We present a method for solubilizing and purifying UDP-Glc-ceramide glucosyltransferase (EC 2.4.1.80; glucosylceramide synthase (GCS)) from rat liver and present data on its substrate specificity. A Golgi membrane fraction was isolated, washed with N-lauroylsarcosine, and subsequently treated with 3-(3-cholamidopropyl)-dimethylammonio-2-hydroxy-1-propanesulfonate to solubilize the enzyme. GCS activity was monitored throughout purification using UDP-Glc and a fluorescent ceramide analog as substrates. Purification of GCS was achieved via a two-step dye-agarose chromatography procedure using UDP-Glc to elute the enzyme. This resulted in an enrichment >10,000-fold relative to the starting homogenate. The enzyme was further characterized by sedimentation on a glycerol gradient, labeling, and SDS-polyacrylamide gel electrophoresis, which demonstrated that two polypeptides (60–70 kDa) corresponded closely with GCS activity. Purified GCS was found to require exogenous phospholipids for activity, and optimal results were obtained using dioleoyl phosphatidylcholine. Studies of the substrate specificity of the purified enzyme demonstrated that it was stereospecific and dependent on the nature and chain length of the N-acyl-sphingosine or -sphinganine substrate. UDP-Glc was the preferred hexose donor, but TDP-glucose and CDP-glucose were also efficiently used. This study provides a basis for molecular characterization of this key enzyme in glycosphingolipid biosynthesis.

Glycosphingolipids (GSLs) are amphipathic molecules that contain the hydrophobic moiety, ceramide, and a hydrophilic oligosaccharide residue. They are found in the plasma membranes of all eukaryotic cells and play important roles in cell recognition, cell proliferation and differentiation, immune recognition, and signal transduction (for reviews, see Refs. 1–5).

The biochemical pathways for GSL synthesis are well established (reviewed in Refs. 6–9), but not all of the enzymes involved in GSL synthesis have been purified and/or cloned (for review, see Ref. 10). One such enzyme is UDP-Glc-ceramide glucosyltransferase (EC 2.4.1.80; glucosylceramide synthase (GCS)), which catalyzes the formation of glucosylceramide (GlcCer) from ceramide and UDP-Glc. This enzyme is of particular interest for a number of reasons. First, it has been shown that there is a correlation between tumor progression and cell surface GSLs (1, 12). Since many complex acidic and neutral GSLs are derived from GlcCer, regulation of GCS activity could have a profound effect on cell growth activity.

EXPERIMENTAL PROCEDURES

Materials

Male Sprague-Dawley rats (5 weeks old) were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). N-[7-(4-Nitrobenzo-2-oxa-1,3-diazole)-6-aminocapryloyl-o-erythro-sphingosine (C6-NBD-Cer) and N-[5-(5,7-dimethyl-1-oxo-1,3-benzoxadiazol-2-yl)-1-pentanoyloxy-o-erythro-sphingosine (C5-DMB-Cer) were obtained from Molecular Probes. Four stereoisomers of C6-NBD-Cer were synthesized and purified as described (18). Other fluorescent and radioactive ceramide analogs were prepared by N-acylation of sphingosine (or sphinganine) using N-hydroxysuccinimide fatty acids (18, 19), o-threo-PDMP was from Matreya, Inc. (Pleasant Gap, PA). CHAPSO was from Pierce. Green dye-Sepharose CL-2B, hereafter referred to as “dye-agarose,” was prepared from active Green 19 dye (Sigma) and Sepharose CL-2B (Pharmacia Biotech Inc.) as described (20). Phospholipids were from Avanti Polar Lipids, Inc. (Alabaster, AL). Bovine liver catalase, equine heart cytochrome C, rabbit muscle lactate dehydrogenase, and porcine heart malate dehydrogenase were obtained from Calbiochem. 1H] was from DuPont NEN.
GCS activity was assayed as described previously (16), with the following modifications. Enzyme fractions were preincubated in screw cap tubes in 50 mM Hepes (pH 7.4), 25 mM KCl, 5 mM MnCl₂, 5 mM UDP-Glc in a final volume of 0.5 ml for 5 min at 37 °C. The enzyme reaction was initiated by addition of 5 nmol of C₂₀-NBD-Cer-bovine serum albumin complex prepared as described (18, 26). After 15 min at 37 °C with constant stirring, the reaction was stopped by addition of 3 ml of chloroform/methanol (1:2, v/v). Lipids were extracted (27) and separated by thin layer chromatography (26). Individual spots on the TLC plates were identified by comparison with fluorescent standards and quantified by image analysis as described (28). In some cases, the reaction tubes were precipitated with phospholipids (usually 0.1 mM DOPC) by first adding phospholipid dissolved in chloroform to screw cap tubes and evaporating the chloroform under a stream of N₂ for the experiment shown in Fig. 5, the reaction tubes were also precoated with d-threo-PDMP using an ethanolic stock solution of the inhibitor.

**Analytical Methods**

The polypeptide composition of various fractions was assessed by SDS-PAGE (29). In some cases, the enzyme fractions were concentrated by precipitation with trichloroacetic acid in the presence of sodium deoxycholate prior to electrophoresis (30). Polypeptides were visualized using a silver staining kit (Bio-Rad) as described (31). To visualize the polypeptides in samples with very low protein concentrations, samples were preblotted with 0.5–1 ml of 0.25T with the chloramine-T method (32). Radioactive gels were analyzed using a phosphorimager (Molecular Analytical GS-363, Bio-Rad) and by autoradiography. Immunoprecipitation of rat lamin was performed as described (33) using a polyclonal antirat lamin antiserum from Cappel/Organon Teknika (Durham, NC). The Golgi marker enzyme, galactosyltransferase, was measured as described (35). Electron microscopy of Golgi membranes was performed as described (21). Protein concentrations were measured using Coomassie Blue dye reagent (Bio-Rad) with bovine serum albumin as a standard (36).

**RESULTS**

**Purification of GCS**

Preliminary studies showed that GCS in Golgi membranes could be solubilized with CHAPSO (1% w/v) with recovery of ~70% of the activity in the extract (data not shown). We also found that addition of 0.1–1.0 mM DOPC and 0.1 mM UDP-Glc to the solubilized GCS improved its stability at 4 °C (data not shown). Thus, these protective agents were added during some purification steps. Finally, we noted that after chromatographic procedures, GCS had specific phospholipid requirements for optimal activity (see below). Thus, most activity assays of solubilized GCS fractions were performed in the presence of 0.1 mM DOPC.

We used rat liver Golgi membranes as a starting material for the purification of GCS, because the previous studies showed that these membranes were highly enriched in GCS activity (16). The Golgi fractions prepared in the present work were enriched 60–80-fold in galactosyltransferase, a trans-Golgi marker protein, with ~35% recovery of this enzyme. In addition, electron microscopy of the Golgi samples revealed numerous intact stacks comprised of 4 and 5 cisternae (data not shown). Thus, these Golgi preparations were similar in enrichment and morphology to highly purified Golgi fractions prepared by established methods (for review, see Ref. 37).

Golgi membrane fractions were enriched >60-fold in GCS activity compared to crude rat liver homogenate with a yield of >50% (Table I). Since our enzyme assay used a fluorescent ceramide analog, which is also a substrate for sphingomyelin synthase (18, 38, 39), we were also able to determine that the Golgi membranes were enriched 35–40-fold in sphingomyelin synthase activity with a recovery of ~40% (data not shown). This observation is in agreement with the differential localization of GCS and sphingomyelin synthase in the Golgi apparatus (16, 17, 21, 40).

**Detergent Solubilization**—We found that pretreatment of...
The detergent-solubilized enzyme was loaded onto a dye-agarose column (A) in the presence of 1 mM UDP-Glc as described under "Experimental Procedures." After washing with 50 ml of buffer B and 50 ml of 0.15 M KCl in buffer B without UDP-Glc, the column was eluted with 1 mM KCl in the same buffer as indicated by the arrow. The unbound fractions, indicated by the horizontal bar, were pooled, subjected to gel filtration, and loaded onto a second dye-agarose column equilibrated in buffer B without UDP-Glc (B). The column was then washed in the same buffer and sequentially eluted (at arrows) with 0.15 mM KCl (1), 20 mM UDP-Glc plus 20 mM NADH (2), and 1 mM KCl (3) in buffer C. The fractions eluted from the second dye-agarose column, which were very low in protein, aliquots were radiiodinated. When a radiolabeled aliquot of the 0.15 mM KCl-eluted fraction was electrophoresed and visualized by autoradiography, the profile obtained (Fig. 2, lane 5) showed a similar pattern to that of the fractions eluted from the homogenate and Golgi membranes were measured without CHAPSO.

Two-step Dye-Agarose Column Chromatography—We attempted to purify GCS by affinity chromatography using various bound ligands (UDP-hexanolamine, UDP-GlucUA, ceramide, sphingosine, and PDMP), but these efforts were unsuccessful. We next explored dye adsorption chromatography and found that GCS activity could be released from a dye-agarose column by UDP-Glc, while most other dye-binding proteins were elutable only with salt. Thus, we devised a two-step purification procedure. First, CHAPSO-solubilized Golgi membranes were loaded onto the dye-agarose column in the presence of 1 mM UDP-Glc as described under "Experimental Procedures." After washing with 50 ml of buffer B and 50 ml of 0.15 M KCl in buffer B without UDP-Glc, the column was eluted with 1 mM KCl in the same buffer as indicated by the arrow. The unbound fractions, indicated by the horizontal bar, were pooled, subjected to gel filtration, and loaded onto a second dye-agarose column equilibrated in buffer B without UDP-Glc (B). The column was then washed in the same buffer and sequentially eluted (at arrows) with 0.15 mM KCl (1), 20 mM UDP-Glc plus 20 mM NADH (2), and 1 mM KCl (3) in buffer C. The fractions eluted from the second dye-agarose column, which were very low in protein, aliquots were radiiodinated. When a radiolabeled aliquot of the 0.15 mM KCl-eluted fraction was electrophoresed and visualized by autoradiography, the profile obtained (Fig. 2, lane 5) showed a similar pattern to that of the fractions eluted from the homogenate and Golgi membranes were measured without CHAPSO.

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same sample visualized by silver staining in lane 4. Three major bands (45, 60, and 66 kDa) were visible in this fraction (Fig. 2, lane 5). The 45-kDa polypeptide was noticeably diminished in the UDP-Glc-eluted fraction, while the 60- and 66-kDa forms continued to predominate on the gel (Fig. 2, lane 6).

In an attempt to further purify GCS following dye-agarose chromatography, UDP-Glc-eluted GCS, including a radiolabeled aliquot, was centrifuged through a glycerol gradient. Preliminary experiments showed that only ~50% of the starting GCS activity was preserved after a 12-h ultracentrifugation in a glycerol gradient with 0.5 mM UDP-Glc present, preventing a quantitative evaluation of GCS enrichment. Nevertheless, the discrete peak of GCS activity in the glycerol gradient allowed us to assess the apparent relative size of the enzyme under nondenaturing conditions. Based on a standard curve of marker enzymes sedimented in the same tube, we estimated the sedimentation coefficient of GCS as ~4.2 s (Fig. 3A). The peak of GCS activity migrated to position immediately preceding malate dehydrogenase, which has a molecular mass of 70 kDa. Fig. 3B shows the glycerol gradient distribution of the radiolabeled 45-, 60-, and 66-kDa polypeptides present in dye-agarose purified GCS. The intensity of the 45-kDa polypeptide peaked earlier in the glycerol gradient than the peak of GCS activity (Fig. 3, B and C). Most of the radioactivity associated

FIG. 2. SDS-PAGE analysis of GCS at various steps of purification. Protein samples from different steps in the purification of GCS from rat liver Golgi membranes were subjected to SDS-PAGE under reducing conditions using a 10% gel. Following electrophoresis, the gel was either silver stained (lanes 1–4) or 125I-labeled proteins were visualized by autoradiography (lanes 5 and 6). Lane 1, CHAPSO extract; lane 2, unbound fraction from dye-agarose (column I); lane 3, unbound fraction from dye-agarose (column II); lanes 4 and 5, 0.15 M KCl elution from dye-agarose (column II); lane 6, UDP-Glc/NADH elution from dye-agarose (column II). Approximately 5 μg of protein was loaded in each of lanes 1–3 and ~1.5 μg in lane 4. Arrows indicate the positions of molecular weight standards.

FIG. 3. Glycerol gradient sedimentation of purified GCS. A concentrated sample of dye-agarose-purified GCS (250 μl) including an aliquot (~4 × 10⁶ cpm), which had been radiolabeled with 125I, was layered onto the top of a 6–25% glycerol density gradient (5 ml) and centrifuged at 250,000 × g for 12 h as described under “Experimental Procedures.” The gradient was then fractionated into 30 samples, and the fractions were assessed for polypeptide composition, GCS activity, and the positions of marker proteins (cytochrome C, 14 kDa; malate dehydrogenase (MDH), 70 kDa; lactate dehydrogenase (LDH), 140 kDa; and catalase, 250 kDa) as described under “Experimental Procedures.” A, standard curve showing the migration of marker proteins and GCS activity. B, 10% SDS-PAGE of glycerol gradient fractions in which 125I-labeled proteins were visualized by a phosphorimager. AS, applied sample. Numbers beneath the gel correspond to glycerol gradient fractions. Position of molecular mass markers are shown at the right. Arrow at left marks position of the ~60-kDa polypeptide. C, relative GCS activity plotted versus glycerol gradient fraction number. Also shown plotted versus fraction number are the relative intensities of the 45-, 60-, and 66-kDa polypeptides determined by phosphorimager analysis.
with the 66-kDa band was found to be rat albumin by immunoprecipitation; however, this technique did not completely remove the radioactivity in this region of the gel (data not shown). Furthermore, both the 60- and the 66-kDa bands peaked in intensity at fraction 11, which coincided with the peak of GCS activity (Fig. 3, B and C). Thus, although we cannot definitively rule out the possibility that another polypeptide is responsible for GCS activity, our data suggest that the ~60- and/or ~66-kDa polypeptides are the GCS protein.

Characterization of GCS

Purified fractions obtained after the second dye-agarose column were used to examine the enzymatic characteristics and substrate specificity of GCS. GCS activity was previously reported to be stimulated by phospholipids (41, 42). Thus, we first investigated effects of phospholipids on GCS activity. Purified GCS showed an almost absolute requirement for phospholipids for activity (Table II, Fig. 4). In contrast, GCS activity in the Gdgi and crude CHAPSO extract was not stimulated significantly by phospholipids (data not shown). The optimal phospholipid concentration for stimulating GCS activity in purified fractions was 0.1 mM, with higher concentrations causing a relative suppression of activity (Fig. 4). Of the phospholipids tested, phosphatidylcholines showed the greatest ability to stimulate GCS activity, although phosphatidylethanolamine and phosphatidylglycerol were less effective. Phosphatidylinositol and phosphatidylserine were poor substrates with 21% glucosylation compared to C6-NBD-Cer (Table III). We also tested the effects of D- and L-threo-PDMP, a known specific inhibitor of GCS (43). PDMP inhibited the activity of GCS.

![Purification of Rat Liver Glucosylceramide Synthase](http://www.jbc.org/)
Finally, we evaluated the ability of GCS to use various donor substrates to glycosylate C6-NBD-Cer. Of the UDP-hexoses, GCS was able to utilize UDP-Glc efficiently but had little or no activity using UDP-glucuronic acid, UDP-galactose, UDP-N-acetylglucosamine, UDP-mannose, or UDP-xyllose as hexose donors (Table IV). UDP-Glc was the best glucose donor among diphosphoglucose nucleotides, but TDP-glucose and CDP-glucose also were efficient glucose donors (Table IV). Surprisingly, ADP-glucose was also used as a substrate by GCS, leading to about 6% glucosylation of C6-NBD-Cer compared to UDP-Glc.

**DISCUSSION**

We report here for the first time a method for the purification of GCS. Several features of the method were critical for the successful purification of this enzyme. First, the modifications that we introduced in the homogenization and Golgi fractionation procedure (see “Experimental Procedures”) significantly improved the enrichment and recovery of GCS activity relative to our previous work (16). Second, we found that inclusion of UDP-Glc and DOPC as protective agents improved the stability of GCS. Similarly, UDP and phospholipids were reported to stabilize UDP-Glc: dolichyl-phosphate glucosyltransferase (44); however, UDP had no protective effect on GCS activity (data not shown). Finally, we found that green d-agarose could be used in a two-step procedure to purify GCS based on the selective binding of GCS to the dye-agarose in the absence, but not in the presence, of UDP-Glc. This procedure led to a ~20-fold enrichment of GCS in the UDP-Glc-eluted fractions and was the only chromatographic procedure that we tried that produced any enrichment in the enzyme. Dye-agaroses have been used previously in the purification of numerous proteins such as dehydrogenases, kinases, and serum proteins (for review, see Ref. 45) as well as UDP-Glc: dolichyl-phosphate glucosyltransferase (46). The observation that the binding of GCS to dye-agarose is inhibited by UDP-Glc suggests a competition between UDP-Glc and dye molecules for the active site of the enzyme. Similar results were described for another glucosyltransferase purified on dye-agarose (47).

Using the solubilized and purified GCS, we also obtained new information about the enzymatic characteristics of this protein. First, we found that purified GCS had almost no activity in the absence of exogenous phospholipid. Activity was restored by the addition of phospholipids, with the highest enhancement of activity observed with low concentrations (0.1 mM) of unsaturated, long chain (C16:1 or C18:1) phosphatidylcholine (Table II). In contrast, phospholipids had little effect on GCS activity in the Golgi or CHAPSO-solubilized Golgi membranes. Presumably, endogenous phospholipids present in Golgi membranes were sufficient to stimulate maximal activity in the Golgi fractions. These endogenous phospholipids may have been depleted during purification of the enzyme by dye-agarose chromatography, causing an almost complete loss of GCS activity, which could be restored by exogenous phospholipids. A stimulating effect of exogenous phospholipids on glycosyltransferase activities was documented previously (42, 48–50). More recently, stimulation of solubilized GCS activity by phosphatidylcholine was reported (51); however, the concentrations reported for optimal activity were ~100-fold higher (8–10 mM) than the value that we report here (0.1 mM).

Our results on the specificity of purified GCS toward ceramide analogs are in good agreement with previously published studies concerned with GCS activity in cultured cells or membrane preparations. First, we found that GCS is stereospecific, utilizing erythro- but not three-C6-NBD-Cer as a substrate, similar to results reported using cultured fibroblasts (18). We also found that ω-erythro-C6-NBD-dihydroceramide was glucosylated to about 25% of control values obtained with C6-NBD-Cer (Table III). This result supports recent observations on the metabolism of C6-Cer analogs in Chinese hamster ovary cells (52). Second, we found that C6-Cer and C7-ceramides were better substrates than ceramides with longer or shorter N-acyl chains, confirming observations reported for GCS activity in microsomal preparations (41). Finally, we found that C6-NBD-Cer is a better substrate for GCS than is [14C]C6-Cer. By contrast, C5-DMB-Cer, containing a different fluorophore, was poorly glucosylated relative to C6-NBD-Cer (Table III), consistent with previous findings in cultured cells (53). We also examined the nucleotide specificity of GCS and found that, surprisingly, GCS is able to efficiently utilize CDP-Glc and TDP-Glc as glucose donors (see Table IV). While CDP-Glc and TDP-Glc are not naturally occurring glucose donors, earlier studies have also described UDP-Glc: glucosyltransferases that are able to utilize CDP-Glc and TDP-Glc (54, 55).

In summary, we have presented for the first time a method for the purification of GCS, applied this method to isolate a ~10,000-fold enriched GCS fraction from rat liver, identified two polypeptides (60–70 kDa) as likely candidates for the GCS protein, and further characterized the purified enzyme. This
information provides a basis for future molecular studies of this key enzyme in GSL biosynthesis.

Acknowledgments—We thank the members of the Pagano laboratory for encouragement and critical reading of the manuscript.

Note Added in Proof—Recently Ichikawa et al. (34) have isolated a cDNA encoding human GCS by expression cloning using GM-95 cells lacking the enzyme as a recipient. The open reading frame encodes a protein containing 394 amino acids (predicted molecular mass of 44.9 kDa).

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Pascal Paul, Yasushi Kamisaka, David L. Marks and Richard E. Pagano

J. Biol. Chem. 1996, 271:2287-2293.
doi: 10.1074/jbc.271.4.2287

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