Oligomerization and Topology of the Golgi Membrane Protein Glucosylceramide Synthase⁎

(Received for publication, May 21, 1998, and in revised form, September 18, 1998)

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Glucosylceramide synthase (GCS) catalyzes the transfer of glucose from UDP-glucose to ceramide to form glucosylceramide, the precursor of most higher order glycosphingolipids. Recently, we characterized GCS activity in highly enriched fractions from rat liver Golgi membranes (Paul, P., Kamisaka, Y., Marks, D. L., and Pagano, R. E. (1996) J. Biol. Chem. 271, 2287–2293), and human GCS was cloned by others (Ichikawa, S., Sakiyama, H., Suzuki, G., Hidari, K. I.-P. J., and Hirabayashi, Y. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4638–4643). However, the polypeptide responsible for GCS activity has never been identified or characterized. In this study, we made polyclonal antibodies against peptides based on the predicted amino acid sequence of human GCS and used these antibodies to characterize the GCS polypeptide in rat liver Golgi membranes. Western blotting of rat liver Golgi membranes, human cells, and recombinant rat GCS expressed in bacteria showed that GCS migrates as an ~38-kDa protein on SDS-polyacrylamide gels. Trypsinization and immunoprecipitation studies with Golgi membranes showed that both the C terminus and a hydrophilic loop near the N terminus of GCS are accessible from the cytosolic face of the Golgi membrane. Treatment of Golgi membranes with N-hydroxysuccinimide ester-based cross-linking reagents yielded an ~50-kDa polypeptide recognized by anti-GCS antibodies; however, treatment of ~10,000-fold purified Golgi GCS with the same reagents did not yield cross-linked GCS forms. These results suggest that GCS forms a dimer or oligomer with another protein in the Golgi membrane. The migration of solubilized Golgi GCS in glycerol gradients was also consistent with a predominantly oligomeric organization of GCS.

Glucosylceramide is synthesized by UDP-glucose:ceramide glucosyltransferase (glucosylceramide synthase (GCS)) (1), a resident integral membrane protein of the cis/medial-Golgi membrane (2–4). Glucosylceramide is the common precursor of most higher order glycosphingolipids, which are important cell membrane constituents and have been implicated as important factors in development, differentiation, tumor progression, and pathogen/host interactions (5–11). Thus, GCS may play significant roles in several biological processes by regulating the overall synthesis of glucosylceramide-derived glycosphingolipids. However, surprisingly little is known about the polypeptide responsible for GCS activity.

We recently solubilized and partially purified (~10,000-fold) enzymatically active rat liver GCS (12). We found that detergent-solubilized GCS peaked in glycerol gradients at an apparent molecular mass of ~60 kDa, but were unable to conclusively identify the GCS polypeptide on SDS-polyacrylamide gels. Since then, GCS was cloned from a human cDNA library by rescue of a mutant mouse cell line deficient in GCS activity (13). The predicted amino acid sequence of the cloned enzyme encodes a protein with a calculated molecular mass of ~45 kDa, but the cloned enzyme was not visualized by SDS-polyacrylamide gel electrophoresis. The difference between the predicted molecular mass of GCS based on its amino acid sequence and its apparent size in glycerol gradients suggests that GCS may be organized in a dimer or oligomer; however, this possibility has not yet been investigated. In addition, no homology was found between the predicted amino acid sequence of GCS and any previously known protein. Thus, except for the identification of a predicted N-terminal membrane-spanning domain (13) and observations by us (2) and others (3, 4) that the active site of GCS is accessible to proteases applied to the cytosolic face of the Golgi membrane, no information is available on the secondary structure of GCS.

Given the unresolved issues concerning the GCS polypeptide, we prepared polyclonal antibodies against peptides based on the predicted amino acid sequence of human GCS and used these antibodies to identify the GCS polypeptide, to investigate the topology of GCS in rat liver Golgi membranes, and to provide evidence that GCS is organized as a dimer or oligomer in the Golgi membrane.

EXPERIMENTAL PROCEDURES

Preparation of Rat Liver Golgi Fractions—Golgi membrane fractions were prepared from male Sprague-Dawley rats (5 weeks old) as described previously (12, 14, 15) with the following modifications. Livers were homogenized (25%, w/v) in 0.25 M sucrose in buffer A (50 mM Tris-HCl, pH 7.4, and 25 mM KCl) with protease inhibitors (10 μg/ml each leupeptin, tosylarginylmethyl ester, and aprotinin; 1 μg/ml each pepstatin and antipain; and 25 mM 4-amidophenylmethanesulfonyl fluoride (all from Sigma)) using a Polytron (Brinkmann Instruments) at 10,000 × g for 5 min.

The abbreviations used are: GCS, glucosylceramide synthase; NLS, N-lauroylsarcosine; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; BS, 1-sulfosuccinimidyl) suberate; DSS, di-N-hydroxysuccinimide ester-based cross-linking reagents; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulfonate; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); DSP, dithiobis(succinimidyl propionate); SMS, sphingomyelin synthase.
setting 1 for ~30 s. The homogenate was filtered through cheesecloth and then adjusted (150 parts homogenate and 95 parts 2 m sucrose in buffer A (v/v)) to a final concentration of 1.07 M sucrose. The adjusted homogenate (19 ml/tube) was loaded into Beckman SW 28 tubes; 9 ml each of 0.9 and 0.2 m sucrose in buffer A were then sequentially overlaid above the homogenate. At 200,000 × g, the membranes were resuspended at one-twentieth of their original volume in 0.25 M sucrose in buffer B. N-Lauroylsarcosine (NLS)-washed Golgi membranes were prepared as described previously (12). In some experiments, NLS-washed Golgi membranes were solubilized with either 1% Igepal CA-630 (a Nonidet P-40 equivalent from Sigma) or 0.5% Triton X-100 in buffer B. The extract was then centrifuged at 200,000 × g, and the pellet was discarded. Fractions (~10,000-fold enriched in GCS) were prepared from NLS-washed Golgi membranes using dye-agarose columns as described previously (12) and then concentrated and equalized with buffer B containing 1% Igepal CA-630 using Centricon 50 filters (Amicon, Inc., Beverly, MA).

Preparation of Polyclonal Antibodies—Four synthetic peptides were synthesized on hydrophilic regions of the predicted regions of the full-length human GCS (13). The peptides used were CTISWRTGRYR-LRCGTAEEILDV (referred to as GCS-1), AMQNSGSYSISISQFQSMNKG-NH₂ (GCS-2), KLKPXDLNPNLLETTFELDCY-NH₂ (GCS-5), and TRRLHNLKDAKDPKYSLKPGC-NH₂ (GCS-6), which correspond to amino acids 372–394, 257–280, 57–78, and 33–52, respectively, of the human GCS sequence (13), except that cysteine was added to the N terminus of GCS-1, and cysteine-NH₂ was added to the C terminus of GCS-2, -5, and -6 to facilitate peptide conjugation. Fractions of each peptide were conjugated to keyhole limpet hemocyanin. To prepare polyclonal antibodies, New Zealand White rabbits (two rabbits/peptide) were injected with peptides (500 µg of conjugate plus 100 µg of the corresponding free peptide for initial injection) in Freund’s adjuvant and boosted (100 µg of conjugate of each peptide) every 20 days. Bleeds were tested for immunoreactivity to GCS by immunoprecipitation assays and Western blotting. For some studies, antibodies were affinity-purified using the corresponding peptide conjugated to Amino-Link Plus columns (Pierce).

Immunoprecipitation and Immunopurification Methods—For immunoprecipitation and immunopurification of active GCS, Golgi samples were solubilized in 0.5% CHAPS and 0.2% Triton X-100 in buffer B. For immunoprecipitation to be followed by Western blotting, samples were solubilized in 150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 8.0 (radioimmune precipitation assay buffer) (16), or in 1.0% Triton X-100, 0.2% SDS, 150 mM NaCl, 0.5 mM EDTA, and 10 mM Tris-HCl, pH 8.0 (17). Samples were incubated for 2 h at room temperature with affinity-purified antibodies conjugated to protein A-Sepharose CL-4B beads (Sigma) prepared using dimethyl pimelimidate (16). The samples were then centrifuged; the supernatant was saved; and the pellet was washed three times in the solubilizing buffer. Pellets and supernatants were analyzed by GCS activity assays or by Western blotting (see below). Human GCS was immunoprecipitated from lysates of differentiated cultured human keratinocytes as described (10). For immunopurification, solubilized rat Golgi GCS was eluted from antibody-bead complexes with 3 M MgCl₂ (Golgi GCS was eluted from antibody-bead complexes with 3 M MgCl₂).

Glycerol Gradient Fractionation—Aliquots of N-lauroylsarcosine-washed Golgi membranes (0.5 ml) were solubilized with 1% Igepal CA-630 (see above); adjusted to 5% glycerol; and loaded onto 10.5 ml 8–25% glycerol gradients prepared in 0.3% Igepal CA-630, 50 mM HEPES, pH 7.4, 50 mM KCl, 2 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml tosylarginylmethyl ester, 1 µg/ml pepstatin, and 25 mM 4-amidophenyl-methanesulfonfluoride. The gradients were centrifuged for 18 h at 200,000 × g in a Beckman SW 41 rotor at 4 °C. After centrifugation, gradient fractions (0.8 ml) were collected and analyzed for GCS activity or by Western blotting. In some cases, Igepal CA-630-solubilized samples were treated with cross-linking reagents either before or after gradient fractionation.

Results

Development of Polyclonal Antibodies against GCS—To develop antibodies against the GCS protein, we synthesized four peptides (referred to as GCS-1, -2, -5, and -6, see Fig. 1A) based on hydrophilic regions of the predicted amino acid sequence of human GCS and injected these into rabbits. Antibodies against peptides GCS-1, -5, and -6 each recognized an ~38-kDa polypeptide in rat liver Golgi membrane fractions and in fractions of enzymatically active GCS purified from solubilized Golgi membranes on an anti-GCS-1 antibody affinity column (Fig. 1C), slightly lower than the predicted molecular mass (44.5 kDa) of human GCS (15). The migration of this immunoreactive band was unchanged by the addition of reducing agents (data not shown). Antiserum made against the GCS-2 peptide did not recognize the ~38-kDa band or other specific bands in Western blotting or immunoprecipitations (data not shown). Western blots showed that anti-GCS-1 and anti-GCS-6 antibodies immunoprecipitated the ~38-kDa band from Golgi fractions under mildly denaturing conditions (radioimmune

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Anti-GCS antibodies recognize rat and human GCS proteins. Polyclonal antibodies were prepared by injection of peptides (GCS-1, -2, -5, and -6) into rabbits as described under “Experimental Procedures.” For Western blots, samples were run on SDS-polyacrylamide gels (10% acrylamide) and transferred to polyvinylidene fluoride membrane, and blotting was performed using anti-GCS antibodies. Antibodies to GCS (GCS-1, -2, -5, and -6) were synthesized and injected into rabbits as described under “Experimental Procedures.” For Western blots, samples were run on SDS-polyacrylamide gels. GCS was not detected by Western blotting in crude liver fractions (homogenates or total microsomes; WG). However, the anti-GCS-5 antibody detected GCS proteins on SDS-polyacrylamide gels. GCS was detected by Western blotting with the anti-GCS-5 antibody. Similar results were seen with the anti-GCS-1 antibody (data not shown). 1. N-lauroylsarcosine-washed rat liver Golgi membranes; 2, the entire rat GCS coding region expressed in E. coli; 3, a rat GCS truncation mutant that lacks the first 10 amino acids at the N terminus expressed in E. coli; 4, GCS from cultured differentiated human keratinocytes. Fractions 2–4 are GCS immunoprecipitates from crude lysates.

Precipitation assay buffer; however, the anti-GCS-5 antibody immunoprecipitated the 38-kDa band only under strongly denaturing conditions (data not shown). These data demonstrate that rat Golgi GCS runs as an 38-kDa polypeptide on SDS-polyacrylamide gels. GCS was not detected by Western blotting in crude liver fractions (homogenates or total microsomes) loaded at 20 μg/lane (Fig. 1B), probably due to the low overall expression of GCS in liver.

To further characterize the GCS polypeptide, we compared the migration on SDS-polyacrylamide gels of rat Golgi GCS with that of recombinant rat GCS expressed in Escherichia coli and GCS immunoprecipitated from differentiated human keratinocytes, a cell type that expresses a high level of GCS (10). Rat GCS is 97% identical to human GCS at the amino acid level, with a predicted molecular mass of 44.8 kDa. Western blotting showed that rat Golgi GCS migrated identically (~38 kDa) on SDS-polyacrylamide gels compared with rat GCS expressed in E. coli and GCS immunoprecipitated from human keratinocytes. Immunoprecipitation and Western blotting with the anti-GCS-1 antibody, which recognizes the C-terminal amino acids of GCS, indicated that the C terminus of GCS is present in the mature rat and human proteins (Fig. 1). To investigate the possibility that the N-terminal region of GCS is missing (due to either post-translational cleavage or GCS translation initiating from a second methionine (amino acid 11 of the rat GCS sequence), we expressed a deletion mutant that lacks the first 10 amino acids of GCS. This mutant form of GCS ran lower on Western blots than wild-type recombinant rat GCS (Fig. 1C) and, in addition, had only 4% of the activity of the wild-type form. Together, these data indicate that both rat and human GCS proteins migrate anomalously as ~38-kDa polypeptides and that the full amino acid sequence, including the N terminus, is present in the mature forms.

GCS Topology—We then used the anti-GCS antibodies to further investigate the topology of GCS in the Golgi membrane. Golgi membrane fractions were incubated with a low concentration of trypsin over time; samples were then tested for GCS and SMS activities, and the integrity of the GCS polypeptide was assessed by Western blotting. In agreement with our previous study (2), GCS activity decreased rapidly over time, whereas SMS activity was relatively unaffected (Fig. 2A). When incubations were performed in the presence of 0.02% Triton X-100, SMS was also rapidly degraded (data not shown).
DTSSP. GCS in Golgi membranes was treated with DTSSP (a thiol reagent cleavable cross-linker), BS, or 5% MeSO alone (polypeptides were detected with the anti-GCS-6 antibody, but not with nonimmune controls (data not shown).

Liver Golgi GCS fractions, DSS results (data not shown) were similar to those shown for BS. Note the occurrence of an anti-GCS-1 and anti-GCS-5 antisera (diluted 1:2000 each). For Igepal CA-630-solubilized NLS-washed Golgi fractions and dye-agarose-purified rat liver Golgi GCS fractions were treated with 5 mM cross-linker (BS or DSS) or 10% MeSO alone (control) and then Western-blotted for GCS. DTSSP (minus dithiothreitol +DTT) induced the formation of an ~50-kDa form (XL1) recognized by the anti-GCS-5 antisera. Some higher molecular mass forms were also generated. Similar cross-linked polypeptides were detected with the anti-GCS-6 antibody, but not with nonimmune controls (data not shown).

Western blotting using three different anti-GCS antibodies showed that GCS was rapidly degraded by trypsin, with little of the intact ~38-kDa form present after 5 min of incubation (Fig. 2B). The anti-GCS-1 antibody recognized only intact GCS and detected no lower molecular mass forms after trypsin treatment, suggesting that the GCS-1 epitope is readily accessible to the protease. At 5 min and later time points, an ~35-kDa GCS fragment was recognized by both the anti-GCS-5 and antiGCS-6 antibodies (Fig. 2B). Finally, a third fragment (~33 kDa), which peaked in appearance at 10 min, was recognized by only the anti-GCS-5 antibody (Fig. 2B). At later time points, the ~33-kDa fragment was apparently degraded as well. These results show that both the GCS-1 and GCS-6 epitopes may be cleaved by trypsin, leaving the ~33-kDa fragment, and suggest that this fragment may be partially protected from proteolysis by its secondary structure.

We noted that after 10 min of proteolysis, almost no intact GCS was recognized by the anti-GCS-1 antibody, although large fragments recognized by the other antibodies were present, and GCS activity had decreased to ~35% of initial levels (Fig. 2). These results suggest that the C-terminal region recognized by the anti-GCS-1 antibody may be important for optimal GCS activity. Expression of a recombinant GCS mutant lacking the 8 last amino acids of the C terminus showed that it had only ~4% activity compared with wild-type GCS, supporting the hypothesis that the C terminus of GCS is important for activity.

These results demonstrate that, under the experimental conditions used here, the Golgi membranes are predominantly nonpermeable to trypsin and are right-side out (GCS, but not SMS, is accessible to trypsin).

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We then tested the ability of different anti-GCS antibodies to interact in situ with GCS in right-side-out Golgi membranes. GCS-1 immunoprecipitated most (~80%) of the GCS activity in Golgi vesicles; GCS-6 partially immunoprecipitated activity (~50%); but the anti-GCS-5 antibody did not immunoprecipitate activity (Fig. 2C). Taken together with the controlled proteolysis studies, these data support a model for GCS in which the C terminus (GCS-1 epitope) extends into the cytosol from the outer face of the Golgi membrane and the GCS-6 epitope forms a loop or hinge that is also partially accessible to the cytosol, whereas the GCS-5 epitope is somewhat protected by the protein secondary structure or interactions with the Golgi membrane.

GCS Oligomerization—We next began studies to determine if GCS is organized in oligomers. Experiments in which a series of bifunctional cross-linking agents were reacted with Golgi membranes demonstrated that cross-linkers based on N-hydroxysuccinimide esters (BS, DSS, DSP, and DTSSP) consistently induced the appearance of an ~50-kDa polypeptide recognizable by anti-GCS antibodies on Western blots (Fig. 3). Additional higher cross-linked forms were also detectable, especially with the anti-GCS-1 antibody (Fig. 3A). In general, cross-linking caused an increase in the overall signal of immunoreactive material on blots, possibly due to changes in immunoreactivity after protein derivatization by cross-linkers or increased efficiency of electrophoretic transfer of higher molecular mass derivatized forms relative to the unaltered GCS monomer.

When Golgi fractions were incubated with the cleavable cross-linker DTSSP, the ~50-kDa band formed was recognized by the anti-GCS-5 antibody, but not by GCS-1 (Fig. 3B). How-
ever, cleavage of DTSSP with dithiothreitol in these samples eliminated the ~50-kDa band and restored the anti-GCS-1 antibody-immunoreactive monomeric GCS band. These results are consistent with the hypothesis that the ~50-kDa form is a dimer or oligomer that contains GCS, rather than an artifact caused by reaction of the cross-linker with some other protein. Additional control experiments demonstrated the following. 1) The cross-linked forms were not recognized by nonimmune sera; and 2) higher molecular mass forms were not generated by reaction of Golgi proteins with sulfosuccinimidyl 6-(biotinamido)hexanoate, a reagent with reactivity (i.e. primary amines) similar to that of the bifunctional cross-linkers used, but that does not form cross-links (data not shown).

We next compared the cross-linking of GCS in four different Golgi-derived fractions. We found that reaction of BS or DSS with intact Golgi membranes, Golgi membranes washed with N-lauroylsarcosine (~75% of total proteins but little GCS) are removed by this step (12), and Igepal CA-630 extracts of NLS-washed Golgi membranes all yielded similar patterns of GCS-immunoreactive cross-linked forms (Fig. 3C). Treatment of dye-agarose-purified GCS with cross-linking reagents, however, did not result in the formation of the ~50-kDa form or other immunoreactive cross-linked species (Fig. 3C). Similarly, reaction of Igepal CA-630-solubilized recombinant rat GCS expressed in bacteria with cross-linkers also did not generate any cross-linked GCS forms (data not shown). These results suggest that GCS in Golgi membranes specifically cross-links to a closely associated protein, which is absent in dye-agarose-purified GCS and bacterially expressed GCS (i.e. GCS in the Golgi membrane is a heterodimer or heterooligomer).

To provide further characterization of GCS oligomers, we fractionated detergent-solubilized Golgi membranes on a glycerol gradient and then cross-linked each sample with 5 mM BS after gradient fractionation to “trap” any oligomeric GCS in higher molecular mass forms that would be visible by Western blotting. Without cross-linking, only the GCS monomer (~38-kDa form) was visible by Western blotting; this form peaked early in the gradient (approximately fractions 3–5) and coincided with the peak of GCS activity (Fig. 4). Note that without cross-linking, any GCS oligomers present in these fractions are dissociated by the conditions of SDS-polyacrylamide gel electrophoresis so that only the GCS monomer is visualized on Western blots.

In cross-linked fractions (Fig. 4A, +BS), the GCS monomer peaked earlier (fraction 3) in the glycerol gradient than the peak of GCS activity, and most GCS-immunoreactive material appeared as cross-linked ~50- or ~70- and ~120-kDa forms that peaked later in the gradient (fractions 5 and 6). The higher molecular mass forms, including aggregated immunoreactive material present at the top of the gel, were not recognized by nonimmune antisera, but were recognized by both anti-GCS-1 (Fig. 4A) and anti-GCS-6 (data not shown) antibodies, confirming that they represent cross-linked forms of GCS. These data show that a significant portion of solubilized GCS migrated farther in the gradient than the monomer and was cross-linkable with BS, consistent with an oligomeric structure.

**DISCUSSION**

This study reports the development of peptide-specific anti-GCS antibodies and their use to characterize the GCS protein in rat liver. We have used the anti-GCS antibodies to visualize GCS by Western blotting for the first time and show that it runs as an ~38-kDa protein on SDS-polyacrylamide gels. We used Western blotting and activity assays for GCS in conjunction with controlled proteolysis and immunoprecipitation of intact Golgi vesicles to generate new information on the topology of GCS in the Golgi membrane. Finally, we used cross-linking reagents and glycerol gradients to provide evidence that GCS is organized as a heterodimer or heterooligomer.

**Recognition of GCS by Antibodies**—The predicted molecular mass of both the human (13) and rat GCS polypeptides is ~45 kDa. We demonstrate here that rat Golgi GCS, recombinant rat GCS expressed in bacteria, and human GCS run as ~38-kDa polypeptides on SDS-polyacrylamide gels as detected by Western blotting with peptide-specific antibodies against GCS. We have shown that the C terminus of GCS is present in these polypeptides by the use of an antibody (GCS-1) against the C-terminal 23 amino acids of GCS. We also expressed a mutant rat GCS lacking the first 10 amino acids at its N terminus. This

![Fig. 4](image-url)
mutant ran lower than wild-type GCS on SDS-polyacrylamide gels and had little activity, in contrast to the wild-type form, suggesting that the N terminus of GCS must also be present in the native forms. Thus, we have ruled out the possibility that the inconsistency between the predicted and empirical molecular masses of GCS is due to the loss of a portion of the N- or C-terminal region of GCS during the processing of the protein. A likely explanation for the discrepancy in molecular mass is that, because GCS is an extremely hydrophobic protein, it runs anomalously on SDS-polyacrylamide gels, as do some other hydrophobic proteins (e.g. see Ref. 24).

GCS Topology—Previous results from our laboratory (2) and others (3, 4) have established that the active site of GCS is on the cytosolic face of the Golgi membrane by showing that the GCS activity of right-side-out Golgi fractions is readily accessible to proteases. Here we extend these data by demonstrating that, in right-side-out Golgi vesicles, the GCS-1 epitope at the carboxyl terminus of the protein is rapidly degraded by trypsin and is accessible to antibodies (for immunoprecipitation of Golgi vesicles). The GCS-6 epitope was slightly less accessible to trypsin and could also be used to immunosolubilize Golgi vesicles, although with lower efficiency. Finally, the GCS-5 epitope appeared to be somewhat protected against trypsin and was not accessible to antibodies for immunoprecipitation except under denaturing conditions. These results suggest that the carboxyl terminus of GCS (GCS-1 epitope) and a loop (containing the GCS-6 epitope) just after the putative N-terminal transmembrane domain of GCS protrude into the aqueous environment on the cytosolic face of the Golgi membrane, whereas the region containing the GCS-5 epitope is shielded from the aqueous environment by other portions of the protein or membrane interactions.

GCS Oligomerization—We have shown that bifunctional cross-linking reagents shift the size of GCS to ~50 kDa and higher forms. The ~50-kDa form could be generated from both solubilized and intact Golgi membranes, suggesting that GCS is organized in dimers/oligomers; however, treatment of dye-agarose-purified GCS or bacterially expressed rat GCS with crosslinkers did not yield any cross-linked GCS products. These results suggest that there is a specific association of GCS with a small (~15 kDa) polypeptide normally present in Golgi membranes, but absent in the dye-agarose-purified GCS fractions. In preliminary experiments, we attempted to identify such a GCS-associated polypeptide by trying to immunoprecipitate GCS (and associated proteins) from both metabolically 35S-labeled proteins from cultured cells and 125I-labeled rat liver Golgi proteins; however, we could not visualize GCS or associated proteins above background radioactivity in either case (data not shown), presumably because of the low levels of GCS expression. Thus, the isolation of GCS-associated polypeptide(s) will probably require an affinity purification approach using overexpressed recombinant GCS.

The organization of GCS in heterodimers or heterooligomers may play a significant role in GCS localization and/or function. The oligomerization of Golgi membrane proteins has been suggested to be involved in their retention in the Golgi complex (25–29). In addition, several other glycosyltransferases have been shown to have a dimeric or oligomeric structure, suggesting a functional role of oligomerization in such enzymes (30–33). We plan future studies using site-directed mutagenesis and truncation mutations to identify the oligomerization domain(s) of GCS and to explore the role of GCS oligomerization in Golgi targeting.

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