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UDP galactose:ceramide galactosyltransferase and glutamate/aspartate transporter

Copurification, separation and characterization of the two glycoproteins

Stephan SCHULTE and Wilhelm STOFFEL

Institute of Biochemistry, Medical Faculty, University of Cologne, Germany

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The oligodendrocyte-specific UDP-galactose:ceramide galactosyltransferase (CGT) is the key enzyme involved in the biosynthesis of the oligodendrocyte- and myelin-specific cerebrosides. The galactosyltransferase was isolated and purified to homogeneity from Triton-X-100-solubilized rat brain microsomes by ion exchange, dye ligand and lectin affinity chromatography as a 64-kDa protein homogenous in SDS/PAGE. It copurified with the brain-specific Na^+-dependent high-affinity L-glutamate/aspartate neurotransmitter transporter (GLAST-1) of the central nervous system. Differential lentil lectin affinity chromatography led to the separation of two glycoproteins with very similar physical properties. CGT was identified as a high-mannose glycoprotein and GLAST-1 as a hybrid glycoprotein, both with a molecular mass of 64 kDa. Deglycosylation reduced the molecular mass of the two proteins to 59 kDa. A 70-kDa isoform of GLAST-1 was isolated from whole brain by wheat germ lectin affinity chromatography. Deglycosylation again reduced the molecular mass to 59 kDa. Therefore the 70-kDa isoform differs only in the degree of glycosylation from the 64-kDa GLAST-1 isoform. The two isoproteins form homodimers of 130 and 140 kDa, respectively. They were isolated and characterized with protein-chemical and immunological methods. Oligonucleotides derived from respective peptide sequences of CGT and GLAST-1 were successfully applied to the cloning of CGT and the first high-affinity glutamate neurotransmitter transporter (GLAST-1) in glia of the central nervous system as well.

Keywords: ceramide galactosyltransferase; myelination; L-glutamate transporter; neurotransmitter; glycoprotein separation.

The myelin membrane of the central and peripheral nervous systems contains a high ratio of lipid/protein (4:1) and is particularly rich in glycosphingolipids and cholesterol. Cerebrosides (galactosylceramide) and sulfatides are specific lipid components of myelin. Unravelling the assembly of the specific protein and lipid constituents by oligodendrocytes to form the myelin membrane of axons of the central nervous system requires information about key enzymes of the biosynthesis of myelin lipids, none of which has so far been characterized on the molecular level.

We report here the isolation and purification of the oligodendrocyte-specific cerebroside-synthesizing enzyme UDP-galactose:ceramide galactosyltransferase (CGT) from myelinating rat brain and of 64-kDa and 70-kDa isoforms of the Na^+-specific high-affinity L-glutamate transporter (GLAST-1).

Differential lentil lectin affinity chromatography separated two glycoproteins with molecular mass 64 kDa from the protein extract of brain microsomes of myelinating rats. CGT bound to the lentil lectin column with a low affinity and the 64-kDa isoform of GLAST-1 with higher affinity.

The purification steps yielded a single protein band of CGT homogenous in SDS/PAGE with an apparent molecular mass of 64 kDa and a specific enzyme activity of 13 kU. Deglycosylation with glycopeptidase F reduced the molecular mass to that of the 59-kDa core protein. The N-terminal sequence of CGT was determined by Edman degradation. Tryptic digestion of CGT, separation and sequence analysis yielded eight peptides suitable for oligonucleotide screening of rat brain cDNA libraries. This led to the isolation of cDNA clones which encoded the UDP galactose:ceramide galactosyltransferase.

In addition, the first L-glutamate neurotransmitter transporter (GLAST-1), also a glycoprotein, was discovered to copurify with the transferase due to its very similar physicochemical properties (Schulte and Stoffel, 1993). CGT is a high-mannose glycoprotein whereas GLAST-1 is a hybrid-type glycoprotein. We have also isolated the 70-kDa isoform of GLAST-1 in abundant amounts from whole rat brain by wheat germ lectin affinity chromatography. This isoform is the mature L-glutamate transporter glycoprotein. Both the 64-kDa and 70-kDa isoforms of GLAST-1 differ in the complexity of their glycan structure. They form stable homodimers.

In previous reports about the isolation and characterization of UDP-galactose:ceramide galactosyltransferase from rat brain a molecular mass of 53 kDa protein was assigned to this enzyme (Neskovic et al., 1974, 1981). These data are at variance with

Correspondence to W. Stoffel, Institut für Biochemie, Medizinische Fakultät, Universität zu Köln, Joseph-Stelzmann-Str. 52, D-50931 Köln, Germany

Abbreviations. CGT, UDP galactose:ceramide galactosyltransferase; GLAST-1, high-affinity Na^+-specific L-glutamate/L-aspartate transporter; NaCl/P_i, phosphate-buffered saline containing 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.2 g KH_2PO_4, in 1 l adjusted to 7.2; ORF, open reading frame.

Enzymes. UDP galactose:ceramide galactosyltransferase (EC 2.4.1.45); glycopeptidase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase (EC 3.5.1.52); Lys C, lysyl endopeptidase (EC 3.4.21.50); Glu C, glutamyl endopeptidase (EC 3.4.21.19).

our results. We have characterized and identified this 53-kDa protein unambiguously as β-tubulin.

MATERIAL AND METHODS

Reagents. Keyhole limpet hemocyanin, bovine serum albumin, 2-mercaptoethanol, methyl β-mannoside, *N*-acetylglucosamine, Cibachron blue Sepharose, lentil lectin Sepharose and wheat germ agarose were purchased from Sigma. DEAE-cellulose (DE-52) was obtained from Whatman, Triton X-100 from Serva, potassium thiocyanate and glycerol from Fluka. The glycan differentiation kit, glycopeptidase F, Lys-C and Glu-C were purchased from Boehringer, Mannheim.

Isolation and purification of CGT and the glycosylation intermediate of GLAST-1. All purification steps were performed at 4°C. 100 brains of 20-day-old Wistar rats were homogenized with a Potter-Elvehjem homogenizer in 1000 ml 0.32 M sucrose. The homogenate was centrifuged at 15000 *g* for 20 min and the supernatant decanted. The pellet was resuspended in 500 ml 0.32 M sucrose and again centrifuged. The combined supernatants were centrifuged at 105000 *g* for 60 min. The microsomal pellet was resuspended and stirred for 30 min in buffer A (50 mM Tris pH 8.0, 20% glycerol, 10 mM 2-mercaptoethanol and 0.5% Triton X-100), then centrifuged at 105000 *g* for 60 min. The pellet was discarded. The final protein concentration of the supernatant was adjusted to 5 mg/ml. The supernatant was added to a 150-ml batch of DEAE-cellulose previously equilibrated with buffer A for 15 min. The suspension was separated from the DEAE-cellulose over a fritted glass funnel and the filtrate immediately passed over a 75-ml column of Cibachron blue Sepharose. The column was washed with 0.1 M KSCN in buffer A and eluted with 0.5 M KSCN in buffer A. The eluate (200 ml) was dialysed against 2 l buffer A and applied to a 25-ml lentil lectin column. After washing with 10 vol. 0.5 M NaCl in buffer A, CGT was eluted with 15 mM methyl glucoside in 60 ml buffer A and the glycosylation intermediate of GLAST-1 with 500 mM methyl glucoside in 60 ml buffer A.

Isolation of the mature form of GLAST-1. 50 brains of adult Wistar rats were homogenized with a Waring blendor in 400 ml buffer A and stirred for 30 min. The homogenate was centrifuged at 48000 *g* for 30 min and the pellet discarded. The DEAE-cellulose and the Cibachron blue Sepharose steps were the same as described above. The dialysed eluate from the Cibachron blue Sepharose (100 ml) was applied to a 10-ml wheat germ agarose column. The column was washed with 10 vol. 0.5 M NaCl in buffer A and the mature form of GLAST-1 eluted with 0.1 M *N*-acetylglucosamine in buffer A.

SDS/PAGE was performed according to Laemmli (1971) on 10% slab gels and for peptides according to the procedure described by Schägger and von Jagow (1987). Protein concentration was estimated by the method of Sedmak and Grossberg (1977) with BSA as standard. The glycan structure of GLAST-1 was determined with the glycan differentiation kit from Boehringer, Mannheim.

***Immunological* methods.** The following peptides were synthesized: GLAST-1-specific peptide: Lys-Gly-Thr-Lys-Glu-Asn-Met-Tyr-Arg-Glu-Gly-Lys-Ile-Val-Gln-Val-Thr-Val-Ala-Ala; CGT-specific peptides: Asn-Thr-Lys-Leu-Ile-Glu-Trp-Leu-Pro-Gln-Asn-Asp-Leu-Leu-Gly-His-Ser-Asn-Ile-Arg and Gly-Asp-His-Tyr-Asp-Thr-Met-Thr-Arg-Val-Gln-Ala-Lys-Gly-Met-Gly-Ile-Leu. For conjugation, 5 mg keyhole limpet hemocyanin was incubated at room temperature with 25 mg synthetic peptide in the presence of 25 mg ethyl(3-dimethylaminopropyl)-carbodiimide for 1 h. The conjugate was extensively dialysed against NaCl/P_i. For immunization 2 mg conjugate in 200 µl NaCl/P was mixed with 200 µl complete Freund's adjuvant and injected intraperitoneally into rabbits. Rabbits were boostered up to four times with 2 mg of the conjugate in incomplete Freund's adjuvant. Antibodies were affinity-purified from the antiserum. Peptides (25 mg) were coupled to BrCN-activated Sepharose according to the manufacturer's instructions. Separate antibodies from the antiserum were eluted from 4-ml columns of the immobilized peptides with 0.1 M glycine pH 2.8 and immediately neutralized with 2 M Tris/HCl pH 9.0.

For Western blot analysis the proteins were electrophoretically transferred from SDS/polyacrylamide gels to nitrocellulose membrane as described by Towbin et al. (1979). The nitrocellulose strips were blocked overnight with 3% BSA in Tris-buffered saline and subsequently incubated with the respective peptide antibody overnight. After extensive washing with Tris-buffered saline the strip was incubated with the anti-IgG–rabbit-alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolylphosphate, and nitrotetrazolium as substrates.

RESULTS

Separation and purification of CGT and GLAST-1. During the purification of CGT, we obtained a protein fraction apparently homogenous in SDS/PAGE with a molecular mass of 64 kDa. Differential lentil lectin affinity chromatography (Gerard, 1990; Lis and Sharon, 1986; Lotan and Nicolson, 1979; Merkle and Cummings, 1987; Osawa and Tsuji, 1987) revealed that another glycoprotein with an identical molecular mass of 64 kDa was copurified. CGT was desorbed from the affinity column with 15 mM methyl glucoside (Schulte and Stoffel, 1993) whereas the unknown glycoprotein was eluted with 500 mM methyl glucoside. This glycoprotein, shown to be the first brain-specific high-affinity L-glutamate transporter (GLAST-1), was purified to homogeneity and subsequently characterized by molecular biological techniques (Storck et al., 1992). We exploited the different subcellular location of the two proteins for their physical separation. CGT, a lipid-synthesizing enzyme, should reside in the smooth endoplasmatic reticulum. Typical proteins resident in the endoplasmic reticulum (e.g. β-hydroxy-β-methyl glutaryl-coenzyme A reductase and ribophorin (Atkinson and Lee, 1984; Liscum et al., 1983) are not exposed to glycosyltransferases located in the *cis*, *medial* or *trans* Golgi cisternae and should therefore possess an Asn-linked high-mannose-type glycan structure (Hirschberg and Snider, 1987). In addition CGT, like others, might have a typical C-terminal endoplasmic reticulum membrane retention signal. GLAST-1, on the other hand, is a neurotransmitter transporter responsible for the controlled uptake of the excitatory neurotransmitter L-glutamate from the synaptic cleft. It is expected to be a plasma-membrane-resident glycoprotein. The glycan structure of plasma membrane glycoproteins is the result of the processing to the hybrid glycan structures when traversing the *cis*, *medial* and *trans* Golgi network. Our separation strategy therefore made use of lectins with specific affinities to the presumptive different carbohydrate groups of CGT and GLAST-1. *Lens culinaris* lectin has a high affinity to Asn-linked oligosaccharides with an α(1-6)-linked fucose at the innermost *N*-acetylglucosamine residue and a lower affinity to α-linked mannose residues. Since the corresponding fucosyltransferase is localized in the *medial* Golgi, only GLAST-1 should contain this α(1-6)-fucosyl residue. Indeed, CGT interacted only weakly with lentil lectin Sepharose and was eluted as a 64-kDa protein with 15 mM methyl glucoside whereas homogenous GLAST-1 was eluted with 500 mM methyl glucoside.

Table 1. Amino acid sequences of N-terminus of 64-kDa CGT and tryptic peptides.

Peptide	Sequence
N-terminus, 1	Ala-Lys-Ile-Ile-Ile-Val-Pro-Pro-Ala-Met-Phe-Gln-Ser-His-Leu-Tyr-Ile-Phe-Lys
Tryptic peptides, 2	Val-Ile-Asn-Asn-Pro-Ser-Tyr-Arg
3	Val-Ile-Trp-Arg
4	Leu-Pro-Gln-Lys
5	Leu-Ile-Gln-Trp-Leu-Pro-Gln-Asn-Asp-Leu-Leu-Gly-His-Ser-Asn-Ile-Arg
6	Tyr-Pro-Gly-Ile-Phe-Xaa-Ser-Thr-Thr-Ser-Asp-Ala-Phe-Leu-Gln
7	Tyr-Ala-Val-Phe-Ser-Thr-Gly-Leu-Trp-Tyr-Pro-Ala-Gln-Val-Gly-Ala-Pro-Ala-Pro
8	Ser-Thr-His-Ser-Thr-Val
9	Asn-Leu-Glu-Xaa-Asn-Thr-Lys

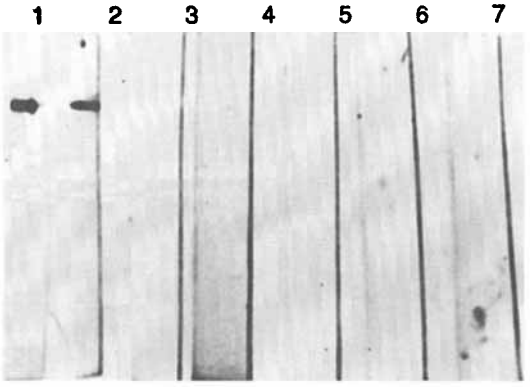

Fig. 1. Lectin staining of UDP-galactose:ceramide galactosyltransferase (CGT). SDS/PAGE (12.5%) of CGT and blot transfer to nitrocellulose followed by staining with specific lectin digoxigenin conjugates. Carbohydrate specificities of the respective lectins are added in brackets. Lane 1, concanavalin A [recognizes mannose-containing carbohydrate structures]; lane 2, *Galanthus nivalis* [recognizes terminal mannose α(1-3), α(1-6) or α(1-2)-linked to mannose]; lane 3, *Datura stramonium* [recognizes Galβ(1-4)GlcNAc]; lane 4, *Maackia amurensis* [recognizes sialic acid linked α(2-3) to galactose]; lane 5, *Sambucus nigra* [recognizes sialic acid linked α(2-6) to galactose]; lane 6, peanut agglutinin [recognizes the core disaccharide Galβ(1-3)Gal NAc]; lane 7, wheat germ lectin.

The identity and purity of the two proteins was verified immunochemically. Affinity-purified antibodies against synthetic peptides of the CGT and GLAST-1 sequences recognized only the 64-kDa CGT and monomeric GLAST-1 of the same molecular mass in their respective fractions eluting from the affinity column. Affinity-purified CGT and GLAST-1 were separated from methyl glucoside and from Triton X-100 by acetone precipitation. For protein sequencing by Edman degradation, lentil lectin which bled from the Sephadex matrix was separated by preparative SDS/PAGE (10%).

Carbohydrate structure of CGT. The differential desorption of CGT from the lentil lectin stationary phase with 15 mM methyl glucoside supported the assumption that CGT is a mannose-rich glycoprotein residing in the smooth endoplasmic reticulum. Further proof came from a lectin blot of the UDPgalactose:ceramide galactosyltransferase (Fig. 1). The CGT band is only affinity-labeled with concanavalin A and *Galanthus nivalis* agglutinin. The latter recognizes specifically terminal α(1-2), α(1-3) and α(1-6) mannosyl residues in oligosaccharide structures. No reaction occurred with *Sambucus nigra* which recognizes sialic acids linked α(2-6) ketosidically to terminal galactose. This underlines that CGT carries no O-glycosidic linkages but is a mannose-rich glycoprotein. Deglycosylation with glycopeptidase F (Elder and Alexander, 1982; Trimble and Maley, 1984) reduced the molecular mass to the 59-kDa core polypeptide of CGT.

The cDNA-derived sequence reveals three putative glycosylation sites at Asn78, Asn333 and Asn442. Only Asn78 and Asn333 are glycosylated. Edman degradation did not detect two Asn residues in two of the eight sequenced tryptic peptides of CGT (marked as Xaa in sequences 6 and 9 in Table 1). These positions correspond to Asn78 and Asn333 in the complete amino acid sequence of CGT, which are putative glycosylation sites in the cDNA-derived CGT amino acid sequence. The loss of two oligosaccharide groups accounts for the reduction of the molecular mass by approximately 6 kDa. Asn442 in close proximity to the transmembrane domain is apparently not N-glycosylated.

Purification and characterization of two isoforms of L-glutamate transporter GLAST-1. Two distinct forms of GLAST-1, monomeric proteins with apparent molecular masses of 64 and 70 kDa, were isolated, the 64-kDa isoform from the microsomal fraction of rat brain but the 70-kDa isoform only from total rat brain. Starting the purification procedure with whole rat brain instead of microsomes, a 70-kDa monomeric form of GLAST-1 was extracted and fractionated up to the Cibachron blue Sepharose step (Schulte and Stoffel, 1993). Lectin blot analysis of the glycan structure is able to discriminate distinctly between the two forms of GLAST-1. The 64-kDa GLAST-1 protein was identified as a high-mannose glycoprotein. It reacted with concanavalin A which rather nonspecifically recognizes glycopyranosyl residues with unmodified hydroxy groups at C3, C4 and C6, and *Galanthus nivalis* agglutinin which specifically and strongly binds to terminal mannose. The 64-kDa isoform of GLAST-1 also bound strongly to lentil lectin Sepharose, a lectin which recognizes fucose α(1-6)-linked to the innermost *N*-acetylglucosamine (GlcNAc). The 70-kDa GLAST-1 isoform has no affinity toward lentil lectin Sepharose but binds strongly to wheat germ lectin. This facilitated the separation of abundant amounts of the adsorbed GLAST-1 glycoprotein from contaminating proteins. Wheat germ lectin recognizes terminal dimeric and trimeric glycostructures containing GlcNAc and sialic acid. The 70-kDa form of GLAST-1 stained positively in the lectin blot of concanavalin A, *G. nivalis* and *Datura stramonium* agglutinins which recognizes Galβ(1→4)GlcNAc. The reaction with the *Sambucus nigra* and the *Maackia amurensis* lectins which recognize α(2-6)- and α(2-3)-linked *N*-acetylneuraminic acid residues was negative (Fig. 2). Therefore the abundant 70-kDa isoform is a hybrid glycoprotein. We conclude from the glycosylation pattern that the abundant 70-kDa form of GLAST-1 is the mature and fully glycosylated transporter protein whereas the minor 64-kDa isoform is an incompletely glycosy-

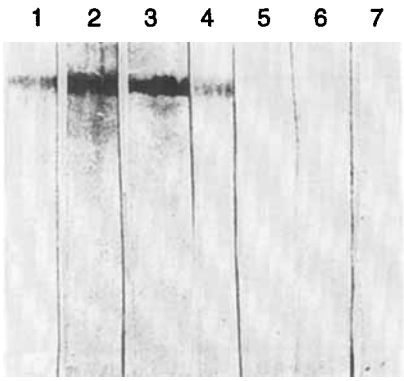

Fig. 2. Immunoblot and lectin staining of glutamate/aspartate transporter (GLAST-1). SDS/PAGE (12.5%) of GLAST-1 and blot transfer to nitrocellulose followed by staining with specific lectin digoxigenin conjugates. Carbohydrate specificities of the respective lectins are added in brackets. Lane 1, antipeptide antibody (Western blot) against GLAST-1; lane 2, *Galanthus nivalis* [recognizes terminal mannose α(1-3), α(1-6) or α(1-2) linked to mannose]; lane 3, concanavalin A (recognizes mannose-containing carbohydrate structures); lane 4, *Datura stramonium* [recognizes Galβ (1-4) GlcNAc]; lane 5, *Maackia amurensis* [recognizes sialic acid linked α(2-3) to galactose]; lane 6, peanut agglutinin [recognizes the core disaccharide Galβ(1-3)GalNAc]; lane 7, *Sambucus nigra* [recognizes sialic acid linked α(2-6) to galactose].

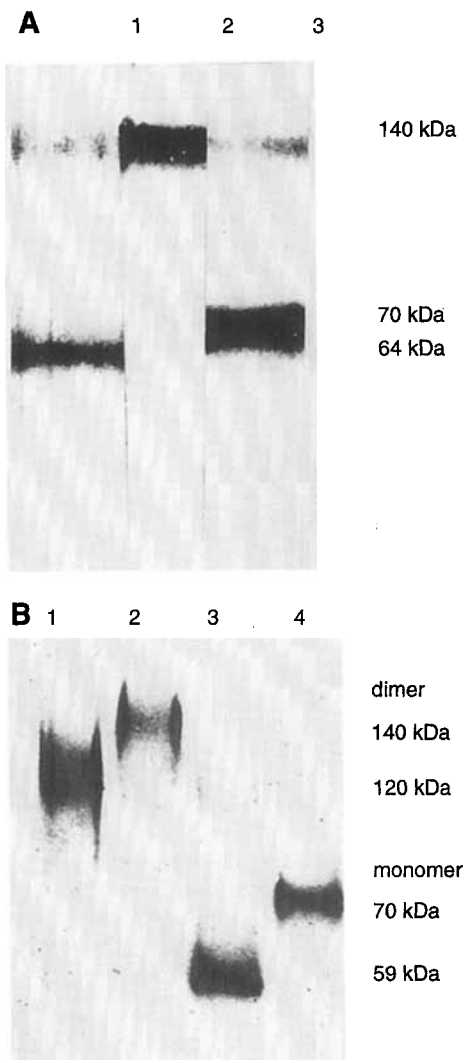

Fig. 3. (A) SDS/PAGE and (B) Western blot analysis of different GLAST-1 forms. (A) SDS/PAGE (12.5%) with Coomassie blue staining. Lane 1, GLAST-1 isolated with lentil lectin (64 kDa); lane 2, GLAST-1 in dimeric form isolated with wheat germ lectin (PAGE-purified); lane 3, GLAST-1 monomer form, 70 kDa, isolated with wheat germ lectin. (B) Western blot analysis of the two isoforms of GLAST-1 before and after deglycosylation. Lane 1, dimer of 70-kDa GLAST-1, deglycosylated with glycopeptidase F (120 kDa); lane 2, dimer, 140 kDa; lane 3, monomer (59 kDa), deglycosylated with glycopeptidase F; lane 4, monomer, 70 kDa. Staining with anti-(GLAST-1 peptide) antibodies and alkaline phosphatase coupled to second antibody (antirabbit Ig antibodies).

lated, transient form of GLAST-1 isolated from the endoplasmic reticulum (microsomal fraction) of myelinating rat brains.

Glycopeptidase F treatment of the 64-kDa and 70-kDa GLAST-1 isoforms reduced the apparent molecular mass to 59 kDa (Fig. 3B). Therefore the difference between the two forms is due to the glycosylation. Quantitative ELISA for GLAST-1 in rat brain extracts suggests that the 70-kDa GLAST-1 isoform is present in rat brain in 1000-fold higher concentration than the 64-kDa isoform.

In Coomassie blue staining, significant amounts of the 64-kDa and the 70-kDa GLAST-1 isoproteins are visible as dimers (Fig. 3A, lanes 1 and 3). They were detected at 130 and 140 kDa, respectively. The dimeric 140-kDa form was isolated by preparative PAGE. Glycopeptidase F treatment reduced the molecular mass of the monomeric (70 kDa) and of the dimeric form (140 kDa) to approximately 59 and 120 kDa, respectively (Fig. 3B) as shown in the Western blot (Fig. 3B). GLAST-1-specific antibodies detected the monomeric and dimeric forms in Western blot analysis.

The affinity-purified isoforms were proteolytically cleaved, the resulting peptide mixture was separated by HPLC as described for CGT. Eight tryptic peptide sequences of the GLAST-1 protein (20%) were determined by Edman degradation. They are listed in Table 2 and were used for the synthesis of respective oligonucleotides for screening cDNA libraries of brains of myelinating rats (Storck et al., 1992). Two additional mammalian L-glutamate transporter with identities of about 55% to GLAST-1 have been subsequently cloned and characterized (Kanai and Hediger, 1992; Pines et al., 1992). These two L-glutamate transporters also belong to the new family of GLAST-1 neurotransmitter uptake systems. In preliminary experiments we addressed the question of whether heterodimers between these transporters are present as dimers. The gel-purified monomeric and dimeric GLAST-1 isolated by wheat germ lectin chromatography from whole rat brain were separately digested with endoproteinases Glu-C and Lys-C. The peptides of these digests were separated on SDS/PAGE (Schägger and von Jagow, 1987) and the peptide pattern of the dimeric and monomeric forms compared (Fig. 4). The peptide footprint of both forms is identical. So far we have no indication that GLAST-1 forms heterodimers with one of the two other L-glutamate transporters but forms homodimers by homophilic interaction in a specific manner.

Characterization of the 53-kDa protein. The Cibachron blue Sepharose chromatography fraction has been taken as the final step in the purification of CGT in previous studies (Neskovic et al., 1976, 1981, 1986). However, this fraction still contains several protein bands in silver-stained gels among which the 53-kDa protein component has been regarded as CGT. A comparison of the enzyme activity of this fraction (50–700 U/mg protein) with the activity of our 64-kDa CGT purified by the lectin affinity chromatography (13 kU/mg protein) revealed another discrepancy. We isolated the 53-kDa protein band by preparative SDS/

Table 2. Amino acid sequence of eight tryptic peptides of 64-kDa GLAST-1.

Peptide	Sequence	
1	(Glu)	Met-Lys-Lys-Pro-Tyr-Gln-Leu-Ile-Ala-Gln-Asp-Asn
2	(Glu)	Met-Val-Pro-Val-Pro-Gly-Ser-Val-Asn-Gly-Val-Asn-Ala
3	(Glu)	Gly-Lys-Ile-Val-Gln-Val-Thr-Ala-Ala-Asp-Ala-Phe
4	(Glu)	Thr-Lys-Ser-Leu-Gly-Val-Asp
5	(Lys)	Ala-Ser-Gly-Lys-Met-Gly-Met
6	(Arg/Lys)	Phe-Gln-Gln-Gly-Val-Arg
7	(Arg/Lys)	Asp-Val-Glu-Met-Gly-Asn-Ser-Val-Ile-Glu-Glu-Asn-Glu-Met-Lys
8	(Arg/Lys)	Pro-Tyr-Gln-Leu-Ile-Ala-Gln-Asp-Asn-Glu-Pro-Glu-Lys

Table 3. Comparison of amino acid sequences of tryptic peptides of the 53 kDa protein with the fragments of rat β-tubulin. Tryptic peptides 1, 2 and 3 of the 53-kDa protein have been aligned with fragments of β-tubulin; the numbers underneath indicate the position in the full sequence of β-tubulin.

Peptide or fragment	Sequence
Peptide 1	Phe-Trp-Glu-Val-Ile-Ser-Xaa-Xaa-His
Fragment 1	Phe-Trp-Glu-Val-Ile-Ser-Asp-Glu-His
	530
Peptide 2	Asn-Ser-Ser-Tyr-Phe-Val-Glu-Trp-Ile-Pro-Asn-Asn-Val-Lys
Fragment 2	Asn-Ser-Ser-Tyr-Phe-Val-Glu-Trp-Ile-Pro-Asn-Asn-Val-Lys
	850
Peptide 3	Ala-Ile-Leu-Val-Asp-Leu-Glu-Pro-Gly-Thr-Met-Asp-Xaa-Val-Arg-Thr
Fragment 3	Ala-Ile-Leu-Val-Asp-Leu-Glu-Pro-Gly-Thr-Met-Asp-Ser-Val-Arg-Ser
	570 580

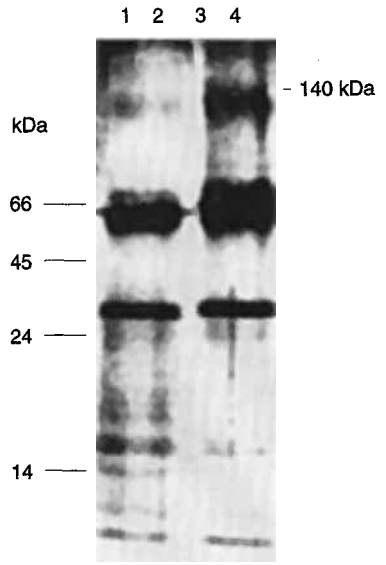

Fig. 4. High-resolution PAGE of proteolytic fragments. Lanes 1 and 3, monomeric and dimeric GLAST-1 treated with Lys-C protease; lanes 2 and 4, monomeric and dimeric GLAST-1 treated with Glu-C protease. The method of Schägger and von Jagow (1987) was used.

PAGE. Tryptic cleavage, separation of the peptides by HPLC and Edman degradation yielded the amino acid sequences of the three peptides listed in Table 3. Computer-assisted similarity screening of the available protein libraries unambiguously identified them as partial amino acid sequences of the 53-kDa β-tubulin (Ginzburg et al., 1985) the alignments of which are shown in Table 3.

DISCUSSION

We report here the isolation and purification to homogeneity of the key enzyme of the glycolipid synthesis in oligodendrocytes UDP-D-galactose:ceramide-galactosyl transferase (cerebroside synthetase, CGT). The enzyme has a molecular mass of 64 kDa and a specific activity of 13 kU/mg. The enzyme is a glycoprotein. Deglycosylation with glycopeptidase F leaves a deglycosylated core protein of 59 kDa which is still enzymically active. The pivotal purification step consisted in lentil lectin affinity chromatography with differential desorption by which the enzyme is separated from the copurifying L-glutamate transporter (GLAST-1) with very similar physical properties. The experimental data reported here suggest that CGT is an enzyme resident in the smooth endoplasmic reticulum and therefore should be a mannose-rich glycoprotein. The L-glutamate transporter (GLAST-1) however is located in the plasma membrane and is expected to be a hybrid glycoprotein after passage through the Golgi apparatus. The different types of glycosylation of CGT and GLAST-1 isoforms facilitated their separation and characterization on the protein level.

The protein purification and analysis of CGT provided ample peptide sequence information, including the N-terminal signal sequence, for its molecular characterization by cloning strategies and the expression reported recently from this laboratory (Schulte and Stoffel, 1993). Its 4.2-kb mRNA has an open-reading frame (ORF) of 1623 nucleotides encoding a 541-amino-acid polypeptide with a putative signal sequence. Edman degradation of the N-terminus of the mature CGT (Table 2) indeed confirmed that a 20-residue sequence precedes the sequence of mature CGT, which is processed during cotranslational translocation. Furthermore a 20-residue transmembrane domain at the C-terminus is apparent from the hydropathy plot and serves as an anchor of the enzyme in the membrane of the endoplasmic

reticulum. In addition the ERRXR (where X =V) motif characteristic of proteins resident in the endoplasmic reticulum is present.

CGT has three potential glycosylation sites in positions Asn78, Asn333 and Asn442. The peptide sequencing data suggest that only the Asn78 and Asn333 (Xaa in sequences 6 and 9 of Table 1) anchor the mannose-rich carbohydrate groups. *In situ* hybridization studies on brain sections of developing young rats proved that CGT is oligodendrocyte-specific. It is only weakly expressed in kidney. CGT expression is time-regulated and peaks around postnatal days 15−25 similar to myelin basic protein in the rat brain.

Earlier studies assigned a molecular mass of 53 kDa to CGT isolated from rat brain (Neskovic et al., 1974, 1976, 1981, 1986; Warren et al., 1976). Not only the assignment of the transferase activity to the 53-kDa protein of this fraction is at variance with the 64-kDa glycoprotein described in this report but also the specific activity (500−700 U/mg) of this 53-kDa protein as compared to the transferase activity (13 kU/mg) of the 64-kDa glycoprotein described here. The peptide sequences (Table 3), which we obtained from this 53-kDa protein purified by peparative SDS/PAGE leave no doubt that this protein is β-tubulin.

The oligodendrocyte-specific CGT is a member of a protein family of UDP-glucuronyl and glucosyl transferases present in the endoplasmic reticulum of liver and kidney. These enzymes transfer glucuronic acid in detoxification reactions to lipophilic acceptors such as bilirubin. The cloned CGT shares considerable amino acid and DNA sequence similarities with this family of glycosyl transferases, but also shares the following properties: (a) the signal sequence for translocation into the endoplasmic reticulum; (b) a C-terminal transmembrane domain, and (c) a highly conserved 50-residue C-terminal sequence motif: WXXQXX[LIVM]LXHXXXXAF[LIVM][STAG]GXX[STAG]XXXX[STAG][LIVM]XXX[LIVM]P[LIVM]XXXP[LIVMF][LIVMF][STAG][DE]Q. CGT differs from the other members by its unique expression restricted to oligodendrocytes of the central nervous system and only weakly expressed in kidney.

Besides the CGT, we isolated and identified a 64-kDa intermediate of the neurotransmitter transporter protein GLAST-1. The different degree of glycosylation of the two GLAST-1 isoforms facilitated not only the separation of CGT from the 64-kDa GLAST-1 but also of the two GLAST-1 isoforms with molecular masses of 64 and 70 kDa by differential lectin affinity chromatography. GLAST-1 travels from the endoplasmic reticulum through the Golgi apparatus to its target plasma membrane exposed to the array of glycosyl transferases of the *cis*, *medial*, and *trans* Golgi system.

Small amounts of immature 64-kDa GLAST-1, with its high-mannose glycan structure in which fucose is $\alpha(1\rightarrow6)$-linked to the inner *N*-acetylglucosamine in the N-glycosidic linkage to asparagine (Gerard, 1990; Merkle and Cummings, 1987), were isolated by lentil lectin affinity chromatography. The mature GLAST-1, binding with high affinity to the wheat germ lectin matrix, has a mass of 70 kDa. This difference is due to a higher degree of glycosylation. Glycopeptidase F treatment yielded the core protein of identical size (59 kDa). This finding is also supported by the cDNA structure.

The peptide sequences of the proteolytic fragments of the two GLAST-1 isoproteins described here allowed the cloning of their cDNAs by oligonucleotide screening of rat brain cDNA libraries. There is an ORF of 1629 nucleotides of the 4.5-kb mRNA encoding the 543-amino-acid GLAST-1 polypeptide with two putative N-glycosylation sites at Asn206 and Asn216. GLAST-1 is brain-specific as proven previously by Northern blot hybridization. *In situ* hybridization suggests that it is resident in the plasma membrane of glia cell.

We derived a model for the membrane integration of the hydrophobic transporter with six α-helical transmembrane domains and an undefined C-terminus which allows different topological assignments.

GLAST-1 has a high tendency to dimerize to the 140-kDa form very likely due to the hydrophobic properties of GLAST-1. The dimeric 140-kDa form is eluted from wheat germ lectin columns with high concentrations (100 mM) of *N*-acetylglucosamine together with the monomeric protein in homogenous form. The 140-kDa band in SDS/PAGE is a homodimer as far as preliminary protein analyses suggest. Comparative studies on the proteolytic cleavage products of the monomer and dimer of GLAST-1 with endoproteases of different specificities (Lys C und Glu C) yielded only identical peptide patterns in high-resolution SDS/PAGE (Schägger and von Jagow, 1987). So far there is no indication of heterodimeric L-glutamate transporters. However, more sensitive studies are under way to find out whether heterodimeric glutamate transporters, consisting of monomers of GLAST-1 and Glt-1 or EAAT-1, can be formed, if simultaneously expressed in one cell type of the central nervous system, thus expanding the plasticity of its glutamate transporter systems.

The protein chemical data of CGT and GLAST-1 gained during these studies together with their specific cDNAs cloned into expression vectors will facilitate numerous studies regarding the role of CGT in the differentiation of oligodendrocytes and the concerted intracellular transport of cerebrosides and derived sulfatides during the assembly of the compact multilayer myelin membrane during myelination. The reaction product of CGT is the widely used Gal C antigen applied to surface labeling in lineage studies (ffrench-Constant and Raff, 1986; Gard and Pfeiffer, 1990; Raff et al., 1984).

The simultaneous isolation and characterization of GLAST-1from rat brain reported here has lead to its cloning from rat brain and the functional analysis of this new family of Na^+-dependent neurotransmitter transporters. Its transport properties were characterized by functional expression in *Xenopus laevis* oocytes. The information about these proteins summarized in these studies should facilitate investigations on the divergently discussed topology of this polytopic plasma membrane protein (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992), its cotranslational translocation, post-translational modifications and, most importantly, the domains involved in neurotransmitter and Na^+ binding and transport.

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