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Lactosylceramide is synthesized in the lumen of the Golgi apparatus

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Abstract

Recently, synthesis of lactosylceramide has been described to occur on the cytosolic face of the Golgi [(1991) J. Biol. Chem. 266, 20907–20912]. The reactions following in the biosynthesis of higher glycosphingolipids are known to take place in the lumen of the Golgi. For our understanding of the functional organization of the multiglycosyltransferase system of glycosphingolipid synthesis in the Golgi, the knowledge of the topology of individual reactions is a prerequisite. We have developed a simple and quick assay system for sphingolipid biosynthesis and have obtained evidence that lactosylceramide is synthesized in the lumen of the Golgi. Because lactosylceramide is generated by galactosylation of glucosylceramide which, in turn, is synthesized from ceramide and UDP-Glc on the cytosolic surface of the Golgi apparatus, further efforts will be directed to the characterization of a glucosylceramide-translocator in the Golgi membranes rather than a lactosylceramide-translocator.

Key words: Glycosphingolipid; Lactosylceramide synthase; Golgi; Semi-intact cell

1. Introduction

Biosynthesis and transport of plasma membrane constituents in mammalian cells involves the coordinated action of various organelles along the secretory pathway: endoplasmic reticulum, the individual stacks of the Golgi apparatus, and the plasma membrane.

In addition to glycosylation, trimming and processing of glycoproteins, these organelles cooperate in the biosynthesis of sphingolipids [1,2]. In order to understand the mechanisms underlying sphingolipid synthesis and transport at a molecular level, we need to precisely determine the locations of the individual enzymes involved in sphingomyelin (Sph) and glycosphingolipid biosynthesis.

Glycosphingolipids of the ganglioside type are made up from ceramide by the stepwise addition of individual sugars from their nucleotide activated precursors. There is convincing evidence that the first step in this series of transfer reactions, synthesis of glucosylceramide (GlcCer) by the addition of Glc from UDP-Glc to ceramide (Cer), is localized to the cytosolic face of the Golgi apparatus [3–6]. A glycosphingolipid cannot simply flip through biological membranes, but shows a strictly vectorial orientation to the outer leaflet of the plasma membrane. Thus, a translocator must exist to make part of the GlcCer available for the enzymes that are involved in the biosynthesis of higher glycosphingolipids located in the lumen of the Golgi apparatus, and to help express the remainder GlcCer on the cell surface. This view has recently been challenged by a report that not only GlcCer-synthase, but also LacCer-synthase is located to the cytosolic side of the Golgi membrane [5]. If that were true, a GlcCer translocator must be postulated for the GlcCer found on the cell surface, and in addition, a translocator would be needed in the Golgi membrane to allow access to the lumen for LacCer, a substrate for subsequent ganglioside biosynthesis. In order to solve this problem, we have investigated the site of LacCer synthesis in isolated Golgi membranes and in semi-intact cells. To this end we have developed assay systems based on those that had recently allowed us to allocate sphingomyelin biosynthesis to the lumen of the cis Golgi [7,8] and GlcCer synthesis to the cytosolic surface of various subsites of the Golgi [6]. In this paper evidence is presented that LacCer is synthesized in the lumen of the Golgi apparatus.

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This paper is dedicated to Prof. Dr. H. Schimassek on the occasion of his 70th birthday.

Abbreviations: GlcCer, glucosylceramide; LacCer, lactosylceramide; Sph, sphingomyelin; t-Cer, (truncated) C8,C8-ceramide; t-GlcCer, (truncated) C8,C8-glucosylceramide; t-LacCer, (truncated) C8,C8-lactosylceramide; t-Sph, (truncated) C8,C8-sphingomyelin; MDCK-cells, Madin Darby canine kidney cells; CHO cell, Chinese hamster ovary cells; SIC, semi-intact cells.

2. Materials and methods

2.1. Isolation of Golgi membranes

Intact Golgi membranes from livers of fasted rats (Wistar male, 180 g) were isolated according to [9]. In order to assess the degree of enrichment, the following enzyme activities were determined: esterase

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as a marker for endoplasmic reticulum [10,11], (protein)-galactosyltransferase as a marker for Golgi organelles [12] according to [13], and sphingomyelin as a marker for Golgi [7] according to [6]. Golgi membranes in the isolated fraction are enriched by a factor of more than 50 compared to a post nuclear supernatant. This is in good agreement with the enrichment factors described in the literature [6,9,14]. The resulting fraction is shown to be intact by electron microscopy revealing a stacked appearance of the organelles [15,16], and by proteolytic digestion experiments in which luminal enzyme activities like sphingomyelin synthase and Galactosyl transferase are protected against proteolytic deactivation [6].

2.2. Determination of lactosylceramide and glucosylceramide synthesis

A standard assay to follow t-GlcCer- and t-LacCer synthesis in Golgi membranes similar to that described by Jeckel et al. [6] contained in a total volume of 10 μl 5–20 μg intact Golgi membranes 20 μM [^{3}H]t-Cer (specific activity 0.21 μCi/nmol), prepared according to [8], 50 mM NaCl, 5 mM UDP-Glc and 0.26 mM castanospermin, in 250 mM sucrose/10 mM Tris-maleate, pH 7.4, and for t-Lac-Cer-synthesis 20 μM [^{3}H]t-GlcCer (specific activity 0.21 μCi/nmol), prepared according to [8], 10 mM $MgCl_2$, 50 mM NaCl, 0.26 mM castanospermin, 5mM UDP-Gal in 250 mM sucrose/10 mM Tris-maleate, pH 7.4. These mixtures were incubated at 37°C for 60 min under shaking. The reaction was stopped by the addition of an equal volume of i-propanol, the samples centrifuged (10,000 × *g*, 5 min) and an aliquot of the supernatants analyzed by TLC on Whatman silica gel plates LK 6 in chloroform/methanol/0.22% $CaCl_2$ in water = 65:35:8. The chromatograms were evaluated by an automatic TLC-2D-analyzer (digital autoradiograph, Berthold, Wildbad, Germany).

2.3. Treatment of Golgi with pronase E

Pronase E treatment of membranes was as desribed by Jeckel et al. [6], with the following modifications: 250 μg membrane protein in 10 mM Tris-maleate, pH 7.4 containing 50 mM NaCl and 250 mM sucrose was incubated with the protease (protein/protease = 5:1), in a total volume of 60 μl at 37°C for various time intervals. After incubation the samples were diluted threefold with ice-cold buffer, loaded on a cushion of 1 ml 10 mM Tris-maleate, pH 7.4, containing 500 mM sucrose and 2 mg BSA/ml, and the membranes were pelleted by centrifugation (30 min at 170,000 × *g* and 4°C). The pellets were resuspended in 250 mM sucrose in 10 mM Tris-maleate, pH 7.4, and aliquots used for t-SPH- and t-GlcCer- as well as t-LacCer-synthesis.

2.4. Preparation of semi-intact cells

Semi-intact cells were prepared exactly as described by Beckers et al. [17] using the swelling technique. In short, after washing a nonconfluent cell culture with 50 mM HEPES, 90 mM KCl, pH 7.4, cells were swollen with a hypotonic buffer (10 mM HEPES, 15 mM KCl, pH 7.4). After 18 min, the hypotonic buffer was replaced by 3 ml of 50 mM HEPES, 90 mM KCl, pH 7.4, and cells were scraped immediately using a rubber policeman. The cells were washed once with 3 ml of 50 mM HEPES, 90 mM KCl, pH 7.4. The washed cells were resuspended in 0.25 ml of 50 mM HEPES, 90 mM KCl, pH 7.4/10-cm dish and used within 1 h. For this study, the mutant CHO cell line 15B was used because the most effective and reproducible permeabilization was obtained with these cells. We routinely found that 90% of the cells became accessible to Trypan blue.

2.5. Biosynthesis and secretion of truncated [^{3}H]glycosphingolipid analogs in permeabilized cells

Incubation of semi-intact cells at various temperatures (20°C and 37°C) was carried out as described in [18]. A typical incubation contained the following components: 10 μl of semi-intact cells, (10^5 cells) 2 mM $MgCl_2$, 1.3 mM ATP, 6.6 mM creatine phosphate, 0.07 U of rabbit muscle creatine phosphokinase, 0.53 mM UDP-Gal and 3.3 μM [^{3}H]t-GlcCer (specific activity 0.21 μCi/nmol). The total volume was adjusted to 15 μl with 50 mM HEPES, 90 mM KCl, pH 7.4. The ATP regenerating system according to [19] was freshly prepared by mixing 10 μl of creatine phosphokinase (2,000 U/ml), 100 μl of 200 mM creatine phosphate, and 100 μl of a mixture containing 40 mM ATP and 50 mM $MgCl_2$ (neutralized with KOH).

The vials containing the mixed components were kept at 0°C, and the incubation was started by transferring the tubes to a waterbath at 37°C. At the times indicated, the incubations were stopped by centrifugation for 2 min in a cooled (2°C) Microfuge at 10,000 × *g* to separate the medium from the cells. The cell pellets were extracted with 15 μl 50% i-propanol in water and centrifuged for 2 min in a Microfuge. Aliquots (5 μl) of the media and of the cell extracts were analyzed by TLC on Whatman silica gel plates LK6 in chloroform/methanol/0.22% $CaCl_2$ in water = 65:35:8. The chromatograms were evaluated by an automatic TLC-2D-analyzer (digital autoradiograph, Berthold, Wildbad, Germany).

2.6. Lactosylceramide synthesis by the mutant CHO clone 13 cells

Preparation of semi-intact CHO Clone 13 cells was carried out under the conditions described for CHO 15 B cells. For control experiments (to prove the presence of active LacCer synthase) semi-intact CHO clone 13 cells either contained 2% (w/v) digitonin in the test mix or were pretreated by repeated (three times) freezing in liquid nitrogen and thawing to room temperature in order to permeabilize the Golgi membranes. Effective permeabilisation was assessed by the determination of LacCer synthase activity after additional treatment with pronase E: 100 μg pronase E was added to 4 × 106 permeabilized cells in 50 μl 50 mM HEPES, 90 mM KCl, pH 7.4, and incubated for 15 min at 37°C. Thereafter the cells were separated from the protease by dilution with 200 μl ice-cold 50 mM HEPES, 90 mM KCl, pH 7.4, and centrifugation at 10,000 × *g* for 3 min at 4°C. The cells were resuspended in 50 mM HEPES, 90 mM KCl, pH 7.4, and the enzyme activities determined by addition of the substrates UDP-Gal and [^{3}H]t-Glc-Cer, and an ATP regenerating system. The LacCer synthase activity was fully destroyed under these conditions. Likewise, the activity of the luminal marker sphingomyelin synthase was decreased below 10% of the original activity. As a luminal control, the activity of sphingomyelin synthase assessed in semi-intact CHO clone 13 cells after pronase treatment was fully retained.

3. Results

3.1. LacCer-synthase assay

We have established a simple assay system to determine the activity of LacCer synthase in its natural environment, i.e. in intact membranes that need not be permeabilized with detergents. In brief: a radioactively labelled water-soluble GlcCer analog with a truncated ceramide residue that contains only 8 C atoms in both its sphingosin and its fatty acid chain is added to Golgi membranes or to permeabilized cells, in the presence of UDP-Gal. Reaction products are analyzed by thin layer chromatography and subsequent quantification with a two dimensional radioactivity scanner. In Fig. 1, fluorograms of the products formed from [^{3}H]t-GlcCer by intact Golgi membranes are shown.

Depending on the presence of UDP-Gal an additional spot is observed, marked with an arrow, in lanes 3 and 5, Fig. 1A. This material was isolated from the thin layer plate and subjected to digestion with β-galactosidase. The original spot was completely abolished, and a novel spot appeared that migrates with an authentic t-GlcCer standard, as shown in Fig. 1B, lane 4. From the conversion of t-GlcCer to a substance that migrates more slowly (Fig. 1A, lanes 3 and 5), and from regeneration of t-GlcCer from this substance by digestion with β-galactosidase we conclude that the material marked with an arrow in Fig. 1A represents t-LacCer.

t-GlcCer is also converted into t-Cer by Golgi membranes (upper spot in Fig. 1A, lane 2), and part of this

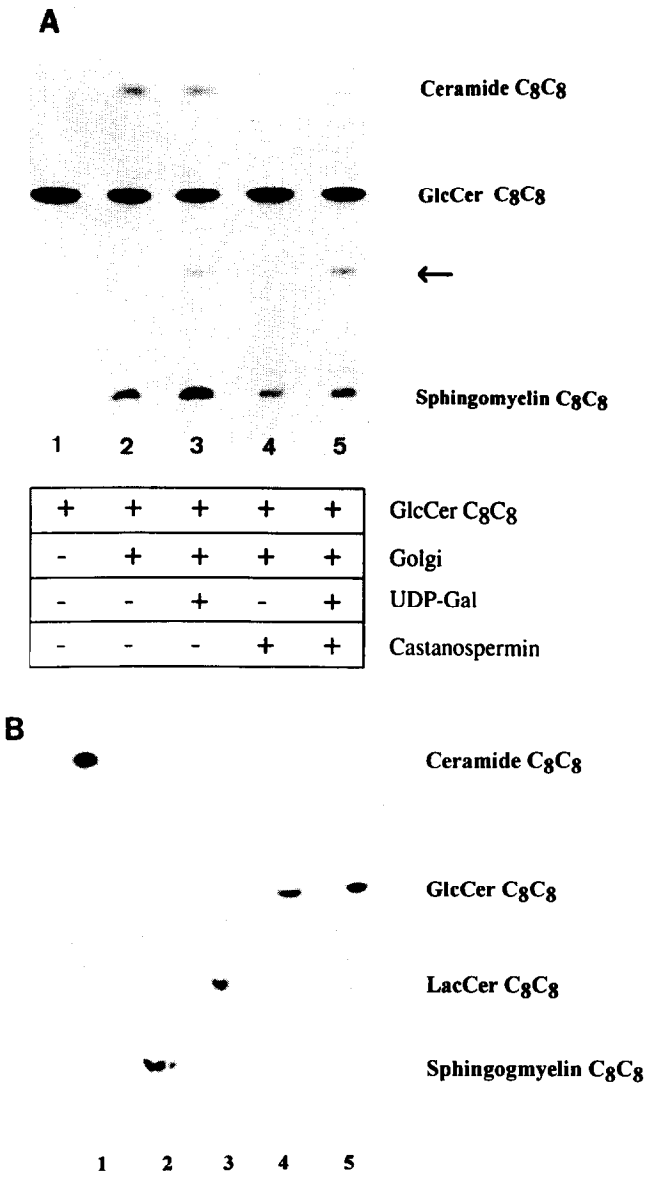

Fig. 1. Analysis of [^{3}H]t-LacCer. (A) Analysis of the products formed from [^{3}H]t-GlcCer by intact Golgi-enriched membranes. Autoradiograph of a thin layer chromatogram. Sample compositions are indicated in the bottom box. Incubations were performed for 60 min at 37°C. For further details see section 2. (B) [^{3}H]t-LacCer is synthesized from [^{3}H]t-GlcCer by intact Gogi enriched membranes in the presence of UDP-Gal. Analysis of the material formed from t-GlcCer and UDP-Gal by digestion with β-galactosidase and subsequent TLC. A corresponding autoradiograph ist shown. Lane 1, [^{3}H]t-Cer; lane 2, [^{3}H]t-SPH; lane 3, eluted material indicated with an arrow in Fig. 1. A, lane 5; lane 4, sample as in lane 3 after digestion with β-galactosidase; lane 5, [^{3}H]t-GlcCer standard.

t-Cer gives rise to t-SPH (lower spot in Fig. 1A, lane 2). Therefore we have aimed to inhibit the hydrolase activity by the addition of castanospermin, an inhibitor of α- and β-glucosidases as well as β-glucocerebrosidase [20]. In the presence of castanospermin, the hydrolysis of t-GlcCer is decreased (compare Fig. 1A, lanes 2 and 4).

This GlcCer hydrolase activity might interfere with an optimal formation of [^{3}H]t-LacCer. Correspondingly, the amount of t-LacCer formed by Golgi membranes from UDP-Gal and t-GlcCer is slightly increased in the presence of the inhibitor (compare Fig. 1A, lanes 3 and 5). Therefore, and because the β-glucocerebrosidase activity varied from Golgi to Golgi preparations, kinetic studies were performed in the presence of castanospermin.

In order to investigate whether this synthesis of t-LacCer by Golgi membranes may be used as an assay system for LacCer-synthase, we have followed the kinetics of t-LacCer formation by intact enriched Golgi membranes. A linear time dependence of t-LacCer synthesis for more than one hour was found under our standard conditions.

3.2. *LacCer-synthase is protected against proteolytic attack on isolated intact golgi membranes*

Sphingomyelin synthase is known to be located to the lumen [8,18,21] and GlcCer-synthase to the cytosolic face of the Golgi [3,4,6]. Thus, these activities can be used as internal controls in experiments to investigate the accessibility to proteases of LacCer-synthase. The three enzyme activities were determined at various times in isolated Golgi membranes in the presence of pronase E. As shown in Fig. 2, the marker activity for the cytosolic side, GlcCer synthase, is drastically decreased after 5 min, and nearly abolished after 10 min. In contrast, LacCer synthesis is not strongly affected after 10 min, and is quite stable even after 15 and 20 min. Sphingomyelin synthase, the marker for the lumen of the Golgi shows a similar stability. This protection against proteolytic attack strongly indicates that LacCer synthase is located

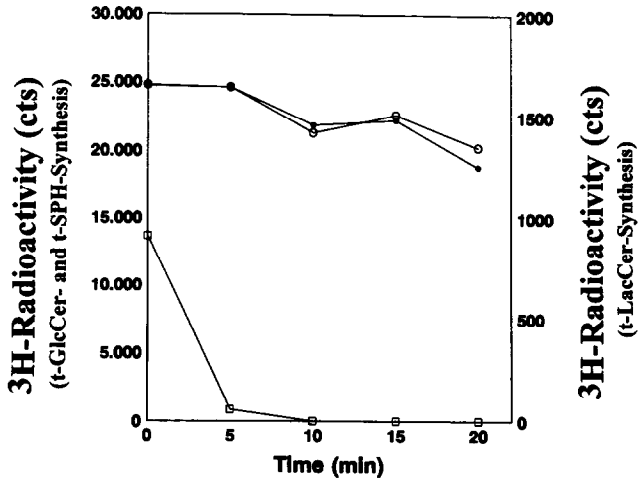

Fig. 2. [^{3}H]t-LacCer formation is not abolished by protease treatment. Intact isolated Golgi-enriched membranes were incubated with Pronase E for the times indicated. Thereafter, membranes were centrifuged through a sucrose layer, and the activity of [^{3}H]t-SPH formation as a luminal marker (●-●), of [^{3}H]t-GlcCer formation as a cytosolic marker (□-□), and of [^{3}H]t-LacCer synthesis (○-○) was determined as described in section 2.

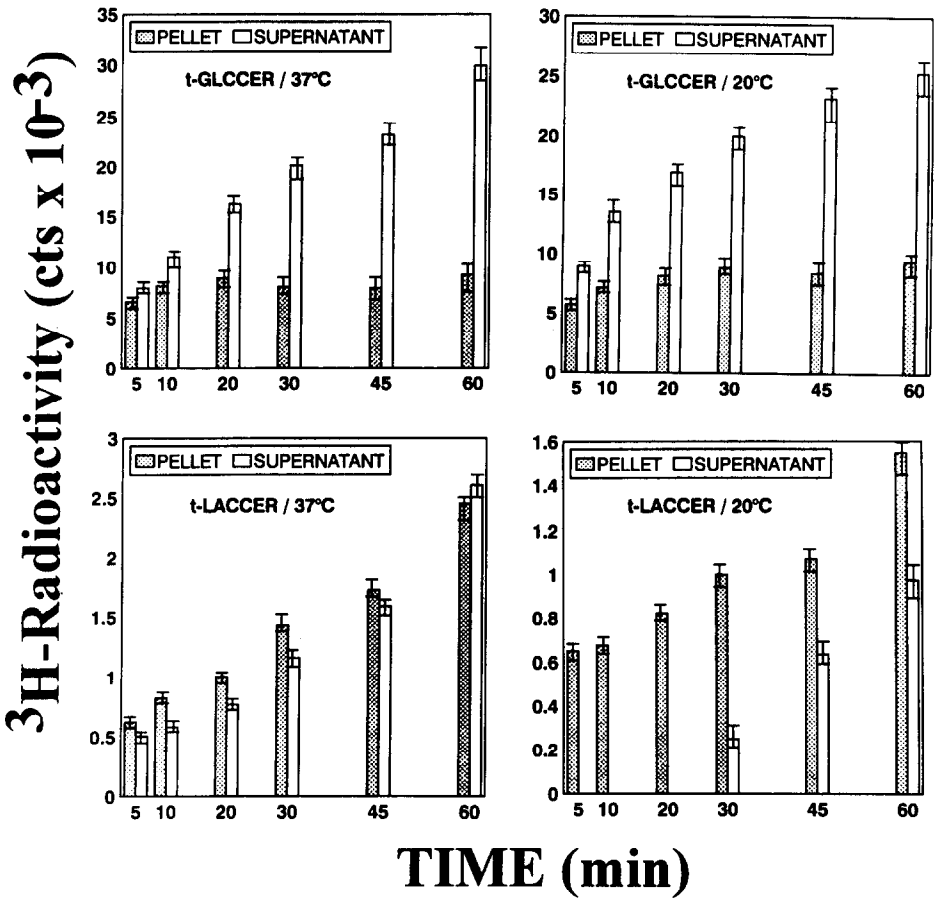

Fig. 3. [^{3}H]t-LacCer is retained in semi-intact cells at lowered temperature. For the synthesis of [^{3}H]t-GlcCer semi-intact CHO cells were incubated with [^{3}H]t-Cer and UDP-Glc (upper panels). For the synthesis of [^{3}H]t-LacCer, semi-intact cells were incubated with [^{3}H]t-GlcCer and UDP-Gal (bottom panels). Left panels, incubation at 37°C; right panels, incubation at 20°C. At the times indicated aliquots were taken, centrifuged, and the amount of [^{3}H]t-GlcCer and [^{3}H]t-LacCer formed was determined by TLC as described in section 2. Hatched columns, [^{3}H]t-glycolipid in the pellets; open columns, [^{3}H]t-glycolipid in the supernatants. The columns represent the means of three independent experiments and the bars represent the lowest and highest deviations.

to the lumen of the Golgi. However, the possibility cannot be fully excluded that a cytosolic enzyme domain is more stable against proteolytic attack than the enzyme used as a marker. Therefore in addition to the enzyme activity we have investigated the location of the product t-LacCer. This can be achieved by use of semi-intact cells as described below.

3.3. Truncated LacCer is retained in semi-intact cells at lowered temperature

Any membrane-impermeable substance of the Golgi lumen (or lipid with luminal orientation) is expected to be delivered to the medium (or to be expressed at the cell surface) by transport vesicles that ultimately fuse with the plasma membrane. Although to date vesicles have been characterized in more detail only with respect to protein transport [22], a variety of conditions are established under which biosynthetic transport vesicle formation or fusion is blocked. This holds for the formation of vesicles from the trans Golgi network, that is strongly decreased at 20°C [23]. We have applied this condition to semi-intact cells to investigate whether t-LacCer is retained in these cells at lowered temperature, like the luminally made truncated sphingomyelin [6,18], or flows out of the perforated cells as does t-GlcCer described in [6]. Semi-intact cells were incubated with [^{3}H]t-GlcCer and UDP-Gal, and total formation of [^{3}H]t-LacCer was followed with time. In Fig. 3, lower left hand panel, the partition of newly synthesized t-LacCer is shown between the semi-intact cells and the surrounding medium at 37°C. Aliquots of the semi-intact cells were centrifuged, and the content of [^{3}H]t-LacCer determined in the cell pellets (hatched columns) and the supernatants (light columns). At all time points, we found about half of the [^{3}H]t-LacCer in the medium. In contrast, at 20°C the [^{3}H]t-LacCer is retained in the semi-intact cells, as depicted in Fig. 3, lower right hand panel. Even after 60 min, the cellular content of the analog predominates. In contrast, the cytosolic marker [^{3}H]t-GlcCer, when freshly synthesized by semi-intact cells, very efficiently diffuses

Fig. 4. [^{3}H]t-LacCer synthesis from [^{3}H]t-GlcCer in UDP-Gal-translocator deficient CHO cells clone 13. Comparison of [^{3}H]t-LacCer synthesis in semi-intact cells from clone 15B and clone 13. For details, see section 2.

into the supernatant at both temperatures (Fig. 3, upper panels). Probably the chance of this water soluble compound to contact a GlcCer translocator is greatly diminished due to the dilution of the cytosolic space with the medium surrounding the semi-intact cells [6].

3.4. LacCer-synthesis is abolished in UDP-Gal-translocator deficient mutant CHO clone 13 cells

If LacCer synthesis takes place in the lumen of the Golgi, it should be dependent on translocation of the substrate UDP-Gal into the Golgi. The CHO mutant cell line clone 13 is known to be deficient in the UDP-Gal-translocator [24] and as a consequence cellular lipids are depleted of galactose [25]. We prepared semi-intact cells from this mutant and investigated its capacity to synthesize the [^{3}H]t-LacCer analog from [^{3}H]t-GlcCer and UDP-Gal. The result is shown in Fig. 4. In contrast to semi-intact control cells (CHO clone 15B) that are functional in UDP-Gal transport, no t-LacCer synthesis could be observed in the semi-intact clone 13 cells. If this lack of activity is due to a luminal orientation of the enzyme and thus to a lack of substrate, perforation of the Golgi should reestablish [^{3}H]t-LacCer synthesis. Clearly, LacCer synthesizing activity is established in the mutant Golgi, either by treatment with digitonin [26] or by disintegration of the membranes by repeated freezing and thawing. This is in accordance with the results by Briles et al. [25], who found that LacCer synthase activity is not affected in CHO clone 13 cells.

4. Discussion

The three different kinds of experiments described here, together clearly indicate that lactosylceramid synthase is located to the lumen of the Golgi. This finding is in conflict with a previous report [5] that describes a cytosolic orientation of LacCer-synthase based on the following findings: (i) no differences in the activities of GlcCer-synthase and LacCer-synthase were observed when the enzymes were either tested in Golgi preparations in the presence of detergent, or when the lipid-substrates were added packed in liposomes to 'intact' Golgi membranes. This does not exclude, however, that GlcCer is translocated from the liposomes to the lumen of the Golgi-apparatus via a tentative GlcCer-translocator, and subsequently is converted into LacCer; (ii) incubation of Golgi-membranes with either pronase E or with trypsin lead to a complete loss of all enzyme activities tested in these preparations. This result is surprising because several authors used much higher protease concentrations in order to specifically destroy cytosolically oriented GlcCer synthase, at the same time leaving luminal activities of the Golgi intact [3,4,6,21]. Furthermore, the activity of sphingomyelin synthase, an established marker for the Golgi lumen, was not assayed as an internal control in these proteolytic probing experiments; (iii) Sepharose-linked ceramide and glucosylceramide were used as substrates for glycolipid synthesis with 'intact' Golgi preparations. The authors observed not only glucosylation of Sepharose-ceramide, but also galactosylation of Sepharose-glucosylceramide. At this time we cannot explain that discrepancy, but we cannot exclude that the membrane fractions investigated were damaged by the treatment with the substrate-Sepharose beads.

Our results shown here are in agreement with the observation that in CHO-clone 13 cells [25] and in a polarized MDCK-cell mutant [27] both deficient in the Golgi-UDP-Gal-transporter, the amounts of galactose containing glycosphingolipids on the plasma membrane were strikingly depressed.

Our finding allows the prediction that no LacCer translocation is needed to allow for the subsequent synthesis of higher glycosphingolipids that takes place in the lumen of the Golgi [1]. By contrast, a GlcCer translocator must exist. This translocator may either be present solely in Golgi membranes, or an additional carrier might exist for the expression of GlcCer as a plasma-membrane glycolipid.

In all likelihood, biosynthetic transport of these lipids occurs in the membranes of vesicular carriers [1,2]. Recently we have assessed the half times of transport for the

luminal bulk from the Golgi to the plasma membrane [8]. A striking difference was observed between the mean residence time of t-Sph as a bulk marker for the early Golgi [7] and the mean residence time of t-GlcCer, ≈ 12 and ≈ 4 min, respectively [8].

The shorter residence time of the GlcCer analog may indicate either a shorter way for GlcCer (less cycles of vesicle formation and fusion) from its origin to the plasma membrane, in agreement with preliminary findings that GlcCer is synthesized in the late Golgi (C. Bünning et al. unpublished), or that a GlcCer-translocator exists in the plasma membrane, in addition to the Golgi membranes. In any case assaying the activity of LacCer synthase by adding t-GlcCer and UDP-Gal to isolated intact Golgi membranes, we make use of this translocator activity. In contrast to semipermeable cells, where t-LacCer synthesis from exogenously added t-GlcCer is dependent on the inclusion of an ATP-generating system, no ATP needs to be added to isolated intact organelles. This effect cannot be explained to date. We aim to use the assay system described here for a biochemical characterization of the proposed GlcCer-translocator.

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