Molecular Cloning and Expression of a Fifth Type of α2,8-Sialyltransferase (ST8Sia V)

ITS SUBSTRATE SPECIFICITY IS SIMILAR TO THAT OF SAT-V/III, WHICH SYNTHESIZE
G_D1c, G_T1a, G_Q1b AND G_T3∗

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The cDNAs encoding a new α2,8-sialyltransferase (ST8Sia V) were cloned from a mouse brain cDNA library by means of a polymerase chain reaction-based method using the nucleotide sequence information on mouse ST8Sia I (G_D3 synthase) and mouse ST8Sia III (Sia2,3Galβ1,4GlcNAc2,8-sialyltransferase), both of which exhibit activity toward glycolipids. The predicted amino acid sequence of ST8Sia V shows 61.6% identity to those of mouse ST8Sia I and III, respectively. The recombinant protein A-fused ST8Sia V expressed in COS-7 cells exhibited an α2,8-sialyltransferase activity toward G_D1b, G_T1a, G_D3, and G_T3 and synthesized G_D1c, G_T1a, G_Q1b, and G_T3, respectively. The apparent K_m values for G_D1b, G_T1a, G_D3, and G_T3 were 1.1, 0.082, 0.070, and 0.28 mM, respectively. However, ST8Sia V did not exhibit activity toward G_Ma. Thus, the substrate specificity of ST8Sia V is different from those of ST8Sia I and III, both of which exhibit activity toward G_Ma. Transfection of the ST8Sia V gene into COS-7 cells, which express G_D1a as a major glycolipid, led to the expression of determinants for mononclonal antibody 4F10, which recognizes G_T1a and G_Q1b, suggesting that ST8Sia V exhibits activity toward gangliosides G_D1a and/or G_T1b in vivo. The expression of the ST8Sia V gene was tissue- and developmental stage-specific, and was clearly different from those of other α2,8-sialyltransferase genes. The ST8Sia V gene was strongly expressed in the brain and weakly in other tissues such as the liver. In addition, its expression was greater in the adult than fetal brain. These results strongly indicate that ST8Sia V is a candidate for SAT-V, the α2,8-sialyltransferase involved in G_D1c, G_T1a, G_Q1b, and G_T3 synthesis.

Gangliosides comprise a structurally diverse subset of sialylated glycosphingolipids that are abundantly expressed in the brain (1). The biological roles of gangliosides are becoming increasingly appreciated, particularly in regard to intercellular adhesion, immune modulation, growth control, and receptor function (2). Ganglioside biosynthesis takes place in the Golgi apparatus where, starting with Glc-Cer, it progresses with the sequential addition of Gal, GalNAc, and Sia to the growing oligosaccharide chain. These reactions are catalyzed by specific glycosyltransferases. Many of these enzyme activities have been studied and partially characterized in rat liver Golgi membrane fractions. These studies suggested a scheme for ganglioside biosynthesis (Scheme 1, Table I) (3–5). G_Ma, G_D3, and G_T3 synthase activities (SAT-I, -II, and -III) can be discriminated on the basis of the enzymatic characteristics. However, a competition-based assay suggested that each of galactosyltransferase II (GalT-II), GalNac transferase, SAT-IV and -V activity is due to a sole enzyme, respectively. In addition, SAT-V activity was reported to synthesize G_T3 from G_D3 (5).

To clarify the biosynthesis of gangliosides, it is necessary to clone, express, and characterize the enzymes corresponding to the activities in the Golgi membrane fractions. In the case of GalNac-T, the results for knockout mice as to GalNac-T clearly suggested that GalNAc-T activity is due to a sole enzyme. However, the situation in the case of α2,8-sialyltransferase groups is more complicated. In 1994, three groups including ours cloned G_D3 synthase (ST8Sia I) by the expression cloning method (6–8). One group reported that G_D3 synthase exhibits SAT-V activity (9). Recently, G_T3, synthase was cloned by the expression cloning method and its nucleotide sequence was shown to be identical to that of G_D3 synthase, indicating that G_D3 and G_T3 are synthesized by a single enzyme (10). These observations may lead to the conclusion that only one α2,8-sialyltransferase, ST8Sia I, is responsible for the biosynthesis of all α2,8-sialic acid linkages in gangliosides. However, the following evidence suggests that plural α2,8-sialyltransferases are responsible for the activities of SAT-II, -III, and -V, respectively. (i) ST8Sia III also exhibits activity toward G_D3 and G_D3 in vitro (11). (ii) In the rat liver Golgi membrane fractions, the activities of SAT-II and -V were clearly distinguished enzymatically (4). Furthermore, as shown in this paper, the apparent K_m values of mouse ST8Sia I for G_D1a, G_T1b, and G_D3 are much higher than those observed for the Golgi membrane fractions. Thus, it is possible that there are other α2,8-sialyltransferases that synthesize G_T3, G_D1a and/or G_Q1b, more specifically, like

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1 The nomenclature for gangliosides follows the system of Svennerholm (26). The abbreviated nomenclature for cloned sialyltransferases follows the system of Tsuji et al. (27). The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); 2,3-SPG, sialylparagloboside (Sia2,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Cer); HPTLC, high performance thin layer chromatography; RT-PCR, reverse transcription polymerase chain reaction; G3PDH, glyceraldehyde-3-phosphate dehydrogenase gene; SSC, saline-sodium citrate solution.

2 K. Furukawa, personal communication.
the activity of SAT-V, even though GD3 synthase can transfer all α2,8-linked sialic acid residues in gangliosides. According to this consideration, we tried to clone new ganglioside-specific α2,8-sialyltransferases, using the polymerase chain reaction (PCR)-based approach and the information on mouse ST8Sia I and ST8Sia III, both of which exhibit activity toward glycolipids. In this article, we will describe evidence for the occurrence of an α2,8-sialyltransferase similar to SAT-VIII.

**Experimental Procedures**

**Materials**—Some glycosphingolipids (lactosylceramide, GM3, GM1, Gb3, GD1a, GD1b, GT1b, Gq1b, and asialo-GM1) and Triton-CF-54 were purchased from Sigma, CMP-[14C]NeuAc (11.8 GBq/mmol) was from Amersham Corp, IgG-Sepharose was from Pharmacia Biotech Inc., LipofectAMINE was from Life Technologies, Inc., NANase II was from Amersham Corp, IgG-Sepharose was from Pharmacia Biotech Inc., and sialidase from *Vibrio cholerae* was from Boehringer Mannheim. Gb3 (Siaα2,3Galβ1,4GlcNAcβ1,4Galβ1,4Glcβ1,1Cer) was synthesized from asialo-Gb3α1,2 by the enzymatic reaction of recombinant mouse ST3Gal I, which exhibits activity toward Galα1,4GlcNAcβ1,4Galβ1,4Glcβ1,1Cer (2,3-Sing3). The scheme is based on the results obtained for the rat Golgi apparatus (3–5). Glc, glucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Sia, sialic acid; Cer, ceramide.

**Scheme 1. Postulated pathway for ganglioside biosynthesis.** The scheme is based on the results obtained for the rat Golgi apparatus (3–5). Glc, glucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Sia, sialic acid; Cer, ceramide.

**Table I.** Enzymes for representative ganglioside biosynthesis.

| Reaction | Name of enzyme (detected in Golgi fractions) | Corresponding cloned sialyltransferase(s) suggested by in vitro assay |
|----------|---------------------------------------------|---------------------------------------------------------------|
| (α2,3-sialyltransferase; GM3 synthase) | SAT-I | not cloned |
| (α2,8-sialyltransferase; GD3 synthase) | SAT-II | ST8Sia I(6-8) > III(11) |
| (α2,6-sialyltransferase; GT3 synthase) | SAT-III | ST8Sia V > III(11) > I(10) |
| (GalNAc transferase) | GalNAc-T | -- |
| (Gal transferase) | GalT-II | -- |
| (α2,3-sialyltransferase) | SAT-IV | ST3Gal II(25) > I(12) |
| (α2,8-sialyltransferase) | SAT-V | ST8Sia V > I(9) |

**Table II.** Enzymes for representative ganglioside biosynthesis.
Saline-sodium phosphate-EDTA solution, 5 mM Denhardt's, 0.5% SDS, 0.25% sodium lauryl sarcosine, and 10 mg/ml denatured salmon sperm DNA at 37°C for 2 h. Hybridization was performed overnight at 45°C with a 32P-labeled DNA probe made by the random priming method. The full-length ST8Sia V-M cDNA (1128 bp) was used as a probe. The membrane was washed twice in 2x SSC, 0.1% SDS at 65°C, in 0.5x SSC, 0.1% SDS at 65°C, and finally in 0.2x SSC, 0.1% SDS at 65°C.

RT-PCR was performed with cDNA synthesized from total RNA (5 μg), and 5'- and 3'-primers for ST8Sia V and G3PDH (ST8Sia V 5'-primer P4C1, 5'-GCGGGAAGGAGATCAACAGCGCT-3', nucleotides 641–663; ST8Sia V 3'-primer, 5'-CAGGAGTCGAGACACCCATG-GCAGGCCTGGT-3', complementary to the ST8Sia V coding strand nucleotides 1243–1273; G3PDH 5'-primer, 5'-ACCACAGTCCATGCCTGCTGCTATCGGTCGCTGCT-3', nucleotides 526–547; G3PDH 3'-primer, 5'-TCCACCAC-CTGCTGCTGCTATCGGTCGCTGCTGCT-3', complementary to the G3PDH coding strand nucleotides 958–977). The cycling parameters for PCR for ST8Sia V and G3PDH were 94°C for 40 s, 55°C for 40 s, and 72°C for 1 min for 35 cycles or 18 cycles, respectively.

RESULTS

Cloning and Nucleotide Sequencing of a New Sialyltransferase cDNA—Recently, four types of α2,8-sialyltransferase genes were cloned (6–8, 11, 14–16), two of which, ST8Sia I (GD3 synthase) and ST8Sia III, exhibit activity toward α2,3-sialylated glycosphingolipids. To isolate the gene encoding a new α2,8-sialyltransferase involved in ganglioside biosynthesis, we conducted PCR cloning experiments with two degenerate oligonucleotide primers based on two highly conserved regions, sialyl motifs L and S, of mouse ST8Sia I and ST8Sia III (11), and a mouse cDNA library. In order to exclude fragments of ST8Sia I and III, we digested the amplified fragment with BglII and HindIII, whose restriction sites are present in the amplified fragments (0.5 kb) of ST8Sia I and ST8Sia III, respectively, and then another PCR was performed using the same primers and the BglII/HindIII-resistant fragment as a template. The PCR product corresponding to a 0.5-kb fragment was subcloned and sequenced. Among several clones, one clone, pCRP4, encoded a peptide exhibiting 47.9% and 22.5% identity to mouse ST8Sia I and ST8Sia III, respectively. This fragment, pCRP4, was used as a probe to screen an adult mouse brain cDNA library. Several overlapping clones were obtained and sequenced (Fig. 1). cDNAs of three different lengths were cloned, named P4-L, P4-M, and P4-S. Among nine clones, six clones were P4-M, two clones were P4-L, and only one clone was P4-S. P4-L, -M, and -S encode proteins of 412, 376, and 345 amino acids, respectively, each of which exhibits a type II transmembrane topology, consisting of a NH2-terminal cytoplasmic tail, a transmembrane domain, a proline-rich stem region, and a large COOH-terminal active domain, and contains two highly conserved regions, namely sialyl motifs L and S, like all so far cloned sialyltransferases. The predicted amino acid sequence encoded by P4-M exhibits 36.1% and 15.1% identity to the mouse ST8Sia V and III genes, respectively. No similarity, except in sialyl motifs L and S, was observed as compared with other types of sialyltransferases. As described in the following sections, P4-L, -M, and -S encode the α2,8-sialyltransferases, which exhibit the same substrate specificities, and we designated them as ST8Sia V-L, -M, and -S.
The only difference between the three cDNAs was found in the putative stem region of the proteins (Fig. 1), deletions of 36 and 67 amino acids in the stem region of ST8Sia V-L (residues 44–79 and residues 44–110) being observed in ST8Sia V-M and -S, respectively. The nucleotide sequences in other regions of the three cDNAs were completely the same (Fig. 1).

Expression of the ST8Sia V Gene in Mouse Tissues—In order to compare the quantity of gene expression among the three kinds of ST8Sia V in adult mouse brain, RT-PCR was performed using cDNA synthesized from total RNA (5 µg) as templates, and 5’- and 3’-primers for ST8Sia V (5’-primer P4C1 and 3’-primer P4Tail) and G3PDH. The bands for ST8Sia V and G3PDH are 633 and 452 bp, respectively.

To examine the in vitro activity of ST8Sia V, the ST8Sia V gene was transiently transfected into COS-7 cells, which express Gβδ1 as a major ganglioside, and then cells were stained with a monoclonal antibody, 4F10, which equally recognizes GT1a and GQ1b (17). As shown in Fig. 5, some of the ST8Sia V gene-transfected cells were positively stained with 4F10, whereas the cells transfected with the same vector without an insert were not stained.

Comparison of the Substrate Preferences of the Three α2,8-Sialyltransferases in Vitro—It has been reported that Gαδ3 synthase (ST8Sia I) cloned from human exhibited Gβδ1 synthase activity in addition to Gαδ synthase activity in vivo and in vitro (6–8, 10). On the other hand, ST8Sia III can also produce Gαδ1 and Gαδ1 in 2W, and 7-week (7W) mouse brains (B). The hybridization probe was made from the full-length fragment (1128 bp) of ST8Sia V-M cDNA C, RT-PCR was performed using cDNA synthesized from total RNA (5 µg) as templates, and 5’- and 3’-primers for ST8Sia V (5’-primer P4C1 and 3’-primer P4Tail) and G3PDH. The bands for ST8Sia V and G3PDH are 633 and 452 bp, respectively.

As shown in Fig. 4, the product co-migrated with authentic GbQ1b was resistant to the treatment with a2,3- and a2,6-specific sialidase, but was completely digested by the treatment with V. cholerae sialidase, which cleaves all types of sialic acid linkages, suggesting that the incorporated sialic acids contained a2,8-linkages.

The substrate specificities of the other forms (ST8Sia V-L and -S) were compared with that of ST8Sia V-M. Soluble recombinant ST8Sia V-L and -S also exhibited activity toward GbQ1b, GbT1b, and GbQ1b, respectively, on HPTLC with two different solvent systems (chloroform/methanol/0.02% CaCl2 (55:45:10) and n-propanol/28% ammonia solution/water (75:5:25)). ST8Sia V-M also exhibited activity toward GbQ1b (Siaα2,3Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1,1Cer), 2,3-sialylparagloboside (2,3-SPG, Siaα2,3Galβ1,4GlcNaCβ1,3Galβ1,4Glcβ1,1Cer), and probably toward GQ1b, in addition to GbQ1a, GbT1b, and GbT1b (Fig. 3). However, it was not surprising that ST8Sia V did not exhibit activity toward GbM3 (Siaα2,3Galβ1,4Glcβ1,1Cer) (Fig. 3). Since ST8Sia I and III were shown to exhibit the activity toward GbM3 as an acceptor together with other glycolipids including 2,3-SPG (9, 11), ST8Sia V was enzymatically distinguishable from ST8Sia I and III.

Comparison of the Substrate Preferences of the Three α2,8-Sialyltransferases in Vitro—It has been reported that Gαδ3 synthase (ST8Sia I) cloned from human exhibited Gβδ1 synthase activity in addition to Gαδ synthase activity in vivo and in vitro (6–8, 10). On the other hand, ST8Sia III can also produce Gαδ1 and Gαδ1 in 2W, and 7-week (7W) mouse brains (B). The hybridization probe was made from the full-length fragment (1128 bp) of ST8Sia V-M cDNA C, RT-PCR was performed using cDNA synthesized from total RNA (5 µg) as templates, and 5’- and 3’-primers for ST8Sia V (5’-primer P4C1 and 3’-primer P4Tail) and G3PDH. The bands for ST8Sia V and G3PDH are 633 and 452 bp, respectively.

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Expression of the ST8Sia V Gene. A and B. Northern blot analyses (lower) and ethidium-bromide-stained gels (upper). Total RNAs (5 µg) were prepared from various mouse adult tissues (A) Br, brain; He, heart; Li, liver; Lu, lung; Ki, kidney; Sp, spleen; S.G., salivary gland; Th, thymus; Te, testis; Pl, placenta. Total RNAs (5 µg) were also prepared from 7-day postcoital mouse embryo (E7), mouse fetal kidney (E14), 4-day (4d), 7-day (7d), 10-day (10d), 2-week (2W), 3-week (3W), 4-week (4W), and 7-week (7W) mouse brains (B). The hybridization probe was made from the full-length fragment (1128 bp) of ST8Sia V-M cDNA C, RT-PCR was performed using cDNA synthesized from total RNA (5 µg) as templates, and 5’- and 3’-primers for ST8Sia V (5’-primer P4C1 and 3’-primer P4Tail) and G3PDH. The bands for ST8Sia V and G3PDH are 633 and 452 bp, respectively.
III, and V showed broad activities toward most gangliosides tested. However, the \( K_m \) value for each substrate was characteristic of each enzyme. In the case of ST8Sia V, the \( K_m \) values for GD1a and GT1b were very low (70–80 \( \mu \)M), that for GD3 was medium (about 300 \( \mu \)M), and those for GM1b and 2,3-SPG were relatively high (over 1000 \( \mu \)M), indicating that ST8Sia V exhibits a substrate preference for GD1a and GT1b rather than GD3, 2,3-SPG, or GM1b. On the other hand, the \( K_m \) values for GD1a, GT1b, and 2,3-SPG of ST8Sia I were very high (2–5 mM) compared to that for GM3 (30 \( \mu \)M). ST8Sia III exhibited only very low activity toward GD1a and GT1b, showing the lowest \( K_m \) value toward 2,3-SPG. Thus, GD3 was a much more suitable acceptor for ST8Sia I than GM3, GD1a, or GT1b, and 2,3-SPG served as the best acceptor for ST8Sia III in vitro. The \( V_{\max}/K_m \) values indicated that GD1a and GT1b served as much better acceptors for ST8Sia V than ST8Sia I or III. In addition, the apparent \( K_m \) values of the three sialyltransferases for GD1a (280 \( \mu \)M for ST8Sia V, and 3–5 \( \mu \)M for ST8Sia I and III) indicated that ST8Sia V is a candidate for GT1 synthase. 

**DISCUSSION**

We cloned three cDNAs encoding new \( \alpha2,8\)-sialyltransferases (ST8Sia V-L, -M, and -S) from a mouse brain cDNA library by a PCR-based approach using the sequence information on the sialyl motifs of ST8Sia I and III, which exhibit activity toward gangliosides. The putative amino acid sequences revealed that the three types of ST8Sia V had putative stem regions of different length. The different lengths of the stem region may result of alternative splicing. Several genomic organization of sialyltransferase genes have been reported and some of them occur the splicing at stem region (ST6Gal I, ST6GalNAc II, ST3Gal I and IV, and ST8Sia III) (18–22). The length of the stem region may affect the substrate specificity and/or preference, but, as far as seen in an in vitro assay, there are no differences in substrate specificity and/or preference within experimental error (Table II). Northern blot analysis indicated that expression of the ST8Sia V gene was completely different from that of the ST8Sia I and III genes. ST8Sia V was strongly expressed in brain with age, but low in other tissues, as shown in Fig. 2.

Enzymatic analysis with the protein A-fused soluble ST8Sia V revealed that mouse ST8Sia V exhibited similar activity to SAT-V observed in the rat liver Golgi fractions, as follows. (i) ST8Sia V synthesizes GT1a, GQ1b, GD1c, and GT3 from GD1a, as shown in Fig. 2. (ii) The apparent \( K_m \) values of the recombinant soluble ST8Sia V for GD1a (82 \( \mu \)M), GT1b (70 \( \mu \)M), GM1b (1.1 mM), and GD3 (280 \( \mu \)M) were comparable with those observed in the rat liver Golgi fractions (10–30 \( \mu \)M for GM1a and GT1b, about 500 \( \mu \)M for GM1b, and 130 \( \mu \)M for GT1a) (4, 5). (iii) Transient transfection of the mouse ST8Sia V gene into COS-7 cells led to the expression of GT1a/GQ1b. Comparison of the substrate specificities of ST8Sia I, III, and V demonstrated that the in vitro specificities of these cloned \( \alpha2,8\)-sialyltransferases for glycolipids were rather broader than those of other sialyltransferases so far cloned, and almost completely overlapped each other, most \( \alpha2,3\)-sialylated glycolipids serving as in vitro acceptor substrates for the three enzymes. It has been demonstrated that human ST8Sia I (GD3 synthase) also exhibits SAT-V activity (9). Mouse ST8Sia I as well as mouse ST8Sia V actually synthesized Gt1a, Gq1b, and Gd3 from Gd1a, and GT1b, and mouse ST8Sia III exhibited weak activity toward Gd1a and GT1b in vitro. However, the apparent \( K_m \) values for Gd1a and GT1b of
In vitro enzyme activity and the expression of GT3 through the gene was transfected into mouse melanoma B16 cells, which expressed G\textsubscript{M3} but not the ST8Sia III or V gene, with a strong virus promoter, G\textsubscript{T3} as well as G\textsubscript{D3} was expressed on the cell surface. Furthermore, the mouse ST8Sia III gene transfection also led to the expression of G\textsubscript{D3} and G\textsubscript{T3} on the cell surface. Similarly, G\textsubscript{T1b}/G\textsubscript{Q1b} was expressed on the surface of COS-7 cells by the transfection of not only the mouse ST8Sia V gene but also the mouse ST8Sia I and III genes.\textsuperscript{3} Since the substrate specificities of the cloned α2,8-sialyltransferases are rather broad in vitro, it is possible that multiple enzymes co-function in a single step of the ganglioside pathway in the Golgi network. To confirm the in vivo specificity of cloned enzymes, especially sialyltransferases, it is important to determine the localization of the cloned enzymes in the Golgi network in addition to expression of specific carbohydrates through transfection of glycosyltransferase genes.

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### TABLE III

| Acceptor       | K\textsubscript{m} | V\textsubscript{max} | V\textsubscript{max}/K\textsubscript{m} |
|----------------|---------------------|---------------------|-----------------------------------|
| **ST8Sia V**   |                     |                     |                                   |
| G\textsubscript{M3} | ND                  | ND                  | ND                                |
| G\textsubscript{T3} | 0.28                | 16                  | 57                                |
| G\textsubscript{M1b} | 1.1                 | 67                  | 61                                |
| G\textsubscript{D1a} | 0.082               | 60                  | 730                               |
| G\textsubscript{T1b} | 0.070               | 100                 | 1400                              |
| SPG            | 1.0                 | 80                  | 80                                |
| **ST8Sia I**   |                     |                     |                                   |
| G\textsubscript{M3} | 0.030               | 100                 | 3300                              |
| G\textsubscript{M1b} | NT                  | NT                  | NT                                |
| G\textsubscript{T3} | 5.0                 | 50                  | 10                                |
| G\textsubscript{D1a} | 5.0                 | 78                  | 16                                |
| G\textsubscript{T1b} | 2.0                 | 78                  | 39                                |
| SPG            | 2.2                 | 52                  | 24                                |
| **ST8Sia III** |                     |                     |                                   |
| G\textsubscript{M3} | 0.59                | 40                  | 68                                |
| G\textsubscript{M1b} | NT                  | NT                  | NT                                |
| G\textsubscript{T3} | 3.3                 | 66                  | 20                                |
| G\textsubscript{D1a} | NC                  | NC                  | NC                                |
| G\textsubscript{T1b} | 0.082               | 100                 | 1200                              |

\textsuperscript{3} M. Kono, N. Kojima, and S. Tsuji, unpublished observation.

\textsuperscript{2} M. Kono, N. Kojima, and S. Tsuji, unpublished observation.