Molecular Cloning and Functional Expression of Two Members of Mouse NeuAca2,3Galβ1,3GalNAc GalNAca2,6-Sialyltransferase Family, ST6GalNAc III and IV*

(Received for publication, August 4, 1998, and in revised form, January 15, 1999)

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Two cDNA clones encoding NeuAca2,3Galβ1,3GalNAc GalNAca2,6-sialyltransferase have been isolated from mouse brain cDNA libraries. One of the cDNA clones is a homologue of previously reported rat ST6GalNAc III according to the amino acid sequence identity (94.4%) and the substrate specificity of the expressed recombinant enzyme, while the other cDNA clone includes an open reading frame coding for 302 amino acids. The deduced amino acid sequence is not identical to those of other cloned mouse sialyltransferases, although it shows the highest sequence similarity with mouse ST6GalNAc III (43.6%). The expressed soluble recombinant enzyme exhibited activity toward NeuAca2,3Galβ1,3GalNAc, fetuin, and GM1b, while no significant activity was detected toward Galβ1,3GalNAc or asialofetuin, or the other glycoprotein substrates tested. The sialidase sensitivity of the 14C-sialylated residue of fetuin, which was sialylated by this enzyme with CMP-[14C]NeuAc, was the same as that of ST6GalNAc III. These results indicate that the expressed enzyme is a new type of ST6GalNAc2,6-sialyltransferase, which requires sialic acid residues linked to Galβ1,3GalNAc residues for its activity; therefore, we designated it mouse ST6GalNAc IV. Although the substrate specificity of this enzyme is similar to that of ST6GalNAc III, ST6GalNAc IV prefers O-glycans to glycolipids. Glycolipids, however, are better substrates for ST6GalNAc III.

Sialic acids are key determinants of carbohydrate structures that play important roles in a variety of biological functions, like cell-cell communication, cell-substrate interaction, adhesion, and protein targeting. The transfer of sialic acids from CMP-Sia to the terminal positions of the carbohydrate groups of glycoproteins and glycolipids is catalyzed by a sialyltransferase. Although roles of sialic acids have been proposed in the regulation of many biological phenomena, the purpose of this structural diversity remains largely obscure. To determine the meaning of the diversity of and the regulatory mechanism for the sialylation of glycoconjugates, it is necessary to obtain information on the enzymes themselves and the gene structure of sialyltransferases. Each sialyltransferase exhibits strict specificity for acceptor substrates and linkages (3–6). Although three linkages, Siaβ2,6Gal, Siaβ2,3Gal, and Siaβ2,6GalNAc, are commonly found in glycoproteins (7), and two, Siaβ2,3Gal and Siaβ2,8Sia, occur frequently in gangliosides (8), each of these linkages has been found in both gangliosides and glycoproteins (8–10).

So far, the cloning of three members of the α2,6-sialyltransferase family (ST6GalNAc I, II and III) has been reported (11–14). The cDNAs of ST6GalNAc I and II were cloned from both chick (11, 12) and mouse (13, 62).2 The overall amino acid sequence identity of chick ST6GalNAc I is 30.5% to chick ST6GalNAc II, 43.2% to mouse ST6GalNAc I, and 33.6% to mouse ST6GalNAc II, and that of mouse ST6GalNAc I is 29.6% to mouse ST6GalNAc II and 28.3% to chick ST6GalNAc II, and that of chick ST6GalNAc II is 57.3% to mouse ST6GalNAc II. ST6GalNAc III has been cloned from rat (14), and exhibits very low amino acid sequence identity (8.2–9.8%) to mouse and chick ST6GalNAc I and II.

As far as seen with the expressed recombinant enzymes, the substrate specificity of chick ST6GalNAc I is almost the same as that of the mouse one, and also chick ST6GalNAc II exhibits similar substrate specificity to the mouse enzyme. ST6GalNAc I exhibits the broadest substrate specificity for the following structures: GalNAc-O-Ser/Thr, Galβ1,3GalNAc-O-Ser/Thr, and NeuAca2,3Galβ1,3GalNAc-O-Ser/Thr (11). On the other hand, the

1 The abbreviations used are: Sia, sialic acid; NANAse, N-acetylneuraminidase; ST6GalNAc I, GalNAc α2,6-sialyltransferase (EC 2.4.99.3); ST6GalNAc II, Galβ1,3GalNAc GalNAcα2,6-sialyltransferase (EC 2.4.99.7); ST6GalNAc III, the first type of Siaβ2,3Galβ1,3GalNAc GalNAcα2,6-sialyltransferase (EC 2.4.99.7); NeuAca, N-acetylneuraminic acid; CMP-NeuAca, cytidine 5'-monophospho-N-acetylneuraminic acid; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s); E, embryonic day; RACE, rapid amplification of cDNA ends; MES, 4-morpholineethanesulfonic acid.

2 The accession number of the cDNA sequence of mouse ST6GalNAc I is Y11274.

* This work was supported by Grants-in-aid 10152963 and 1078104 for Scientific Research on Priorities Areas and Grant-in-aid C 09680639 for Scientific Research from the Ministry of Education of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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ST6GalNAc II exhibits a narrower substrate specificity, requiring β-galactosides linked to GalNAc residues, whereas sialic acid residues linked to galactose residues are not essential for its activity, i.e., this enzyme exhibits activity toward Galβ1,3GalNAc-O-Ser/Thr and NeuAcα2,3Galβ1,3GalNAc-O-Ser/Thr (12, 13). Both genes are expressed in secretory organs, such as the submaxillary and mammary glands, so these enzymes are considered to be involved in the biosynthesis of O-glycans of mucin (11–13). On the other hand, rat ST6GalNAc III exhibits the most restricted substrate specificity, only utilizing the NeuAcα2,3Galβ1,3GalNAc sequence as an acceptor (14). This enzyme can transfer sialic acid to both NeuAcα2,3Galβ1,3GalNAc-O-Ser/Thr and ganglioside GM1b, suggesting that it cannot discriminate between α- and β-linked GalNAc (14). Incidentally, two types of α2,3-sialyltransferases (ST3Gal I and II) have been cloned that exhibit activity toward Galβ1,3GalNAc but which have different substrate preferences for glycoproteins and glycolipids, i.e., ST3Gal I prefers glycoproteins to glycolipids, but II prefers glycolipids (15–18). According to these observations, there may be two scenarios; one is that ST6GalNAc III synthesizes almost all the NeuAcα2,3Galβ1,3( NeuAcα2,6,6GalNAc residues, and the other is that there may be another member of the ST6GalNAc family that has a different substrate preference from that of ST6GalNAc III. To solve this problem, we have extensively performed polymerase chain reaction (PCR) cloning. Comparison of sialyltransferases cloned thus far has revealed highly conserved regions, named sialylmotifs I, S, and VS (11, 19–21), not found in other glyco- syltransferases. From the conservation of these sialylmotifs, it was expected that other members of the sialyltransferase gene family have the same motifs. The PCR-based approach with degenerate primers deduced on the conserved sequence in the sialylmotif has resulted in the isolation of several new members of the sialyltransferase gene family (22).

As a result, we have cloned two members of the ST6GalNAc family from mouse, which synthesize NeuAcα2,3Galβ1,3( NeuAcα2,6,6GalNAc residues. A new member, named ST6GalNAc IV, exhibits strong activity toward NeuAcα2,3Galβ1,3GalNAc of O-glycans. The other is a homologue of rat ST6GalNAc III. Here, we report the cloning of the cDNAs encoding the two NeuAcα2,3Galβ1,3GalNAcα2,6-sialyltransferases.

EXPERIMENTAL PROCEDURES

Materials—Fetuin, asialofetuin, bovine submaxillary mucin, O-acid glycoprotein, CMP-NeuAc, Galβ1,3GalNAcI-benzyl, Galβ1,3GalNAc, Galβ1,3GlcNAc, Galβ1,4GlcNAC, lacto-N-tetraose, benzyl-GalNAc, N-acetyllyctosamine and Triton CF-54 were from Sigma. CMP-(11C) NeuAc (11Bq/mmol) was from Amersham Pharmacia Biotech. NeuAcα2,3Galβ1,3GalNAc was from Seikagaku Co. Nanase I, Newcastle disease virus sialidase, and sialidase from Vibrio cholerae were from Oxford Glycosystems and Roche Molecular Biochemicals, respectively. Synthetic primers were obtained from Espec Oligo Service (Japan). The restriction endonucleases were from Takara (Japan) and Toyobo (Japan). Galβ1,3GalNAcα2,6 was sialylated using the secreted form of ST3Gal I expressed in COS-7 cells (16). NeuAcα2,3Galβ1,3GalNAc was purified by preparative TLC (ethanol/1-butanol/pyridine/water/acetate 100/10/10/30/1) and subsequent DEAE-Sephadex A-25 anionic exchange chromatography. NeuAcα2,3Galβ1,3( NeuAcα2,6,6GalNAcα2,6,6 was prepared by the reduction of NeuAcα2,3Galβ1,3( NeuAcα2,6,6GalNAcα2 (23).

PCR Cloning with Degenerate Oligonucleotides—A mouse ST6GalNAc III cDNA fragment was prepared by PCR amplification. The primers used were rat ST6GalNAc III cDNA, 5′-ATCATAGGCCGATCTGCT- GCATC-3′ (nucleotides –12 to 12), and 5′-TCACAGGTCAAGCACA CGCATCA-3′ (complementary to the rat ST6GalNAc III coding strand; nucleotides 916–939). The amplified 940-bp cDNA was subcloned into the EcoRV site of a pBluescript SK+ vector (Stratagene). A mouse brain cDNA library was constructed and screened using the PCR-amplified mouse ST6GalNAc III cDNA as described previously (11), and full-length mouse ST6GalNAc III cDNA was isolated by rapid amplification of 5′-cDNA ends (RACE-PCR).

To isolate the new sialyltransferases, PCR was performed as described previously (11) with two primers (5′-primer ST-107, 5′-TGGGCTTGGG(Imo)(AC)AGGTGGCTGGTGG-3′, and 3′-primer ST-205, 5′-AGCCAAATGGTGATTGTGTGA(T/ACCCGATC-3′) deduced from the conserved region in mouse ST6Gal I and II. The standard molecular cloning techniques described by Ausubel et al. (25) were used. PCR RACE—Amplification of the 5′-end of mouse ST6GalNAc III cDNA was performed as described previously (13). cDNA was synthesized by reverse transcription (Superscript II, Life Technologies, Inc.) of 5 μg of mouse brain poly(A)+ RNA and NBlAI poly(A)+ RNA using primer RT-181, 5′-TTAGGCCCTGCTCCAAAGCAGTATG-3′ (complementary to the ST6GalNAc III coding strand, nucleotides 181–204), and was A-tailed. Two consecutive PCRs were performed with two nested sets of primers, Nor-I(d)T (Amersham Pharmacia Biotech) and RT-181, and then following primers, 5′-AACCTGAAGAATTCGGGCACGAGA-3′, and RT-91, 5′-CCTGAGGATGACGCGCTACACCAGTGATG-3′ (complementary to the ST6GalNAc III coding strand, nucleotides –9 to 18). The cDNA was amplified through 35 cycles of a step protocol (94 °C, 30 s; 55 °C, 30 s; 72 °C, 40 s). The amplified products were subcloned and sequenced.

Preparation of Soluble ST6GalNAc Proteins Fused with Protein A—A truncated form of ST6GalNAc III and IV, lacking the first 28 and 36 amino acids of the open reading frame, respectively, was prepared by PCR amplification using 5′-primers containing an EcoRI site and 3′-primers containing a XhoI site, i.e., 5′-primer, 5′-CCCTTGGATCATGACGATTCC-3′, 3′-primer, CCACAGCTCGAGTGTAGCTTGGCGG-3′, ST6GalNAc IIITAIL; complementary to the coding strand, nucleotides 930–953 for ST6GalNAc IV. The resulting amplified 878- and 803-bp fragments were subcloned into the EcoRV sites of pBluescript SK(+). The inserted fragments were cut out by digestion with EcoRI and XhoI, and then inserted into the EcoRI and XhoI sites of expression vector pcDLA (25).

The insert junctions were confirmed by restriction enzyme and DNA sequencing. The resulting plasmids consisted of the IgM signal peptide sequence, the protein A IgG binding domain, and a truncated form of ST6GalNAc III (pCDB8ST) and IV (pCDR1ST), respectively. Each expression plasmid (pcDB8ST and pCDR1ST; 20 μg) was transiently transfected into COS-7 cells on a 150-mm plate using LipofectAMINE reagent (Life Technologies, Inc.). Each protein A-fused ST6GalNAc III and IV expressed in the medium was absorbed to an IgM antibody gel (Amersham Pharmacia Biotech) and eluted, 50 μl of culture medium/50 ml of culture medium; Ref. 25) and used as the enzyme source.

We also constructed an expression vector containing the whole coding region of ST6GalNAc III, in which a 1337-bp fragment from the NH2-terminus was inserted into the pcDL-SRα vector, named pcDL-SRα8ST for kinetic analysis. The vector was transiently transfected into COS-7 cells as described above. After 5 h of transfection, the culture medium was changed to Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. After 48 h, the COS-7 cells were collected and the membrane-bound proteins were extracted by sonication in 20 mM MES buffer (pH 6.4) containing 0.3% Triton CF-54. After centrifugation of the cell lysate at 10,000 × g for 15 min, the resultant supernatant was used as the enzyme source. In this case, equivalent amounts of protein from COS cells stably transfected with pcDL-SRα were assayed in parallel as control experiment, and then the values obtained were subtracted from those obtained with cells expressing the full-length enzyme.

Sialyltransferase Assays and Linkage Analysis—Sialyltransferase assays were performed as described previously (13). In brief, enzyme activity was measured in 50 mM MES buffer (pH 6.0), 1 mM MgCl2, 1 mM CaCl2, 0.5% Triton CF-54, 100 μM CMP-[14C] NeuAc (10.2 kBq), an acceptor substrate, and an enzyme preparation, in a total volume of 10 μl. As acceptor substrates, 10 μg of proteins, 5 μg of glycolipids, or 10 μg of oligosaccharides were used. The enzyme reaction was performed at 37 °C for 2 h.

For linkage analysis of sialic acids, [14C] NeuAc-incorporated fetuin was incubated with ST3Gal I (16), ST6GalNAc III (12), ST8Sia II (26), ST6GalNAc III, and IV. The sialylated fetuin was then analyzed with the linkage-specific sialidase, NANase I (specific for α2,3-linked sialic acids), NANase II (specific for α2,3-, α2,8-linked sialic acids), and Newcastle disease virus sialidase (specific for α2,3- and α2,8-linked sialic acids) (27). After the sialidase treatment, the desialylated glycoprotein was subjected to SDS-polyacrylamide gel electrophoresis (gradient gel, 5–20%).
To obtain oligosaccharide portion of 14C-sialylated fetuin, the sialylation of fetuin was carried out essentially as described, but on a 10-fold larger scale. To maximize the product yield, the incubation period was extended to 24 h. The incubation mixture was then treated with 0.1 N NaOH, 1 μl NaBH₄ at 37 °C for 48 h, and neutralized by the gradual addition of acetic acid in an ice bath. A sample was then desalted with Sephadex G-25 chromatography (1.3 × 25 cm). The 14C-sialylated oligosaccharide alditol and reference oligosaccharide NeuAc2,3Galβ1,3GalNAc-ol were treated with various kinds of sialidases. A radioactive sample containing at least 10,000 cpm or a sialylated sample containing at least 5 μg of sialic acid was spotted onto a TLC plate (Merck, Darmstadt, Germany) and then developed with 1-propanol/aqueous ammonia/water/5% ethanol/1-butanol/pyridine/water/acetic acid (100/10/10/30/3 (21) or 1-propanol/aqueous ammonia/water = 6/1/2.5 (28). The chromatogram was visualized with a BAS2000 radio image analyzer for 14C-sialylated sample (Fuji Film) or the resorcinal method for nonradioactive sample (29).

**Analysis of ST6GalNAc III and IV Gene Expression**—The level of the ST6GalNAc III transcript was determined by competitive PCR (30). For the construction of a competitor DNA, PCR was performed with the ST6GalNAc III gene-specific primers, 5'-ATGGATACATAAATAGTGGCGCC-3' (nucleotides 185–207) and 5'-TGCGATGTACGCTTGAAATCC-3' (complementary to the ST6GalNAc III coding strand; nucleotides 677–699), with ST6GalNAc III cDNA as the template. The amplified fragment (515 bp) was subcloned into pKF18k (Takara, Japan), and then subjected to site-directed mutagenesis using a mutagenic primer, 5'-TGGAGAGATCTCGGTACATG-3' (nucleotides 119–235), exhibits 20.0–60.0% identity. The overall amino acid sequence identity of this protein is 11.9% to mouse ST6GalNAc I (62), 2 10.3% to mouse ST6GalNAc II (13), and 43.0% to mouse ST6GalNAc III, respectively (Table I). These results suggest that the cloned gene belongs to the sialyltransferase gene family. In fact, the following results revealed that it is a member of the ST6GalNAc family, so it was named ST6GalNAc IV.

**Both the ClonedDNAs Encode GalNAc α2,6-Sialyltransferase**—To facilitate functional analysis of the enzyme, it was desirable to produce a soluble and condensable form of enzyme that could be secreted from the cells. First of all, sequences corresponding to the putative stem and active domains of ST6GalNAc III and IV were fused to the immunoglobulin signal peptide sequence followed by the IgG binding domain of protein A (pCDBSSST and pCDR1ST). After transfecting each of the constructs into COS-7 cells, the enzyme secreted into the medium was condensed with IgG-Sepharose and used for further experiments. As shown in Table II, among the glycolipids examined in this study, only GM1b, i.e. not asialoGM1, served as an acceptor substrate for ST6GalNAc III and IV. ST6GalNAc III showed higher activity toward GM1b, of which the product comigrated with authentic GD1a, which has the NeuAc

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**Table I**

| Amino acid-identity (%) of members of the ST6GalNAc family | Mouse ST6GalNAc | I | II | III | IV |
|---|---|---|---|---|---|
| ST6GalNAc | | | | | |
| I | Mouse<sup>a</sup> | 29.6 | 10.8 | 11.9 | |
| | Chick (11) | 43.2 | 33.6 | 1.3 | 8.2 |
| | Mouse (13) | 7.9 | 10.3 | |
| | Chick (12) | 28.3 | 57.3 | 8.2 | 8.6 |
| II | Mouse | 9.8 | 8.2 | 94.4 | 44.7 |
| III | Rat (14) | 9.8 | 8.2 | 94.4 | 44.7 |

<sup>a</sup> See Ref. 62 and footnote 2.

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*Fig. 1. Nucleotide and deduced amino acid sequences of mouse ST6GalNAc III and IV. The nucleotide and amino acid sequences of mouse ST6GalNAc III (A) and IV (B) are numbered from the presumed start codon and initiation methionine, respectively. The double underlined amino acids correspond to a putative transmembrane domain. Sialylmotifs L and S are boxed by solid and dashed lines, respectively. The positions of the PCR primers are indicated by arrows.*
oligosaccharide was a poor acceptor substrate for ST6GalNac III. When the relative enzyme activities of ST6GalNac III and IV toward fetuin were, respectively, set to 100%, the activity to oligosaccharide NeuAc2,3Galβ1,3GalNAc was 290% in case of ST6GalNac IV, while only 0.5% of the activity was detected in case of ST6GalNac III. ST6GalNac IV enzyme activities toward non-sialylated Galβ1,3GalNAc and disialylated NeuAc2,3Galβ1,3( NeuAc2,6)GalNAc were almost negligible (Table III).

The truncated form of ST6GalNac III exhibited enzyme activity that was too low (18 fmol/h/ml of medium) to determine kinetic parameters. Therefore, the full-length ST6GalNac III was also subcloned into expression vector pCDL-SRα to yield pcDL-SRαB8ST. The enzyme fraction (3.6 pmol/h/μl of cell lysate) was then obtained as described under “Experimental Procedures.” The COS cell lysates intrinsically exhibit strong ST3Gal I and II, and significant ST6Gal I activities; thus, this enzyme fraction is not suitable for analyzing substrate specificity but for estimating K_m and relative V_{max}/K_m values for acceptor substrates. The apparent K_m value for GM1b was 200 μM, which was lower than those for fetuin (8,000 μM) and NeuAc2,3Galβ1,3GalNAc-benzyl (670 μM). The relative V_{max}/K_m value for GM1b was 1.0, which was higher than those for fetuin (0.31) and NeuAc2,3Galβ1,3GalNAc-benzyl (0.019). These results suggested that the expressed ST6GalNac III prefers glycolipids to glycoproteins.

**Linkage Specificity of the Two Sialyltransferases**—For linkage analysis, [14C]NeuAc-incorporated fetuin, which was synthesized with ST3Gal I (16), ST6GalNac I (12), ST8Sia II (26), and ST6GalNac III and IV, respectively, and each sialylated fetuin was treated with linkage-specific sialidases, i.e., NANase I (specific for α2,3-linked sialic acids), NANase III (specific for α2,3-, α2,6-, and α2,8-linked sialic acids), and Newcastle disease virus sialidase (specific for α2,3- and α2,8-linked sialic acids) (27) (Fig. 3). The resulting patterns of the ST6GalNac III and IV enzymes were virtually identical to that of ST6GalNac I. The [14C]NeuAc residue of fetuin sialylated by both the enzymes was removed by the NANase III treatment but not by the NANase I or Newcastle disease virus sialidase treatment (Fig. 4). These results indicate that the incorporated sialic acids each contain an α2,6-linkage, and the expressed enzymes are the mouse homologue of rat ST6GalNac III, and a new member of the ST6GalNac family, ST6GalNac IV, respectively.

To confirm the linkage specificity of ST6GalNac III and IV, the following experiments were performed. Although we report here the results of ST6GalNac IV, the results for ST6GalNac III is the same as those for IV. The [14C]-sialylated oligosaccharide alditol was prepared by β-elimination of the sialylated fetuin with ST6GalNac IV. A desalted sample was then spotted onto a TLC plate and developed with ethanol/1-butanol/pyridine/water/acetic acid = 100/10/30/3. All of the radioactive product migrated as a low molecular compound, i.e. no radioactivity remained at the origin, suggesting that [14C]-sialylation occurred exclusively on O-linked glycan chains of fetuin (data...
not shown). Furthermore, more than 70% of the radioactivity comigrated with the reference oligosaccharide, NeuAcα2,3Galβ1,3GalNAc (data not shown). This radioactive material was isolated by preparative TLC and used for linkage analysis (Fig. 4). The reference oligosaccharide, NeuAcα2,3Galβ1,3GalNAc-ol, was also used for comparison. The 14C-sialylated oligosaccharide alditols were detected by BAS2000 radioimage analyzer (Fig. 4, lanes 1–4). In case of reference oligosaccharide, resorcinol reagent was used for detection (Fig. 4, lanes 5–8). On NANase I or Newcastle disease virus digestion, NeuAcα2,3Galβ1,3GalNAc-ol (oligo 1, lane 5) was converted to Galβ1,3-(NeuAcα2,6)GalNAc-ol (oligo 2, lanes 6 and 7). With these sialidase treatments, the radioactive band that co-migrated with oligo 1 (lane 1) migrated to the same position as oligo 2 (lanes 2 and 3). It should be noted that the digestion with NANase I and Newcastle disease virus sialidase was partial for some reason. When NeuAcα2,3Galβ1,3GalNAc-ol (oligo 1) was treated with V. cholerae sialidase (α2,3, α2,6, and α2,8 linkage-specific sialidase), it was converted to Galβ1,3GalNAc-ol, which was not detectable with the resorcinol method (lane 8). With this treatment, the radioactive band comigrated with neither oligo 1 nor oligo 2, but with NeuAc, indicating that the linkage type of the 14C-NeuAc residue in the oligosaccharide alditol is α2,6 (lane 4). The reason that 14C-NeuAc band in lane 4 (Fig. 4A) was remarkably sharp could be because the prepared 14C-sialylated oligosaccharide alditol contained more salts, which sometimes affect the migration pattern on TLC plate. Taken together, the results strongly suggest that the 14C-sialylated oligosaccharide alditol derived from fetuin is NeuAcα2,3Galβ1,3GalNAc-ol. Thus, the cloned enzyme is a new member of the ST6GalNAc family, named ST6GalNAc IV.

Expression of the ST6GalNAc III and IV Genes in Mouse Tissues—To examine the expression of the mouse ST6GalNAc III gene in various tissues, Northern blot hybridization was performed using 5 μg of poly(A)+ RNA from various adult mouse tissues, which gave only faint signals and could not be used to examine the specific gene expression of the ST6GalNAc III gene. Therefore, we performed competitive reverse transcription-PCR to determine in which tissues the ST6GalNAc III gene is expressed. In adult tissues, significant levels of expression that could not be detected on Northern blot analysis but could be detected on reverse transcription-PCR were observed in brain, lung, and heart, followed by lower levels of expression in kidney, mammary gland, spleen, thymus, and testis (Fig. 5A). We also estimated the amount of the ST6GalNAc III transcript in brain during development (Fig. 5B). The amount of the ST6GalNAc III transcript was highest at E12, although whole embryos at E7, E11, and E12 were used to isolate poly(A)+ RNA. The amount of the ST6GalNAc III transcript in brain was lower at E16 than at embryonal stages (E11 and E12), and then kept almost similar levels during mouse development, suggesting that the mouse ST6GalNAc III gene may be highly transcribed in tissues other than brain at

| Acceptor Relative activity |
|-----------------------------|
| Galβ1,3GlcNAc | % |
| Galβ1,4GlcNAc | 0 |
| Galβ1,5GalNAc | 0 |
| Galβ1,3GalNAc | 0.3 |
| NeuAcα2,3Galβ1,3GalNAc | 100* |
| NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAc | 3 |

* When the relative activity toward NeuAcα2,3Galβ1,3GalNAc was set to 100%, the activity in case of fetuin was 34%.
embryonic stages.

The mRNA size and distribution of the ST6GalNAc IV gene were determined by Northern blot analysis (Fig. 5, C and D). Three transcripts (1.9, 2.2, and 3.6 kb) were observed in ICR mouse tissues (8-week-old mice). Strong signals were observed in brain and colon, and moderate ones in lung, heart, thymus, and spleen (Fig. 5C). The expression in brain was developmentally regulated. Analysis of RNAs from embryonal stage (E12), and 1-day (P1), 3-week (3w), and 8-week (8w) ICR mouse brains and livers. The hybridization probe was prepared from the NH2-terminal truncated fragment (nucleotides 109–111) of ST6GalNAc IV.

**DISCUSSION**

In this study, we have described the isolation and characterization of cDNAs for encoding third and fourth types of mouse GalNAc a2,6-sialyltransferase (ST6GalNAc III and IV). The mouse ST6GalNAc III shared very high sequence similarity (94.4%) at the amino acid level with the rat one (14). The mouse ST6GalNAc IV cloned in this study turned out to be encoded for a novel type of sialyltransferase. The cDNAs were isolated by PCR cloning method based on the highly conserved regions from previously cloned sialyltransferases (11, 19–21). The cDNA for ST6GalNAc IV was also isolated independently by mRNA differential display method by comparing the gene expressions between native and activated CD8+ cells.3

Based on the following observations, we concluded that the cDNAs we isolated were indeed for ST6GalNAc III and IV, which transfer CMP-NeuAc with an a2,6-linkage to a GalNAc residue on NeuAc a2,3Galb1,3GalNAc of glycoproteins and glycolipids. First, fetuin, which contains the O-glycosidically linked NeuAc a2,3Galb1,3GalNAc sequence (32), was shown to serve as a good acceptor for both enzymes. However, both asialofetuin (contains the Galb1,3GalNAc sequence) and asialo-bovine submaxillary mucin (5% of the total carbohydrate chains contain Galb1,3GalNAc sequences) (33) served as much poorer acceptors compared with fetuin. Second, the study of the sensitivity of14C-sialylated oligosaccharide alditol to various sialidase including NANase I, III, _V. cholerae_ sialidase, and 3

3 M. Kaufmann, C. Blaser, S. Takashima, S. Tsuji, and H. Pircher (1999) _Int. Immunol._, in press.
Newcastle disease virus sialidase revealed that the sialylated product was NeuAc\(^{a}\)\(^{2,3}\)Gal\(^{b}\)\(^{1,3}\)(NeuAc\(^{a}\)\(^{2,6}\)GalNAc and the linkage type of the \([\text{14C}]\text{NeuAc}\) was \(\text{a}^{2,6}\). The \([\text{14C}]\text{sialylated oligosaccharide alditol}\) was derived from the fetuin sialylated by both enzymes with CMP-\([\text{14C}]\text{NeuAc}\). Furthermore, GM1\(^{b}\) could be served as an acceptor for both enzymes, and the product was GD1\(^{a}\).

Similar to other glycosyltransferases, ST6GalNAc \(^{IV}\) has a type II membrane protein topology, a short NH\(_{2}\)-terminal cytoplasmic tail, a hydrophobic signal-anchor domain, a proteolytically sensitive stem region, and a large COOH-terminal active domain (6). The location of the transmembrane domain was determined by hydropathy plot according to the Kyte and Doolittle method (34). The transmembrane domain was 23 amino acids long from position 14 to 36. We also noticed that ST6GalNAc \(^{IV}\) was the smallest protein (302 amino acid) among all cloned sialyltransferases. This was mainly because of the very short stem region of ST6GalNAc \(^{IV}\). The size of stem regions among members of the ST6GalNAc family varies to a great degree. ST6GalNAc \(^{IV}\) had only 38 amino acid residues between the transmembrane region and sialylmotif \(L\), while ST6GalNAc \(^{I}\), \(^{II}\), and \(^{III}\) have 261, 123, and 53 amino acid residues (13, 62),\(^{2}\) respectively. These differences may have important implications for the \(in\ vivo\) functions of individual enzymes, although this remains to be clarified.

The four members belonging to ST6GalNAc family can be classified into two subfamilies according to the sequence similarity and substrate specificity differences (Fig. 6 and Tables I and III). ST6GalNAc \(^{I}\) and \(^{II}\) belong to one subfamily, and \(^{III}\) and \(^{IV}\) belong to the other. A dendrogram constructed by the method of Higgins and Sharp (35) suggested that one subfamily (\(^{III}\) and \(^{IV}\)) is separated from other sialyltransferase families, suggesting a great difference in domain structure (Fig. 6). The dendrogram also showed that the other ST6GalNAc subfamily (\(^{I}\) and \(^{II}\)) is near the group of ST3Gal families. As reported previously (13, 15–17, 36–43), mouse ST3Gal family contains four cysteine residues, which are conserved in both chick and mouse ST6GalNAc \(^{I}\) and \(^{II}\). This structure is the so-called Kurosawa motif, Cys-Xaa\(^{75–82}\)-Cys-Xaa\(^{1–2}\)-Cys-Ala-Xaa-Val-Xaa\(^{150–160}\)-Cys (Xaa denotes any amino acid residue). However, mouse and rat ST6GalNAc \(^{III}\) and \(^{IV}\) do not contain this motif, nor do any members

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**FIG. 6. Dendrogram of the cloned mouse sialyltransferases.** After analyzing the deduced amino acid sequences of cloned mouse sialyltransferases according to Higgins and Sharp (35), a dendrogram was constructed. Note that one subfamily of the ST6GalNAc family (\(^{III}\) and \(^{IV}\)) is separated from other sialyltransferase families, and that the other ST6GalNAc subfamily (\(^{I}\) and \(^{II}\)) is near the group of ST3Gal families. Parentheses indicate the accession number of GenBank\(^{TM}/\)EBI data base.

**TABLE IV**

**Comparison of the substrate specificities of members of the ST6GalNAc family**

A, B, and C, significant enzyme activity could be detected. The activity strength order is A > B > C. —, enzyme activity could not be detected or was negligible.

| Substrate | ST6GalNAc |
|-----------|-----------|
|           | I Chick (11), mouse (62) | II Chick (12), mouse (13) | III Mouse, rat (14) | IV Mouse |
| NeuAc\(^{a}\)\(^{2,3}\)Gal\(^{b}\)\(^{1,3}\)(GalNAc\(^{a}\)\(^{2,6}\)-O-Thr/Ser | B | A | A | B |
| Gal\(^{b}\)\(^{1,3}\)(GalNAc\(^{a}\)\(^{2,6}\)-O-Thr/Ser | A | B | — | — |
| NeuAc\(^{a}\)\(^{2,3}\)Gal\(^{b}\)\(^{1,3}\)(GalNAc\(^{a}\)\(^{2,6}\)-O-Thr/Ser | C | — | — | — |
| NeXuc\(^{a}\)\(^{2,3}\)Gal\(^{b}\)\(^{1,3}\)(GalNAc\(^{a}\)\(^{2,6}\)-O-Thr/Ser | — | — | — | — |
| NeuAc\(^{a}\)\(^{2,3}\)Gal\(^{b}\)\(^{1,3}\)(GalNAc\(^{a}\)\(^{2,6}\)-O-Thr/Ser | — | — | — | — |

\(^{a}\) See also Footnote 2.

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4 In addition to the references, the following data also support it: EMBL accession nos. X66667, AF039321, AF033290, and Y15003.
family will help us to understand the sialylglycoconjugates’ biological functions during the course of development.

Acknowledgments—We thank Dr. James C. Paulson for the valuable discussion and for giving us the cDNA sequence of rat ST6GalNAc III prior to publication, and Dr. Yoshitaka Nagai, Director of the Glycobiology Research Group, and Dr. Tomoya Ogawa, Coordinator of the Group, Frontier Research Program of the Institute of Physical and Chemical Research (RIKEN), for their support in this work.

REFERENCES

1. Svennerholm, L. (1964) J. Lipid Res. 5, 145–155
2. Tsuji, S., Datta, A. K., and Paulson, J. C. (1990) Glycobiology 6, v–vii
3. Schnaar, R. L. (1991) Glycobiology 1, 477–485
4. Wasserman, P. M. (1987) Annu. Rev. Cell Biol. 3, 199–1409
5. Kimber, S. J. (1989) Biochim. Biophys. Acta 1595, 23–27
6. Feizi, T. (1985) Nature 314, 53–57
7. Hakomori, S. (1981) Annu. Rev. Biochem. 50, 733–764
8. Fishman, P., and Brady, R. O. (1976) Science 194, 906–915
9. Shortman, H. (1967) J. Biol. Chem. 242, 3852–3859
10. Svennerholm, L. (1980) in Structure and Function of Gangliosides (Svennerholm, L., Mandel, P., Dreyfus, H., and Urban, P., eds) Vol. 125, pp. 533–544, Plenum Press, New York
11. Kurosawa, N., Hamamoto, T., Lee, Y.-C., Nakaoka, T., and Tsuji, S. (1994) J. Biol. Chem. 269, 14493–14499
12. Kurosawa, N., Kojima, N., Inoue, M., Hamamoto, T., and Tsuji, S. (1994) J. Biol. Chem. 269, 19048–19053
13. Kurosawa, N., Inoue, M., Yoshida, Y., and Tsuji, S. (1996) J. Biol. Chem. 271, 15109–15116
14. Sjoberg, E. R., Kitagawa, H., Ghasaika, J., van Halbeek, H., and Paulson, J. C. (1996) J. Biol. Chem. 271, 7450–7459
15. Gillissey, W., Ke, S., and Paulson, J. C. (1992) J. Biol. Chem. 267, 21004–21010
16. Lee, Y.-C., Kurosawa, N., Hamamoto, T., Nakaoka, T., and Tsuji, S. (1995) Eur. J. Biochem. 230, 377–385
17. Lee, Y.-C., Kojima, N., Hamamoto, T., Kurosawa, N., and Tsuji, S. (1994) J. Biol. Chem. 269, 15104–15109
18. Kojima, N., Lee, Y.-C., Hamamoto, T., Kurosawa, N., and Tsuji, S. (1994) Biochemistry 33, 5772–5779
19. Drickamer, K. (1993) Glycobiology 3, 2–5
20. Geremia, R. A., Harduin-Lepers, A., and Delannoy, P. (1997) Glycobiology 7, 17–21
21. Livingstone, B. D., and Paulson, J. C. (1993) J. Biol. Chem. 268, 11504–11507
22. Tsuji, S. (1996) J. Biochem. (Tokyo) 120, 1–13
23. Hardy, M. R., and Townsend, R. (1994) Methods Enzymol. 230, 208–225
24. Ausebel, F. M., Breni, R., Kangas, O., Moore, D. D., Steinman, J. G., and Strahl, K. (1989) Current Protocols in Molecular Biology, Green Publishing Associates, New York
25. Sasaki, K., Kurata, K., Kojima, N., Kurosawa, N., Ohta, S., Hanai, N., Tsuji, S., and Nishi, T. (1994) J. Biol. Chem. 269, 15950–15956
26. Kojima, N., Yoshida, Y., Kurosawa, N., Lee, Y.-C., and Inoue, S. (1995) FEMS Lett. 306, 1–4
27. Paulson, J. C., Weinstein, J., Dorland, L., van Halbeek, H., and Viegenthart, J. F. G. (1982) J. Biol. Chem. 257, 12734–12738
28. Kitaume, S., Kitaiguma, K., Inoue, S., and Inoue, Y. (1992) Anal. Biochem. 202, 25–34
29. Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604–611
30. Siebert, P. D., and Larrick, J. W. (1992) Nature 359, 557–558
31. Kozak, M. (1984) Nature 308, 241–246
32. Spies, R. G., and Bhoyo, V. D. (1974) J. Biol. Chem. 249, 5704–5717
33. Tsuji, T., and Otsawa, T. (1966) Carbohydr. Res. 151, 391–402
34. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
35. Higgins, D. G., and Sharp, P. M. (1988) Gene 67, 35–52
36. Lee, Y.-C., Kojima, N., Wada, E., Kurosawa, N., Hamamoto, T., and Tsuji, S. (1994) J. Biol. Chem. 269, 10028–10033
37. Kojima, N., Lee, Y.-C., Hamamoto, T., Kurosawa, N., and Tsuji, S. (1994) Biochemistry 33, 5772–5779
38. Drickamer, K. (1993) Glycobiology 3, 2–5
39. Geremia, R. A., Harduin-Lepers, A., and Delannoy, P. (1997) Glycobiology 7, 17–21
40. Kojima, N., Yoshida, Y., Kurosawa, N., Lee, Y.-C., and Inoue, S. (1995) FEMS Lett. 306, 1–4
41. Hanai, N., Nishi, T., and Hasegawa, M. (1993) J. Neurochem. 60, 931–938
42. Kato, H., Hanai, N., and Hasegawa, M. (1992) J. Biol. Chem. 267, 21634–21640
43. Hanai, N., and Hasegawa, M. (1993) J. Biol. Chem. 268, 32782–32787
44. Kato, H., Hanai, N., and Hasegawa, M. (1993) J. Biol. Chem. 268, 22782–22787
49. Yoshida, Y., Kojima, N., Kurosawa, N., Hamamoto, T., and Tsuji, S. (1995) *J. Biol. Chem.* 270, 14628–14633
50. Zeng, G., Gao, L., and Yu, R. K. (1997) *Gene (Amst.)* 187, 131–134
51. Eckhardt, M., Muehlenhoff, M., Bethe, A., Koopman, J., Frosch, M., and Gerardy-Schahn, R. (1995) *Nature* 373, 715–718
52. Nakayama, J., Fukuda, M. N., Fredette, B., Ransch, B., and Fukuda, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 7021–7025
53. Yoshida, Y., Kojima, N., and Tsuji, S. (1995) *J. Biochem. (Tokyo)* 118, 658–664
54. Kim, Y.-J., Kim, K.-S., Do, S.-I., Kim, C.-H., Kim, S.-K., and Lee, Y.-C. (1997) *Biochem. Biophys. Res. Commun.* 235, 327–330
55. Kurosawa, N., Kawasaki, M., Hamamoto, T., Nakaoka, T., Lee, Y.-C., Arita, M., and Tsuji, S. (1994) *Eur. J. Biochem.* 219, 375–381
56. Hamamoto, T., Kawasaki, M., Kurosawa, N., Nakaoka, T., Lee, Y.-C., and Tsuji, S. (1990) *Bioorg. Med. Chem. I*, 141–145
57. Aasheim, H. C., Aas-Eng, A., Deggerdal, A., Blomhoff, H., Funderud, S., and Smeland, E. (1993) *Eur. J. Biochem.* 213, 467–475
58. Bast, B. J., Zhou, L., Freeman, G. J., Colley, K. J., Ernst, T. J., Munro, J., and Tedder, T. F. (1992) *J. Cell Biol.* 116, 423–435
59. Grundmann, U. G., Nerlich, C., Rein, T., and Zettlmeissl, G. (1990) *Nucleic Acids Res.* 18, 667–667
60. Furuya, S., Irie, F., Hashikawa, T., Nakazawa, K., Kozaki, A., Hasegawa, A., Sudo, K., and Hirabayashi, Y. (1994) *J. Biol. Chem.* 269, 32418–32425
61. Ishikawa, D., Taki, T., Nakajima, M., and Handa, S. (1995) *Glycoconjugate J.* 12, 523
62. Kurosawa, N., Takashima, S., Ikehara, Y., Kono, M., Liu, H., Inoue, M., Tachida, Y., Taguchi, A., Y., Ogata, S., Itakowitz, S., Arita, M., Narimatsu, H., and Tsuji, S. (1999) *J. Biochem. (Tokyo)* , in press