Histidine-193 of rat glucosylceramide synthase resides in a UDP-glucose- and inhibitor (D-*threo*-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol)-binding region: a biochemical and mutational study

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Glucosylceramide synthase (GCS) catalyses the transfer of glucose from UDP-glucose (UDP-Glc) to ceramide to form glucosylceramide, the common precursor of most higher-order glycosphingolipids. Inhibition of GCS activity has been proposed as a possible target of chemotherapeutic agents for a number of diseases, including cancer. Design of new GCS inhibitors with desirable pharmaceutical properties is hampered by lack of knowledge of the secondary structure or catalytic mechanism of the GCS protein. Thus we cloned the rat homologue of GCS to begin studies to identify its catalytic regions. The histidine-modifying agent diethyl pyrocarbonate (DEPC) inhibited recombinant rat GCS expressed in bacteria; this inhibition was rapidly reversible by hydroxylamine and could be diminished by pre-incubation of GCS with UDP-Glc. These data suggest that DEPC acts on histidine residues within or near the UDP-Glc-binding site of GCS. Mutant proteins were expressed in which the eight histidine residues in GCS were individually replaced by other amino acids. H193A ($His^{193} \rightarrow Ala$) and H193N ($His^{193} \rightarrow Asn$) mutants were unaffected by 0.1 mM DEPC, a concentration that inhibited other histidine mutants and the wild-type enzyme by at least 60%. These results indicate that His^{193} is the primary target of DEPC and is at, or near, the UDP-Glc-binding site of GCS. His^{193} mutants were also insensitive to the GCS inhibitor D-*threo*-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol, at concentrations which inhibited the wild-type enzyme by $>$ 80%. These results have significance for both an understanding of the GCS active site and also for the possible design of new and specific inhibitors of GCS.

Key words: diethyl pyrocarbonate, glycosphingolipids, glycosyltransferase, Golgi apparatus, D-*threo*-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol.

INTRODUCTION

Glucosylceramide synthase (GCS) catalyses the transfer of glucose from UDP-glucose (UDP-Glc) to ceramide to form glucosylceramide (GlcCer). Because of the reported involvement of GlcCer and higher-order glycosphingolipids derived from GlcCer in multiple pathological processes (e.g., tumorigenesis, drug resistance and host–pathogen interactions) [1–3], GCS has been suggested as a potential target for therapeutic drugs [4–6]. Indeed, relatively specific inhibitors of GCS such as D-*threo*-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol (PDMP) and related compounds have been shown to have several potentially useful effects, including growth suppression in cultured cancer cells [7–9] and keratinocytes [10], decrease in tumour growth in mice and rats [5], inhibition of shedding of immunosuppressive gangliosides in neuroblastoma cells [11,12] and interference with cell adherence [4,13].

Although the activity of GCS has been known for three decades [14,15], almost no structural or mechanistic information is available about the enzyme. Human and mouse GCS have been cloned and found to possess nearly identical amino acid sequences [16,17]; however, no significant sequence similarity has been found between GCS and any other previously identified proteins, and thus no predictions can be made regarding the active site or catalytic mechanism of GCS. A hydrophobic region near the N-terminus (amino acids 11–32) of GCS was suggested to be a transmembrane domain [17]. We recently showed that the carboxy tail and a hydrophilic loop near the putative transmembrane domain of GCS are accessible to the cytosol [18], consistent with previous reports by us and others that the active site of GCS is on the cytosolic face of the Golgi membrane [19–21]. No studies have been performed, however, which identify the active site or substrate-binding regions of GCS.

To begin to identify catalytic and/or substrate-binding regions within GCS, we cloned GCS from rat brain and conducted biochemical and molecular studies on the role of histidine residues in its activity. Our findings show that His^{193} in GCS is responsible for the sensitivity of GCS to the histidine-modifying reagent diethyl pyrocarbonate (DEPC) and suggest that His^{193} is within the binding region for both UDP-Glc and the GCS inhibitor PDMP.

MATERIALS AND METHODS

Rat brain cDNA library screening

A 476 bp GCS cDNA fragment was obtained by reverse-transcription (RT-) PCR of human skin fibrobast (GM5659C; The Coriell Institute, Human Genetic Mutant Cell Repository, Camden, NJ, U.S.A.) total RNA using GCS-specific primers [5′-

Abbreviations used: C_6-NBD-Cer, *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminohexanoyl-D-*erythro*-sphingosine; Cer, ceramide; DEPC, diethyl pyrocarbonate; GalCer, galactosylceramide; GlcCer, glucosylceramide; GCS, glucosylceramide synthase; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol; RACE, rapid amplification of cDNA ends; RT-PCR, reverse-transcription PCR; UDP-Gal, UDP-galactose; UDP-Glc, UDP-glucose; LB, Luria broth.

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The amino acid sequence reported here and corresponding nucleotide sequence have been submitted to the EMBL, GenBank* and DDBJ Nucleotide Sequence Databases under the accession number AF047707.

GCTTTGCTGCCACCTTAGA-3′ (sense) and 5′-GACACCCCYGAGTTGAATG-3′ (antisense)] based on the human GCS cDNA sequence [17] and cloned into the PCR II vector (Invitrogen, San Diego, CA, U.S.A.). An [α-^{32}P]dCTP-labelled probe was prepared from the human GCS cDNA fragment using a random priming kit (Boehringer Mannheim Corporation, Indianapolis, IN, U.S.A.) and used to screen a Lambda ZAP II Sprague–Dawley rat brain cDNA library (Stratagene, La Jolla, CA, U.S.A.). Among 1.32×10^6 plaques screened, two positive bacteriophages containing a 2.1 kb insert were isolated. The inserts were cloned into pBluescript (Stratagene) and sequenced using a combination of vector-based and internal sequence-based primers.

Rapid amplification of rat brain GCS cDNA 5′-ends (5′-RACE)

To obtain the 5′ portion of the encoding region of rat GCS, two rounds of 5′-RACE were performed using a commercial rat cDNA pool with tailed adaptor sequences at the 5′ and 3′ ends (Marathon-Ready Sprague–Dawley rat brain cDNA) and Advantage cDNA polymerase mix (both from Clontech, Palo Alto, CA, U.S.A.). GCS-gene specific primers used for the 5′-RACE were 5′-TAAACTGGCAACAAAGCATTCTGA-3′ (first round of 5′-RACE), 5′-TTCCTCATCAAGCAAGACATCC-3′ (nested PCR of the first round of 5′-RACE) and 5′-ACCACGAAGAGCACGAAGCCGAACAAG-3′ (second round of 5′-RACE). The first two primers were based on the sequence of the 2.1 kb GCS rat brain GCS cDNA clone, whereas the third primer was based on sequence of the product from the first 5′-RACE.

Assembly of the full encoding region of rat brain GCS

The nucleotide sequences of the 2.1 kb rat brain GCS cDNA insert and the products of the two rounds of 5′-RACE were overlapped and aligned using the MacVector 4.5.0KS program (Eastman Kodak Company, New Haven, CT, U.S.A.). A 2.6 kb GCS cDNA insert with an open reading frame of 1182 nucleotides was generated. To assemble a clone with the full encoding region of GCS, a 517 bp/*Eco*RI + *Sna*BI fragment of the first 5′-RACE product and a 740 bp fragment/*Sna*BI + *Alw*NI of the 2.1 kb insert from the library clone were ligated together at the *Sna*BI site with T_4 ligase. The ligated cDNA fragment (1.2 kb) was used as a template for PCR with a pair of primers [5′-AATACCATGGCGCTGCTGGACCTGGCCCAG-3′ (sense) and 5′-TCGGATCCACAGAGGCCTTGTTACACA-3′ (antisense)]. This PCR reaction also added a *Nco*I site at the ATG initiation codon and a *Bam*HI site beyond the TAA stop codon of GCS. The PCR product was cloned into the TA vector and subcloned into the pET-3d expression vector (Novagen Inc., Madison WI, U.S.A.) at the *Nco*I and *Bam*HI sites, yielding pET-3d-GCS. pET-3d-GCS was transformed into INVαF′ cells (Invitrogen), plasmid DNA was prepared and sequenced once again, and finally transformed into *Escherichia coli* expression strain BL21-DE3 (Novagen).

Preparation of GCS histidine mutants

Site-directed mutagenesis of pET-3d-GCS was performed using the QuikChange-site-directed mutagenesis kit (Stratagene) to prepare mutant sequences in which each of the eight histidine residues within GCS was individually altered to other amino acids. GCS inserts were transformed into Epicurian Coli XL1-Blue supercompetent cells (Stratagene) and then sequenced to verify that they contained the expected mutations.

Expression of recombinant GCS

BL21-DE3 bacterial colonies carrying the pET-3d vector with wild-type or mutant GCS inserts were transferred into 2 ml of Luria broth (LB) medium containing ampicillin (50 μg/ml), incubated with shaking at 37 °C until an attenuance (D_{600}) of 0.6 was reached, and then stored at 4 °C overnight. The bacteria were then pelleted, resuspended in 2 ml of fresh LB medium and then added to 50 ml of LB medium with ampicillin (50 μg/ml), which was then incubated with shaking at 37 °C. When the D_{600} reached 0.6, GCS protein expression was induced with 0.4 mM isopropyl β-thiogalactopyranoside. After 30 min, bacterial cell pellets were harvested by centrifugation and then stored at −70 °C until use.

GCS enzyme assays and biochemical characterization

Bacterial pellets derived from 1 ml of BL21-DE3 cells in culture containing GCS inserts in the pET-3d vector ($D_{600} = 0.6$) were resuspended in 0.5 ml of GCS stabilizing buffer [50 mM Hepes (pH 7.4)/100 mM KCl/20 % glycerol/1 mM EDTA, plus protease inhibitors (10 μg/ml each of tosylarginine methyl ester, leupeptin and aprotinin, 1 μg/ml each of antipain and pepstatin, and 25 μM 4-amidophenylmethanesulphonyl fluoride; all from Sigma)] and lysed by probe sonication (twice for 8 s each). GCS activity was measured by incubating lysate samples (equivalent to 100 μl of bacterial culture) with 10 μM *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminohexanoyl-D-*erythro*-sphingosine (C_6-NBD-ceramide; Molecular Probes, Eugene, OR, U.S.A.) and 5 mM UDP-Glc (Sigma) in GCS assay buffer [50 mM Hepes (pH 7.4)/25 mM KCl/5 mM $MnCl_2$], for 30 min at 37 °C, followed by lipid extraction and TLC as previously described [22]. In one experiment, UDP-galactose (UDP-Gal; Sigma) was substituted for UDP-Glc or UDP-hexoses were omitted.

For studies of DEPC inhibition, GCS samples were treated with various concentrations of DEPC (Sigma) for 10 min at room temperature in 100 μl of stabilizing buffer prior to dilution with assay buffer and measurement of activity. For UDP-Glc protection against DEPC inhibition, the samples were pre-incubated with 1 mM UDP-Glc for 15 min at room temperature prior to adding DEPC as described above. Hydroxylamine reversal of DEPC was performed by incubating samples with 0.1 mM DEPC in 50 μl of GCS stabilizing buffer and then adding 50 μl of 1 M hydroxylamine at various time points before dilution with assay buffer and assaying for activity. To study the effects of PDMP, the compound (obtained from Matreya, Pleasant Gap, PA, U.S.A.) was dissolved in ethanol and aliquots were dried under vacuum within the assay tubes. GCS samples (50 μl) and assay buffer were added to the tubes, vortex-mixed and then incubated at room temperature for 15 min before assaying for GCS activity.

Immunoprecipitation and Western blotting

Polyclonal antibodies prepared against peptides based on regions of the human GCS amino acid sequence [18] were used for immunoprecipitation and Western blotting. For immunoprecipitation, bacterial pellets (from 10 ml of cultures) were solubilized in 150 mM NaCl/1.0 % Igepal CA-630 (Sigma)/0.5 % sodium deoxycholate/0.1 % SDS/50 mM Tris/HCl, pH 8.0 [23]. Immunoprecipitation and Western blotting of GCS were performed as recently described [18,24]. Western blots were revealed on XAR film (Eastman Kodak) using horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Boehringer Mannheim) and a chemiluminescence kit (New England Nu-

clear). Scanned images of Western blots were quantified using the NIH Image program.

RESULTS

Cloning and expression of rat GCS

Screening of a rat brain cDNA library with a 476-bp human GCS cDNA probe yielded two identical 2.1 kb positive clones which contained the C-terminal $\approx 60\,\%$ of the GCS coding region plus a 1353 bp 3′-untranslated region (results not shown). The rest of the coding region of rat GCS was obtained by two rounds of 5′-RACE (results not shown). Alignment of the different cloned GCS sequences yielded a 2.6 kb cDNA sequence with a 394-amino-acid open reading frame (Figure 1A). The rat GCS amino acid sequence was $97\,\%$ identical with the published human sequence [17] (Figure 1A). The recently reported amino acid sequence of mouse GCS [25] is also $> 95\,\%$ identical with rat GCS.

We next expressed the full-length rat GCS sequence in bacteria using the pET-3d vector and BL21-DE3 *E. coli* cells. GCS expressed in bacteria was highly active when UDP-Glc was present (Figure 1B). We also found that GCS was able to use UDP-galactose (UDP-Gal) to synthesize galactosylceramide with $\approx 10\,\%$ of the efficiency with which it utilizes UDP-Glc (Figure 1B). The ability of GCS to utilize UDP-Gal has also been recently demonstrated by Sprong and colleagues [26] in GCS-negative mouse cells transfected with human GCS.

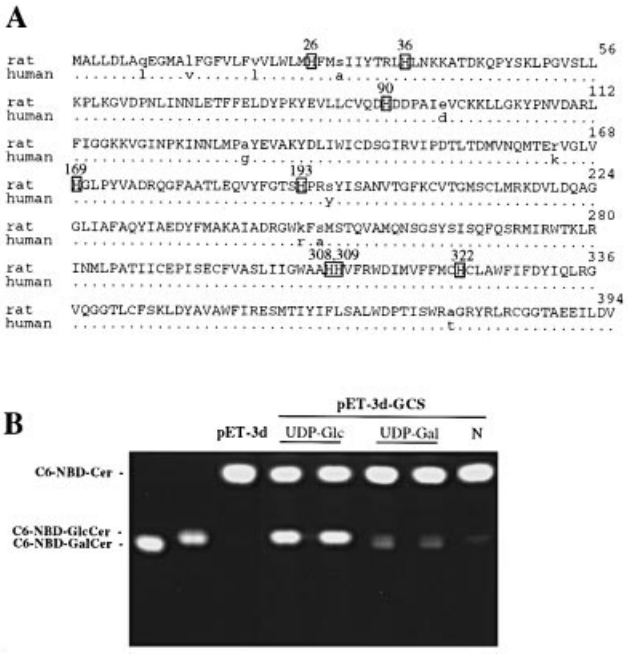

Figure 1 Cloning and expression of rat GCS

(**A**) Amino acid sequence of rat GCS compared with human GCS. Rat GCS cDNA was cloned from a rat brain cDNA library and by 5′-RACE of rat brain cDNA. cDNA sequences were aligned and overlapped and the amino acid sequence shown was predicted from the open reading frame. Histidine residues within rat GCS are boxed and their positions numbered. For human GCS (from [17]), only the amino acids which differ from the rat are shown. (**B**) Enzyme activity of rat GCS expressed in bacteria. The encoding region of rat GCS was assembled by restriction digest and ligation into the pET-3d vector and expressed in BL21-DE3 cells. GCS activity of bacterial lysates was measured with C_6-NBD-Cer as described [22] in the presence of 2.5 mM UDP-Glc or UDP-Gal, or with no UDP-hexose (N). Shown is a fluorescent image of a TLC plate demonstrating the formation of C_6-NBD-GlcCer and smaller amounts of C_6-NBD-GalCer by bacterially expressed GCS. Bacteria expressed with vector alone (pET-3d) show no activity.

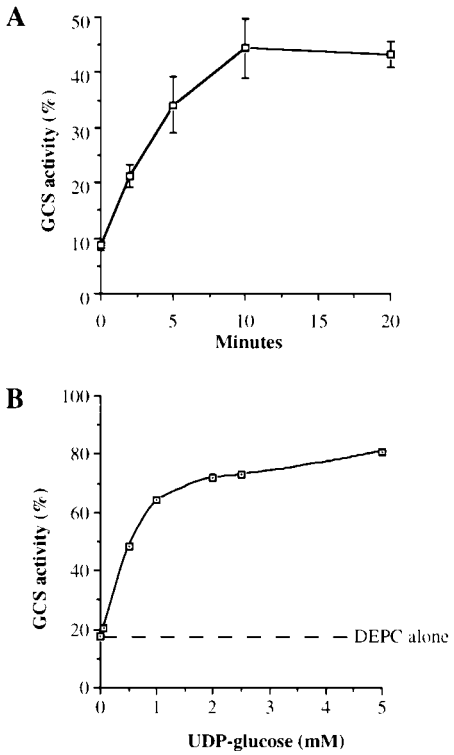

Figure 2 DEPC inhibition of recombinant GCS, reversibility by hydroxylamine and protection by UDP-Glc

Samples of GCS expressed in bacteria (equal to 100 μl of bacterial cultures at $D_{600} = 0.6$) were incubated with or without 0.1 mM DEPC for 10 min at room temperature. All samples were assayed for GCS activity as in Figure 1 and quantified by image analysis. Results are expressed as percentage of control activity without DEPC. (**A**) Reversibility of DEPC inhibition by hydroxylamine. Samples were treated with 0.5 M hydroxylamine for the times indicated after DEPC treatment. Values are means $\pm$ S.E.M. (**B**) Protection against DEPC inhibition by preincubation with the substrate UDP-Glc. Samples were incubated with UDP-Glc at room temperature for 15 min at room temperature prior to treating with DEPC and measuring GCS activity as described above. Values are means for duplicate experiments.

DEPC inhibition of GCS and protection by UDP-Glc

Preliminary studies showed that GCS in rat liver Golgi was strongly inhibited by tosylphenylalanylchloromethane (‘TPCK’) and DEPC (results not shown), suggesting a possible role for histidine residues [27–29] in GCS activity. Thus we tested the effects of DEPC on recombinant GCS. We found that recombinant GCS was inhibited $> 80\,\%$ by 0.1 mM DEPC (Figures 2A and 2B). Further, inhibition by DEPC was rapidly reversible by treatment with 0.5 M hydroxylamine (Figure 2A). Rapid reversal of DEPC-induced inhibition by hydroxylamine suggests strongly that DEPC inhibits GCS via alkylating histidine residues rather than other amino acid residues [29]. In addition, we found that GCS inhibition by DEPC was decreased in a dose-dependent manner by prior incubation with the GCS substrate UDP-Glc (Figure 2B). Preincubation with UDP-Gal (2.5 mM) or *N*-hexanoyl-D-*erythro*-sphingosine [C_6-ceramide (Matreya)] (up to 1.5 mM) had no significant protective effect against DEPC inhibition (results not shown). Taken together, these data suggest

Table 1 Enzyme activity, inhibition by DEPC and protection from DEPC inhibition by UDP-Glc of wild-type and mutant GCS proteins expressed in bacteria

GCS proteins were expressed in BL21 bacteria using the pET-3d vector as described in the Materials and methods section. GCS activity was measured in lysates of bacterial pellets using C_6-NBD-Cer as a substrate [22]. Values are means ± S.E.M. for three or more replicates.

GCS form expressed	Activity (%)*	Inhibition by DEPC (%)†	Protection by UDP-Glc (%)‡
Wild-type	100.0 ± 0.0	86.04 ± 4.9	48.6 ± 5.6
H26A	5.2 ± 0.4	nd§	nd
H26D	10.5 ± 1.5	nd	nd
H26N	44.5 ± 9.5	nd	nd
H26R	118.5 ± 10.4	93.6 ± 1.4	42.9 ± 10.2
H36A	103.2 ± 3.3	91.5 ± 2.5	46.6 ± 8.6
H90A	119.3 ± 12.7	81.4 ± 6.3	48.5 ± 12.7
H169A	26.4 ± 10.6	74.9 ± 11.0	56.3 ± 12.4
H193A	33.0 ± 7.3	12.5 ± 8.8	4.4 ± 2.7
H193D	0.0 ± 0.0	nd	nd
H193N	23.0 ± 4.9	14.4 ± 8.5	6.7 ± 3.6
H193R	0.0 ± 0.0	nd	nd
H308A	35.2 ± 4.3	79.5 ± 4.5	42.7 ± 4.7
H309A	69.1 ± 2.8	87.4 ± 3.7	56.5 ± 9.9
H308A,H309A	10.8 ± 3.8	nd	nd
H322A	3.8 ± 1.6	nd	nd
H322D	2.6 ± 1.0	nd	nd
H322N	49.8 ± 7.6	97.3 ± 1.7	40.0 ± 10.3
H322R	0.0 ± 0.0	nd	nd

* GCS enzyme activities are expressed as a percentage of wild-type activity normalized for relative levels of protein expression as measured by quantitative Western blotting; levels of expression were similar for wild-type and mutant proteins.

† Samples were pretreated with 0.1 mM DEPC for 10 min at room temperature prior to measurement of GCS activity; results are expressed as percentage inhibition relative to controls for each GCS form assayed without DEPC.

‡ Samples were preincubated with 1 mM UDP-Glc for 15 min before treating with DEPC and assaying GCS activity as described above; activities of samples treated with DEPC ± UDP-Glc were calculated as a percentage of control activity (i.e., for the same sample but without DEPC or UDP-Glc); the results shown are the differences between activities of DEPC-treated samples ± UDP-Glc (i.e., the extent of protection by UDP-Glc against DEPC-induced inhibition).

§ nd, not determined.

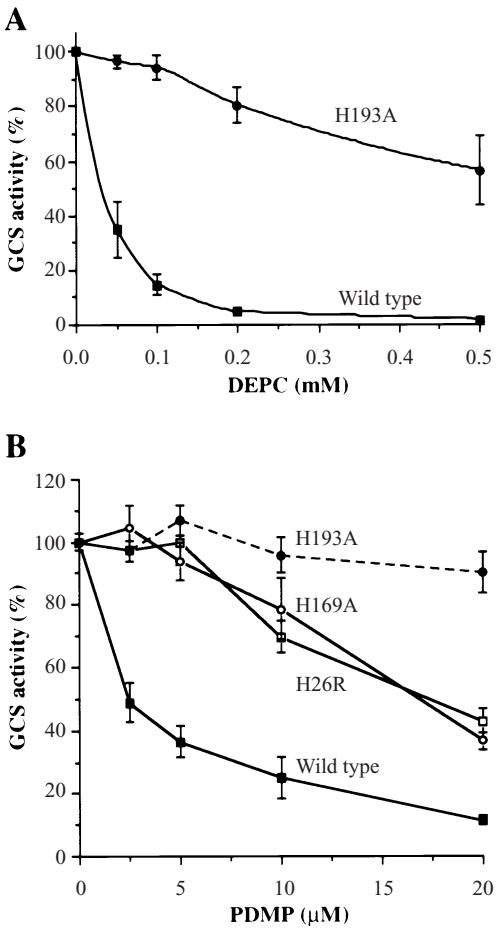

Figure 3 Comparison of histidine mutant and wild-type GCS inhibition by DEPC and PDMP

GCS activity of bacterial lysates were measured as in Figure 1, quantified by image analysis and expressed as percentage of the activity of the same sample without inhibitor. Results are means ± S.E.M.. (**A**) Relative insensitivity of H193A mutant to DEPC. Samples of H193A mutant and wild-type GCS were treated with DEPC as in Figure 2, except that the DEPC concentration was varied. (**B**) Altered PDMP-sensitivities of H193A, H169A and H26R mutant compared with wild-type GCS. Samples of mutant and wild-type GCS were preincubated with PDMP for 15 min at room temperature before assaying for activity.

that DEPC inhibits GCS via one or more histidine residues located at or near the UDP-Glc-binding site of GCS.

Expression and biochemical characterization of histidine-substituted GCS mutants

Given the potential importance of histidine residues in GCS activity, we expressed mutant proteins in which each of the eight histidine residues in GCS (see Figure 1A) was individually substituted with alanine. GCS activity of each mutant form was tested and the results compared with the level of activity in wild-type recombinant rat GCS. The results of these comparisons were normalized to GCS expression levels as measured by quantitative Western blots and are shown in Table 1. Alanine substitution of His^{26} and His^{322} yielded proteins with $< 6\%$ activity compared with wild-type GCS; however, substitution of His^{26} or His^{322} with asparagine restored $\approx 50\%$ activity, and substitution of His^{26} with arginine restored full activity (Table 1). These results suggest that residues 26 and 322 may be important for GCS catalysis or secondary structure, but that histidine resdiues are not specifically required at these locations for activity. A double mutant in which both His^{308} and His^{309} were substituted with alanine had only 10% of wild-type activity; however, individual mutants at these residues were more active (Table 1). It is possible that location of a histidine in this region is important, but that either His^{308} or His^{309} is sufficient to preserve activity. Substitution of His^{169} and His^{193} with alanine yielded proteins which were $\approx 30\%$ as active as the wild type enzyme (Table 1). Replacement of His^{193} with asparagine yielded an enzyme with decreased activity ($\approx 20\%$ of wild-type), and mutants in which this histidine was substituted with aspartate or arginine were devoid of activity (Table 1).

Next, we tested mutant GCS forms for DEPC inhibition and UDP-Glc protection from DEPC. We found that only the H193A ($His^{193} \rightarrow Ala$) and H193N ($His^{193} \rightarrow Asn$) mutants were insensitive to DEPC (Table 1 and Figure 3A). The H193A mutant was not significantly inhibited by 0.1 mM DEPC, a concentration which inhibits wild-type GCS by $> 85\%$ (Figure 3A). Even at 0.5 mM DEPC, which almost completely inactivates wild-type GCS activity, the H193A mutant was only inhibited by $\approx 40\%$ (Figure 3A). All other mutants remained inhibitable by DEPC, and this inhibition could be protected against by 1 mM

UDP-Glc (Table 1). These results indicate that His^{193} is the primary residue involved in DEPC inhibition of wild-type GCS.

Finally, we tested the sensitivity of each mutant to the GCS inhibitor PDMP. Surprisingly, the H193A mutant was almost completely insensitive to PDMP at levels up to 20 μM, a concentration which inhibits wild-type GCS by $\approx 90\%$ (Figure 3B). The H193N mutant was similarly insensitive to PDMP as the H193A mutant (results not shown). H26R ($His^{26} \rightarrow$ Arg) and H169A ($His^{169} \rightarrow$ Ala) mutants also showed decreased sensitivity to PDMP, although less than observed for the His^{193} forms (Figure 3B). All other histidine substitution mutants remained equally sensitive to PDMP as compared with the wild-type GCS (results not shown). These results suggest that His^{193} may play an important role in the binding of PDMP to GCS.

DISCUSSION

We have cloned the rat homologue of GCS and have shown that it is $\approx 95\%$ identical with human and mouse GCS at the amino acid level. Bacterially expressed GCS was highly active in lysates, found to be inhibitable by a histidine-modifying reagent, DEPC, and this inhibition could be decreased by preincubation with the GCS substrate, UDP-Glc. Site-directed mutagenesis of histidine residues within GCS showed that some amino acid substitutions caused partial loss of activity, but no histidine residues were absolutely required for GCS activity. Substitution at one residue, His^{193}, eliminated the sensitivity of the expressed protein to both DEPC and the GCS inhibitor PDMP. These results suggest that His^{193} resides within a UDP-Glc and PDMP-binding region and represent the first evidence toward the identification of substrate- or inhibitor-binding sites within GCS.

Recombinant GCS was inhibited by low concentrations of DEPC, confirming preliminary observations made with rat liver Golgi GCS. DEPC inhibition was rapidly reversible with hydroxylamine and diminished by preincubation with UDP-Glc, suggesting that the DEPC acts on histidine residue(s) within the UDP-Glc-binding region of GCS. Biochemical studies of mutants in which each of the eight histidine residues within GCS were replaced with other amino acids further indicated that His^{193} is the target of DEPC-induced inactivation.

Investigation of the PDMP-sensitivity of histidine mutants of GCS demonstrated that H193A, and to a lesser extent H169 and H26R, were relatively insensitive to PDMP compared with the wild-type enzyme. PDMP is believed to act as a GlcCer (or UDP-Glc/Cer transition state) analogue in which the glucopyranose ring is replaced by a morpholine ring and the long alkene chain of the sphingosine backbone is replaced with a phenyl group [5,30]. The finding that the His^{193} mutants are insensitive to PDMP suggests that this residue may interact with PDMP as well as the substrate, UDP-Glc. The reduced effectiveness of PDMP in the H169A mutant suggests that this residue may also reside within a PDMP and/or substrate-binding region. The decreased sensitivity of the H26R mutant to PDMP is unexpected, because this residue occurs within the putative transmembrane domain of GCS. However, other observations, including the low activity in the H26A mutant ($\approx 4\%$ of wild type; Table 1), and the total loss of activity when the N-terminal portion of GCS, including the transmembrane region, is deleted (K. Wu, D. M. Marks and R. E. Pagano, unpublished work), suggest that this portion of the protein is important for GCS activity as well. Since one substrate of GCS, ceramide, resides in the membrane, the transmembrane region could possibly play a role in substrate interaction.

Our data showing that GCS His^{193} mutants were resistant to DEPC, which acts on a UDP-Glc-protectable site in GCS, and to PDMP, a proposed active-site-directed inhibitor of GCS [5,8,30], provide strong evidence that His^{193} is within a substrate-binding region of GCS. However, thus far our studies do not discriminate between the alternative possibilities that: (1) UDP-Glc binds directly to His^{193}, thus protecting against DEPC, or (2) UDP-Glc protects against DEPC by steric hindrance after binding to a different residue positioned near His^{193} within the substrate-binding region of GCS. These alternatives can only be resolved by future studies with the purified enzyme. For example, the binding of purified recombinant GCS mutant proteins to radiolabelled UDP-Glc could be measured directly, or a UDP-Glc photoaffinity probe could be used to label UDP-Glc-binding residues of purified GCS.

Our findings are a first step in the identification of the substrate- and inhibitor-binding sites of GCS. Inhibition of GCS has been proposed as a target for chemotherapeutic drugs for several applications, including treatment of glucosphingolipidoses, dermatological disorders, infections and cancer [4–6,31]. Among the existing inhibitors for GCS, PDMP and similar compounds have the limitations of rapid degradation and neurological side effects, whereas *N*-butyldeoxynojirimycin inhibits α-glycosidase I and II as well as GCS [5,32]. It is hoped that our present findings coupled with future studies will allow an understanding of the GCS catalytic site and eventually the design of new inhibitors with greater tolerability, stability and specificity for evaluation as therapeutic agents.

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