounts of this compound, a marked decrease in the formation of radiolabeled glucosylceramide from UDP[U-^{14}C]glucose was noted: at 80 μM, a 70% inhibition of glucosylceramide synthesis was reached. The fact that we observed the same degree of inhibition on endogenous acceptors and on exogenous acceptors strongly suggests that the ceramide glucosyltransferase itself was accessible from the cytoplasmic face of the Golgi vesicles. In contrast, galactose transfer on exogenous acceptors was not affected when the Golgi vesicles have been preincubated with DIDS.

Disruption of the Golgi vesicles by Triton X-100 (0.25%) or by sonication and subsequent treatment with DIDS resulted in a substantial inhibition of ovomucoid-β-D-galactosyltransferase (respectively, 50 and 75% at 80 μM). This inhibition ruled out the possibility of a specific insensibility of galactosyltransferase to DIDS. Therefore, the lack of inhibition of galactosyltransferase by DIDS, unless the vesicles have been disrupted, was due to its specific orientation into the lumen of the Golgi vesicles.

The fact that galactosyltransferase on endogenous acceptors was weakly inhibited by DIDS treatment indicated that this inhibitor did not target the UDPgalactose carrier protein.

Glucosylceramidase as a topological probe for cytoplasmic orientation of glucosylceramide

In order to determine the transbilayer orientation of the newly synthesized glucosylceramide, the β-glucosidase from almonds (Sigma) was assayed without any result. Therefore, we have purified a specific glucosylceramidase (see Materials and Methods) which splits the glucose from glucosylceramide. The glucosylceramide hydrolysis was measured in sealed or disrupted Golgi vesicles. It is apparent from the data shown in Table III that under conditions where the vesicles remained sealed, more than 30% of the newly synthesized glucosylceramide was degraded after 3 h incubation by externally added glucosylceramidase.

With Golgi vesicles subjected to pretreatment by pronase, we observed an increase of glucosylceramide hydrolysis (up to 45%) without decrease of galactosyltransferase latency. This suggests that we have increased the accessibility of glucosylceramide by this proteinase treatment,

TABLE II

EFFECT OF DIDS ON CERAMIDE GLUCOSYLTRANSFERASE AND GLYCOPROTEIN GALACTOSYLTRANSFERASE IN INTACT GOLGI VESICLES FROM PORCINE SUBMAXILLARY GLANDS

The vesicles were preincubated with DIDS for 20 min at 25°C prior to the addition of sugar nucleotides. Incubation of vesicles with UDP[^{14}C]glucose or UDP[^{14}C]galactose were carried out under standard conditions without detergent for endogenous activities or with detergent for exogenous activities. The average values of three experiments are shown.

DIDS (μM)	% inhibition of enzyme activity			
	ceramide glucosyltransferase		glycoprotein galactosyltransferase	
	endogenous acceptor	exogenous acceptor	endogenous transfer	exogenous acceptor
20	52	53	0	0
40	62	58	2	0
80	69	68	16	0

TABLE III

CLEAVAGE OF NEWLY SYNTHESIZED GLUCOSYLCERAMIDE FROM SEALED AND DISRUPTED GOLGI VESICLES BY GLUCOSYLCERAMIDASE

Golgi vesicles were preincubated in the presence of UDP[U-^{14}C]glucose to synthesize labeled glucosylceramide. Intact, sonication-disrupted or pronase-treated vesicles were incubated for 1–3 h with glucosylceramidase (75 nmol/min per mg protein). The average values of two experiments are shown; only one experiment for 1 h sonication.

Pretreatment	Glucosylceramide hydrolysis (%)		Intactness of vesicles (%)
	1 h	3 h	
None	18	36	90
Pronase	19	45	90
Sonication	8	30	20

whereas no loss of membrane integrity occurred. Furthermore, when Golgi vesicles were disrupted by sonication, no more glucosylceramide was accessible to cleavage. In the same conditions, 60% of free glucosylceramide was degraded in a lipid extract from Golgi membranes (see Materials and Methods).

All these results suggested that the newly synthesized glucosylceramide was oriented towards the cytoplasmic face of the Golgi apparatus.

Discussion

In a previous study, we have provided evidence that the enzyme UDPglucose-ceramide glucosyltransferase (EC 2.4.1.80) was associated with membranes of the Golgi apparatus [1] like other glycosyltransferases involved in the synthesis of common gangliosides starting from glucosylceramide.

Two lines of evidence that have been presented indicate that the biosynthesis of glucosylceramide occurred on the cytoplasmic face of the Golgi apparatus: (1) by using proteolysis of Golgi vesicles by trypsin or pronase, most of the enzyme involved in the synthesis of glucosylceramide can be inactivated. (2) DIDS, that has been shown to be a non-penetrating agent for intact erythrocytes membranes [24] and Golgi vesicles [25] as well as for artificial phospholipid protein vesicles [26] was found to cause a pronounced decrease in the conversion of UDPglucose to glucosylceramide. In contrast, the glycopeptide galactosyltransferase was unaffected by these treatments. This result is in complete agreement with the luminal orientation of this enzyme in Golgi vesicles [6,7]. This result also provides evidence that our Golgi vesicles are mainly sealed and 'right-side-out'.

We have also shown that the newly synthesized glucosylceramide could be hydrolyzed by addition of purified glucosylceramidase to intact Golgi vesicles. This result suggested that the glucosylceramide was oriented towards the cytoplasmic face of the Golgi apparatus. All these results led us to conclude to a cytoplasmic orientation of the glucosylceramide biosynthesis in Golgi apparatus.

It is of interest to note that this orientation is in agreement with the presence of cytoplasmic glucosylceramide transfer protein [27] which may thus recognize this glycolipid and carry it to other intracellular membranes.

Yusuf et al. [11] suggested that the biosynthesis of gangliosides G_{M2} and G_{M1} takes place in the lumen of Golgi vesicles. In this paper, we demonstrated that glucosylceramide biosynthesis takes place on the cytoplasmic side of Golgi vesicles. Therefore, if this glucosylceramide acts as precursor for G_{M2} and G_{M1} biosynthesis, a translocation must occur before ganglioside biosynthesis. The topology of lactosylceramide, the second step in ganglioside biosynthesis, is studied in our laboratory in order to solve this important question.

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