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Ceramide UDPgalactosyltransferase from myelinating rat brain: Purification, cloning, and expression

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ABSTRACT **Cerebrosides and sulfatides are major glycosphingolipids of the lipid bilayer of the myelin sheath assembled by oligodendrocytes and Schwann cells during myelination. Cerebrosides are synthesized by ceramide UDPgalactosyltransferase [CGT; 2-hydroxyacylsphingosine 1-β-galactosyltransferase; UDPgalactose:2-(2-hydroxyacyl)sphingosine 1-β-D-galactosyltransferase, EC 2.4.1.45] with UDPgalactose and ceramide as substrates. Here we describe a purification method from microsomes of myelinating rat brains that includes ion exchange, dye ligand, and lectin affinity chromatography. The enzyme was identified as a 64-kDa high-mannose glycoprotein. A CGT-specific cDNA clone was isolated from a rat brain cDNA library using CGT oligonucleotides derived from peptide sequences. The cDNA insert encodes a polypeptide of 541 amino acid residues with a molecular weight of 61,126. The polypeptide has three putative glycosylation sites and one hydrophobic domain at the C terminus. A 20-residue N-terminal signal sequence is lost during cotranslational translocation. Northern blot analysis demonstrates that CGT expression is restricted to brain tissue and is time dependent, correlating with myelin basic protein expression. *In situ* hybridization reveals that CGT expression is restricted to the oligodendrocyte-containing cell layers of cerebrum and cerebellum, which also express myelin basic protein. The amino acid sequence of CGT shows significant homology to mammalian UDPglucuronyltransferases, which suggests a common evolutionary origin of these enzymes.**

The myelin sheath of the central nervous system (CNS) and peripheral nervous system (PNS) is a highly ordered multilayer membrane system consisting of 70–80% lipids and 20–30% proteins. During the rather short period of myelination, oligodendrocytes of the CNS and Schwann cells of the PNS synthesize and assemble these components into the myelin membrane (1, 2). In the CNS, cerebrosides and sulfatides are highly enriched in white matter (3) and contribute to the insulating properties of the myelin membrane. The main pathways for biosynthesis of these complex lipids are well known (4), and the corresponding enzymatic activities responsible for their synthesis increase rapidly during myelination and decrease markedly when myelination ceases (5, 6). Ceramide UDPgalactosyltransferase [CGT; 2-hydroxyacylsphingosine 1-β-galactosyltransferase; UDPgalactose:2-(2-hydroxyacyl)sphingosine 1-β-D-galactosyltransferase, EC 2.4.1.45] is the key enzyme in biosynthesis of cerebrosides (7) and catalyzes the transfer of galactose from UDPgalactose to ceramide (8). The enzyme is found in rat brain microsomes (9), Golgi-enriched fractions (10), and myelin (11). Numerous attempts have been made to isolate and purify this membrane-bound enzyme (12–15).

Here we report purification of CGT from rat brain microsomes, its characterization at the cDNA and protein levels, and its expression in myelinating brain.† Northern blot hybridization indicates that CGT is expressed as a brain-specific transcript of 3.2 kb and is time dependently expressed correlating with the process of myelination. Comparative *in situ* hybridization with myelin basic protein (MBP) mRNA demonstrates that the CGT transcript is expressed in the same cellular layers that contain mostly oligodendrocytes.

MATERIALS AND METHODS

Purification of CGT. Microsomes of 100 rat brains (20-day-old Wistar rats) were prepared as described (15) and extracted with 400 ml of 50 mM Tris·HCl, pH 8.0/0.5% Triton X-100, reduced (Aldrich)/0.1% 2-mercaptoethanol/20% (vol/vol) glycerol (TMG buffer). The suspension was centrifuged at 107,000 × *g* for 1 hr and the supernatant was stirred with 50 g of DE-52 cellulose (Whatman) previously equilibrated with TMG buffer. The protein solution was separated from DE-52 cellulose with a sintered glass filter and was immediately passed over a Cibacron blue Sepharose column (Sigma; bed vol, 75 ml). The affinity column was washed with 150 ml of TMG containing 0.1 M KSCN (Fluka), and the enzyme was eluted and reconstituted as described (15). After dialysis against TMG buffer, the enzyme solution was loaded on a lentil lectin-Sepharose column (Sigma; bed vol, 25 ml). The column was washed with TMG containing 0.5 M NaCl and the enzyme was desorbed with 15 mM methyl glucoside in TMG/0.05% phosphatidylcholine. The purity of the enzyme was verified by silver-stained (16) SDS/PAGE.

Enzyme Assay. Enzymatic activity was determined as described (15) using synthetic D-2-hydroxyoctanoylsphingosine and UDP[^{14}C]galactose (Amersham).

Deglycosylation. Purified CGT (5 μg) was incubated for 12 hr at 37°C in 50 μl of incubation buffer (100 mM sodium phosphate, pH 7.5/0.1% SDS/0.5% Triton X-100) with 0.2 unit of Endoglycosidase F (Boehringer Mannheim).

Determination of Glycan Structure with Specific Lectins. Purified CGT (5 μg in each lane) was subjected to SDS/PAGE and subsequently transferred to nitrocellulose by the semidry blot technique (17). After blocking with 2% bovine serum albumin the nitrocellulose was cut into strips and treated with lectins according to the instructions of the manufacturer (Boehringer Mannheim).

Protein Sequencing. Purified CGT (50 μg) was cleaved with 1 μg of trypsin (Boehringer) at 30°C in 50 mM Tris·HCl, pH 8.0/0.01% SDS for 6 hr. The resulting peptide mixture was separated by HPLC on a C_{18} reversed-phase column and sequenced by automated Edman degradation using a model 477A sequencer (Applied Biosystems).

Cloning of CGT. The degenerated oligonucleotides (C/T)TI CCI CA(A/G) AA(A/G) GTI ATI TGG (C/A)G and C(G/T) (A/G)TA (G/A)CT IGG (A/G)TT (A/G)TT IAT IAC derived

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Abbreviations: CGT, ceramide UDPgalactosyltransferase; MBP, myelin basic protein.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. 21698).

from the peptide sequences Leu-Pro-Gln-Lys-Val-Ile-Trp-Arg and Val-Ile-Asn-Asn-Pro-Ser-Tyr-Arg, respectively, were synthesized for PCR amplification (18) of a 327-bp fragment from a λgt10 rat brain library (19). The PCR fragment was ^{32}P labeled by the random-priming method and used for screening the same library. The 2.6-kb insert of 1 of the 10 different clones isolated was subcloned into the *Eco*RI site of pGEM3Z (Promega) and sequenced by the dideoxynucleotide chain-termination method (20) applying the T7 sequencing kit (Pharmacia).

Northern Blot Analysis. Poly(A)$^+$ RNA (21) (3 μg) from cerebrum, cerebellum, liver, kidney, heart, and skeletal muscle of 20-day-old Wistar rats or total RNA (10 μg) from rat brain prenatal day −5; postnatal days 5, 10, 15, 20, and 25; and adult were electrophoresed on a 1% agarose gel containing formaldehyde, transferred to a nylon membrane (NEN), and probed with the ^{32}P-labeled, randomly primed 327-bp PCR fragment (CGT) or an 800-bp *Eco*RI/*Hin*dIII fragment (MBP) (22).

***In Situ* Hybridization.** *In vitro* transcription of the 327-bp cDNA fragment of CGT or a 612-bp fragment from MBP in pGEM3Z by using the SP6 or T7 RNA polymerase (BRL) and uridine 5′-[α-[^{35}S]thio]triphosphate (Amersham) yielded ^{35}S-labeled sense and antisense RNA probes. CGT and MBP antisense (or the corresponding sense probes as control) were hybridized to 6-μm cryosections of 20-day-old rat brains previously fixed in 4% paraformaldehyde and processed for autoradiography (23).

RESULTS

Purification and Cloning of CGT. Based on the results of Neskovic *et al.* (15), we developed a simple and reproducible three-step purification procedure for isolation of CGT from microsomes of myelinating rat brain. The sequential application of ion exchange, dye ligand, and lectin affinity chromatography results in a homogeneous enzyme preparation (Table 1). The purification method yielded a single protein band with an apparent molecular mass of 64 kDa in SDS/PAGE and a final specific activity of 13,000 units/mg. Total enrichment from microsomes was 1530-fold. Deglycosylation with Endoglycosidase F decreased the molecular mass to 58 kDa as demonstrated by SDS/PAGE (Fig. 1). Analysis of the glycan structure using specific lectins revealed that CGT is a high-mannose glycoprotein. The N-terminal sequence of the purified enzyme and sequences of nine tryptic peptides were determined by Edman degradation (sequences are underlined in Fig. 2). Three peptide sequences showed significant homologies to mammalian UDPglucuronyltransferases. Degenerated oligonucleotides corresponding to these peptide sequences were used for PCR amplification of a rat brain-derived cDNA library yielding a 327-bp DNA fragment. The radiolabeled PCR product was used as a hybridization probe for screening a cDNA library of myelinating rat brains. A 2.6-kb cDNA clone was isolated from a λgt10 library. Fig. 2 shows the nucleotide and deduced amino acid sequence of CGT.

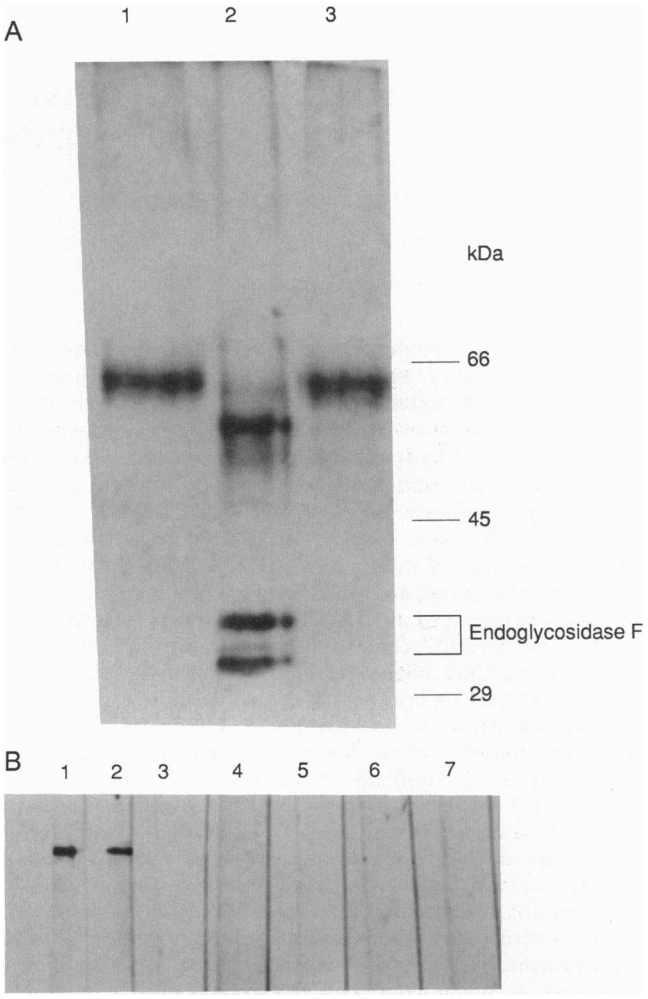

FIG. 1. Purification and characterization of CGT. (*A*) SDS/10% PAGE. Lanes: 1 and 3, purified CGT; 2, purified CGT, deglycosylated with Endoglycosidase F. (*B*) Electrotransfer of purified CGT to nitrocellulose by the blot technique, staining with different lectin–digoxigenin conjugates. Carbohydrate specificities of the respective lectins are in parentheses. Agglutin lanes: 1, concanavalin A (recognizes mannose-containing carbohydrate structures); 2, *Galanthus nivalis* [recognizes terminal mannose (α1→3), (α1→6), or (α1→2) linked to mannose]; 3, *Datura stramonium* [recognizes Gal(β1→4)-GlcNAc]; 4, *Maackia amurensis* [recognizes sialic acid (α2→3)Gal]; 5, *Sambucus nigra* [recognizes sialic acid (α2→6)Gal]; 6, peanut [recognizes the core disaccharide Gal(β1→3)GalNAc]; 7, wheat germ (recognizes GlcNAc-containing carbohydrate structures).

A computer-aided search of available data bases revealed a significant sequence similarity of CGT to a group of enzymes that catalyze the transfer from glucose or glucuronic acid from the corresponding UDP carbohydrates to membrane-bound lipophilic substrates. Amino acid comparison between CGT and UDPglucuronyltransferase sequences revealed 37% identity. Lower, but still significant, homologies

Table 1. Purification steps of UDPgalactosyl ceramide galactosyltransferase (substrate, D-2-hydroxyoctanoylsphingosine)

Purification step	Vol, ml	Total enzyme activity, nmol/h	Total protein, mg	Specific activity, nmol per h per mg of protein	Enrichment factor	Yield, %
Microsomes	100	13,600	1600	8.5	1	100
Microsomal extract	500	9,016	980	9.2	1.1	66.3
DE-52 cellulose	520	9,256	520	17.8	2.1	68.1
Blue-Sepharose	180	4,350	17.2	252.9	29.8	31.2
Lentil lectin-Sepharose	90	1,040	0.08	13,000	1530	7.6

FIG. 2. Nucleotide sequence of CGT cDNA and deduced primary structure. Position 1 refers to the first nucleotide and amino acid residue of the predicted CGT coding region. N-Glycosylation site consensus motifs are double-underlined. The C-terminal hydrophobic amino acid sequence is marked with a dotted line. Amino acid sequences determined by Edman degradation of purified peptide fragments released by tryptic cleavage of the purified CGT protein are underlined.

(22% identical amino acids) exist between CGT and ecdysteroid UDPglucosyltransferase from baculovirus (24) and a flavanol UDPglucosyltransferase from plants (25). No similarity to the group of UDPgalactosyltransferases responsible for transfer of galactose to other carbohydrate residues could be detected.

Analysis of the cDNA-deduced amino acid sequence reveals the presence of an N-terminal signal peptide of 20 amino

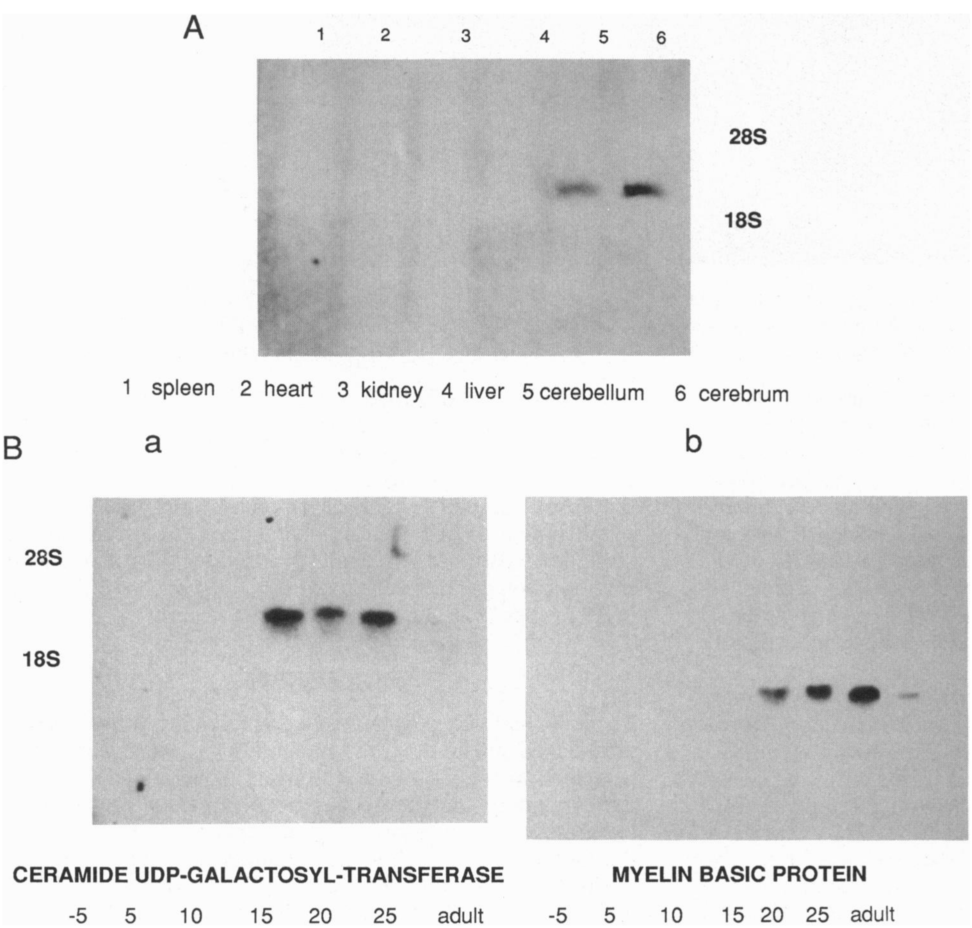

FIG. 3. CGT expression and tissue specificity. (*A*) Northern blot analysis of RNA of different rat tissues. Positions of 2- and 5-kb markers are indicated. Position of CGT mRNA is a 3.2 kb. RNA (10 μg) of spleen (lane 1), heart (lane 2), kidney (lane 3), liver (lane 4), cerebellum (lane 5), and cerebrum (lane 6). (*B*) Time-regulated expression of CGT (*a*) and MBP (*b*) in rat brain. Total RNA of rat brain of prenatal day −5; postnatal days 5, 10, 15, 20, and 25; and adult was separated by an agarose/formaldehyde gel for hybridization and autoradiography.

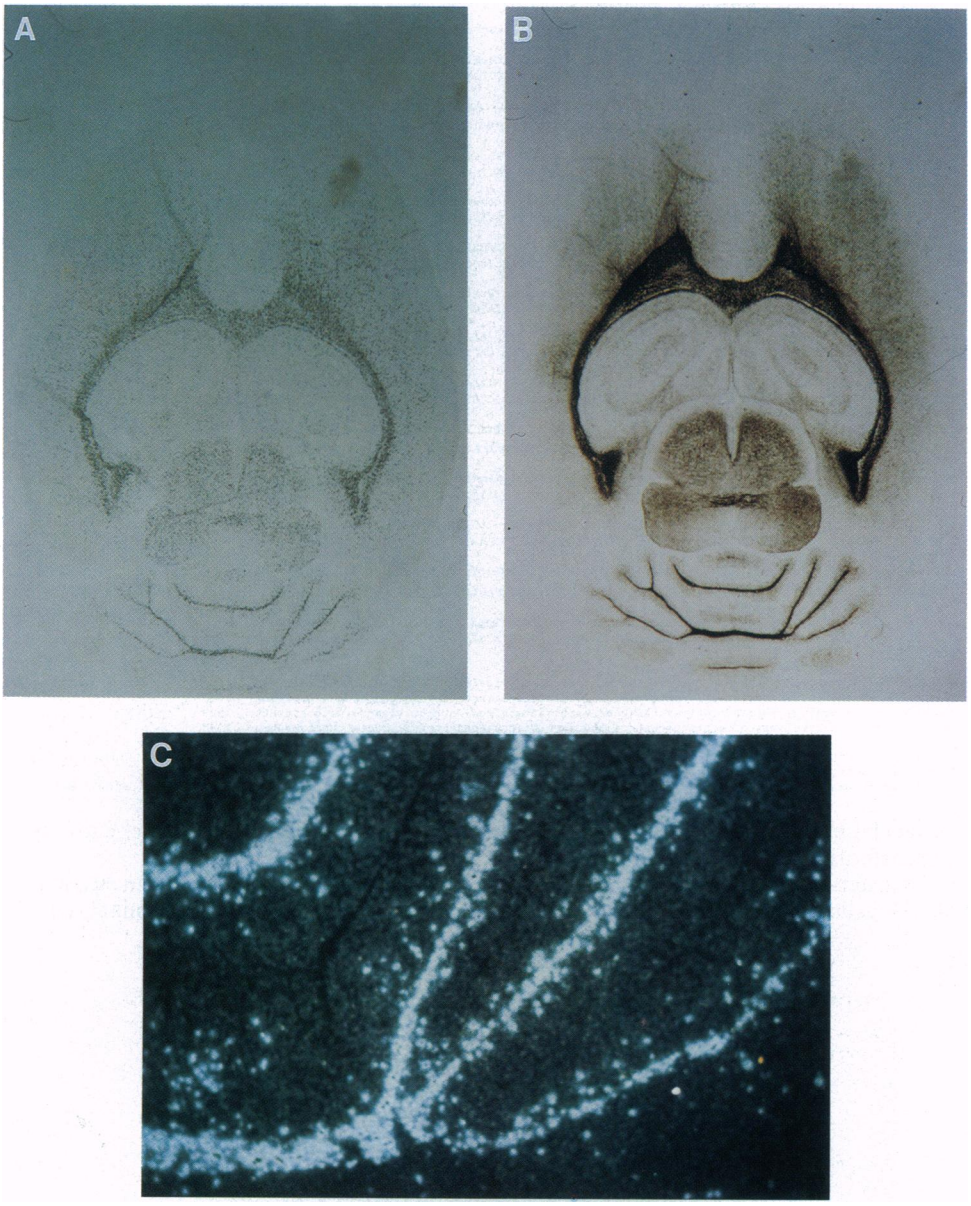

FIG. 4. Comparative *in situ* hybridization analysis of CGT and MBP expression in rat brain. Distribution of CGT and MBP messages in rat brain was examined by *in situ* hybridization of horizontal 6-μm cryosections using CGT antisense complementary RNA (*A* and *C*) and MBP antisense complementary RNA (*B*). [*A* and *B*, bright-field image (×5.6); *C*, dark-field image (×35) of enlarged section of cerebellum of *A*.]

acids that precede the N terminus of the mature sequence as determined by microsequencing. A second hydrophobic segment of 20 amino acid residues is detectable near the C terminus. The mature protein starts with the previously determined N-terminal sequence and contains 521 amino acids with a molecular weight of 58,838, which is in good agreement with the size estimated by SDS/PAGE of the deglycosylated, purified protein (Fig. 1). The protein sequence contains three putative sites for N-glycosylation at positions 78, 333, and 442 in the predicted primary structure (double-underlined in Fig. 2).

Localization of CGT mRNA Expression. The distribution of CGT mRNA in different rat tissues was examined by Northern blot analysis (Fig. 3). The results demonstrate that CGT is specifically expressed in brain as a transcript of 3.2 kb. Comparative *in situ* hybridization of frozen rat brain sections using *in vitro* transcribed antisense CGT RNA and MBP RNA revealed a common pattern of expression in brain. The hybridization signal is restricted to white matter areas in brain (Fig. 4). Bright-field microscopy at higher magnification illustrates the localization of CGT RNA in the white matter of cerebellum. Control experiments using the respective sense RNA revealed no significant accumulation of silver grains (data not shown).

Expression of CGT in rat brain is time regulated and parallels that of MBP during myelination. This was demonstrated by Northern blot analysis of total RNA isolated from rat brain tissue at different ages using the respective CGT and MBP probes (Fig. 3).

DISCUSSION

The main pathway of galactosylceramide biosynthesis is well established and involves the transfer of galactose from UDP-galactose to the C-1 hydroxyl group of ceramide (26). The corresponding transferase has been proposed to be firmly integrated in the membranes of endoplasmic reticulum or of the Golgi apparatus. Several attempts have been made to purify the CGT, with limited success. Neskovic *et al.* (15) describe the isolation and identification of a 53-kDa protein

as CGT. Their purification method resulted in only a 65-fold enrichment of enzymatic activity from microsomes with a specific activity of 700 units/mg after the last purification step. A Coomassie-stained SDS/polyacrylamide gel showed three prominent protein bands migrating at 20, 53, and 68 kDa. Antibodies raised against the 53-kDa protein recognized the protein in Western blot analysis but were unable to immunoprecipitate the enzymatic activity.

We isolated CGT to homogeneity by an improved three-step purification method. The enzyme was isolated as a 64-kDa glycoprotein with a specific enzymatic activity of 13,000 units/mg. Starting from rat brain microsomes, a 1530-fold purification was achieved. Using the lectin blot technique, the enzyme was identified as a high-mannose glycoprotein, suggesting that CGT is an enzyme residing in the endoplasmic reticulum like the homologous glucuronyltransferases and other lipid-synthesizing enzymes [e.g., HMG-CoA reductase (27)].

Several peptide sequences derived from proteolytic fragments of the purified enzyme allowed identification of cDNA clones encoding CGT. An open reading frame of 1740 bp was identified in the cloned cDNA. As the previously determined N-terminal sequence of the mature CGT protein is located at base position 177 in the 2.6-kb cDNA clone, we suppose that the methionine codon at base position 117 serves as translation start for CGT and that the N-terminal peptide of 20 amino acid residues matches a typical signal sequence required for the translocation of newly synthesized proteins through the endoplasmic reticulum membrane (28). This is supported by the homologies to the UDPglucuronyltransferases. They also contain N-terminal signal peptides, which are removed by a peptidase upon translational translocation. The methionine at position 117 (Fig. 2) matches the properties of a typical Kozak sequence (29).

The sequence similarity between CGT and a group of UDPglucuronyltransferases suggests a common evolutionary origin of the brain-specific CGT and the detoxification enzymes of the liver. Both groups of enzymes catalyze glycosylation of lipophilic compounds (30, 31). Analysis of the hydrophobicity (32) and hydrophobic moment (33) of the amino acid sequences shows that these proteins share a common structure with CGT.

The glycan structure of CGT was identified to be of the high-mannose type by its lectin specificity. This strongly suggests that CGT is located in the endoplasmic reticulum of oligodendrocytes. The presence and the processing of the signal sequence suggest a localization of the mature protein in the lumen of endoplasmic reticulum. CGT has only one C-terminal hydrophobic segment at positions 456–476 serving as membrane anchor. Three putative sites for N-glycosylation are present at the presumably luminally oriented N-terminal part at positions Asn-78, Asn-333, and Asn-442 of the CGT protein.

Northern blot analysis demonstrates that CGT is a brain-specific 3.2-kb transcript. The cellular location in rat brain was documented by comparative *in situ* hybridization with MBP mRNA. CGT mRNA is associated with white matter-containing structures and it shows the same cellular distribution as MBP. The time-regulated expression of CGT in rat brain was investigated by Northern blot hybridization of total RNA isolated at prenatal days −5; postnatal days 5, 10, 15, 20, and 25; and adult brain. Expression of CGT correlates clearly with myelination and agrees with previously determined enzymatic activities of CGT during this period (5, 6).

The CGT described here is involved in synthesis of a major lipid class of myelin. It will be a valuable marker for studies on the synthesis and assembly of the myelin membrane and special forms of dysmyelinoses.

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