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A novel glycosyltransferase with a polyglutamine repeat; a new candidate for GD1α synthase (ST6GalNAc V)[1]

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Abstract The fifth type GalNAcα2,6-sialyltransferase (mST6GalNAc V) was cloned from a mouse brain cDNA library. mST6GalNAc V exhibited type II transmembrane topology containing a polyglutamine repeat, which showed 42.6% and 44.8% identity to mouse ST6GalNAc III and IV, respectively. Northern blot analysis revealed that the *mST6GalNAc V* gene was specifically expressed in forebrain and cerebellum. mST6GalNAc V exhibited GD1α synthetic activity from GM1b the same as mST6GalNAc III and IV. The activity ratio of GM1b toward fetuin and the expression pattern were completely different among the three ST6GalNAcs. Interestingly, the polyglutamine repeat number was different from that of inbred mice. We report the first glycosyltransferase with a polymorphic polyglutamine repeat.

Key words: GalNAcα2,6-sialyltransferase; ST6GalNAc V; GD1α synthase; Polyglutamine repeat; Central nervous system

1. Introduction

Carbohydrate antigens containing sialic acids appear in the course of carcinogenesis [1]. Their appearance seems to correlate with the characteristics of cancers such as poor prognosis or metastatic potential [2–5]. Sialyl-Tn (STn) antigen is characterized as one of the cancer-associated carbohydrate antigens, whose expression has been correlated with poor prognosis in gastric cancer patients [4,5]. To elucidate the function of STn antigen in the progression of gastric cancer, as a first step, we recently cloned STn antigen synthetic enzyme (human GalNAcα2,6-sialyltransferase I (hST6GalNAc I)) from gastric mucosa [6]. The gene expression of *hST6GalNAc I* was well correlated with STn antigen positive gastric intestinal metaplasia and gastric cancer. The result clearly indicated that *hST6GalNAc I* takes part in STn antigen synthesis in gastric mucosa. However, further analysis of gene expression in other organs revealed little correspondence with the expression of the *hST6GalNAc I* gene and STn antigen [7], suggesting the occurrence of unidentified GalNAcα2,6-sialyltransferases. Thus, we tried molecular cloning of a new *GalNAcα2,6-sialyltransferase*. We performed BLAST analysis on a mouse expressed sequence tags (EST) database with the amino acid sequence of sialyl motifs L and S of the mST6GalNAc family and identified two clones containing a sequence most similar to ST6GalNAc IV in the mST6GalNAc family. We report here the substrate specificity and gene expression pattern of the newly cloned ST6GalNAc V, which is a new candidate for GD1α antigen synthase.

2. Materials and methods

2.1. Materials

Fetuin, asialofetuin, bovine submaxillary mucin, bovine submaxillary asialomucin, α_1-acid glycoprotein, holotransferrin, GM1a, GD1a, GD1b, GT1b, GM3, paragloboside, asialo-GM1, CMP-NeuAc, and Triton CF-54 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). CMP-[^{14}C]NeuAc (300 mCi/mmol) was from Amersham-Pharmacia Japan (Tokyo, Japan). Synthetic primers were synthesized by Gibco-BRL (Gibco-BRL, Rockville, MD, USA). Restriction endonucleases were from New England Bio Labs Inc. (Beverly, MA, USA). HPTLC plate silica gel 60 was purchased from Merck (Darmstadt, Germany). C3H-HeNCrj (C3H) and C57BL-6Ncrj (B6-N) were from Charles River Japan, Inc. (Kanagawa, Japan) and Slc:CD1 (ICR) mice were from SLC, Inc. Japan (Shizuoka, Japan). C57BL-6J (B6-J) mouse substrain was from Jackson's Lab. (Charles River Japan, Inc.). Monoclonal antibody (mAb) KA17 specific for GD1α [8] (IgM) was kindly gifted by Dr. Y. Hirabayashi (Laboratory for Glyco Cell Biology, Frontier Research Program, RIKEN).

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[1] The 2780 bp nucleotide sequence of *mST6GalNAc V* cDNA was registered by us in DDBJ/EMBL/GenBank with the accession number AB028840.

Abbreviations: NeuAc, *N*-acetylneuraminic acid; GalNAc, *N*-acetylgalactosamine; ST6GalNAcs, GalNAcα2,6-sialyltransferases; ST6GalNAc I, GalNAcα2,6-sialyltransferase I (EC 2.4.99.3); ST6GalNAc II, Galβ1,3GalNAc specific GalNAcα2,6-sialyltransferase; ST6GalNAc III, the first type of NeuAcα2,3-Galβ1,3GalNAc specific GalNAc-α2,6-sialyltransferase III (EC 2.4.99.7); ST6GalNAc IV, the second type of NeuAcα2,3-Galβ1,3GalNAc specific GalNAc-α2,6-sialyltransferase IV (EC 2.4.99.7); ST3Gal I, Galβ1,3GalNAcα2,3-sialyltransferase (EC 2.4.99.4); CMP-NeuAc, cytidine 5′-monophospho-*N*-acetylneuraminic acid; ORF, open reading frame; EST, expressed sequence tags; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TLC, thin layer chromatography; HPTLC, high performance thin layer chromatography; mAb, monoclonal antibody; NANase, *N*-acetylneuraminidase; The nomenclature for gangliosides follows the system of Svennerholm [26]; The abbreviated nomenclature for cloned sialyltransferases follows the system of Tsuji et al. [27]

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2.2. Probe preparation

We performed BLAST analysis on a mouse EST database with sialyl motifs L and S of ST6GalNAcs for the queries, and clones AU035329 from brain cDNA library and AA462934 from mammary gland were expected to contain a homologous sequence in ST6GalNAc IV sialyl motif L. Polymerase chain reaction (PCR) primers 5′ primer YI-1: 5′-CCCAAAATGAAGACCCTGATG-3′ and 3′ primer YI-2: 5′-GGCATCATTCATGCGGAATAAC-3′ were set on AU035329 nucleotide sequence to obtain the cDNA fragment with the reverse transcription polymerase chain reaction (RT-PCR) method. Template cDNA was synthesized from adult ICR mouse brain total RNA with the Superscript Preamplification System for First Strand cDNA Synthesis (Gibco-BRL). It was diluted 50-fold with H_2O, 10 μl of which was applied for template cDNA. Thus, a total volume of 50 μl of PCR reaction mixture consisted of 0.2 μM of the primer set, YI-1 and YI-2, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 μM of each dNTP, 0.1% Triton X-100, Ampli Taq Gold (Roche Molecular Systems, Branchburg, NJ, USA) and 10 μl of the diluted synthesized cDNA. After preheating for 10 min at 94°C, 40 cycles of PCR were run for 1 min at 94°C, 1 min at 60°C as an annealing temperature, and 1 min at 72°C. The amplified 387 bp cDNA fragment was subcloned in pCR II vector (pCR II-387bp frag.) (TA Cloning Kit, Invitrogen, Carlsbad, CA, USA) and purified with Qiaprep Spin Miniprep Kit (Qiagen K.K., Tokyo, Japan) to determine the nucleotide sequence. The *Nsi*I-*Eco*RI 151 nucleotide fragment without CAG repeat from the pCR II-387bp fragment was used as a hybridization probe.

2.3. Isolation of cDNA from adult mouse brain cDNA library

The adult mouse brain cDNA library was previously constructed [9,10]. We performed phage plaque, plaque lift, hybridizing screening, and in vivo excision according to the instruction manual (Stratagene, La Jolla, CA, USA).

2.4. Construction of phylogenetic tree of sialyltransferases

The 1005 bp open reading frame (ORF) encoding a protein was contained in the isolated cDNA clone, U1, whose start codon was assigned by Kozak consensus sequence. The putative whole amino acid sequence (U1 protein) showed only 44.8% and 42.6% identity to mouse ST6GalNAc IV [11] and ST6GalNAc III [11]. We constructed multiple alignments with sialyl motifs L and S of 16 sialyltransferases [11–16] and U1 protein, to predict which substrate specificities it exhibits. The phylogenetic tree was constructed with the neighbor joining method [17] which had been applied for construction of the fucosyltransferase family in our previous study [18].

2.5. Plasmid construction of the isolated U1 cDNA clone for expression of the soluble form enzyme

U1 cDNA was subcloned into our prepared expression vector pcDSA [9,13,19], for the purpose of detecting accurate enzyme activity of the U1 protein as in our previous works [9–13]. The pcDSA vector was constructed to fuse the target cDNA with the immunoglobulin M secretory signal peptide and the protein A immunoglobulin binding site sequentially, and the expressed soluble form protein in medium was collected with IgG beads for the enzyme source. The soluble form isolated cDNA was prepared by PCR amplification with primers (5′ primer YI-3: 5′-TACAGCAGCCTCGAGATGCCAGAAGG-3′, 3′ primer YI-2: 5′-CTGCCACACAGCGTGGACACC-3′) as the pBlueScript cDNA clone for a template. The amplified cDNA fragment was subcloned into the pCR II vector and excised with *Xho*I endonuclease enzyme with the *Xho*I site of the YI-3 primer and pCR II vector to subclone into the *Xho*I site of pcDSA (pcDSA-U1). Twenty μg of the expression plasmid pcDSA-U1, the previously prepared expression vectors pCDB8ST (mST6GalNAc III) [11], pCDR1ST (mST6GalNAc IV) [11] and the mock transfection vector pcDSA, were transiently transfected into COS-7 cells as in our previous works [9–13].

2.6. Acceptor substrate preparation

Asialo α_1-acid glycoprotein was prepared by 0.1 N H_2SO_4, 85°C for 15 min mild acid hydrolysis of α_1-acid glycoprotein [6]. The acceptor glycoproteins were lysed in Milli Q water at a concentration of 10 mg/ml. GM1b was prepared with recombinant ST3Gal I from asialoGM1 in the same manner as in our previous report [9]. In brief, asialoGM1 was sialylated with the secreted form of mouse ST3Gal I in COS-7 cells and purified with preparative TLC (chloroform:methanol:0.2% $CaCl_2$ = 55:45:10).

2.7. Sialyltransferase assay

Sialyltransferase assays were performed as described previously [6,9–11,13]. They were performed in the presence of 0.3% Triton CF-54, 50 mM 4-morpholineethanesulfonic acid (pH 6.4), 10 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM CMP-[^{14}C]NeuAc (10.2 kBq), 1 mg/ml glycoprotein or 0.5 mM ganglioside acceptor substrates, and enzyme source in a final volume of 20 μl. When glycosphingolipid substrates were used, they were subjected to HPTLC with two solvent systems, chloroform:methanol:0.2% $CaCl_2$ = 55:45:10 and chloroform:methanol:2.5 N NH_4OH = 60:40:9. When glycoprotein substrates were used, the reaction mixtures were applied on SDS-PAGE (5–20% acrylamide gel).

TLC immunostaining was performed according to Myoga et al. [20] with the primary antibody KA17 specific for GD1α for 90 min at room temperature.

2.8. Identification of linkage between sialic acid and acceptor glycosyl residues

Using sequential sialidase treatments as previously described [6,11], we identified the linkage between the acceptor glycosyl residues and sialic acid on the product of the sialyltransferase assay. By the pcDSA-U1 expressed in media of transfected COS-7 cells, [^{14}C]NeuAc incorporating GM1b was prepared. [^{14}C]NeuAc incorporating GM1b was subjected to sequential digestion with linkage specific sialidases, Newcastle disease virus (NDV) sialidase [21] (specific for α2,3- and α2,8-linkage; Oxford Glycosystems Ltd., Bedford, MA, USA), NANase I (specific for α2,3-linkage; NeuAc Linkage Analysis Kit, Glyco, Inc., Novato, CA, USA), and *Vibrio cholerae* sialidase [22] (specific for α2,3-, α2,6- and α2,8-linkage; Boehringer Mannheim Co. Japan, Tokyo, Japan).

2.9. Northern blot analysis

Ten μg total RNAs or 2 μg poly(A) fraction RNAs was separated on 1.0% agarose-formaldehyde gel, then transferred onto a membrane (Optitran BA-S 85 Reinforced NC; Schleicher and Schuell, Dassel, Germany). The ST6GalNAc V *Bam*HI-*Eco*RI fragment (449–1608) radiolabeled with High Prime (Boehringer Mannheim) was used for the probe. Using QuikHyb Hybridization Solution (Stratagene), hybridization was performed and membranes were washed according to the instruction manual.

2.10. Identification of CAG repeat numbers in inbred mouse strains

The isolated cDNA included CAG repeat in the ORF, which encoded polyglutamine. To know beforehand whether the CAG repeat numbers are polymorphic or not among mouse strains, three inbred mouse strains (C3H, B6-N and B6-J) were investigated. Total RNAs were prepared from forebrain as described above and RT-PCR was performed with the primer set YI-1 and YI-2. PCR products were purified and the nucleotide sequence determined.

3. Result

3.1. Identification and sequence of a new sialyltransferase cDNA clones from mice

A BLAST search was performed with the sialyl motif S and L amino acid sequences of the ST6GalNAc family, and then AU035329 and AA462934 were refined to contain a partial cDNA sequence of the new sialyltransferase gene. They contained a putative amino acid sequence similar to ST6GalNAc IV sialyl motif L. Using corresponding cDNA fragments, we succeeded in isolating four independent clones from a previously prepared adult ICR mouse brain cDNA library. All of the clones had a 2.8 kb cDNA insert (cDNA clone U1; Fig. 1) and contained a 1005 bp ORF whose start codon was assigned by Kozak consensus sequence encoding a protein. A polyadenylation signal AATAAA 185 nucleotide upstream from the poly(A) sequence is present at the 3′ end. The putative U1 protein indicated a molecular mass of 38.3 kDa and had a hydrophobic cluster (underlined uppercase in Fig. 1) in the amino-terminal region according to the hydropathy profile

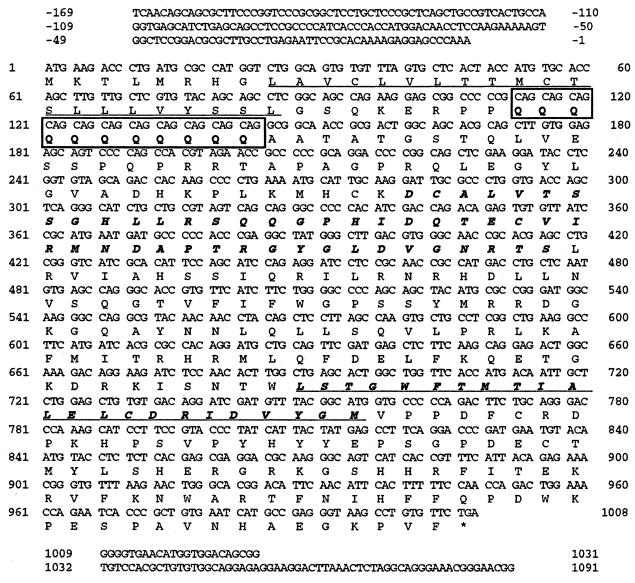

Fig. 1. Nucleotide and deduced amino acid sequence of ICR mouse ST6GalNAc V. The whole nucleotide sequence is described in accession number AB028840. The nucleotide and amino acid sequences of mouse ST6GalNAc V are numbered from the presumed start codon and initiation methionine, respectively. The deduced transmembrane domain is underlined, and CAG repeats are boxed. Bold italic shows sialyl motif L and bold italic underlined indicates sialyl motif S.

by the Kyte and Doolittle method [23]. The putative U1 protein showed type II membrane protein topology with a short cytoplasmic tail, a transmembrane spanning domain (21 amino acid peptide in this protein), a long stem region and a large enzyme active domain the same as other glycosyltransferases (Fig. 1) [24]. It showed 42.6% identity to mouse ST6GalNAc III, and 44.8% to mouse ST6GalNAc IV, but no other showed a similarity of more than 30%. We constructed a multiple alignment with the sialyl motifs S and L of 17 sialyltransferases including the putative U1 protein. ST6GalNAc III, ST6GalNAc IV and putative U1 protein form one group out of the other sialyltransferases on the constructed phylogenetic tree (data not shown) and these results indicated that it was the fifth member of the ST6GalNAc subfamily.

3.2. Cloned cDNA acceptor substrate specificity

The COOH-terminal portion of U1 was fused with the IgG binding domain in an expression plasmid (pcDSA-U1) and expressed in media to determine the enzymatic activity. pcDSA-U1 exhibited strong activity toward GM1b (Fig. 2A) but not toward any other glycolipids and glycoproteins (Fig. 2A) (paragloboside, asialo-GM1, GM1a, GD1a, GD1b, GT1b, GM3, asialofetuin, mucin from bovine submaxillary gland, asialomucin from bovine submaxillary gland, holotransferrin and asialotransferrin). The faint activity toward fetuin, α_1-acid glycoprotein and asialo-α_1-acid glycoprotein were detected at relative activity levels of 8%, 2% and 4%, respectively, of the activity toward GM1b. The activity toward GM1b was not detected in the absence of 10 mM $MgCl_2$ and 5 mM $CaCl_2$. Based on the following result, we assigned the [^{14}C]NeuAc transferred GM1b by pcDSA-U1 for GD1α. pCDB8ST (mST6GalNAc III) and pCDR1ST (mST6GalNAc IV) can also transfer from NeuAc-CMP to the 6-position of GalNAc glycoside residues on GM1b or fetuin, and we have proved that they synthesize GD1α from GM1b [11]. These GD1α synthetic activities are visualized and confirmed with HPTLC and BAS2000 imaging analyzer in Fig. 2B. Both [^{14}C]NeuAc incorporated GM1b by pcDSA-U1 and by two GD1α synthase positioned in the same area using two solvent systems, chloroform:methanol:0.2% $CaCl_2$ = 55:45:10 (Fig. 2B) and chloroform:methanol:2.5 N NH_4OH = 60:40:9 (data not shown). The TLC plates were continuously subjected to TLC immunostaining, and [^{14}C]NeuAc incorporating GM1b by pcDSA-U1 was stained with mAb KA17 specific for GD1α [8], the same as by mST6GalNAc III and mST6GalNAc IV (Fig. 2B). Thus, we demonstrated that a new sialyltransferase synthesized GD1α from GM1b and was a new GD1α synthase. As regards V_{max}, the substrate specificities of mST6GalNAc III, IV and V were different from glycoprotein and glycolipid, respectively. mST6GalNAc III and IV exhibited 80% and 500% relative activity toward fetuin as compared to the incorporation of [^{14}C]sialic acids into GM1b as a substrate but mST6GalNAc V exhibited

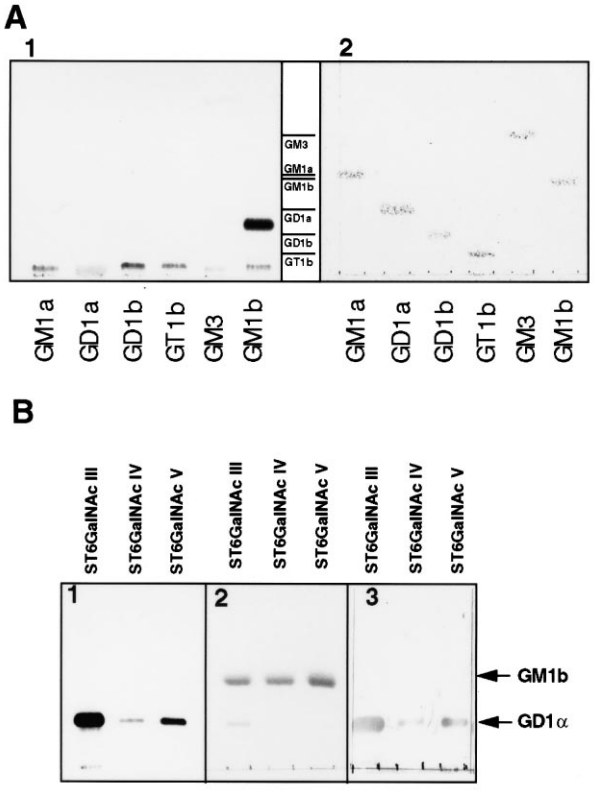

Fig. 2. Enzyme acceptor specificity of mST6GalNAc V Incorporation of [^{14}C]NeuAc into glycosphingolipids by cloned sialyltransferase. The resulting glycosphingolipids were analyzed by HPTLC with a solvent system of chloroform:methanol:0.2% $CaCl_2$ = 55:45:10. The radioactive materials were visualized with a BAS 2000 radio image analyzer (A1, B1), with orcinol-H_2SO_4 (A2, B2) and immunostained with mAb KA17 specific for GD1α (B3). A: Enzyme activity in relation to several glycosphingolipids (GM1a, GD1a, GD1b, GT1b, GM3, GM1b). The enzyme source was prepared from the culture media from mST6GalNAc V (pcDSA-mST6GalNAc V) transfected COS-7 cells. B: Enzyme activity of ST6GalNAc III (pCDB8ST), ST6GalNAc IV (pCDR1ST) and mST6GalNAc V (pcDSA-mST6GalNAc V) toward GM1b. [^{14}C]NeuAc incorporating GM1b was confirmed for GD1α by TLC immunostaining with mAb KA17 specific for GD1α (B3).

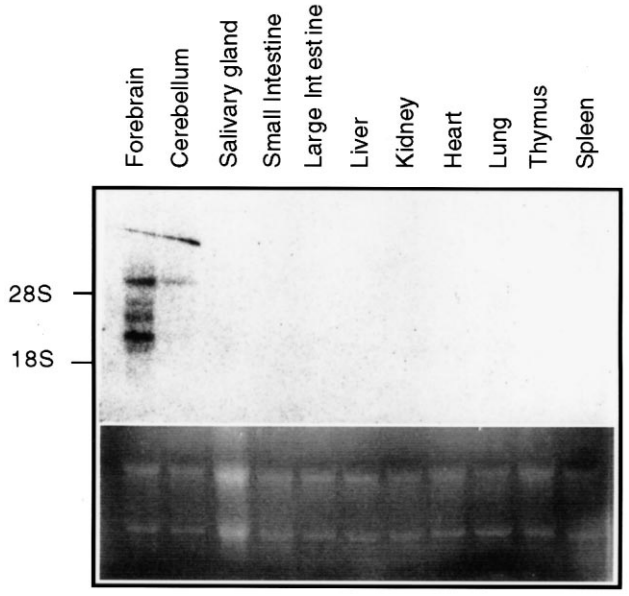

Fig. 3. Northern blot analysis of ST6GalNAc V gene transcription. RNAs from various B6-N Crj mouse tissues were separated on agarose gel and then transferred onto nitrocellulose membrane. Hybridization was performed as described in Section 2. Each 10 μg of total RNA was examined to detect ST6GalNAc V transcription with ^{32}P-labeled *Bam*HI-*Eco*RI digest fragment from isolated cDNA clone (upper panel). Lower panel is ethidium bromide staining.

only 8% relative activity toward fetuin. The sequential linkage analysis for [^{14}C]NeuAc incorporating GM1b indicated that [^{14}C]NeuAc was incorporated into GM1b with α2,6-linkage. NDV sialidase treatment, specific for α2,3- or α2,8-linkage [21], was no longer effective, and NANase I treatment, specific for α2,3- linkage, resulted in only two radioactive bands without total radioactivity reduction. On the contrary, *V. cholerae* sialidase treatment, which exhibits activity for α2,3-, α2,6- and α2,8-linkage [22], reduced total radioactivity (data not shown). Therefore, these result strongly suggest that the incorporated sialic acids contained α2,6-linkage but not either α2,3- or α2,8-linkage. Thus, we named U1 mST6GalNAc V. The 2780 bp nucleotide sequence of *mST6GalNAc V* cDNA was registered by us in DDBJ/EMBL/GenBank with the accession number AB028840.

3.3. Expression of the ST6GalNAc V gene in mouse tissues

The expression of the ST6GalNAc V gene was examined in several mouse tissues with Northern blot analysis. Four strong bands corresponding to about 7 kb, 5 kb, 3 kb and 2 kb were detected in adult mouse forebrain. A moderately intense single signal was detected in cerebellum (Fig. 3). The other tissues showed no signal. The same result was obtained from poly(A) RNA enriched fractions (data not shown).

3.4. Polymorphism of CAG repeats number

To determine whether CAG repeat numbers were common in inbred mouse strains, we performed RT-PCR with YI-1 and YI-2 primers and determined the nucleotide sequence of the amplified fragment. The inbred mouse strains C3H, B6-N and B6-J possessed 12, 11 and 10 CAG repeats, respectively.

4. Discussion

The fifth type of α*2,6-sialyltransferase* (*mST6GalNAc V*) was successfully cloned from a mouse brain cDNA library, which exhibited GD1α synthetic activity from GM1b.

mST6GalNAc V exhibited 42.6% and 44.8% identity at the amino acid level to mST6GalNAc III and IV, respectively. A phylogenetic tree constructed by the neighbor joining method revealed that mST6GalNAc V belongs to the ST6GalNAc family, and is especially closely related to ST6GalNac III and V (data not shown). *mST6GalNAc* cDNA encoded a protein of 335 amino acids, which contained highly conserved regions, sialyl motifs L and S, the same as other sialyltransferases so far cloned. The *mST6GalNAc V* gene was highly expressed in adult mouse forebrain and moderately in cerebellum. No other organs tested, such as salivary gland, intestine, liver, kidney, heart, lung, thymus, and spleen, expressed the *mST6GalNAc V* gene. Four species of mRNAs (about 2.0, 2.8, 4.0 and 6.0 kb) were detected in Northern blot. The length of all cloned cDNAs was 2.8 kb. Further analysis of the expression of various transcripts should be performed. Now genomic analysis is in progress. Interestingly, *mST6GalNAc V* carried CAG repeats encoding polyglutamine in the putative stem region, different from the other glycosyltransferases so far cloned. Moreover, the CAG repeat number of *mST6GalNAc V* cDNA varied among the inbred mouse strains of C3H (12 repeats), B6-N (11 repeats) and B6-J (10 repeats). We will examine whether the CAG repeat exists in a human *ST6GalNAc V* gene or not.

The following evidence suggests that mST6GalNAc V is the third candidate for GD1α synthase. First, the substrate specificity was highly restricted to GM1b. The recombinant soluble mST6GalNAc V fused with protein A, expressed in culture media of COS-7 cells, exhibited activity toward GM1b, and the product appeared at the GD1α position on HPTLC. mST6GalNAc V did not exhibit any activity toward other substrates we tested including glycoproteins and glycolipids. Secondly, linkage analysis using linkage specific sialidases confirmed that sialic acids of the product from GM1b were bound through α2,6-linkage.

Substrate specificity comparison among all cloned GD1α synthetic activities (mST6GalNAc III, IV and V) revealed that the three ST6GalNAcs exhibited characteristic activities toward glycoprotein and glycolipid, respectively [11]. The newly cloned mST6GalNAc V showed higher activity toward glycolipid than glycoprotein in contrast with mST6GalNAc IV, which preferred glycoprotein rather than glycolipid. mST6GalNAc III exhibited activity toward both glycoprotein and glycolipid [11]. The result clearly indicates that three GD1α synthetic enzymes possess different physiological functions, and mST6GalNAc V is a most probable candidate for GD1α synthase. mST6GalNAc V did not show GQ1bα synthetic activity from GT1b. The result indicates that the other α2,6-sialyltransferase synthesizes GQ1bα from GT1b [25]. The mST6GalNAc V gene was highly expressed as compared with mST6GalNAc III and IV genes, as previously reported in our data [11]. Otherwise, GD1α expression in the adult mouse central nervous system is extremely low [8]. The kinetic analysis of divalent cation requirement revealed divalent cation is essential for mST6GalNAc V activity. The contradiction between expression levels of gene expression and GD1α may be caused by the divalent cation requirement in vivo. Further

analysis of the enzyme kinetics is necessary. Analyses of gene expression and kinetic properties of ST6GalNAc III, IV and V will help to understand more about the physiological function of GD1α.

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