A novel member of the mouse CMP-NeuAc: β-N-acetylgalactosaminide α2,6-sialyltransferase (ST6GalNAc) subfamily, designated ST6GalNAc V, was identified by BLAST analysis of expressed sequence tags. The sequence of the longest cDNA clone of ST6GalNAc V encoded a type II membrane protein with 8 amino acids comprising the cytoplasmic domain, 21 amino acids comprising the transmembrane region, and 306 amino acids comprising the catalytic domain. The predicted amino acid sequence showed homology to the previously cloned ST6GalNAc III and IV, with common amino acid sequences in sialyl motifs L and S among these three enzymes. Eleven CAG repeats were found in the stem region. A fusion protein with protein A and extracts from L cells transfected with ST6GalNAc V in an expression vector showed enzyme activity of α2,6-sialyltransferase almost exclusively for GM1b, but not toward glycoproteins. Sialidase treatment and thin layer chromatography immunostaining revealed that the product was GD1α. Northern blotting revealed that three transcripts of the gene were expressed specifically in brain tissues. It is concluded that this enzyme is involved in the synthesis of GD1α in the nervous tissues, and the CAG repeats may have implications in neurodegenerative diseases.

Gangliosides are glycosphingolipids containing sialic acids in the carbohydrate moiety and have been considered critical to a wide variety of cellular events, such as cell-cell interaction, cell adhesion, mediation of invasion of vectors, and protein targeting (1, 2). The sialic acids in particular are thought important to the biological functions of gangliosides. In all the ganglioside structures so far defined, four main linkages of sialic acids are present, i.e. α2,3galactose (Gal),1 α2,6Gal, α2,8sialic acid (Sia), and α2,6N-acetylgalactosamine (GalNAc).

To biosynthesize sialyl compounds containing one or more sialic acids with the linkages described above, a number of sialyltransferases are needed. To date, more than 15 species of sialyltransferase genes have been isolated (3, 4); and six genes for α2,3Gal (ST3Gal), one gene for α2,6Gal (ST6Gal), five genes for α2,8Sia (ST8Sia), and four genes for α2,6GalNAc (ST6GalNAc) have been cloned as sialyltransferase genes involved in the synthesis of sialylated carbohydrates on glycoproteins and glycolipids (4). Some of them act on both glycoproteins and glycolipids, while others utilize either as an acceptor. The expression pattern of these genes varies, i.e. the expression of some genes is restricted to certain tissues or cells or to specific stages of development. However, many sialyltransferase genes are expressed in a ubiquitous manner.

For the glycosyltransferase genes responsible for the synthesis of gangliosides, the majority of cDNAs have been isolated (5). Namely, sialyltransferases designated SAT I, SAT II, SAT III, SAT IV, and SAT V have been cloned and well characterized, although there is some ambiguity as to their identities and specificities (5). However, enzymes to extend the carbohydrate chains, or those to further modify the carbohydrate structures, have not been well characterized as their cDNAs are not available. To analyze the significance of the minor structures present in the ganglioside-series gangliosides and regulatory mechanisms for the restricted and/or universal expression of those enzymes, molecular cloning of the genes is essential.

α-Series gangliosides were defined as a new series of gangliosides containing NeuAc linked to the C6 position of GalNAc of the gangliotetrasyl backbone (6, 7). They have been thought only a minor component (8), and little is known about them. In contrast with O-glycans, α2,6-sialylated GalNAc structures are rarely detected in the carbohydrate moiety of glycosphingolipids. However, the expression of GD1α,2 a typical α-series ganglioside, was restricted to a particular region and a particular population in brain tissues (9), suggesting that the expression level of GD1α is fairly high in some regions.

In the present study, we have isolated a cDNA of GD1α synthase (ST6GalNAc V) gene specifically expressed in the brain, which contains an interesting CAG repeat. Although several ST6GalNAc cDNAs that may synthesize GD1α have been reported (10, 11), ST6GalNAc V is specific for GM1b in

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1 The abbreviations used are: Gal, galactose; Sia, sialic acid; GalNAc, N-acetylgalactosamine; CMP-NeuAc, cytidine 5'-monophospho-N-acytleneuraminic acid; mAb, monoclonal antibody; RT-PCR, reverse transcription polymerase chain reaction; DME, Dulbecco’s modified Eagle’s medium; TLC, thin layer chromatography; BSM, bovine submaxillary mucin.

2 The nomenclature of gangliosides is based on that of Svennerholm (34). The abbreviated nomenclature for cloned sialyltransferases is the same as used in Ref. 13.
contrast with other members of the ST6GalNAc family. Moreover, ST6GalNAc V showed brain-specific expression, suggesting a critical role of ST6GalNAc V in the synthesis of GD1α in brain tissues.

**EXPERIMENTAL PROCEDURES**

**Nomenclature of Cloned Sialyltransferase—**Four members of the GaLNAc α2,6-sialyltransferase (ST6GalNAc) subfamily have been cloned to date: ST6GalNAc I (12), ST6GalNAc II (13, 14), ST6GalNAc III (10, 11), and ST6GalNAc IV (11). The GaLNAc α2,6-sialyltransferase cloned in this study is referred to as ST6GalNAc V according to Tsuji et al. (15).

**Materials—**CMP-NeuAc, LacCer, asialo-GM2 (GA2), GM2, GM1, GD1α, GD1b, GT1b, fetuin, asialofetuin, bovine submaxillary mucin (BSM) and bovine submaxillary asialomucin (asialo-BSM) were purchased from Sigma. GM3 and GD3 were purchased from Snow Brand Milk Products Co. (Tokyo, Japan). [α-32P]dCTP was from ICN (Costa Mesa, CA). GM1b was chemically synthesized as described previously (16). Asialo-GM1 (GA1) was prepared by digestion of GM1 with neuraminidase from Vibrio cholerae (Sigma).

**Isolation of ST6GalNAc V cDNA—**Mouse-expressed sequence tags (GenBank™ accession numbers AU035329, AA462934, and AA968060) with similarity to mouse ST6GalNAc IV were identified using the tBLASTn algorithm against the dbEST data base at the National Center for Biotechnology Information. The expressed sequence tag cDNA clone (AU035329) with the longest 5′-region was obtained from Japanese Collection of Research Resources. To isolate cDNA clones, the reverse transcription-polymerase chain reaction (RT-PCR) method using total RNA from mouse brain was performed. A sense primer containing a XhoI site, 5′-AAGTGGGCTCCTAAGACCATGCTTACTG-3′ (nucleotides 188–204 in Fig. 1A), and an antisense primer containing a SpeI site, 5′-CAGCTGACACAGCCTACTACCC-3′ (nucleotides 1182–1200), were used for the PCR, which was carried out as follows: 94°C for 1 min, 25 cycles of (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min), and 72°C for 1 min. The RT-PCR-amplified product (1010 base pairs) was subcloned into pCR®2.1-TOPO vector (Invitrogen, San Diego, CA). The nucleotide sequence was determined by the dideoxy termination method using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

**Construction of Expression Vector—**An expression vector of the cloned cDNA was prepared by insertion of the subcloned cDNA fragment into the XhoI and SpeI sites of pMIKneo vector (kindly provided by Dr. K. Maruyama at Tokyo Medical and Dental University). To prepare soluble fusion enzyme, a 2% formaldehyde gel, then transferred onto a GeneScreen Plus® membrane (DuPont). After baking, the filter was prehybridized for 2 h at 4°C in a solution consisting of 5 × SSPE (saline/sodium phosphate/EDTA), 50% formamide, 5x Denhardt's solution, 1% SDS, and 10% dextran sulfate. Hybridization was carried out in a mixture containing 1 ml of water. The products were isolated using a C18 Sep-Pak cartridge, dried, and resuspended in 25 μl of 50 mM sodium citrate (pH 6.0) and 100 mM NaCl containing 100 μg/ml bovine serum albumin. Salmonella typhimurium LT2 sialidase (0.85 unit) (New England Biolabs, Beverly, MA) was added to the resultant products, which were then incubated overnight at 37°C. After purification with a C18-Sep-Pak cartridge, the digestion product was further treated with 7 milliunits of bovine testes β-galactosidase (Sigma) in 40 μl of 10 mM potassium acetate (pH 5.0) containing 0.2% sodium taurocholate and 1 mg/ml bovine serum albumin at 37°C for 48 h. For linkage analysis of sialic acids, 0.5 μg of the product was treated with a linkage-specific sialidase, 0.85 units of S. typhimurium LT2 sialidase (Boehringer Mannheim), to release 3′-linked sialic acids and weakly active for α2,3 linkage, 0.85 unit of Clostridium perfringens sialidase (specific for α2,3- and α2,6-sialic acids, New England Biolabs), or 5 milliunits of Newcastle disease virus sialidase (specific for α2,3- and α2,8-linked sialic acids, Roche Molecular Biochemicals) (20). The enzyme reaction was performed at 37°C for 24 h.

**TLC Immunostaining—**Five μg of GM1b was sialylated with ProtA-ST6GalNAc V for 6 h, and purified with a C18-Sep-Pak cartridge, dried, and subjected to TLC. TLC immunostaining was performed as described previously (21) according to the method of Taki et al. (22). In brief, the TLC plate was heat-blotted to a polyvinylidenedifluoride membrane after chromatography of the glycolipids. The membrane was incubated with monoclonal antibody (mAb) KA-17 at a 1:100 dilution for 1 h, washed, and incubated with biotinylated anti-mouse IgG for 1 h. The antibody binding was revealed with ABC-PO (Vector, Burlingame, CA) and HRP-1000 (Konica, Tokyo, Japan) as described previously (23).

**Northern Blot Analysis—**mRNA was isolated from mouse tissues using an mRNA isolation kit (Miltenyi Biotec, Bergisch, Germany) according to the manufacturer’s instructions. Two μg of poly(A) RNA was separated on a 1% agarose, 2% formaldehyde gel, then transferred onto a GeneScreen Plus® membrane (DuPont). After baking, the filter was prehybridized for 2 h at 42°C in a solution consisting of 5 × SSPE (saline/sodium phosphate/EDTA), 50% formamide, 5x Denhardt’s solution, 1% SDS, and 10% dextran sulfate. Hybridization was carried out for 16 h at 42°C in the same solution containing 5 × 10^5 dpm/ml of the 32P-labeled probes. Alternatively, a mouse Multiple Choice™ Northern blot was obtained from OriGene Technologies Inc. (Rockville, MD) and hybridized according to the manufacturer’s instructions. The filters were washed and then exposed to the imaging plate to be analyzed in a FUJIX BIO-Imaging Analyzer BAS 2000.

**RESULTS**

**Isolation of ST6GalNAc V cDNA—**Using the mouse expressed sequence tag data base, we found sequences (GenBank™ accession numbers AA035329, AA462934, and AA968060) with similarity to mouse ST6GalNAc IV and obtained an expressed sequence tag cDNA clone AU035329 from Japanese Collection of Research Resources. Then a corresponding cDNA fragment was obtained by RT-PCR using total RNA from mouse brain. The nucleotide sequence revealed that the cDNA contains an open reading frame encoding a protein of 335 amino acids with a calculated molecular mass of 38,301 daltons, with two potential N-linked glycosylation sites (Fig. A). The initiation codon at the beginning of the open reading frame is embedded within a sequence similar to the Kazak consensus initiation sequence (24, 25). Inspection and hydrophy of the predicted protein sequence suggested that this enzyme molecule has the structural organization of a membrane protein with type II topology, which is commonly detected in glycosyltransferase genes. A single hydrophobic seg.
ment with 21 amino acids was present near the amino terminus. This putative signal anchor sequence would place 8 residues within the cytosolic compartment and 306 amino acids within the Golgi lumen as a catalytic domain (Fig. 1B). Comparison of the primary structure of the newly cloned sialyltransferase and the 16 other cloned sialyltransferases indicated that there is significant similarity in two regions, the L sialyl motif and S sialyl motif (Fig. 2). In particular, this new enzyme has several common amino acid residues specifically conserved among the three members of ST6GalNAc (ST6GalNAc III, IV, and this gene, as shown by gray boxes in Fig. 2), although there were few conserved residues commonly detected among all five ST6GalNAc members. Thus, this new gene was tentatively designated as ST6GalNAc V. These results suggested that ST6GalNAc III, IV, and V share similar functions such as substrate specificity. A most characteristic finding in the primary structure of this gene product was the presence of CAG repeats at Gln38 to Gln48 (totally 11 CAGs) located in the stem region (Fig. 1A).

**Sialyltransferase Activity of the Cloned cDNA Product**—To analyze the sialyltransferase activity of the ST6GalNAc V, the expression vector of the cloned cDNA, pMIKneo-ST6GalNAc V, was transfected into L cells, and the extracts were assayed for sialyltransferase activity using CMP-[14C]NeuAc as a donor. The enzyme sialylated GM1b almost exclusively, but no other asialo or sialosyl compounds were significantly utilized as an acceptor (data not shown). No activity was detected in the extracts prepared from mock-transfected cells. The apparent $K_m$ value for GM1b was 0.65 mM (data not shown).

**Substrate Specificity of Cloned ST6GalNAc V**—To analyze the substrate specificities of ST6GalNAc V, the fusion enzyme in the supernatant, we analyzed the...
sialyltransferase activity for various glycolipids and glycoproteins. As shown in Fig. 3, no glycolipids except for GM1b showed significant acceptor activity. Fetuin and BSM, and their desialylated forms were also completely inactive as an acceptor for ST6GalNAc V (Table I). The fact that GA1 was inactive indicates that a sialic acid linked to galactose at the non-reducing end by an α2,3 linkage was critical for the substrate activity.

**Linkage Analysis by Exoglycosidase Digestion**—To determine the incorporated sialic acid linkage, GM1b was labeled with CMP-[14C]NeuAc using ProtA-ST6GalNAc V and the product was subjected to digestion with *S. typhimurium* LT2 sialidase, which cleaves the α2,3 linkage. The enzyme products obtained with ProtA-ST6GalNAc V were sensitive to this treatment, but the radioactivity was not removed as shown in Fig. 4, lane 2. Then, the products were digested by bovine testes β-galactosidase, resulting in the partial conversion to a more rapidly migrating component. This component was supposed to be a sialic acid linked to galactose at the non-reducing end by an α2,3 linkage. The possibility that it was GM2 seemed unlikely from the TLC pattern of the first product. Subsequently, this product mixture was treated with three different neuraminidases with different specificities; α2,3 linkage-dominant sialidase from *S. typhimurium* LT2, α2,3/α2,6 linkage-specific sialidase from *C. perfringens*, or α2,3/α2,6 linkage-specific sialidase from Newcastle disease virus. As shown in Fig. 4 (right panel), the band at the top was partially digested by α2,3-dominant and by α2,3/α2,6-specific sialidase, but not by α2,3/α2,8-specific sialidase even in the presence of taurocholate. Since the α2,3 linkage-dominant sialidase from *S. typhimurium* LT2 has some activity toward α2,6-linked sialic acids, these results suggested that the structure of the sialylated intermediate product was NeuAcα2,6GalNAcβ1,4Galβ1,4Glc-Cer, and the original product was GD1α.

**TLC Immunostaining**—To confirm that the enzyme product is GD1α, TLC immunostaining of the products using the anti-GD1α mAb KA-17 was performed. As shown in Fig. 5, a product with ProtA-ST6GalNAc V from GM1b was clearly stained as standard GD1α at the same migration site. GM1b itself was faintly stained as previously reported (9). None of the major gangliosides from bovine brain were stained, confirming the specificity of the mAb. Thus, the product was confirmed to be GD1α.

**Expression of the ST6GalNAc V Gene**—To determine the expression pattern and the size of the ST6GalNAc V mRNA, Northern blotting was performed. Among 11 tissues examined, only sample from brain showed three bands at 6.5, 3.0, and 2.3 base pairs (Fig. 6). Only spleen sample showed a very faint band at 6.5 base pairs. Consequently, this ST6GalNAc V gene was expressed in brain tissues in a very restricted manner.

**DISCUSSION**

Four ST6GalNAc genes have been reported to date. Among these four, ST6GalNAc I (12) and ST3GalNAc II (13, 14) were isolated as sialyltransferases which mainly utilize O-glycans as an acceptor. ST6GalNAc I acts toward Galα1-3GalNAc-Ser/Thr, and ST3GalNAc II acts on Galβ1,3GalNAcα1-6Glc-Cer. ST6GalNAc III and IV (11) showed a similar substrate specificity and preferred a terminal sialic acid with an α2,3 linkage on galactose as an acceptor structure. However, ST6GalNAc III better utilizes glycolipid acceptors, while ST6GalNAc IV preferred O-glycans as acceptors. Therefore, the ST6GalNAc V reported in this study is more similar to ST6GalNAc III and IV in terms of major substrate structure than ST6GalNAc I and II. The amino acid sequence alignment of these five ST6GalNAc also demonstrated that ST6GalNAc V is closer to ST6GalNAc III and IV in primary structure (Fig. 2).

ST6GalNAc V is similar to ST6GalNAc III in terms of the nature of acceptor structures they prefer, i.e. NeuAcα2,3Galβ1,4Galα1-3GalNAc on glycolipids. However, their fine substrate specific-

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**Table I**

| Acceptor | Structure(s)* | Relative rate |
|----------|---------------|---------------|
| Fetuin   | NeuAco2,3Galβ1,4Galα1-3GalNAc-Ser/Thr | 0             |
|          | NeuAco2,3Galβ1,4Galα1-3GalNAc-Ser/Thr | 0             |
|          | NeuAco2,6,9Galβ1,4GlcNAcβ1,1,4Glc-Cer/Thr | 0             |
| Asialo-fetuin | GlnAβ1,3,6GalNAc-Ser/Thr | 0             |
| BSM      | NeuAco2,6GalNAc-Ser/Thr | 0             |
| Asialo-BSM | LacCer | Galβ1,4Glcβ1-Cer | 0             |
|          | GA2       | GalNAβ1,4Glcβ1-Cer | 0             |
|          | GA1       | GalNAβ1,3GalNAβ1,4Glcβ1-Cer | 0             |
|          | GM1b      | NeuAco2,3Galβ1,4GalNAβ1,4Galβ1,4Glcβ1-Cer | 100*          |
|          | GM3       | NeuAco2,3Galβ1,4Glcβ1-Cer | 0             |
|          | GM2       | NeuAco2,3Galβ1,4Glcβ1-Cer | 0             |
|          | GM1       | NeuAco2,3Galβ1,4GalNAβ1,4Galβ1,4Glcβ1-Cer | 0             |
|          | GD1a      | NeuAco2,3Galβ1,4GalNAβ1,4NeuAco2,3Galβ1,4Glcβ1-Cer | 0             |
|          | GD3       | NeuAco2,6NeuAco2,3Galβ1,4Glcβ1-Cer | 0             |
|          | GD1b      | NeuAco2,3Galβ1,4GalNAβ1,4NeuAco2,3Galβ1,4Glcβ1-Cer | 0             |
|          | GT1b      | NeuAco2,3Galβ1,4GalNAβ1,4NeuAco2,3Galβ1,4Glcβ1-Cer | 0             |

* Data were from Ref. 41 for fetuin and Ref. 42 for BSM.

* R represents the remainder of the N-linked oligosaccharide chain.

* 372 pmol/h/µl of an enzyme solution.
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FIG. 4. Linkage analysis of incorporated sialic acids by exoglycosidase digestion. GM1β was labeled with CMP-[3H]NeuAc using protA-ST6GalNAc V (lane 1). The labeled products were then subjected to treatment with α2,3 sialidase (lane 2) followed by β-galactosidase digestion (lane 3). The resulting glycolipids were separated on a TLC plate with a solvent system of chloroform/methanol/12 mm MgCl₂ (50: 40:10) and detected with a BAS 2000 radioimage analyzer. The resultant product was treated in the absence of sialidase (lane 7) or the presence of α2,3 linkage-specific sialidase (lane 4), α2,3- and α2,6-specific sialidase (lane 5), or α2,3- and α2,8-specific Newcastle disease virus sialidase (lane 6).

FIG. 5. TLC immunostaining of sialic acid-incorporated products. TLC immunostaining was performed as described under “Experimental Procedures.” Lane 1, standard GD1α; 2.5 μg); lane 3, [3H] NeuAc-labeled sialyl-GM1b using ProtA-ST6GalNAc V. As a control, the same reaction was performed without the enzyme (lane 2). Lane 4 was acidic glycolipidolipids (2.5 μg) extracted from bovine brain (B.B.) containing GM1, GD1α, GD1b, and GTb as major components. Glycolipidolipids were separated by TLC and blotted onto a PVDF membrane. Immunostaining was done using mAb KA-17 to detect GD1α.

FIG. 6. Restricted expression of ST6GalNAc V gene in mouse tissues. Northern blots with poly(A)⁺ RNA from various adult mouse tissues were probed with a mouse ST6GalNAc V cDNA fragment (nucleotides 1-1201 in Fig. 1A) as described under “Experimental Procedures.” The same filters were probed with glyceraldehyde-phosphate dehydrogenase (GAPDH) cDNA after removing the radioactivity. The sizes of ST6GalNAc V transcripts are indicated at the left.

The brain-specific sialyltransferase has a CAG repeat structure is very interesting and may imply that this protein has functions other than that of sialyltransferase activity.

GD1α was reported to be a minor ganglioside in bovine brain tissues (8). It was demonstrated to be accumulated in the proximal dendrites and cell bodies of Purkinje cells in murine cerebellum using a specific mAb (9). It was also detected in macrophages (31) and mammary glands during lactation (32). Furthermore, it was reported that GD1α is a functional molecule on mouse lymphoma cells (33), playing important roles in tumor cell metastasis as an adhesion molecule. Therefore, there may be several tissue specific ST6GalNAc members capable of synthesizing GD1α, and ST6GalNAc V might be a brain-specific isotype responsible for the synthesis of α-series gangliosides in nervous tissues. These results in addition to the characteristics of GD1α synthase as predicted from the cloned cDNA here suggest that GD1α is a critical molecule in the communication and interaction between neuronal cells and their supportive cells, particularly in brain tissues. The availability of the GD1α synthase (ST6GalNAc V) gene would enable us to clearly demonstrate the roles of GD1α in neuronal development and in tumor metastasis.

Most of studies on the α-series of gangliosides have been done with animal tissues or cells. That no human studies on the expression of GD1α have been conducted to date suggests that α-series gangliosides are minor components of human tissues and cells or merely that no rigorous investigation on the presence of α-series gangliosides in human has been done, if in fact they exist. Either way, the use of ST6GalNAc V should enable us to clearly investigate the presence and significances of α-series gangliosides in human bodies, especially in the nervous systems.

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