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Two Polysialic Acid Synthases, Mouse ST8Sia II and IV, Synthesize Different Degrees of Polysialic Acids on Different Substrate Glycoproteins in Mouse Neuroblastoma Neuro2a Cells[1]

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We previously cloned cDNAs encoding two different polysialic acid (PSA) synthases, ST8Sia II and IV, from mouse, and showed that both mouse ST8Sia II and IV can synthesize PSA on the neural cell adhesion molecule (NCAM) as well as other glycoproteins such as fetuin, at least *in vitro* (Kojima, N., Tachida, Y., Yoshida, Y., and Tsuji, S. (1996) *J. Biol. Chem.* 271, 19457-19463]. In the present study, to clarify how the two PSA synthases act differently *in vivo*, we first cloned PSA-expressing cell lines (N2a-II and N2a-IV) by stable transfection of the cDNA encoding either mST8Sia II or IV into mouse neuroblastoma Neuro2a cells, which do not express PSA but express NCAM, then compared the expression of the PSA and NCAM isoforms and *de novo* synthesis of PSA between N2a-II and N2a-IV. Western blotting with an anti-NCAM polyclonal antibody showed that NCAM was expressed as the polysialylated form in both ST8Sia II cDNA-transfected and ST8Sia IV cDNA-transfected Neuro2a cells, but that the polysialylated NCAMs expressed in ST8Sia IV cDNA-transfected clones migrated much slower on SDS-PAGE than those expressed in ST8Sia II cDNA-transfected clones. The slower migration of polysialylated NCAM of the ST8Sia IV cDNA-transfected clone (N2a-IV) than that of the ST8Sia II cDNA-transfected clone (N2a-II) was also observed when cells were metabolically labeled with [^{3}H]glucosamine or pulse-chase labeled with [^{35}S]methionine followed by immunoprecipitation with anti-PSA antibody or anti-NCAM monoclonal antibody. In addition, polysialylated *N*-glycans of PSA-carrying glycoproteins prepared from [^{3}H]glucosamine-labeled N2a-IV by immunoprecipitation with anti-PSA monoclonal antibody were eluted at a much higher salt concentration than those from [^{3}H]glucosamine-labeled N2a-II on an anion-exchange column. These results indicated that the degree of *de novo* polysialylation of NCAM by mST8Sia IV was much higher than that by mST8Sia II. In N2a-IV, NCAM-120, -140, and -180 were expressed as polysialylated forms, while polysialylation was restricted to NCAM-140 and -180, *i.e.*, not NCAM-120, in N2a-ST8Sia II. Metabolic labeling of the cells with [^{3}H]glucosamine, pulse-chase labeling with [^{35}S]methionine followed by immunoprecipitation with anti-PSA antibody, and subsequent sialidase treatment revealed that NCAM-140 and -180 were specifically polysialylated in N2a-II, whereas not only NCAM but also other glycoproteins were *de novo* polysialylated in N2a-IV. The above results demonstrated that the two different PSA synthases, mST8Sia II and IV, synthesize PSA of different lengths on different substrate glycoproteins *in vivo* when the enzymes are expressed in neuroblastoma Neuro2a cells. These differences suggest that mST8Sia II and IV play different roles in the biosynthesis and expression of PSA.

Key words: neural cell adhesion molecule, neuroblastoma cells, polysialic acid, polysialic acid synthase, sialyltransferase.

Polysialic acid (PSA) is a unique linear homopolymer of α2,8-sialic acid residues that is distributed in bacteria to mammals (*1*). In mammalian cells, PSA is known to be mainly associated with the neural cell adhesion molecule

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Abbreviations: The abbreviated nomenclature for the cloned sialyltransferases follows the system of Tsuji *et al.* (*21*). N2a-II, highest PSA-expressing clone derived from ST8Sia II cDNA-transfected Neuro2a cells; N2a-IV, highest PSA-expressing clone derived from ST8Sia IV cDNA-transfected Neuro2a cells; PSA, polysialic acid; NCAM, neural cell adhesion molecule; PSA-NCAM, polysialylated form of NCAM; endo N, endoneuraminidase; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; mAb, monoclonal antibody; pAb, polyclonal antibody; PMSF, phenyl methylsulfonylfluoride; MES, 2-[*N*-morpholino]ethanesulfonic acid.

(NCAM), although the α-subunit of the sodium channel in rat and an undefined intracellular protein in breast cancer and leukemia cell lines have been reported to be polysialylated (*1-3*). The expression of PSA on NCAM is developmentally regulated in the central and peripheral nervous systems. Since the polysialylation of NCAM considerably modifies NCAM-mediated adhesive interactions, PSA plays important roles in pathfinding and targeting on the innervation of axons, migration of neuronal cells and tumor cells, and spatial learning and memory (*2*).

Two cDNAs encoding α2,8-sialyltransferases, ST8Sia II/STX and ST8Sia IV/PST-1, were recently cloned from mouse, rat, hamster, and human, and their *in vitro* enzymatic properties and mRNA expression were individually characterized (*4-10*). The results demonstrated that both ST8Sia II and IV can synthesize PSA on α2,3-linked sialic acids on some glycoproteins without any initiators *in vitro* (*6, 9*), and that the transfection of the cDNAs encoding ST8Sia II and IV into cells leads to the expression of PSA on the cell surface (*4, 5, 9, 10*). Thus, both ST8Sia II and IV are PSA synthases. However, the cloning and characterization of the two different PSA synthases raised the new questions of why these two types of PSA synthases co-exist in tissues and how they act differently. We previously compared the *in vitro* enzymatic activities of mST8Sia II and IV, using recombinant soluble enzymes tagged with protein A (*11*). Our study indicated that both mST8Sia II and IV synthesize PSA on NCAM as well as other glycoproteins such as fetuin, and the enzymatic properties of mST8Sia II and IV are very similar. However, the degree of polysialylation differed between the products of mST8Sia II and V *in vitro*, *i.e.*, the product of mST8Sia IV exhibited much slower mobility on SDS-PAGE than that of mST8Sia II (*11*). In addition, fetuin was almost completely polysialylated by the recombinant mST8Sia IV, while only part of fetuin was polysialylated by the recombinant mST8Sia II, suggesting that mST8Sia II and IV may recognized different substrates *in vitro*. Furthermore, the expression of mST8Sia II mRNA was shown to be well correlated with the expression of PSA and PSA synthase activity during the *in vitro* neuronal differentiation of P19 and MNS-8 cells (*12*). We also showed that only NCAM, *i.e.*, not other glycoproteins, was polysialylated in mST8Sia II cDNA-transfected Neuro2a cells (*11*), suggesting that mST8Sia II specifically synthesizes PSA-NCAM *in vivo*. On the other hand, there is no direct evidence as to whether mST8Sia IV specifically synthesizes PSA-NCAM *in vivo*, although ST8Sia IV is considered to be the only factor required for the polysialylation of NCAM in CHO cells (*13*).

Several reports have demonstrated that both ST8Sia II and IV can potentially participate in the biosynthesis of PSA on NCAM in cells (*4-6, 8-11, 13*), but there has been no comparison of PSA expression and biosynthesis *in vivo* by ST8Sia II and IV. Our previous results (*11*) also raise the questions of whether mST8Sia II and IV synthesized PSAs of different lengths *in vivo* as they did *in vitro* experiments, and whether they recognize and modify the same substrates *in vivo*. To address these questions, in the present study, we compared the expression of the PSA and NCAM isoforms in mST8Sia II or IV cDNA-transfected Neuro2a cells, which express NCAM but not PSA. In addition, the *de novo* synthesis of PSA in these cells was compared after the cells had been metabolically labeled with [^{3}H]glucosamine (for labeling of glycans in glycoproteins) or [^{35}S]methionine (for labeling of proteins) followed by immunoprecipitation with anti-PSA mAb or anti-NCAM mAb.

EXPERIMENTAL PROCEDURES

Materials and Enzyme Assay—Unless otherwise indicated the materials used in this study were the same as those previously reported (*8, 9, 11, 12*). The anti-mouse NCAM monoclonal antibody, H.28 (rat IgG1), which reacts with the 120, 140, and 180 kDa isoforms of mouse NCAM, was from Immunotech, anti-NCAM polyclonal antibody, NA-1206, was from Affinity Research Products, and the anti-PSA monoclonal antibody 735 was kindly provided by Dr. R. Gerardy-Schahn, Inst. für Med. Mikrobiologie, Hanover, Germany (*14*). Endoneuraminidase (endo N) purified from bacteriophage K1F, which only cleaves poly-α2,8-sialic acids, was kindly provided by Dr. F.A. Troy, Univ. of California, Davis, CA (*15*). Neuraminidase F from *Streptococcus* 6646K (*16*), and NANase II, which cleaves α2,3- and α2,6-linked sialic acid residues, were from Seikagaku Kogyo and Glyko, respectively.

PSA synthase activity in cells was measured using soluble recombinant NCAM fused with the Fc region of human IgG as the acceptor substrate and the membrane fraction of the cells as the enzyme source, as described previously (*12*). Briefly, the reaction mixture, comprising 0.1 mM CMP-[^{14}C]Sia (0.25 μCi), 10 mM $MgCl_2$, 25 mM MES, pH 6.0, 0.5 μg of NCAM-Fc, and 50 μg of membrane protein, was incubated at 37°C for 4 h. Then NCAM-Fc in the reaction mixture was recovered by adding protein A-Sepharose, followed by SDS-PAGE. PSA synthase activity was estimated as the difference in radioactivity between before and after treatment with endo N.

Transfection of ST8Sia II and IV cDNAs into Neuro2a Cells—cDNAs containing the full open reading frames of mouse ST8Sia II and IV (*6, 8*) were ligated into the cloning site of the mammalian expression vector, pRc/CMV (Invitrogen), yielding pRc/CMV-mST8Sia II and pRc/CMV-mST8Sia IV, respectively. These plasmids were transfected into mouse neuroblastoma Neuro2a cells by means of lipofectoamine (*11*). After culturing for 72 h in DMEM supplemented with 10% FCS, cells were selected in 1.0 mg/ml G-418. The G-418-resistant colonies were then stained with mAb 735, and PSA-positive colonies were re-cloned as described above. Seven and nine PSA-positive clones were obtained from pRc/CMV-mST8Sia II and pRc/CMV-mST8Sia IV transfected cells, respectively. After determination of PSA expression by flow cytometry, the highest PSA-expressing clones of pRc/CMV-mST8Sia II and pRc/CMV-mST8Sia IV transfected cells, which were designated as N2a-II and N2a-IV, respectively, were examined in this study. The same vector without any insert was transfected into Neuro2a cells and the cells were selected in 1.0 mg/ml G-418, the resulting G-418-resistant cells being used as control Neuro2a cells.

Western Blotting—For immunoblot analysis, cells were sonicated on ice in an extraction buffer (50 mM MES, pH 6.0, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 1 mM PMSF), incubated for 15 min on ice, and centrifuged at 15,000 $\times$ *g* for 15 min, and then the protein concentration in

the supernatant was measured by the BCA method (Pierce). The lysate (5 μg of protein) was subjected to SDS-PAGE on a 5-20% gel before and after treatment with endo N (1:10 dilution) (*9*). The proteins were then transferred to Zetaprobe (Bio-Rad) filter paper. The filter paper was blocked for 60 min with PBS containing nonfat dry milk, then incubated with an anti-NCAM antibody, NA-1206, overnight at 4°C, followed by incubation with an alkaline phosphatase-conjugated anti-mouse IgG rabbit secondary antibody. NCAM was detected by its chemiluminescence using a Phototope™-Star detection kit (New England BioLabs).

Metabolic Labeling of Cells—Control Neuro2a, N2a-II, and N2a-IV cells were cultured in medium supplemented with 10% FCS, then metabolically labeled with [^{3}H]glucosamine (10 μCi/ml) by culturing in DMEM without glucose/DMEM (9:1) supplemented with 1% FCS for 48 h (*11*). After labeling, the cells were harvested and washed with PBS. The cells were then incubated with 200 μl of lysis buffer (50 mM MES, pH 6.0, containing 0.15 M NaCl, 1.0% Nonidet P-40, 5 mM EDTA, and 1 mM PMSF) at 4°C for 30 min, and centrifuged, the supernatant (cell lysate) being collected. The cell lysate was immunoprecipitated with mAb 735 and protein G-Sepharose, and then analyzed by SDS-PAGE on a 5-20% gradient gel before and after treatment with endo N or neuraminidase F (0.5 U/ml) for 3 h, followed by autoradiography.

For pulse-chase labeling with [^{35}S]methionine, cells were cultivated in the wells of a 24-well culture plate (Falcon), washed with methionine-free DMEM, then incubated with the same medium for 30 min. The cells were then pulsed with [^{35}S]methionine (100 μCi/ml) in methionine-free DMEM for 10 min at 37°C. After the pulse-labeling, non-radiolabeled methionine (1 mM) was added to the medium, the medium was changed to DMEM supplemented with 10% FCS and 1 mM methionine, then the cells were cultured for 3 h. After the cells had been washed with PBS twice, they were lysed with 50 mM MES, pH 6.0, containing 1.0% Nonidet P-40, 5 mM EDTA, and 1 mM PMSF. The cell lysate was centrifuged with 15,000 rpm for 20 min. The supernatant was precipitated with non-immunized mouse IgG and protein G-Sepharose for 16 h at 4°C with gentle rotation, and then the resulting supernatant was immunoprecipitated with anti-NCAM mAb H.28 or anti-PSA mAb 735, and protein G-Sepharose. The beads were collected by centrifugation and then subjected to SDS-PAGE on a 5-20% gradient gel before and after treatment with endo N or neuraminidase F. The gels were then dried and radioactivity was detected with a BAS2000 radio image analyzer (Fuji Film).

Preparation of N-Linked Oligosaccharides from Transfected Cells—Cells were labeled with [^{3}H]glucosamine, as described above, then the labeled glycoproteins were extracted from the cells. The labeled NCAM and PSA-carrying glycoproteins were immunoprecipitated with anti-NCAM mAb and anti-PSA mAb, respectively, then subjected to hydrazinolysis followed by *N*-acetylation (*9*). The *N*-linked oligosaccharides derived from NCAM or PSA-carrying glycoproteins were then separated on a DEAE-Toyopearl column with a linear gradient of ammonium acetate (0-0.6 M). Fractions (1 ml) were collected and aliquots of 200 μl were counted with a liquid scintillation counter.

RESULTS

mST8Sia II cDNA- and mST8Sia IV cDNA-Transfected Neuro2a Cells Express PSA-NCAM with Different Degree of Polysialylation on Different Isoforms of NCAM—We previously compared the enzymatic activities of mST8Sia II and IV, and reported that both can synthesize PSA on soluble chimeric NCAM and fetuin *in vitro* but that under these conditions the degree of polysialylation by mST8Sia IV was much higher than that by the recombinant soluble mST8Sia II (*11*). In the present study, to compare the expression and *in vivo* biosynthesis of PSA by mST8Sia II and IV, we transfected cDNAs encoding mST8Sia II and IV, which were inserted into the same vector, into Neuro2a cells. Neuro2a cells express NCAM-180, -140, and -120, but not PSA (Fig. 1). In addition, Neuro2a cells do not express either mST8Sia II or IV mRNA (Fig. 2B). After transfection of the cDNAs, seven and nine individual PSA-positive clones were obtained from mST8Sia II cDNA-transfected and mST8Sia IV cDNA-transfected cells, respectively. Since the major PSA-carrying glycoprotein in mammals is known to be NCAM, we first examined the pattern of polysialylation of NCAMs expressed in three clones each of mST8Sia II cDNA- or mST8Sia IV cDNA-transfected Neuro2a cells by Western blotting with anti-NCAM polyclonal antibody (pAb).

The proteins recognized by the anti-NCAM pAb appeared as polydisperse materials in both mST8Sia II cDNA- and mST8Sia IV cDNA-transfected Neuro2a cells, and the polydisperse materials disappeared on treatment with endo

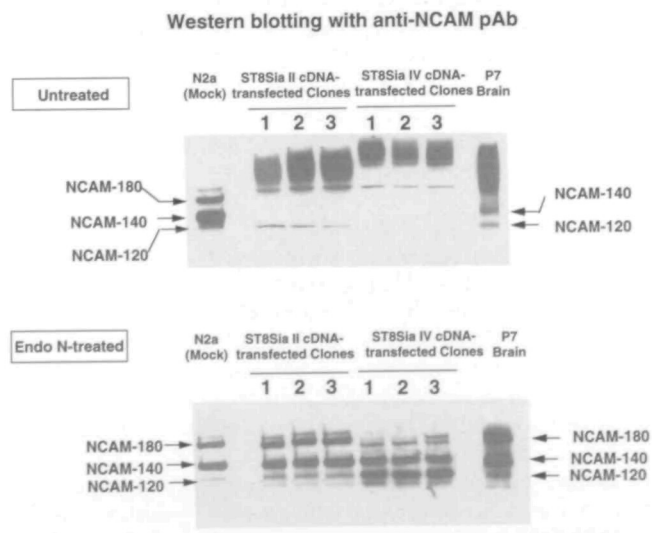
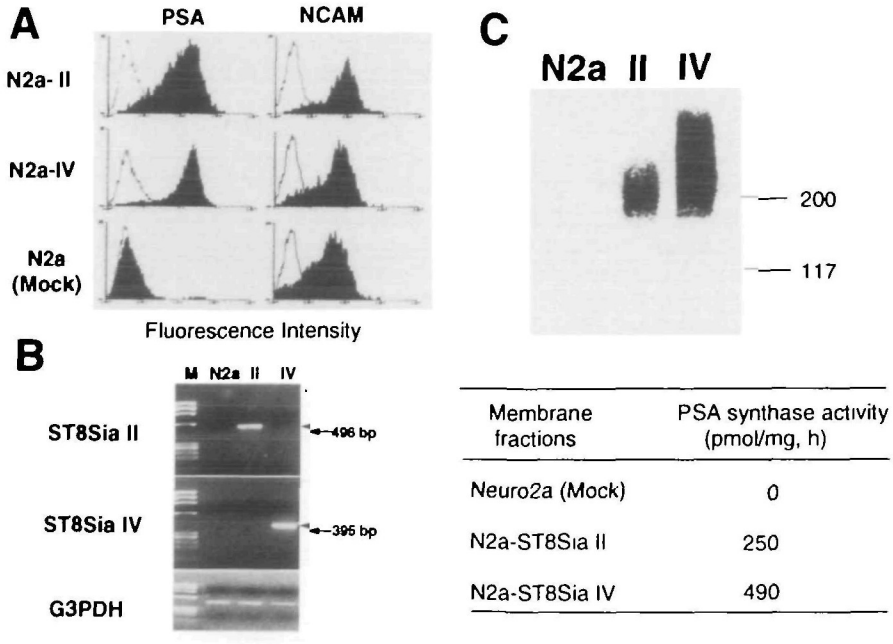


Fig. 1. **Analysis of PSA-NCAM expressed in mST8Sia II cDNA- and mST8Sia IV-cDNA transfected Neuro2a clones.** Three clones of each mST8Sia II cDNA- and mST8Sia IV cDNA-transfected clones were lysed with lysis buffer, and cell lysates (5 μg of protein) were subjected to SDS-PAGE on a 5-20% polyacrylamide gel, before (upper panel) and after (lower panel) treatment with endo N. Membrane proteins isolated from post-natal 7 day (P7) mouse brain were electrophoresed on the same gel. The proteins were blotted onto a nylon membrane, and NCAM was detected using the anti-NCAM polyclonal antibody. N2a denotes the control Neuro2a cells transfected with the same vector without an insert (Mock transfectant).



Membrane fractions	PSA synthase activity (pmol/mg, h)
Neuro2a (Mock)	0
N2a-ST8Sia II	250
N2a-ST8Sia IV	490

Fig. 2. **Characterization of Neuro2a cells transfected with mST8Sia II cDNA (N2a-II) or mST8Sia IV cDNA (N2a-IV).** cDNAs encoding mST8Sia II and IV were stably transfected into Neuro2a cells, the highest PSA-expressing clones being designated as N2a-II and N2a-IV. A: Expression of PSA and NCAM. N2a-II, N2a-IV, and mock transfectants of Neuro2a cells were stained with anti-PSA mAb 735 and anti-NCAM mAb H.28, followed by fluorescein-conjugated anti-mouse IgG and fluorescein-conjugated anti-rat IgG, respectively, then analyzed with a fluorescence activated cell sorter (solid peak). Open peaks indicate the cells stained with nonimmunized (control) mouse IgG or rat IgG. B: Expression of mST8Sia II and IV mRNAs. The mRNAs prepared from the cells were converted to cDNAs, then the same amount of each cDNA (as estimated with 17 cycles of PCR for the G3PDH gene) was subjected to 26 cycles of PCR. The 5′ and 3′ primers were 5′-GGGGTCTTGCTGAACAGCGGCTGT-GG-3′ and 5′-GGTAGATCTGATTGCAGA-AGCGTG-3′ for ST8Sia II, and 5′-ATGTG-GAAAGGAGATTGACAG-3′ and 5′-AGTG-TATACATGAGGAGACCTGT-3′ for ST8Sia IV, respectively. M, N2a, II, and IV indicate the standard markers ($\phi X174$), and control Neuro2a, N2a-I, and N2a-IV cells, respectively. C: Expression of PSA synthase activity in the cells. Membrane fractions were prepared by sonication of cells followed by centrifugation at 100,000 × *g*, and then the polysialic acid synthase activity in the membrane fractions was analyzed. The PSA synthase activity was measured in a reaction mixture comprising 0.1 μM CMP-[^{14}C]Sia (0.25 μCi), 10 mM $MgCl_2$, 25 mM MES, pH 6.0, 0.5 μg of NCAM-Fc, and 50 μg of membrane protein, at 37°C for 4 h. After incubation, NCAM-Fc in the reaction mixture was recovered by adding protein G-Sepharose, followed by SDS-PAGE. PSA synthase activity was estimated as the difference in radioactivity between before and after treatment with endo N (inset table).

N, demonstrating that both types of transfected cells expressed the polysialylated form of NCAM (PSA-NCAM) (Fig. 1). The PSA-NCAMs expressed in the three clones of mST8Sia IV cDNA-transfected cells migrated much slower on SDS-PAGE than those expressed in the three clones of mST8Sia II cDNA-transfected cells. The apparent molecular masses of the PSA-NCAMs expressed in the clones of mST8Sia II cDNA-transfected cells were in the range of 200–1,000 kDa, while those in the clones of mST8Sia IV cDNA-transfected cells ranged between 500 and 2,500 kDa. Therefore, the transfection of the cDNA encoding mST8Sia IV directed the expression of PSA-NCAMs with much higher degree of polysialylation than that of the cDNA encoding mST8Sia II in Neuro2a cells.

NCAMs were expressed as polydisperse materials and gave a single sharp band, that corresponded to a molecular mass of about 120 kDa in three mST8Sia II cDNA-transfected clones (Fig. 1, upper panel). On endo N treatment, proteins corresponding to NCAM-180 and -140 appeared, suggesting that, of the three isoforms expressed in the cells, NCAM-180 and -140 were expressed as polysialylated forms, but NCAM-120 was hardly polysialylated. The ratio of the three NCAM isoforms expressed in mST8Sia II cDNA-transfected Neuro2a clones was almost the same as that in the control cells (Fig. 1, lower panel). On the other hand, the NCAMs in the mST8Sia IV cDNA-transfected clones appeared only as polydisperse materials, there being no proteins corresponding to NCAM-180, -140, and -120, which were expressed in the parental and control Neuro2a cells (Fig. 1, upper panel). On treatment with endo N, proteins corresponding to NCAM-180, -140, and -120 appeared (Fig. 1, lower panel). Thus, all three isoforms were expressed as PSA-NCAM in the mST8Sia IV cDNA-transfected clones. It should be noted that the patterns of the NCAM isoforms in the mST8Sia IV cDNA-transfected clones were markedly different from those of the control Neuro2a and mST8Sia II cDNA-transfected clones. In all three mST8Sia IV cDNA-transfected clones, NCAM-120 and -140 were expressed as the major NCAM isoforms, NCAM-180 being expressed as a minor component, whereas in the parental and control Neuro2a cells, and in all three mST8Sia II cDNA-transfected clones, NCAM-120 was expressed as a minor isoform. An increase in NCAM-120 and a decrease in NCAM-180 were observed in other ST8Sia IV cDNA-transfected Neuro2a cells we isolated (data not shown). These results suggested that the substrate specificity of mST8Sia II was different from that of mST8Sia IV *in vivo*, and that the *in vivo* polysialylation of NCAM by mST8Sia IV cDNAs affected the expression pattern of NCAM isoforms.

To characterize the *in vivo* polysialylation in more detail, the highest PSA-expressing clones of mST8Sia II cDNA- and mST8Sia IV cDNA-transfected Neuro2a cells, designated as N2a-II and N2a-IV, respectively, were examined in the following studies. The expression levels of PSA and NCAM in N2a-II and N2a-IV were almost the same, as judged from the results of flow cytometry with anti-PSA mAb 735 and anti-NCAM mAb H.28, respectively (Fig. 2A). The expression levels of mST8Sia II mRNA in N2a-II and mST8Sia IV mRNA in N2a-V were similar, as judged from the results of RT-PCR (Fig. 2B). The polysialic acid synthase activities of N2a-ST8Sia II and N2a-ST8Sia IV

were 250 and 490 pmol/mg•h, respectively, based on the incorporation of sialic acids into soluble chimeric NCAM (table in Fig. 2) (*12*). The cell lysates of N2a-II and N2a-V synthesized PSAs of different lengths on soluble chimeric NCAM *in vitro* (Fig. 2C), as seen in the case of the purified soluble enzymes (*11*).

Comparison of De Novo Synthesis of PSA-NCAM—As shown in Fig. 1, the pattern of NCAM isoforms expressed in N2a-IV was different from that in control Neuro2a cells or N2a-II. To confirm that the biosynthesis pattern of NCAM isoforms in N2a-IV is different from that in N2a-II, cells were pulse-labeled with [^{35}S]methionine for 10 min and chased for 3 h, then the labeled proteins were analyzed after immunoprecipitation with anti-NCAM mAb, H.28. As shown in Fig. 3, the labeled polydisperse materials from N2a-IV migrated much more slowly than those from N2a-II, as seen on Western blot analysis with anti-NCAM pAb, indicating that the degree of the newly synthesized PSA-NCAM in N2a-IV was also higher than that in N2a-II. In N2a-II, most of the newly synthesized NCAM-120 was immunoprecipitated in non-polysialylated form, as seen in Western blot analysis. The labeled protein bands corresponding to NCAM-140 and -180 were not detected in N2a-II before neuraminidase treatment, but they appeared on neuraminidase treatment, suggesting that NCAM-140 and -180 were almost completely polysialylated within the pulse-chase period. This suggests that NCAM-120 was hardly polysialylated by mST8Sia II *in vivo*, as compared with NCAM-180 and -140. On the other hand, the three non-polysialylated NCAM isoforms were observed in addition to the polydisperse materials in N2a-IV. On treatment with neuraminidase F, the polydisperse materials disappeared, and the labeled proteins corresponding to NCAM-180, -140, and -120 increased. Therefore, all three NCAM isoforms in N2a-IV were partly polysialylated within the pulse-chase period. These results demonstrated that the *in vivo* polysialylation pattern of NCAM isoforms with mST8Sia II was different from that with mST8Sia IV. It should be noted that the patterns of labeled NCAM isoforms were almost the same in the control Neuro2a, N2a-II, and N2a-IV after treatment with neuraminidase F. In these cells, NCAM-140 was synthesized as a major isoform, and NCAM-120 and -180 were synthesized in

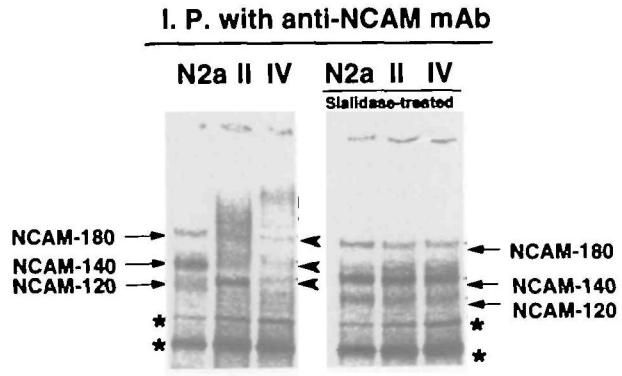


Fig. 3. **Biosynthesis of PSA-NCAM in N2a-II and N2a-IV.** Cells were pulse-labeled with [^{35}S]methionine for 10 min, then chased for 3 h. After the chase, the cells were lysed with lysis buffer, and half of each cell lysate was immunoprecipitated with anti-mouse NCAM mAb, then subjected to SDS-PAGE before and after treatment with neuraminidase F. N2a, II, and IV indicate the control Neuro2a, N2a-II, and N2a-IV cells, respectively. The black arrowheads indicate the proteins corresponding to NCAM isoforms. Asterisks indicate the nonspecifically labeled proteins immunoprecipitated with the anti-NCAM mAb.

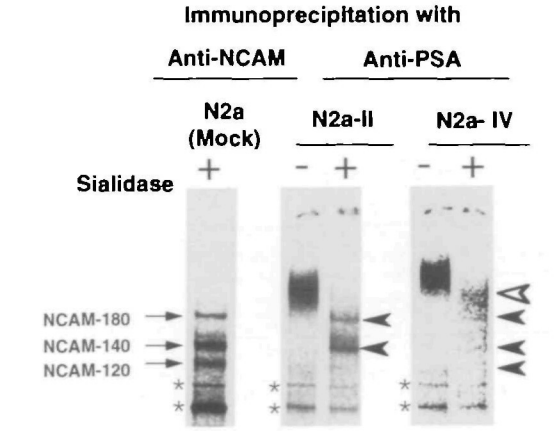


Fig. 4. ***De novo* polysialylation of newly synthesized proteins in N2a-II and N2a-IV.** Half of the [^{35}S]methionine-labeled proteins described in Fig. 3 were immunoprecipitated with the anti-PSA mAb. The polysialylated proteins were separated by SDS-PAGE before and after treatment with neuraminidase F, followed by autoradiography. The black arrowheads indicate the proteins corresponding to NCAM isoforms. The white arrowheads indicate the proteins other than NCAM isoforms. Asterisks indicate the nonspecifically labeled proteins immunoprecipitated with anti-PSA mAb.

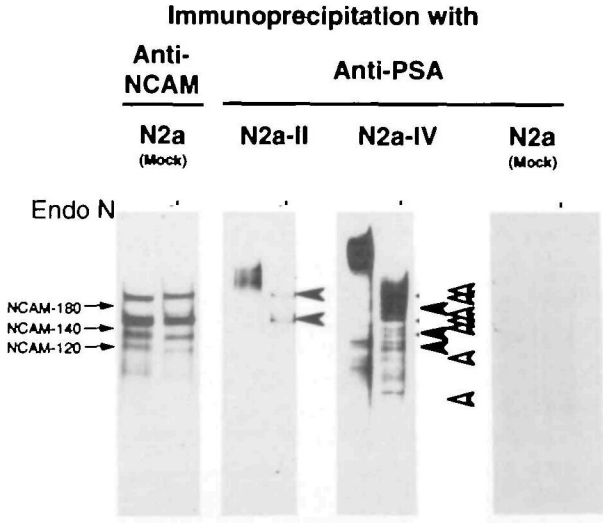


Fig. 5. **Analysis of PSA-carrying glycoproteins expressed in N2a-II and N2a-IV cells.** N2a-II and N2a-IV cells were metabolically labeled with [^{3}H]glucosamine for 48 h. The cells were collected, washed with PBS, and then lysed with lysis buffer. The labeled glycoproteins which carried PSA were immunoprecipitated with anti-PSA mAb 735 from the lysates. At the same time, control Neuro2a cells were labeled with [^{3}H]glucosamine, and the labeled NCAM was isolated by immunoprecipitation with anti-NCAM mAb H.28. The labeled materials were subjected to SDS-PAGE on a 5-20% polyacrylamide gel, before and after treatment with endo N. The gel was dried and radioactivity was detected by autoradiography. The black arrowheads indicate the proteins corresponding to NCAM isoforms. The white arrowheads indicate the proteins other than NCAM isoforms.

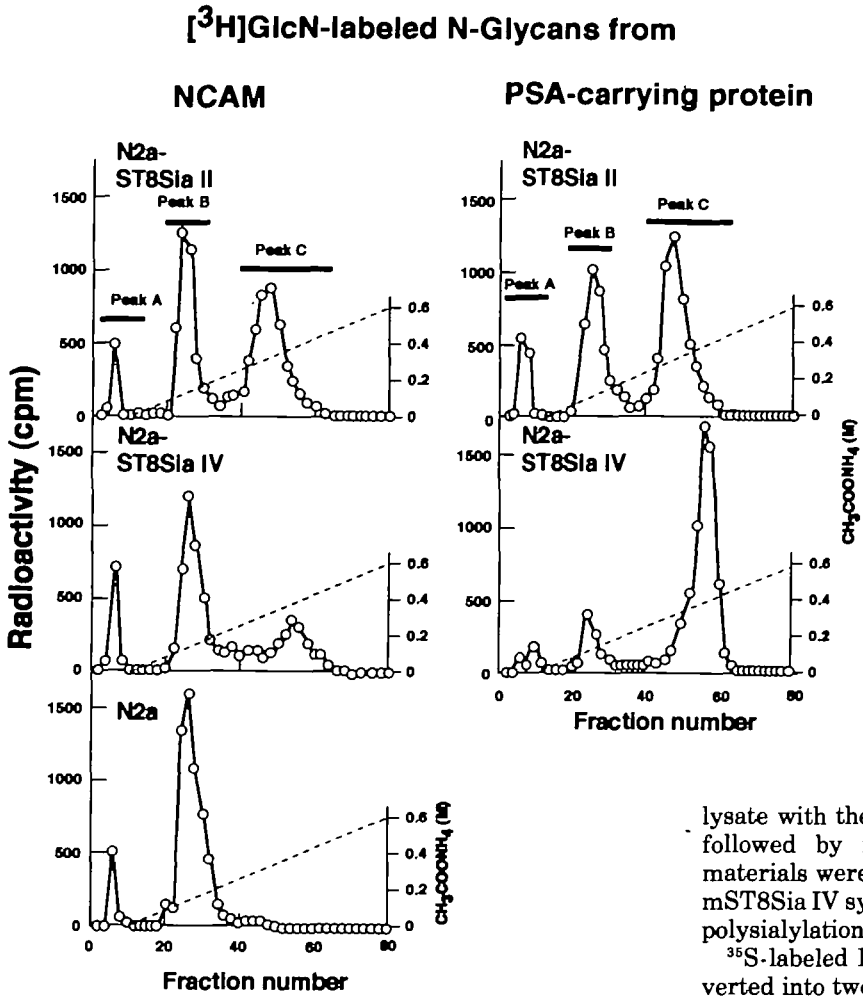


Fig. 6. **Ion-exchange chromatography of *N*-glycans derived from NCAM and PSA-carrying glycoproteins in N2a-II and N2a-IV cells.** Cells were metabolically labeled with [^{3}H]glucosamine for 24 h, then lysed with lysis buffer, and the labeled PSA-carrying glycoproteins and NCAM were immunoprecipitated with anti-PSA mAb 735 and anti-NCAM mAb H.28, respectively, as described in the legend to Fig. 2. Then *N*-glycans were released from the labeled glycoproteins by hydrazinolysis as described under "EXPERIMENTAL PROCEDURES," then subjected to anion-exchange chromatography on a DEAE-Toyopearl column with a linear gradient of 0–0.6 M CH_3COONH_4. PSA and NCAM indicate the *N*-glycans derived from PSA-carrying glycoproteins and NCAM, respectively.

similar amounts. This result suggested that the biosynthesis rates of the NCAM isoforms within the pulse-chase period were similar in these types of cells. Therefore, the difference in the expression pattern of NCAM isoforms between N2a-II and N2a-IV may be due to differences in the degradation or turn-over rates of the isoforms.

Polysialic Acids Were Specifically Synthesized on NCAM in ST8Sia II cDNA-Transfected Cells, but Synthesized on Other Glycoproteins in ST8Sia IV cDNA-Transfected Cells—To determine whether PSA-carrying proteins are restricted to NCAM in the cDNA-transfected cells, PSA-carrying proteins were recovered from the cells by immunoprecipitation with anti PSA mAb, 735, and analyzed. As shown in Fig. 4, when ^{35}S-labeled proteins were immunoprecipitated with the anti-PSA mAb followed by SDS-PAGE, the degree of polysialylation of newly synthesized PSA-carrying glycoproteins in N2a-IV was much higher than that in N2a-II, as seen in the case of NCAM expressed on the cells and ^{35}S-labeled PSA-NCAM. The *in vivo* biosynthesis of PSA with different degree of polysialylation was confirmed by metabolically labeling the cells with [^{3}H]glucosamine for 48 h (Fig. 5). The [^{3}H]glucosamine-labeled polydisperse materials immunoprecipitated with the anti-PSA mAb exhibited molecular weights of about 500-2,500 kDa, *i.e.*, much higher than those in the case of N2a-II cells as seen on Western blotting of the cell lysate with the anti-NCAM pAb and pulse-chase labeling followed by immunoprecipitation. These polydisperse materials were sensitive to endo N treatment. Therefore, mST8Sia IV synthesizes a PSA with much higher degree of polysialylation than mST8Sia II *in vivo*.

^{35}S-labeled PSA-carrying proteins in N2a-II were converted into two proteins corresponding to NCAM-180 and -140 on treatment with neuraminidase F, *i.e.*, NCAM-120 was not derived from PSA-carrying proteins (Fig. 4). The same results were obtained when cells were metabolically labeled with [^{3}H]glucosamine, followed by immunoprecipitation with the anti-PSA mAb (Fig. 5). In this case, other [^{3}H]glucosamine-labeled glycoproteins were not derived from the PSA-carrying glycoproteins in N2a-II. These results demonstrated that the NCAM-140 and -180 isoforms are specifically polysialylated by mST8Sia II *in vivo*.

In contrast, broad bands appeared around 200 kDa as the major radiolabeled proteins derived from the ^{35}S-labeled polydisperse materials in N2a-IV (Fig. 4). When the cells were metabolically labeled with [^{3}H]glucosamine, several faster migrating [^{3}H]glucosamine-labeled glycoproteins, which were not observed for N2a-ST8Sia II or control Neuro2a cells, were immunoprecipitated with the anti-PSA mAb from N2a-IV cells in addition to the polydisperse materials. On treatment with endo N, the majority of the ^{3}H-labeled polydisperse materials gave heterogeneous bands around 200 kDa, which were not coincident with any NCAM isoform (white arrowheads in Fig. 5), although minor bands corresponding to NCAM-180, -140, and -120 also appeared (black arrowheads in Fig. 5). No [^{3}H]glucosamine-labeled material was derived from the control or parental Neuro2a cells on immunoprecipitation with the anti-PSA mAb. These results clearly indicated that not only NCAM but also other glycoproteins were polysialylated in N2a-IV.

Comparison of the Lengths of PSA on N-Glycans—To determine whether the difference in the degree of polysialylation between N2a-II and N2a-IV is due to differences in PSA chain length or differences in the number of PSA chains on the PSA-carrying glycoproteins, PSA-carrying proteins were immunoprecipitated with the anti-PSA mAb from [^{3}H]glucosamine-labeled N2a-II and N2a-IV cells, and then the [^{3}H]glucosamine labeled-*N*-glycans were isolated by hydrazinolysis followed by *N*-acetylation. The resulting labeled *N*-glycans were subjected to chromatography on a DEAE-Toyopearl column. As shown in Fig. 6, the *N*-glycans were separated into three peaks on the DEAE-Toyopearl column: Peak A appeared in the pass-through fractions, Peak B was eluted at the concentrations of 0.1-0.2 M CH_3COONH_4, and Peak C was eluted at the concentrations of 0.35-0.45 M CH_3COONH_4. The recovery of the radioactivity after chromatography was over 90%, suggesting that almost all the labeled *N*-glycans were eluted from the column under these conditions. The materials in Peak B were eluted at the same position as the majority of the *N*-glycans of non-polysialylated NCAM isolated from control Neuro2a cells were eluted, indicating that Peak B contained non-polysialylated *N*-glycans. The materials in Peak C were eluted at almost the same position as colomic acid from *Escherichia coli* and were sensitive to endo N treatment, indicating that Peak C contained PSA-carrying *N*-glycans. The radioactivities in the peaks are summarized in Table I. Over 85% of the radioactivity was recovered in PSA-carrying *N*-glycans in the case of N2a-IV, whereas about 53% was recovered in PSA-carrying *N*-glycans in the case of N2a-II. These results suggested that the average number the PSA-carrying glycoproteins in N2a-IV was greater than that in N2a-II. In addition, the PSA-carrying *N*-glycans derived from N2a-IV were eluted at a slightly higher salt concentration than those from N2a-II, *i.e.*, the PSA-carrying *N*-glycans from N2a-IV were eluted at 0.4 M CH_3COONH_4, whereas those from N2a-II were eluted at 0.35 M CH_3COONH_4 (Fig. 6), suggesting that the PSA chains in N2a-IV were much longer than those in N2a-II. Therefore, both the length of PSA chains and the number of PSA-carrying glycoproteins in N2a-IV seemed to be greater than those in N2a-II. When *N*-glycans were prepared from the materials immunoprecipitated with the anti-NCAM mAb and separated on the same column, about 50 and 25% of the *N*-glycans derived from NCAM expressed in N2a-II and N2a-IV were recovered as PSA-carrying *N*-glycans, respectively. No radioactivity was recovered in Peak C of the *N*-glycans of NCAM expressed in control Neuro2a cells.

TABLE I. **Proportions of polysialylated *N*-glycans in total *N*-glycans carried on PSA-carrying glycoproteins and NCAM in N2a-ST8Sia II and N2a-ST8Sia IV cells.** PSA-carrying glycoproteins and NCAM were isolated from [^{3}H]glucosamine-labeled cells by immunoprecipitation with anti-PSA and anti-NCAM mAbs, respectively, as described under "EXPERIMENTAL PROCEDURES." The radioactive materials eluted in Peaks A, B, and C in Fig. 6 were pooled, and their radioactivity was counted. Peaks A, B, and C contained neutral, non-polysialylated and polysialylated *N*-glycans, respectively. The percentages of recovered radioactivity in Peak C relative to total radioactivity are given in parentheses.

Immunoprecipitated with	Cells	Radioactivity recovered in		
		Peak A	Peak B (cpm)	Peak C
Anti-PSA	N2a-ST8Sia II	950	2,990	4,430 (52.9%)
	N2a-ST8Sia IV	450	810	7,450 (85.5%)
Anti-NCAM	N2a (Mock)	550	7,600	0
	N2a-ST8Sia II	560	3,680	4,070 (48.6%)
	N2a-ST8Sia IV	800	4,760	1,890 (25.4%)

DISCUSSION

We reported in the previous paper that mST8Sia II and IV can synthesize PSA-NCAM *in vitro* by a single enzyme, but the length of PSA synthesized by mST8Sia IV under these conditions was much greater than that by mST8Sia II (*11*). As shown in this study, PSA-expressing clones derived from mST8Sia IV cDNA-transfected Neuro2a cells expressed a PSA-NCAM with much higher degree of polysialylation than the PSA-expressing clones derived from mST8Sia II cDNA-transfected Neuro2a cells (Fig. 2). Since all clones derived from mST8Sia IV cDNA-transfected cells showed a higher degree of polysialylation than all clones derived from mST8Sia II cDNA-transfected cells, the higher and lower degrees of polysialylation were not clone-specific, *i.e.*, there is a common polysialylation manner in mST8Sia IV cDNA-transfected and mST8Sia II cDNA-transfected Neuro2a cells, respectively. Metabolic labeling with [^{3}H]glucosamine and pulse-chase labeling with [^{35}S]methionine indicated the different degrees of PSA biosynthesis *in vivo* between one clone from mST8Sia II cDNA-transfected cells (N2a-II) and one from mST8Sia IV cDNA-transfected cells (N2a-IV). Comparison of the *N*-linked oligosaccharides derived from the two types of cells suggested the difference in the degree of polysialylation was due to differences in both the PSA chain length and the number of PSA chains on the glycoproteins. Both the lengths and the numbers of PSA chains on the PSA-carrying glycoproteins in N2a-ST8Sia IV seemed to be greater than those in N2a-ST8Sia II. Thus, mST8Sia IV synthesizes a PSA with higher degree of polysialylation on NCAM than mST8Sia II *in vivo*.

PSA-NCAM exhibits molecular masses in the range of 200-2,500 kDa in the late embryonic to early postnatal stages (*1, 2*). However, the molecular masses of PSA-NCAM in mST8Sia II cDNA-transfected Neuro2a cells are smaller than in the case of brain PSA-NCAM (see Fig. 2). On the other hand, those of PSA-NCAM in mST8Sia IV cDNA-transfected Neuro2a cells are larger than to those of PSA-NCAM expressed in mouse brain. It is possible that mST8Sia II and IV individually synthesize PSA-NCAM with the different degrees of polysialylation in various types of cells in mouse brain, and therefore the molecular masses of PSA-NCAM in the total brain are in the range of 200-2,500 kDa. In fact, the results of Western blotting for various regions of the P1 mouse CNS revealed that the mobilities of PSA-carrying glycoproteins were slightly different in each region (*17*).

It was surprising that not only NCAM but also other glycoproteins were polysialylated in N2a-IV, while only NCAM-140 and -180 were polysialylated in N2a-II. Since we transfected both mST8Sia II and IV cDNAs with a strong promoter, both the genes and enzymes were probably over-expressed at nonphysiological levels. Thus, polysialylation of other glycoproteins by mST8Sia IV may be explained by over-expression of mST8Sia IV. However, even with the over-expression of mST8Sia II, the poly-

sialylation of the proteins in N2a-II was restricted to NCAM-180 and -140. This limited polysialylation in N2a-II and broad polysialylation in N2a-IV were confirmed in three different ways, *i.e.*, Western blotting, metabolic labeling of cells with [^{3}H]glucosamine (for glycans in glycoproteins), and pulse-chase labeling with [^{35}S]methionine (for proteins). Since there was no significant difference in the polysialic acid synthase activity between the two types of cells, the differences in the polysialylated glycoproteins in N2a-II and N2a-IV may reflect the differences between the *in vivo* substrate specificities of mST8Sia II and IV. Our results strongly suggested that mST8Sia II specifically recognizes NCAM, but that mST8Sia IV exhibits a much broader specificity in Neuro2a cells. The *in vivo* specificities of the two PSA synthases must be confirmed with other cell lines, since ST8Sia IV was shown to be the only factor required for PSA-NCAM synthesis in CHO cells (*13*). It is not yet known what kinds of glycoproteins are polysialylated in N2a-IV. Recently, it was shown that the hamster PST-1, a hamster homologue of mST8Sia IV, is a polysialylated glycoprotein, which is automatically polysialylated (*18*). Thus, one of the unknown PSA-carrying glycoproteins in N2a-IV may be mST8Sia IV itself. However, identification of the PSA carrier in N2a-IV is required for elucidation of the role of ST8Sia IV.

Interestingly, the expression pattern of NCAM isoforms in mST8Sia IV cDNA-transfected cells was markedly different from those in control Neuro2a and mST8Sia II cDNA-transfected clones. NCAM-120 was expressed in a polysialylated form and as the major NCAM isoform in all mST8Sia IV cDNA-transfected clones, and NCAM-180 was expressed as a minor isoform, whereas NCAM-120 was expressed as a minor isoform, and NCAM-180 and -140 were expressed as major components in the mST8Sia II cDNA-transfected clones and control Neuro2a cells. On the other hand, there was no significant difference in NCAM synthesis among them, as judged from the results of the pulse-chase experiment with [^{35}S]methionine, followed by immunoprecipitation with the anti-NCAM mAb and neuraminidase treatment (Fig. 3). In addition, the pattern of the newly synthesized NCAM isoforms was different from that of the expressed ones, *i.e.*, NCAM-140 was synthesized as a major isoform and NCAM-120 and -180 were synthesized similarly. The differences between the patterns of the expressed NCAM isoforms and newly synthesized NCAM-isoforms may be explained by the different stability of each NCAM isoform in the cells. NCAM-120 may be rapidly degraded in Neuro2a and mST8Sia II cDNA-transfected cells, and therefore NCAM-120 expressed as a minor isoform in these cells, whereas in mST8Sia IV cDNA-transfected Neuro2a, NCAM-120 may be more stable, but NCAM-180 becomes unstable, and therefore NCAM-120 is expressed as a major isoform. Although the detailed mechanism by which NCAM-120 is accumulated in mST8Sia IV cDNA-transfected Neuro2a cells is not known, the stabilities and turn-over rates of NCAM isoforms may be affected by the heavy polysialylation by mST8Sia IV.

So far, NCAM and the α-subunit of the voltage-gated sodium channel are known as PSA-carrying glycoproteins in higher vertebrates (*1*). According to the *in vivo* substrate specificities of mST8Sia II and IV, ST8Sia IV may be involved in the PSA synthesis of the α-subunit of the voltage-gated sodium channel. In muscle cells, the GPI-anchored form of NCAM is known to be polysialylated (*19*). It is also possible that the polysialylation of the GPI-anchored form of NCAM may be directed by ST8Sia IV, because mST8Sia IV can synthesize and express the GPI-anchored form of NCAM (NCAM-120) in Neuro2a cells. Since other glycoproteins, in addition to NCAM, can be polysialylated in mST8Sia IV cDNA-transfected Neuro2a cells, other PSA-carrying glycoproteins may occur that are synthesized by ST8Sia IV. In fact, an undefined protein was shown to be polysialylated in breast cancer and leukemia cells (*3*). Since ST8Sia IV mRNA is more abundantly expressed in lung and peripheral blood than ST8Sia II mRNA in mouse and human (*6, 8, 20*), this undefined polysialylated protein may be modified by ST8Sia IV.

In summary, the results presented here strongly indicate that mST8Sia II more specifically recognizes NCAM than mST8Sia IV, but that mST8Sia IV synthesizes a much more highly polysialylated PSA than mST8Sia II *in vivo*. Differences in the expression of mST8Sia II and IV mRNAs in various mouse tissues and CNS regions, and *in vitro* neuronal differentiation (*6, 8, 12, 17*), suggest that mST8Sia II and IV have different roles in the biosynthesis of PSA and the regulation of PSA expression. To understand the biological functions of PSA, it is important to determine the relationship between the difference in the PSA length expressed on the cell surface and the biological phenomena, *e.g.*, cell adhesion, migration, and neurite extension, which are considered to be affected by PSA expression.

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