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Determination of the intracellular sites and topology of glucosylceramide synthesis in rat liver

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We examined the intracellular site(s) and topology of glucosylceramide (GlcCer) synthesis in subcellular fractions from rat liver, using radioactive and fluorescent ceramide analogues as precursors, and compared these results with those obtained in our recent study of sphingomyelin (SM) synthesis in rat liver [Futerman, Stieger, Hubbard & Pagano (1990) J. Biol. Chem. **265**, 8650–8657]. In contrast with SM synthesis, which occurs principally at the *cis/medial* Golgi apparatus, GlcCer synthesis was more widely distributed, with substantial amounts of synthesis detected in a heavy (*cis/medial*) Golgi-apparatus subfraction, a light smooth-vesicle fraction that is almost devoid of an endoplasmic-reticulum marker enzyme (glucose-6-phosphatase), and a heavy vesicle fraction. Furthermore, no GlcCer synthesis was detected in an enriched plasma-membrane fraction after accounting for contamination by Golgi-apparatus membranes. These results suggest that a significant amount of GlcCer may be synthesized in a pre- or early Golgi-apparatus compartment. Unlike SM synthesis, which occurs at the luminal surface of the Golgi apparatus, GlcCer synthesis appeared to occur at the cytosolic surface of intracellular membranes, since (i) limited proteolytic digestion of intact Golgi-apparatus vesicles almost completely inhibited GlcCer synthesis, and (ii) the extent of UDP-glucose translocation into the Golgi apparatus was insufficient to account for the amount of GlcCer synthesis measured. These findings imply that, after its synthesis, GlcCer must undergo transbilayer movement to the luminal surface to account for the known topology of higher-order glycosphingolipids within the Golgi apparatus and plasma membrane.

INTRODUCTION

Although the biochemical pathways of glycosphingolipid (GSL) synthesis are well established (Kishimoto, 1983), little is known about the regulation of synthesis or the mechanisms of intracellular transport of GSLs (Schwarzmann & Sandhoff, 1990). It is thought that the sequential glycosylation of GSLs by glycolipid glycosyltransferases occurs as the maturing GSL moves vectorially from the endoplasmic reticulum (ER) through the Golgi apparatus (Wattenberg, 1990), but few of the glycosyltransferases have been purified to homogeneity or assigned to specific sites along the ER–Golgi-apparatus pathway (Trinchera & Ghidoni, 1989, 1990; Trinchera *et al.*, 1990). The intracellular site and topology of glucosylceramide (glucocerebroside) (GlcCer) synthesis, the precursor of many complex GSLs, has been studied using subcellular fractions from porcine submaxillary glands (Coste *et al.*, 1985, 1986), in which GlcCer synthesis was reported to occur at the cytosolic surface of the Golgi apparatus, whereas previous studies suggested that GlcCer synthesis occurs in smooth microsomes [reviewed by Kishimoto (1983) and Sasaki (1990)]. In the present study we have investigated the site of GlcCer synthesis in well-characterized subcellular fractions from rat liver. We show that the enzyme responsible for GlcCer synthesis, namely UDP-glucose:ceramide glucosyltransferase (ceramide glucosyltransferase, EC 2.4.1.80) (GlcCer synthase), is found in microsomal subfractions, and is distributed among a heavy-vesicle (HV) fraction, a light-smooth-vesicle (LSV) fraction that is essentially devoid of ER contamination, and a *cis/medial*-Golgi-apparatus fraction. Further, we found that the enzyme is located at the cytosolic surface of these vesicles. The implications of these findings with regard to intracellular GSL traffic, sorting and regulation are discussed.

MATERIALS AND METHODS

Materials

Rats were obtained from Charles River Laboratories (Wilmington, MA, U.S.A.). Ultrapure sucrose was purchased from Schwarz–Mann. UDP-D-[6-^{3}H]galactose (20 Ci/mmol), UDP-D-[U-^{14}C]galactose (303 mCi/mmol) and UDP-D-[U-^{14}C]glucose (313 mCi/mmol) were from Amersham Corp. Trypsin (TRL-3 grade) and chymotrypsin (CDI grade) were from Worthington. *N*-{6-[(7-Nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl}-D-*erythro*-sphingosine, (C_6-NBD-Cer) was from Molecular Probes (Eugene, OR, U.S.A.). Antiserum directed against rat liver Golgi-apparatus mannosidase II (Moremen & Touster, 1985) was a gift from Dr. K. Moremen (Massachusetts Institute of Technology, Cambridge, MA, U.S.A.). Solvents were from Burdick and Jackson Laboratories (Muskegon, MI, U.S.A.), and all other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Radioactive lipids

N-(1-[^{14}C]Hexanoyl)-D-*erythro*-sphingosine ([^{14}C]hexanoyl-Cer) and *N*-(1-[^{14}C]hexanoyl)-D-*erythro*-glucosylsphingosine ([^{14}C]hexanoyl-GlcCer) were prepared by *N*-acylation of either sphingosine or glucosylsphingosine respectively using the *N*-

Abbreviations used: Cer, ceramide; ER, endoplasmic reticulum; GlcCer, glucosylceramide; GSL, glycosphingolipid; [^{14}C]hexanoyl-Cer, *N*-(1-[^{14}C]hexanoyl)-D-*erythro*-sphingosine; [^{14}C]hexanoyl-GlcCer, *N*-(1-[^{14}C]hexanoyl)-D-*erythro*-glucosylsphingosine; [^{14}C]hexanoyl-SM, *N*-(1-[^{14}C]hexanoyl)-D-*erythro*-sphingosylphosphocholine; HV, heavy vesicles; LSV, light smooth vesicles; C_6-NBD-Cer, *N*-{6-[(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl}-D-*erythro*-sphingosine; PM, plasma membrane; RM, rough microsomes; SM, sphingomyelin.

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hydroxysuccinimide ester of 1-[^{14}C]hexanoic acid (Schwarzmann & Sandhoff, 1987).

Subcellular fractionation

An intact Golgi-apparatus fraction (Bergeron *et al.*, 1982), a plasma-membrane (PM) fraction (Hubbard *et al.*, 1983) and Golgi-apparatus (microsomal) subfractions (Ehrenreich *et al.*, 1973) were prepared as described by Futerman *et al.* (1990), with some modifications. For the intact Golgi-apparatus isolation procedure, the rotating Potter–Elvehjem homogenizer was used at 2700 rev./min. Five fractions were collected after centrifugation: fraction a, band at the air/0.2 M-sucrose interface; fraction b, intact Golgi apparatus; fraction c, remaining gradient after removal of band b; fraction d, residual load zone; fraction e, pellet (see Table 1 below).

For the Golgi-apparatus (microsomal) subfraction isolation procedure, in addition to collecting enriched light (G_L; fraction 1), intermediate (G_I; fraction 2), and heavy (G_H; fraction 3) Golgi-apparatus subfractions, microsomal fractions were obtained from the 1.0/1.15 M-sucrose interface (fraction 4), the residual load zone (1.15 M-sucrose; fraction 5) and the pellet (fraction 6) (see Table 2 below).

Measurement of GlcCer synthesis and glucosylceramidase activity

Defatted BSA–[^{14}C]hexanoyl lipid and defatted BSA–C_6-NBD-Cer complexes were prepared and stored as described by Futerman *et al.* (1990). GlcCer synthesis was assayed by incubating aliquots of liver fractions with a BSA–Cer complex and UDP-Glc (see the Results section) and analysing the amount of GlcCer produced. Glucosylceramidase activity was assayed by incubating liver fractions with BSA–[^{14}C]hexanoyl GlcCer and analysing the amount of [^{14}C]hexanoyl-Cer produced. Lipids were separated by t.l.c. and recovered from the plates by scraping, and radioactivities were determined by liquid-scintillation counting in a Packard Minaxi (Tri-Carb 4000; Packard Instrument Co., Downers Grove, IL, U.S.A.) liquid-scintillation counter using Beckman Ready Safe scintillant (Beckman Instruments, Fullerton, CA, U.S.A.). Background radioactivity was measured by incubating [^{14}C]hexanoyl-Cer in 25 mM-KCl/50 mM-Tris, pH 7.4 (TK buffer) at 37 °C in the absence of any liver fractions, scraping and counting the area corresponding to [^{14}C]hexanoyl-GlcCer, and subtracting this from the radioactivity in [^{14}C]hexanoyl-GlcCer in the corresponding experimental lane (Futerman *et al.*, 1990). Fluorescent lipid products were quantified after t.l.c., scraping, and extraction (Bligh & Dyer, 1959) by measurement of relative fluorescence intensities using an SLM Aminco 8000C spectrophotofluorimeter.

Proteinase treatment of liver fractions

An aliquot of fractions b or d from the intact Golgi-apparatus isolation procedure was incubated with trypsin or chymotrypsin (liver protein/proteinase ratio 2:1, w/w) at 30 °C for 30 min. The trypsin digestion was stopped by addition of soybean trypsin inhibitor (soybean-trypsin-inhibitor/trypsin ratio 2:1, w/w) before assay of GlcCer synthesis. In control incubations, soybean trypsin inhibitor was added alone. The chymotrypsin reaction was not stopped before assay of GlcCer synthesis.

Translocation of [^{14}C]UDP-Glc and [^{14}C]UDP-Gal

Sugar-nucleotide translocation into Golgi-apparatus vesicles was measured by a centrifugation method (Perez & Hirschberg, 1985), with some modifications. An aliquot of the intact Golgi-apparatus fraction was diluted in TK buffer containing 5 mM-$MnCl_2$ to give a final sucrose concentration of 0.25 M. Samples (1 ml each) (containing 50–70 μg of protein) were incubated with either 0.125 μCi of [^{14}C]UDP-Glc or [^{14}C]UDP-Gal and increasing amounts of unlabelled UDP-Glc or UDP-Gal respectively. To prevent degradation of the sugar nucleotides by nucleotide pyrophosphatase (Spik *et al.*, 1979), either 1 mM-ATP or 1 mM-NADH was included in the incubation buffer (a 2-fold increase in translocation of either [^{14}C]UDP-Glc or [^{14}C]UDP-Gal was observed in the presence of either ATP or NADH; results not shown). After 15 min at 37 °C, the samples were cooled on ice and centrifuged (Beckman TL-100 centrifuge; 263000 $g_{av.}$; 10 min; 4 °C) and the supernatant was discarded. The pellet was gently washed twice with 1 ml of cold 0.25 M-sucrose/25 mM-KCl/50 mM-Tris, pH 7.4 (STK buffer), resuspended in 500 μl of TK buffer containing Triton X-100 (1 %, v/v), and radioactivity measured by scintillation counting (see above).

Miscellaneous procedures

Protein concentrations, and galactosyltransferase, alkaline phosphodiesterase and glucose-6-phosphatase activities were measured as described by Futerman *et al.* (1990). β-*N*-Acetylglucosaminidase activity was determined by the method of Scalera *et al.* (1980).

For electron microscopy, samples were fixed in glutaraldehyde (2 %, v/v)/formaldehyde (0.8 %, v/v)/0.1 M-sodium cacodylate, pH 7.4, before centrifugation (Futerman *et al.*, 1990). Representative thin sections were then prepared from the middle region of the pellet for each membrane fraction.

RESULTS

[^{14}C]Hexanoyl-GlcCer synthesis in liver homogenates

We have recently shown that [^{14}C]hexanoyl-Cer spontaneously transfers between BSA–lipid complexes and rat liver membranes, with subsequent metabolism to *N*-(1-[^{14}C]hexanoyl)-D-*erythro*-sphingomyelin ([^{14}C]hexanoyl-SM) (Futerman *et al.*, 1990). In the present study we demonstrate that incubation of rat liver membranes with [^{14}C]hexanoyl-Cer under similar conditions, but with the addition of UDP-Glc, results in the formation of [^{14}C]hexanoyl-GlcCer as well as [^{14}C]hexanoyl-SM.

In rat liver homogenates and subcellular fractions, [^{14}C]hexanoyl-GlcCer was formed at concentrations as low as 100 μM-UDP-Glc (results not shown). However, all subsequent incubations were performed at saturating concentrations of

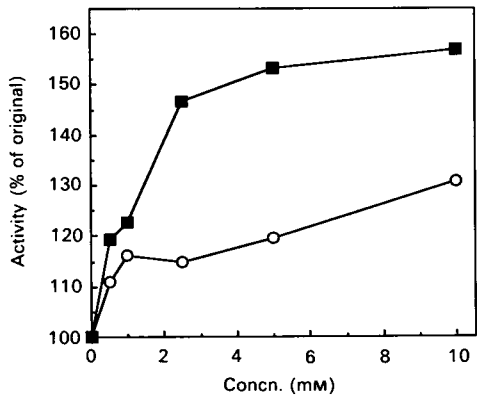

Fig. 1. Effects of Mn^{2+} and Mg^{2+} on [^{14}C]hexanoyl-GlcCer synthesis in a liver homogenate

A rat liver homogenate (500 μg of protein) was incubated with 5 nmol of [^{14}C]hexanoyl-Cer and 5 mM-UDP-Glc in a final volume of 500 μl of TK buffer in the presence of $MnCl_2$ (■) or $MgCl_2$ (○). After 20 min at 37 °C, the reaction was stopped and the synthesis of [^{14}C]hexanoyl-GlcCer determined and expressed as a percentage of the original activity in the homogenate.

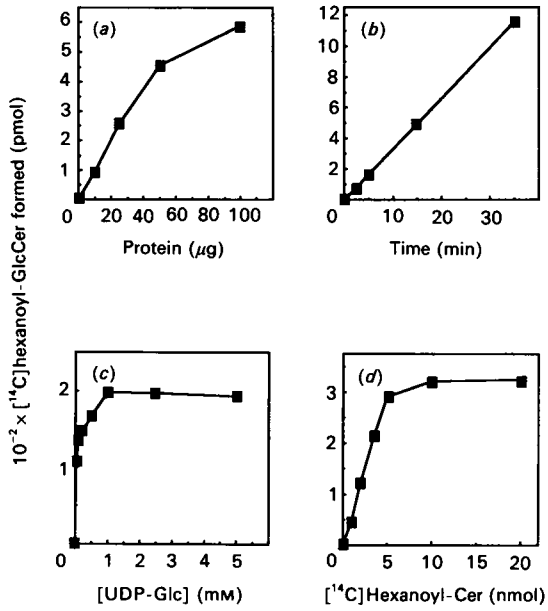

Fig. 2. Analysis of [^{14}C]hexanoyl-GlcCer synthesis in an intact Golgi-apparatus fraction

All assays were performed at 37 °C in 500 μl of TK buffer containing 5 mM-$MnCl_2$. (*a*) Increasing amounts of protein of the Golgi-apparatus fraction were incubated with 5 nmol of [^{14}C]hexanoyl-Cer and 5 mM-UDP-Glc for 15 min. (*b*) Protein (50 μg) was incubated with 5 nmol of [^{14}C]hexanoyl-Cer and 5 mM-UDP-Glc for various times. (*c*) Protein (50 μg) was incubated with 5 nmol of [^{14}C]hexanoyl-Cer for 15 min with increasing amounts of UDP-Glc. (*d*) Protein (50 μg) was incubated with increasing amounts of [^{14}C]hexanoyl-Cer and 5 mM-UDP-Glc for 15 min.

UDP-Glc (5 mM) (see, for example, the effect of increasing UDP-Glc concentrations on [^{14}C]hexanoyl GlcCer synthesis in an intact Golgi apparatus fraction; Fig. 2*c* below), to minimize the effects of nucleotide pyrophosphatase activity (Spik *et al.*, 1979), which degrades UDP-Glc and consequently diminishes GlcCer synthesis at low concentrations (50 μM) of UDP-Glc (Coste *et al.*, 1985, 1986). Inhibitors of nucleotide pyrophosphatase, such as 2-dimercaptopropanol (5 mM) (Faltynek *et al.*, 1981) or NADH (1 mM) (Coste *et al.*, 1985; Shukla & Radin, 1990), had no effect on [^{14}C]hexanoyl-GlcCer synthesis when 5 mM-UDP-Glc was used (results not shown), and therefore were not included in incubation buffers.

[^{14}C]Hexanoyl-GlcCer synthesis was enhanced by $MnCl_2$ (Fig. 1), $MgCl_2$ (Fig. 1), and $CaCl_2$ (results not shown), but was slightly inhibited by EDTA (0.5 mM) (results not shown). Subsequent assays of [^{14}C]hexanoyl-GlcCer synthesis in subcellular fractions of rat liver were therefore performed in the presence of 5 mM-$MnCl_2$.

[^{14}C]Hexanoyl-GlcCer synthesis in fractions obtained by the intact Golgi-apparatus isolation procedure

The distribution of GlcCer synthase in rat liver was examined by measuring [^{14}C]hexanoyl-GlcCer synthesis in fractions obtained by the intact Golgi-apparatus isolation procedure (Bergeron *et al.*, 1982). Standard incubation conditions were chosen for the intact Golgi-apparatus fraction (50 μg of protein; 15 min at 37 °C; 5 mM-UDP-Glc; 5 nmol of [^{14}C]hexanoyl-Cer; Fig. 2) and for the homogenate (250 μg of protein; 30 min at 37 °C; 5 mM-UDP-Glc; 10 nmol of [^{14}C]hexanoyl-Cer; results not shown) by varying protein concentration, time, and substrate concentration. Under these conditions, the rate of formation of [^{14}C]hexanoyl-GlcCer was linear with respect to time and protein concentration and was not limited by the availability of

Table 1. Percentage distribution of protein and enzyme activities in fractions obtained by the intact Golgi-apparatus isolation procedure

GlcCer and SM synthase were assayed at 37 °C in a final volume of 500 μl of TK buffer by incubating fractions a (2–8 μg of protein) or b (50 μg of protein) with 5 nmol of [^{14}C]hexanoyl-Cer and 5 mM-UDP-Glc for 15 min, and by incubating fractions c, d, e or the homogenate (250 μg of protein) with 10 nmol of [^{14}C]hexanoyl-Cer and 5 mM-UDP-Glc for 30 min. Glucosylceramidase activity was assayed by incubating with 2.5 or 5 nmol of [^{14}C]hexanoyl-GlcCer for either 15 or 30 min at 37 °C in a final volume of 500 μl of TK buffer. GlcCer synthase, SM synthase and glucosylceramidase were assayed in the presence of 5 mM-$MnCl_2$. Results are means ± S.D., with the number of measurements (*n*) shown. The organelle in which most of each enzyme activity is found is also noted.

Fraction	Protein ($n = 8$)	GlcCer synthase ($n = 8$)	SM synthase* (*cis/medial*-Golgi) ($n = 6$)	Galactosyltransferase† (*trans*-Golgi) ($n = 5$)	Glucose-6-phosphatase (ER) ($n = 3$)	β-*N*-Acetylglucosaminidase (lysosomes) ($n = 3$)	Glucosylceramidase ($n = 3$)
a‡	0.1±0.0	0.5±0.4	0.4±0.8	1.6±0.7	0.0±0.0	0.2±0.05	0.6±0.2
b	1.1±0.3	42.3±7.0	74.7±5.8	64.6±5.0	0.6±0.2	1.1±0.6	2.9±0.9
c	7.2±1.3	7.5±1.8	3.8±1.1	6.3±1.9	3.3±0.8	2.2±0.2	2.6±1.2
d	33.4±1.7	28.5±8.4	12.9±6.4	13.8±3.5	23.1±2.1	12.8±0.1	19.4±6.2
e	58.2±8.7	21.2±3.0	8.1±1.3	14.0±8.8	73.0±3.4	83.7±0.7	74.5±5.2

* Previously (Futerman *et al.*, 1990) approx. 55% of recovered SM synthase was obtained in fraction b. The higher recovery of SM synthase in fraction b in the current experiments may be due to both increasing the yield of this fraction (see footnote †), and to the fact that SM synthase was measured under conditions optimized for the assay of GlcCer synthesis (see above), not SM synthesis (Futerman *et al.*, 1990).

† Previously (Futerman *et al.*, 1990) approx. 56% of recovered galactosyltransferase activity was obtained in fraction b. The higher recovery of galactosyltransferase in our present experiments was due to slightly increasing the volume and consequently the yield of fraction b. This fraction was still virtually devoid of marker enzymes for other subcellular compartments, indicating little cross-contamination by other membranes. Furthermore, by immunoblotting (Futerman *et al.*, 1990) the majority of mannosidase II, a *cis/medial*-Golgi-apparatus marker (Dunphy & Rothman, 1983), was found in fraction b and was virtually undetectable in the other fractions (results not shown), indicating that the intact Golgi-apparatus fraction contained *cis/medial*- as well as *trans*-Golgi-apparatus elements.

‡ The recovery of protein and enzyme activities in the gradient is given by the sum of the values obtained for fractions a–e (total recovered activity) divided by the amount measured for the homogenate × 100. The percentage recoveries were: protein, 95.7±8.7%; GlcCer synthase, 83.4±7.5%; SM synthase, 145.7±24.8; galactosyltransferase, 96.6±8.8%; glucose-6-phosphatase, 86.9±2.2%; β-*N*-acetylglucosaminidase, 98.0±4.4%; glucosylceramidase, 94.1±2.7%. The distribution of each parameter for a given fraction was calculated as the ratio of the activity in that fraction divided by the total recovered activity × 100.

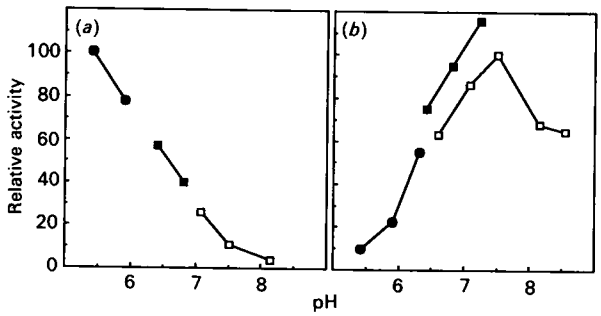

Fig. 3. Effect of pH on [^{14}C]hexanoyl-GlcCer synthesis and degradation

A rat liver homogenate (500 μg of protein) was incubated with (*a*) 2 nmol of [^{14}C]hexanoyl-GlcCer for 45 min at 37 °C or (*b*) 2 or 5 nmol of [^{14}C]hexanoyl-Cer and 5 mM-UDP-Glc. All reactions were performed in a final volume of 500 μl containing 5 mM-$MnCl_2$ and 25 mM-KCl in 50 mM-Mes (●), 50 mM-Mops (■) or 50 mM-Tris (□) buffer at the indicated pH values; the pH of each buffer was measured at 37 °C. Results are means for two independent experiments. The specific activities of GlcCer synthase (pmol of [^{14}C]hexanoyl-GlcCer formed/min per mg of protein) and glucosylceramidase (pmol of [^{14}C]hexanoyl-Cer formed/min per mg of protein) at pH 7.4 in the homogenate were 13.05±4.1 (Table 3; n = 13) and 23.0±0.5 (n = 3) respectively.

substrates. The standard incubation conditions chosen for the homogenate were used to measure [^{14}C]hexanoyl-GlcCer synthesis in fractions c, d and e.

Most (65%) of the recovered galactosyltransferase activity (a *trans*-Golgi-apparatus marker enzyme) and the recovered SM synthase (75%) (a *cis/medial*-Golgi-apparatus enzyme; Futerman *et al.*, 1990) were in the intact Golgi-apparatus fraction (Table 1), but only 42% of the recovered [^{14}C]hexanoyl-GlcCer synthesis was recovered in this fraction (Table 1), with an enrichment of 32-fold relative to the homogenate (see Table 3 below). Significant amounts of [^{14}C]hexanoyl-GlcCer synthesis were detected in fractions d (28.5%) and e (21%), which contained small amounts of SM synthase and galactosyltransferase, with no enrichment relative to the homogenate. Only 50–60% of the synthesis in fractions d and e could be accounted for by Golgi-apparatus membranes present in these fractions, suggesting that [^{14}C]hexanoyl-GlcCer was synthesized at more than one intracellular location. This is in contrast with [^{14}C]hexanoyl-SM synthesis, which occurs almost exclusively at the Golgi apparatus, where synthesis is enriched by 85–98-fold relative to the homogenate (Futerman *et al.*, 1990).

When liver homogenates were incubated with [^{14}C]hexanoyl-GlcCer, some degradation to [^{14}C]hexanoyl-Cer was observed, presumably due to glucosylceramidase (glucosylcerebrosidase) activity. The distribution of glucosylceramidase activity in fractions obtained by the intact Golgi-apparatus isolation procedure was then examined to determine the intracellular location of this activity. Most of the recovered activity (74.5%) was in fraction e, which also contained most (84%) of the lysosomal marker enzyme β-*N*-acetylglucosaminidase (Table 1). In addition, there was no enrichment of glucosylceramidase in an isolated PM preparation (not shown), suggesting that this activity is located in lysosomes (Weinreb *et al.*, 1968) rather than the PM.

Glucosylceramidase activity was decreased by 90% at pH 7.4 compared with pH 5.5 (Fig. 3*a*), suggesting that this activity is an acid rather than a neutral glucosylceramidase. Further, some of the enzymes that degrade sphingolipids with neutral pH optima are affected by bivalent cations (e.g. neutral sphingomyelinase: Hostetler & Yazaki, 1979; Futerman *et al.*, 1990; neutral ceramidase: Slife *et al.*, 1989). However, EDTA (1 mM), $CaCl_2$ (10 mM), $MnCl_2$ (10 mM), and $MgCl_2$ (5 mM) had no effect on glucosylceramidase activity at pH 7.4 in rat liver homogenates (results not shown).

Attempts were made to minimize acid glucosylceramidase activity by performing incubations at pH 8.0 to eliminate the possibility that some newly synthesized [^{14}C]hexanoyl-GlcCer was degraded to [^{14}C]hexanoyl-Cer, thus reducing the apparent synthesis of [^{14}C]hexanoyl-GlcCer in liver fractions. However, at pH 8.0 there were still small, but significant, amounts of

Table 2. Percentage recoveries and distribution of protein and enzyme activities in microsomal subfractions

GlcCer synthase was assayed by incubating equal amounts (35–50 μg) of protein from fractions 1–4 or 250 μg of protein from fractions 5, 6, the microsomal suspension and the homogenate, with 5 nmol of [^{14}C]hexanoyl-Cer and 5 mM-UDP-Glc for 30 min at 37 °C in 1 ml of 50 mM-Tris, pH 7.4, containing 5 mM-$MnCl_2$. SM synthase was measured by incubating fractions with 5 nmol of [^{14}C]hexanoyl-Cer for 30 min at 37 °C in 1 ml of 50 mM-Tris, pH 7.4, containing 0.5 mM-EDTA (Futerman *et al.*, 1990). Glucosylceramidase was assayed by incubating fractions with 5 nmol of [^{14}C]hexanoyl-GlcCer for 30 min at 37 °C in 1 ml of 50 mM-Tris, pH 7.4, containing 5 mM-$MnCl_2$. Results are means±S.D. for five gradients, except for glucosylceramidase (n = 4). The organelle in which most of each enzyme activity is found is noted in parentheses.

Fraction	Protein	GlcCer synthase	SM synthase* (*cis/medial*-Golgi)	Galactosyl-transferase (*trans*-Golgi)	Glucose 6-phosphatase (ER)	Alkaline phospho-diesterase (PM)	β-*N*-Acetyl glucosaminidase (lysosomes)	Glucosyl-ceramidase
%y† ...	16.2±1.1	30.7±3.5	32.5±3.2	38.9±11.0	24.6±2.8	25.0±2.2	13.3±1.9	23.6±2.2
%r‡ ...	106±4.1	112±5	106±13	159±41	92±25	117±7	106±10	109±7
1–3§	1.4±0.2	15.6±3.2	39.2±5.0	30.4±4.5	0.6±0.3	10.2±1.9	4.5±1.3	3.9±2.3
4 (LSV)	4.1±0.7	32.2±7.3	22.2±4.8	22.8±2.5	4.0±0.9	9.1±1.6	2.8±0.7	5.1±3.0
5 (HV)	44.1±3.4	43.2±4.3	29.4±3.7	35.3±1.6	48.5±5.9	42.1±5.1	24.1±3.7	34.3±6.9
6 (RM)	50.4±3.8	8.9±3.4	9.9±3.3	11.4±2.6	46.8±6.2	38.6±5.1	69.2±2.9	56.6±2.9

* The specific activity of SM synthase in individual fractions 1, 2 and 3 varied in these experiments, but paralleled that of the *cis/medial*-Golgi-apparatus marker mannosidase II measured as described by Futerman *et al.* (1990).

† The yield (%y) of protein and enzyme activities in the microsomal suspension is the percentage of the initial homogenate activity present in the microsomal suspension after correcting for recoveries.

‡ The recovery (%r) of protein and enzyme activities in the gradient prepared from the microsomal suspension is given by the sums of the values obtained for fractions 1–6 (total recovered activity) divided by the amount measured for the microsomal suspension × 100.

§ The distribution of each parameter for a given fraction is calculated as the ratio of the activity in that fraction divided by the total recovered activity (footnote ‡) × 100. The distribution of protein and enzyme activities in fractions 1–3 is the sum of the distributions in individual subfractions 1, 2 and 3.

Table 3. Specific activities of GlcCer synthase in subcellular compartments of rat liver

Compartment			Specific activity (pmol/min per mg of protein) (*n*)
Name	Fraction	Table no.	
Homogenate			13±4 (13)
Intact Golgi	b	1	415±208 (11)
Light Golgi subfraction	1	2	100±58 (5)
Intermediate Golgi subfraction	2	2	235±69 (5)
Heavy Golgi subfraction	3	2	334±67 (5)
LSV	4	2	189±41 (5)
HV	5	2	24±5 (5)
RM	6	2	4±1 (5)

glucosylceramidase activity compared with pH 7.4, and [^{14}C]hexanoyl-GlcCer synthesis was reduced by 30–40% (Fig. 3*b*). Since no specific inhibitor of acid glucosylceramidase is available, the distribution of [^{14}C]hexanoyl-GlcCer synthesis could not be determined in the complete absence of acid glucosylceramidase activity. Thus we are unable to exclude the possibility that [^{14}C]hexanoyl-GlcCer synthesis may be underestimated in fractions that contain acid glucosylceramidase activity.

[^{14}C]Hexanoyl-GlcCer synthesis in microsomal subfractions

To examine further the distribution of GlcCer synthase in subcellular compartments of rat liver, [^{14}C]hexanoyl-GlcCer synthesis was measured in Golgi-apparatus and microsomal subfractions. A microsomal suspension was subfractionated on a discontinuous sucrose gradient (Ehrenreich *et al.*, 1973; Futerman *et al.*, 1990), and the distribution of marker enzymes was examined. About 30% of the recovered galactosyltransferase and 39% of the sphingomyelin (SM) synthase was in the pooled Golgi-apparatus subfractions (fractions 1–3, Table 2), which contained similar amounts of glucose-6-phosphatase (< 1%) and alkaline phosphodiesterase (10%), as previously reported (Bergeron *et al.*, 1982; Futerman *et al.*, 1990). Only 16% of GlcCer synthase was in fractions 1–3, with the highest specific activity in the heavy Golgi-apparatus subfraction (fraction 3, G_H) (Table 3), which has the highest specific activity of the *cis/medial*-Golgi-apparatus marker enzyme mannosidase II, and of SM synthase (Futerman *et al.*, 1990).

The distribution and specific activities of SM and GlcCer synthase also differed significantly in fractions 4 and 5. Of the GlcCer synthase, 32% was in fraction 4, which contained 22% of the recovered SM synthase and 23% of galactosyltransferase. The specific activity of GlcCer synthase in fraction 4 was only 1.7-fold lower than in the heavy-Golgi-apparatus subfraction (Table 3), whereas the specific activity of SM synthase in fraction 4 was 4.7-fold lower than in the heavy-Golgi-apparatus subfraction (results not shown). Fraction 4 had a density of 1.135 g/ml and consisted of a mixture of small smooth vesicular and cisternal elements (Fig. 4*a*) [light smooth vesicle (LSV) fraction], and contained only 4% of the ER marker glucose-6-phosphatase. About 43% of the GlcCer synthase and 29% of the SM synthase were in fraction 5, and the specific activities of both GlcCer (Table 3) and SM synthase (results not shown) in fraction 5 were approx. 8-fold lower than in the LSV fraction. Fraction 5 had a density of 1.145 g/ml and consisted of a mixture of small smooth vesicles and small vesicles studded with ribosomes (Fig. 4*b*; see Bretz *et al.*, 1980) [heavy vesicle (HV)

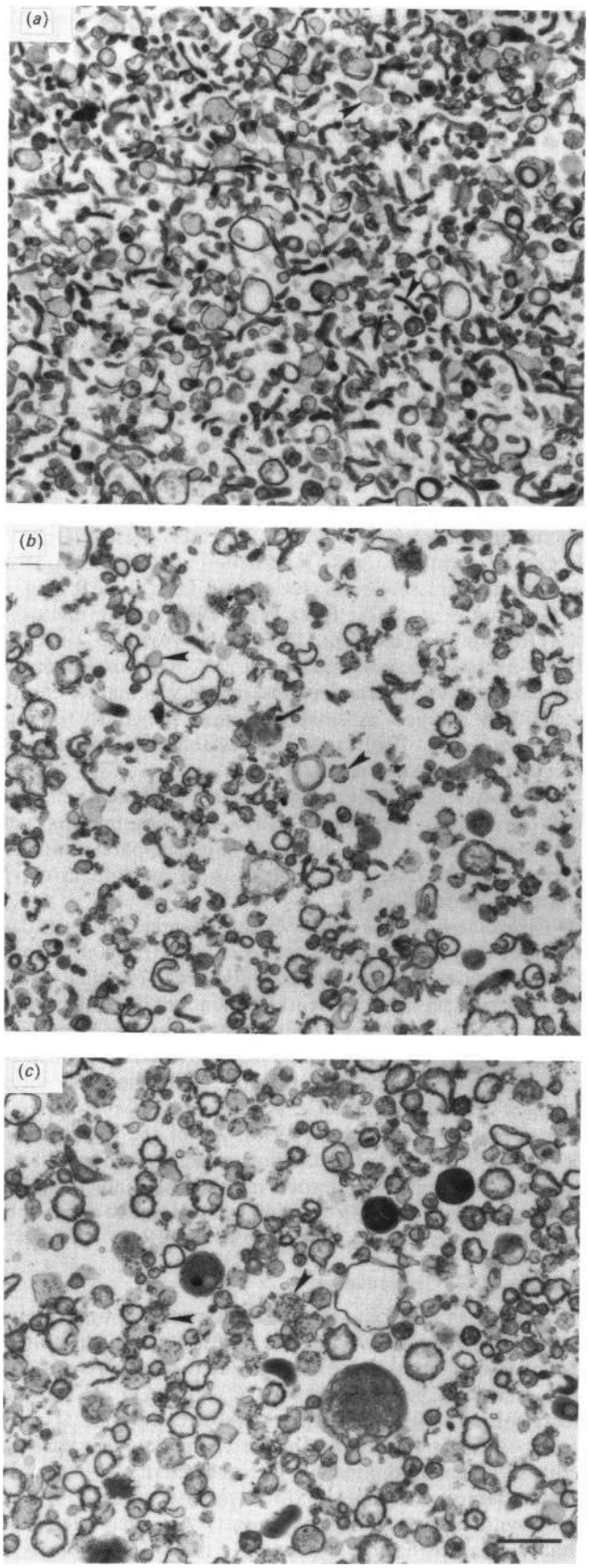

Fig. 4. Electron micrographs of microsomal fractions

(*a*) Fraction 4; arrowheads (◄) indicate cisternal and tubular elements. (*b*) Fraction 5; arrowheads indicate smooth and rough vesicles. (*c*) Fraction 6; arrowheads indicate rough vesicles. The bar represents 500 μm.

Table 4. Effect of proteinase treatment on GlcCer synthesis in the intact Golgi-apparatus fraction

Intact Golgi-apparatus fractions were treated with proteinases as described in the Materials and methods section. [^{14}C]Hexanoyl-GlcCer and C_6-NBD-GlcCer synthesis were measured using standard incubation conditions (Table 1), except that the reaction was allowed to proceed for 30 min. Results are expressed as percentages of the activity of control samples that were not treated with proteinase and are the means for two experiments.

		Percentage activity remaining after proteinase treatment			
		GlcCer synthesis		SM synthesis	
Proteinase	Substrate...	[^{14}C]Hexanoyl-Cer	C_6-NBD-Cer	[^{14}C]Hexanoyl-Cer	C_6-NBD-Cer
Trypsin		12.1	15.6	95.5	118.8
Chymotrypsin		0.6	3.4	107.1	139.4

fraction]. These vesicles are presumably mainly derived from the ER, as 48% of the glucose-6-phosphatase was recovered in this fraction, although significant amounts of a PM marker enzyme, alkaline phosphodiesterase (42%), were also present. Only 9% of GlcCer synthase was in fraction 6, which consisted predominantly of rough vesicles derived from the ER (Fig. 4*c*; see Bergeron *et al.*, 1978; Bretz *et al.*, 1980) [rough microsome (RM) fraction], and contained most of the lysosomes, since 69% of the β-*N*-acetylglucosaminidase and 57% of glucosylceramidase were recovered in this fraction. The recovery of marker enzymes in the RM fraction is similar to that previously obtained (Futerman *et al.*, 1990) using an established RM isolation procedure (Adelman *et al.*, 1973).

We conclude that GlcCer synthesis is distributed among the LSV, heavy Golgi apparatus and HV fractions, and that some GlcCer synthesis in both the heavy Golgi apparatus and HV fractions may be due to contamination by membranes derived from the LSV fraction.

[^{14}C]Hexanoyl-GlcCer synthesis was examined in an isolated PM sheet preparation (Hubbard *et al.*, 1983). Small amounts of [^{14}C]hexanoyl-GlcCer were synthesized (specific activity 111 pmol/min per mg of protein). The amount of synthesis in the PM fraction resulting from contamination by Golgi-apparatus membranes was estimated using the recoveries of protein and marker enzymes in the PM fraction, and using the specific activity of [^{14}C]hexanoyl-GlcCer synthesis in the Golgi-apparatus fraction (see Futerman *et al.*, 1990). All of the [^{14}C]hexanoyl-GlcCer synthesis in the PM fraction could be accounted for by contaminating Golgi-apparatus membranes, indicating that GlcCer synthesis in the HV fraction was not due to residual PM but rather to microsomes derived from the ER.

Topology of GlcCer synthesis

The topology of GlcCer synthase was examined by incubating intact Golgi-apparatus vesicles with either trypsin or chymotrypsin and subsequently assaying the synthesis of GlcCer using either [^{14}C]hexanoyl-Cer or C_6-NBD-Cer as substrate. Treatment with either proteinase almost completely abolished GlcCer synthesis from either of the ceramide (Cer) analogues (Table 4) under conditions in which the activity of the luminal Golgi-apparatus enzymes, SM synthase (Table 4) and galactosyltransferase (results not shown), were unaffected. These results suggested that the catalytic site of GlcCer synthase was located at the cytosolic surface of the Golgi apparatus, although other explanations could not be excluded. Similar results were obtained with fraction d of the intact Golgi-apparatus isolation procedure using [^{14}C]hexanoyl-Cer as substrate (results not shown). We found that GlcCer synthase is an integral membrane protein since, after centrifugation (263000 $g_{av.}$, 10 min), more than 80% of the activity was associated with the high-speed pellet and this value was not significantly altered after treatment with 1 M-NaCl (results not shown). Although some glycosyltransferases can be solubilized by limited proteolytic digestion (reviewed by Paulson & Colley, 1989), our initial attempts to solubilize GlcCer synthase by trypsin or chymotrypsin digestion were unsuccessful.

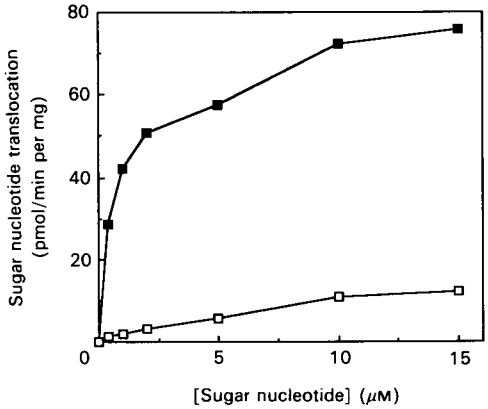

Fig. 5. Translocation of [^{14}C]UDP-Glc and [^{14}C]UDP-Gal into intact Golgi-apparatus vesicles

Measurement of the translocation of sugar nucleotides was performed as described in the Materials and methods section. Samples were incubated with either 0.125 μCi of [^{14}C]UDP-Glc and increasing amounts of UDP-Glc (□) or 0.125 μCi of [^{14}C]UDP-Gal and increasing amounts of UDP-Gal (■). In control experiments, the translocation of [^{14}C]UDP-Glc or [^{14}C]UDP-Gal was decreased 2–4-fold at 4 °C.

The translocation of UDP-Glc into intact Golgi-apparatus vesicles from rat liver was also measured. A small amount of UDP-Glc was translocated into Golgi-apparatus vesicles, with a $V_{max.}$ of 12 pmol/min per mg of protein (Fig. 5). This value is higher than that reported for UDP-Glc translocation into cat liver Golgi vesicles ($V_{max.}$ of 0.15 pmol/min per mg of protein; Persat *et al.*, 1984), into the Golgi apparatus of CHO cells ($V_{max.}$ of 1.2 pmol/min per mg of protein; Deutscher & Hirschberg, 1986) or into rough microsomes from rat liver ($V_{max.}$ of 9.7 pmol/min per mg of protein; Perez & Hirschberg, 1986), but nevertheless substantially lower than the $V_{max.}$ of UDP-Gal translocation into rat liver Golgi-apparatus vesicles ($V_{max.}$ 75 pmol/min per mg of protein, Fig. 5) (see also Yusuf *et al.*, 1983). Thus GlcCer synthesis cannot occur at the luminal surface of the Golgi apparatus, since the specific activity of [^{14}C]hexanoyl-GlcCer synthesis was 415 pmol/min per mg of protein (Table 3).

This rate of GlcCer synthesis would presumably be severely affected by the unavailability of UDP-Glc in the lumen of the Golgi apparatus. Furthermore, UDP-Glc transport was inhibited by only ~ 50% after treatment with trypsin (not shown) under conditions in which [^{14}C]hexanoyl-GlcCer synthesis was completely abolished.

DISCUSSION

In the present study we examined the intracellular sites and topology of GlcCer synthesis in subcellular fractions from rat liver. Since the glucosylation of Cer to GlcCer is the first step in the formation of GSLs, determination of the intracellular sites and topology of GlcCer synthesis is of importance for understanding the regulation of GSL synthesis and transport.

Previous reports using subcellular fractions from porcine submaxillary glands suggested that GlcCer synthase is primarily associated with the Golgi apparatus (Coste *et al.*, 1985, 1986), whereas in the present study we show that GlcCer synthase is not localized primarily in the Golgi apparatus, but is more widely distributed among microsomal subfractions. In the earlier reports, a significant enrichment (20-fold) of the specific activity of GlcCer synthesis in a Golgi-apparatus fraction compared with a postnuclear supernatant was found, but a 6-fold increase in specific activity was also found in a smooth-ER fraction that was not further characterized. No analysis of the distribution of various marker enzymes in the subcellular fractions was provided, so the distribution and recoveries of GlcCer synthesis in subcellular compartments of porcine submaxillary glands were not determined. Other studies have shown that GlcCer synthesis occurs in the microsomal fractions of BHK-21 cells (Suzuki *et al.*, 1984) and of rat brain (reviewed by Kishimoto, 1983), but these fractions contain membranes derived from both the ER and the Golgi apparatus.

In the present study we have shown that significant amounts of GlcCer synthesis in rat liver occurred in an enriched intact Golgi-apparatus fraction (Table 1), with the highest specific activity in a heavy- (*cis/medial*) Golgi-apparatus-subfraction (Table 3). However, significant synthesis also occurred in two microsomal fractions, a light-smooth-vesicle fraction (LSV) and a heavy-vesicle fraction (HV) (Tables 2 and 3). We were unable to characterize the LSV fraction further, but the density of the LSV fraction, and the profile of marker enzymes in this fraction, suggests that the LSV fraction may contain membranes derived from a compartment that is intermediate in density between that of the *cis/medial*-Golgi apparatus and the ER. A compartment of density intermediate between the smooth ER and *cis/medial*-Golgi apparatus has been implicated in a number of cellular functions (see below), but since no marker enzyme is available for this compartment that cross-reacts with rat tissues, we were unable to analyse the LSV fraction, or the extent to which the intact Golgi-apparatus fraction, Golgi-apparatus subfractions and HV fractions were contaminated by membranes derived from the LSV fraction. Consequently we could not determine the precise distribution of GlcCer synthesis among the Golgi apparatus, LSV and HV fractions. Furthermore, no [^{14}C]hexanoyl-GlcCer synthesis could be detected at the PM after accounting for contamination by Golgi-apparatus membranes.

These findings imply that a significant amount of GlcCer synthesis may take place in a pre- or early Golgi-apparatus compartment. This suggestion is consistent with studies of sphingolipid metabolism and transport in cultured cells using (i) a 15 °C temperature block (van Echten & Sandhoff, 1989), in which vesicular transport is arrested between the ER and *cis*-Golgi, and (ii) monensin (Saito *et al.*, 1984; Lipsky & Pagano, 1985; van Echten & Sandhoff, 1989), which disrupts vesicular transport between the *medial*- and *trans*-cisternae of the Golgi apparatus (Griffiths *et al.*, 1983; Quinn *et al.*, 1983). In these studies GlcCer accumulated, implying that it is synthesized early in the ER–Golgi-apparatus pathway. These results, as well as the present ones, strongly suggest that GlcCer synthesis occurs in the smooth ER, a pre-Golgi apparatus compartment, or in an early Golgi-apparatus compartment. This wide distribution of GlcCer synthesis is in marked contrast with SM synthesis in rat liver, which is localized almost exclusively (87%) at the Golgi apparatus, with no detectable synthesis occurring at any other intracellular location, except for small amounts (13%) of synthesis at the PM (Futerman *et al.*, 1990). Determination of the precise intracellular location of GlcCer synthase awaits the isolation of the enzyme, followed by immunolocalization; preliminary steps in this direction have been taken with the solubilization by detergents of GlcCer synthase from porcine submaxillary glands (Durieux *et al.*, 1990).

The possibility that GlcCer is synthesized in a vesicular compartment of density intermediate between the smooth ER and an early Golgi compartment is of particular interest, given recent findings of a 'salvage' or 'intermediate' compartment that may mediate vesicular transport between the ER and the Golgi apparatus (see Groesch *et al.*, 1990). To date, there are no studies on either the lipid composition or the biosynthetic ability of the intermediate compartment. Since vesicle fission, sorting and fusion may depend in part upon the topology and composition of lipids within intracellular vesicles, the synthesis of GlcCer in a compartment of density intermediate between the smooth ER and an early Golgi compartment may provide a means for regulating lipid composition and perhaps the ability of this compartment to mediate vesicular events.

An earlier report (Coste *et al.*, 1986) suggests that GlcCer synthesis occurs at the cytosolic surface of the Golgi apparatus in porcine submaxillary glands. We have confirmed this by demonstrating that proteinases destroy GlcCer synthesis in intact Golgi-apparatus vesicles from rat liver when both radioactive and fluorescent Cer analogues are used as substrates. We have also shown that UDP-Glc translocation into Golgi-apparatus vesicles cannot provide enough UDP-Glc for GlcCer synthesis at the luminal surface of the Golgi apparatus, supporting the conclusion that GlcCer is synthesized at the cytosolic surface of intracellular vesicles. This result is nevertheless surprising, since most GSLs are localized at the external leaflet of the PM (Hakamori, 1981), which is topologically equivalent to the luminal leaflet of the Golgi apparatus where glycolipid glycosyltransferases are believed to be localized (Paulson & Colley, 1989). Thus GlcCer must be translocated across the membranes of the Golgi apparatus to be further glycosylated to form higher-order GSLs. No evidence for GlcCer translocation exists at present, but phospholipid translocases exist in both the ER and PM [reviewed by Pagano (1990) and Zachowski & Devaux (1990)]. The regulation of GlcCer translocation may provide a means for controlling the subsequent steps of GSL synthesis.

The differences in subcellular localization and topology of GlcCer and SM synthesis may be important for the metabolic regulation of the activities of GlcCer and SM synthase. Ultimately, the synthesis of GlcCer and SM may depend upon the transport of Cer from its site of synthesis (presumably the ER) to sites of metabolism.

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