Characterization of Mouse ST8Sia II (STX) as a Neuronal Cell Adhesion Molecule-specific Polysialic Acid Synthase

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We previously showed that mouse ST8Sia II (STX) exhibits polysialic acid (PSA) synthase activity in vivo as well as in vitro (Kojima, N., Yoshida, Y., and Tsuji, S. (1995) FEBS Lett. 373, 119–122, 1995). In this paper, we reported that the neural cell adhesion molecule (NCAM) was specifically polysialylated by a single enzyme, ST8Sia II. PSA-expressing Neuro2a cells (N2a-STX) were established by stable transformation of the mouse ST8Sia II gene. Only the 140- and 180-kDa isoforms of NCAM in N2a-STX cells were specifically polysialylated in vivo, although other membrane proteins of N2a-STX were polysialylated in vitro. A recombinant soluble mouse ST8Sia II synthesized PSA on a recombinant soluble NCAM fused with the Fc region of human IgG1 (NCAM-Fc) as well as fetuin. However, NCAM-Fc served as a 1500-fold better acceptor for ST8Sia II than fetuin. Treatment of NCAM-Fc with Charonia lampas α-fucosidase, which is able to cleave α,1,6-linked fucose, clearly reduced the polysialylation of NCAM-Fc by ST8Sia II. PSA was not synthesized on the N-glycanase-treated NCAM-Fc polypeptide or the free N-glycans of NCAM-Fc. When fetuin and its glycopeptide and N-glycans of fetuin were used as substrates for ST8Sia II, PSA was found to be synthesized on native fetuin and its glycopeptide but not on free N-glycans. These results strongly suggested that core α,1,6-fucose on N-glycans as well as the antennary structures of N-glycans and the polypeptide regions are required for the polysialylation by ST8Sia II. Furthermore, oligo and single α,2,8-sialylated glycoproteins were no longer polysialylated by mouse ST8Sia II. Therefore, the single enzyme, ST8Sia II, directly transferred all α,2,8-sialic acid residues on the α,2,3-linked sialic acids of N-glycans of specific NCAM isoforms to yield PSA-NCAM. Polysialylation did not require any initiator α,2,8-sialyltransferase but did depend on the carbohydrate and protein structures of NCAM.

Polysialic acid (PSA)1 is an unusual carbohydrate associated with the neural cell adhesion molecule (NCAM) and the α-subunit of the voltage-gated sodium channel in mammals and is more abundant in embryonic than adult brain (1–3). PSA is implicated in the reduction of NCAM adhesion through its large negative charge and thereby allows increased neurite outgrowth and cell motility (4–6). It is also reported that the presence of PSA affects cell–cell interactions through other adhesion molecules (7). Although functions of PSA have been described, little is known about the mechanisms by which the synthesis and expression of PSA are regulated.

PSA on NCAM has so far been considered to be synthesized through the actions of at least two distinct enzymes, i.e., an initiator α,2,8-sialyltransferase, which adds a single α,2,8-linked sialic acid to α,2,3-linked sialic acid residues on N-glycans, and a polysialyltransferase, which adds multiple α,2,8-linked sialic acid residues (8). Livingston and Paulson (9) cloned STX/ST8Sia II, a new member of the sialyltransferase gene family from rat. Recently, we showed that ST8Sia II/STX cloned from mouse can synthesize PSA on α,2,3-sialylated N-glycans of glycoproteins without an initiator α,2,8-sialyltransferase in vitro and direct in vivo synthesis of PSA in several cells (10, 11). In addition, we cloned mouse ST8Sia IV by homology cloning using the sequence information on GD3 synthase (12) and STX (9) and showed that the mouse enzyme can synthesize PSA, like ST8Sia II/STX (13). Eckhardt et al. (14) also cloned a sialyltransferase, which is involved in the biosynthesis of PSA on NCAM from hamster, namely PST-1. Hamster PST-1 showed 99.2% identity to mouse ST8Sia IV, indicating that the hamster enzyme is a counterpart of mouse ST8Sia IV. The gene expression of mouse ST8Sia II was restricted in the brain and was dramatically up- and down-regulated during developmental stages of the brain like the expression of PSA, whereas mouse ST8Sia IV was strongly expressed in lung and heart rather than brain, and only little regulation was observed during brain development (10, 13). These observations suggest that ST8Sia II/STX rather than ST8Sia IV/PST-1 is involved in the biosynthesis of PSA associated with NCAM during mouse brain development. However, there is no evidence that the PSA on NCAM is specifically synthesized by ST8Sia II in vivo as well as in vitro, because PSA was synthesized on some glycoproteins such as fetuin in vitro (11), and the transfection of the human STX gene into NCAM-negative cells also caused the expression of PSA on the cell surface (15).

The present study focuses on the biosynthesis of PSA by mouse ST8Sia II using a recombinant soluble NCAM fused with the Fc region of human IgG1 (NCAM-Fc) and comparison of the activities of mouse ST8Sia II and IV. ST8Sia II specifically and directly synthesized PSA on α,2,3-linked sialic acid residues of N-glycans of specific isoforms of NCAM, without acrylamide gel electrophoresis, MES, 4-morpholineethanesulfonic acid; endo N, endoα2,3-endoglucosaminidase; GALNexase I, α,2,3-specific sialidase.
any initiator α,2,8-sialyltransferase. In addition, core α,1,6-linked fucose and the antennary structures of N-glycans and a polypeptide chain are required for the PSA synthesis on NCAM by ST8Sia II.

**EXPERIMENTAL PROCEDURES**

Materials and cDNAs—Unless otherwise indicated the materials used in this study were the same as those previously reported (10, 11, 13). Human chorionic gonadotropin (hCG) α- and β-subunits purified from urine, bovine fetuin, and human α-1 acid glycoprotein were purchased from Sigma. Protein A-Sepharose and protein G-Sepharose were from Pharmacia Biotech Inc. The anti-mouse NCAM monoclonal antibody, H.28 (rat IgG1), which reacts with the 120-, 140-, and 180-kDa isoforms of NCAM, was from Immunotech, and the anti-PSA monoclonal antibody (mAb) 735 was kindly provided by Dr. R. Gerardy-Schmitz. M. m. musculi, which secretes ST8Sia II, was kindly provided by Dr. P. Crocker and D. L. Simmons, Oxford University, Oxford (17). Endo-neuraminidase (endo N) purified from bacteriophage K1F, which degrades only PSA, was kindly provided by Dr. F. A. Troy, University of California, Davis, CA (18). The α-fucosidases purified from Charonia lamarckiana intestine core α,1,6-fucose as well as α,1,4-linked fucose residues, and that from Fusarium oxysporum, which degrades α,1,2- and α,1,4-fucose residues, were from Seikagaku Kogyo. Preparation of Recombinant Proteins—The vector plasmids designated as pCD-SOA-01 and pCD-SOA-05, containing cDNAs encoding protein A-fused soluble mouse ST8Sia II and protein A-fused soluble mouse ST8Sia IV, respectively, were transiently transfected into COS-7 cells and Neuro2a and N2a-STX cells as described previously (10, 12). The media in the transfected cells were collected after 18 h, and the soluble fused enzymes were adsorbed to IgG-Sepharose resin and then used as enzyme preparations, as described previously (10).

A vector plasmid containing cDNA encoding soluble human NCAM fused with the Fc region of human IgG1 was transfected into COS-7 cells. After 48 h, the medium was changed to serum-free medium (Macrophage SFM; Life Technologies, Inc.). After 72 h, the medium was collected and applied to a protein A-Sepharose column (1 × 1.2 cm), the column having been washed with PBS. NCAM-Fc was then eluted with 0.1 M sodium citrate buffer (pH 3.0), neutralized immediately with 1 × Tris-HCl (pH 9.0), concentrated, and then desalted on a Sephadex G-25 column (PD-10 column, Pharmacia) equilibrated with 25 mM MES, pH 6.0. The approximate protein concentration of NCAM-Fc was 52 μg/ml, as estimated by SDS-PAGE followed by densitometry.

Transfection of ST8Sia II Gene into Cells and Metabolic Labeling of Cell Surface Carbohydrates—The cDNA containing the full open reading frame of mouse ST8Sia II was ligated into the cloning site of the mammalian expression vector pBluescript II. The resulting plasmid, pRcCMV-STX, was transfected into murine neuroblastoma Neuro2a cells by the lipofection method with lipofectamine (11). After culturing for 72 h in DMEM supplemented with 10% FCS, the cells were selected in 1.0 mg/ml G418. The G418-resistant colonies were then stained with mAb H.28 or mAb 735, and PSA-positive colonies were re-cloned as described above. Seven PSA-positive lines were then established. One line, Neuro2a, which secretes ST8Sia II, was kindly provided by Dr. P. Crocker and D. L. Simmons, Oxford University, Oxford (17). The plasmid containing the cDNA encoding NCAM-Fc was a gift from Drs. P. Crocker and D. L. Simmons, Oxford University, Oxford (17).

Enzyme activity was measured in the presence of 0.1% sodium cacodylate buffer (pH 6.0), 10 mM MgCl₂, 2 mM CaCl₂, 100 μM CMP-[14C]NeuAc (0.25 μCi), 10 μg of acceptor glycoproteins or 0.1 μg of NCAM-Fc, and 2 μl of enzyme preparation, in a total volume of 1 ml. The reaction mixture and the enzyme were incubated at 37 °C, the reaction was terminated by the addition of 200 μl of water, and then the mixture was centrifuged at 100,000 × g for 5 min, and the supernatant was collected, dried, and subjected to SDS-PAGE followed by autoradiography.

Preparation of Enzyme-treated NCAM-Fc—NCAM-Fc (1 μg) was treated with α-fucosidase (50 mM MES, pH 5.5), N-glycanase (20 mM Tris-HCl, pH 8.0), or α-2,3-specific sialidase (NANase I, 50 mM sodium citrate, pH 5.5) for 16 h at 37 °C. The treated NCAM-Fc was then applied on the protein A-Sepharose column (0.5-ml bed volume) and eluted with 0.1 M sodium citrate, pH 3.0. The eluate was then neutralized immediately with 1 × Tris-HCl (pH 9.0), desalted on a Sephadex G-25 column. For the preparation of diα,2,8-sialylated NCAM-Fc, NCAM-Fc was polysialylated with the soluble ST8Sia II preparation and 1 μM CMP-Sia, and then the polysialylated NCAM-Fc was collected by adding protein A-Sepharose and treated with endo N for 2 h. The endo N-treated NCAM-Fc was then eluted from the protein A-Sepharose resin, neutralized, desalted, and used as diα,2,8-sialylated NCAM-Fc.

Preparation of Oligosaccharides and Gel Filtration—Oligosaccharides were prepared from fetuin by hydrazinolysis (110 °C for 1 h) followed by N-acetylation. The fetuin (1 mg/100 μl) was treated with trypsin (50 μg) overnight. The reaction was terminated by the addition of the reaction mixture to 1 M sodium citrate, pH 3.0. The digest was lyophilized, dissolved in 100 μl of water, and then used as fetuin glycopeptides. SDS-PAGE analysis indicated that fetuin was completely degraded. The same amount of oligosaccharides, fetuin glycopeptides, or native fetuin was sialylated with the enzyme, and then subjected to chromatography on a Sephadex G-50 column (1 × 120 cm) equilibrated with 50 mM ammonium acetate (pH 5.2) after oligosaccharides had been prepared from the fetuin glycopeptides or native fetuin. Fractions of 1 ml were collected, and an aliquot of each (100 μl) was counted with a scintillation counter. The oligosaccharides were then subjected to chromatography on a DEAE-Toyopearl 650 X column (1 × 7 cm) with a linear gradient of ammonium acetate (5–500 mM).

**RESULTS**

NCAM IsSpecifically Polysialylated in Vivo on Transfection of the ST8Sia II Gene—We transfected the mouse ST8Sia II gene into Neuro2a cells, which express NCAM but neither PSA, ST8Sia II, nor ST8Sia IV, and then screened PSA-positive Neuro2a cells (N2a-STX) using the anti-PSA mAb, 735 (Fig. 1A). When the membrane fraction of N2a-STX was incubated with CMP-[14C]Sia, PSA was synthesized on endogenous proteins, but PSA-synthesizing activity was not observed in the parent Neuro2a cells (data not shown). Therefore, the membrane fraction of N2a-STX was incubated with CMP-[14C]Sia and immunoprecipitated with the anti-mouse NCAM mAb, H.28. The nonprecipitated fraction was then immunoprecipitated with the anti-PSA mAb, 735. Both fractions were ana-
ST8Sia II (STX) Is an NCAM-specific PSA Synthase

Recombinant ST8Sia II Synthesizes PSA on Recombinant NCAM—To confirm that mouse ST8Sia II synthesizes PSA on NCAM, a recombinant soluble NCAM fused with the Fc region of human IgG1 (NCAM-Fc) and CMP-[14C]Sia were used as an acceptor and a donor, respectively. As shown in Fig. 2, NCAM-Fc expressed in COS-7 cells served as an in vitro acceptor substrate for ST8Sia II and IV and was detected as polydisperse materials. On treatment with endo N, the materials became depolymerized, indicating that NCAM-Fc was polysialylated by both recombinant mouse ST8Sia II and IV. NCAM-Fc no longer served as an acceptor for either ST8Sia II or IV on treatment with α2,3-specific sialidase (NANase I) or N-glycanase (Fig. 2B), indicating that PSA was synthesized on α2,3-linked sialic acid residues of N-glycans of NCAM-Fc.

The optimum pH for the polysialylation of NCAM-Fc by ST8Sia II was between 6.0 and 7.0, and no significant difference was observed between ST8Sia II and IV. Since PSA synthase activity in chick brain was known to be activated in the presence of Mn2⁺ (20), we investigated the effects of divalent cations for the activities of ST8Sia II and IV. The soluble recombinant ST8Sia II and IV synthesized PSA without cations, but PSA synthesis by ST8Sia II was increased about 1.5-fold in the presence of Mg2⁺ or Mn2⁺, while the activity of ST8Sia IV was enhanced 2-fold in the presence of Ca2⁺ (Table I). To compare the degree of polysialylation, NCAM-Fc and fetuin were incubated with ST8Sia II, III, and IV and subjected to SDS-PAGE on 5% and 5–20% gels, respectively (Fig. 3). We previously showed that PSA was synthesized on fetuin by ST8Sia II and IV but not by ST8Sia III (11, 13, 21). The 14C-sialylated polydisperse materials were synthesized in addition to 14C-sialylated fetuin by ST8Sia II and IV, while such a polydisperse material was not synthesized from fetuin by ST8Sia III. The polydisperse materials were sensitive to endo N treatment, indicating PSA-fetuin (Fig. 4). On the other hand, 14C-sialylated fetuin was resistant to endo N treatment, indicating that 14C-sialylated fetuin carries oligo and single α2,8-sialic acid residues. The degree of polysialylation of fetuin or NCAM-Fc by ST8Sia IV was much higher than that by ST8Sia II, as indicated by the lower average mobility of the polydisperse materials (Fig. 3). Thus, ST8Sia II and IV could be enzymatically distinguished, although the activities of these two PSA synthases were very similar.

Recombinant ST8Sia II Synthesizes PSA on Other Glycoproteins—As shown above, some of the N-glycans of fetuin, which contain triantennary N-glycans as major components (22), were polysialylated by ST8Sia II and IV. Thus, we attempted...
the polysialylation of other proteins that contain tetra-antennary N-glycans (α1-acid glycoprotein) (23) or biantennary N-glycans (hCG α- and β-subunits) (24). All glycoproteins tested were utilized as substrates for ST8Sia II (Fig. 4). However, the incorporation of sialic acids with ST8Sia II and IV into NCAM-Fc was 1500-fold higher than into these glycoproteins (Table II). The polydisperse materials, which disappeared on endo N treatment, were obtained from α1-acid glycoprotein, fetuin, and the hCG β-subunit, indicating that some of the N-glycans in these proteins were polysialylated. Among them, fetuin served as the best acceptor for polysialylation, indicating that triantennary N-glycans are preferentially, not specifically, polysialylated with ST8Sia II. On the other hand, such polydisperse materials were not detected in the case of hCG β-subunit (Fig. 4).

α1,6-Fucose Linked to Core GlcNAc of N-Glycans Is a Signal Structure for PSA Synthesis on NCAM—As shown in Fig. 4, PSA was synthesized on the β-subunit of hCG but not on its α-subunit. The α- and β-subunits of hCG are the structures of which are very similar. The difference in the structures of N-linked oligosaccharides between the α- and β-subunits of hCG is that the N-glycans of the α-subunit do not contain α1,6-linked fucose attached to core GlcNAc residues (24). This result suggests that core α1,6-linked fucose may be one of the signals for polysialylation in addition to the antennary structures. Therefore, NCAM-Fc was treated with C. lampas α-fucosidase, which can cleave α1,6-linked fucose as well as α1,2- and α1,4-linked fucose residues, and F. oxysporum α-fucosidase, which cleaves α1,2- and α1,4-linked fucose residues. As shown in Fig. 5, the polysialylation of NCAM-Fc by ST8Sia II as well as ST8Sia IV after treatment with C. lampas α-fucosidase was greatly reduced as compared with nontreated or F. oxysporum α-fucosidase-treated NCAM-Fc. When [3H]sialic acid-labeled polysialylated NCAM-Fc was treated with C. lampas α-fucosidase, the polysialylated NCAM-Fc was completely resistant toward the treatment (data not shown), indicating no possibility of contamination of sialidase activities in C. lampas α-fucosidase preparation. Therefore, the reduction of polysialylation on NCAM-Fc was due to the elimination of core α1,6-linked fucose by the enzyme, not being due to the action of sialidases. These results strongly suggest that the core α1,6-linked fucose is one of the signals for polysialylation for NCAM.

A Peptide Chain Is Required for PSA Synthesis but Not for Sialyltransferase Activity—To determine whether PSA is synthesized on free N-glycans of NCAM-Fc or not, NCAM-Fc was digested with N-glycanase, and then the mixture of NCAM-Fc polypeptide and free N-glycans was used as a substrate. After incubation, the reaction mixture was centrifuged and protein G-Sepharose was added to the supernatant. The mixture was then centrifuged, and the resin and supernatant were collected. The polypeptide was recovered on the resin and the free N-glycans in the supernatant. To determine the PSA synthesis on free N-glycans, the supernatant was subjected to chromatography on a Sephadex G-50 column. Polysialylated N-glycan was not found in the fraction containing free N-glycans. In addition,
only a negligible amount of radioactivity was incorporated into NCAM-Fc polypeptide (Table III). This result suggested that PSA was not synthesized on the free N-glycans of NCAM-Fc, and the peptide sequence of NCAM was required for PSA synthesis. To further examine this possibility, native fetuin, the glycopeptides of trypsinized fetuin, and NCAM-Fc served as a 1500-fold better acceptor for mouse ST8Sia II as compared with other glycoproteins such as fetuin and NCAM-180 after the mouse ST8Sia II gene was transfected into Neuro2a cells. Very interestingly, only NCAM-180 and NCAM-140, but not other proteins including NCAM-120, were polysialylated in the PSA-positive Neuro2a cells, although the in vitro reaction caused the polysialylation of other membrane proteins. This result coincided with the observation that PSA was found only in NCAM-140 and NCAM-180 in chick embryonic brain (20, 25). Thus, mouse ST8Sia II specifically synthesizes PSA chains on the specific NCAM isoforms, especially in vivo. Transient transfection of the ST8Sia II/STX gene as well as the ST8Sia IV/PST-1 gene caused PSA expression on NCAM-negative cell lines such as COS-7 (15, 26), but the expression of PSA associated with other proteins may be due to over-expression of the PSA synthase genes.

In addition to ST8Sia II, ST8Sia IV has been shown to synthesize PSA in vitro as well as in vivo (13, 14, 26). We compared some enzymatic properties of the two PSA synthases. The activities of ST8Sia II and IV were very similar, but some differences were observed. The PSA synthase activity of mouse ST8Sia II was 1.5-fold stimulated in the presence of Mn$^{2+}$ or Mg$^{2+}$, in agreement with the PSA synthase activity observed in chick embryonal brain (20). In contrast, the activity of mouse ST8Sia IV was stimulated in the presence of Ca$^{2+}$ rather than Mn$^{2+}$ or Mg$^{2+}$. The most striking difference between the two PSA synthases was the degree of polysialylation of the PSA synthesized. The PSA synthesized by mouse ST8Sia IV on NCAM-Fc as well as fetuin seemed to be much more highly polysialylated than that by mouse ST8Sia II (see Fig. 3). However, it was unclear whether the difference in the degree of polysialylation between the two enzymes was due to the difference in PSA length or the numbers of PSA on a molecule. Since the degree of polysialylation on NCAM has been shown to change during the development of chick embryonic brain (27), the degree of polysialylation may be regulated through the different actions of the two PSA synthases.

Mouse ST8Sia II could synthesize PSA on human α1-acid glycoprotein that contains mainly tetra-antennary N-glycans (23), bovine fetuin which contains mainly triantennary N-glycans (22), and the β-subunit of hCG which contains only biantennary N-glycans (24). However, fetuin served as a much better substrate among them for PSA synthesis by ST8Sia II. Therefore, it is likely that mouse ST8Sia II preferentially adds sialic acids to triantennary N-glycans rather than tetra- or biantennary N-glycans like the case of ST8Sia IV/PST-1, as reported previously (26). Interestingly, PSA was not synthesized on the α-subunit of hCG, whose N-glycans are similar to those of β-subunit, but which does not contain α1,6-linked fucose attached to core GlcNAc (24). In addition, treatment

**DISCUSSION**

The aim of the present study was to reveal the mechanism underlying PSA synthesis by the recently characterized PSA synthase, called STX or ST8Sia II. In this study, NCAM-Fc expressed in COS-7 cells was used as the acceptor, and recombiant soluble mouse ST8Sia II was used as the enzyme source. NCAM-Fc served as a 1500-fold better acceptor for mouse ST8Sia II as compared with other glycoproteins such as fetuin and was fully polysialylated. In addition, NCAM clearly carried PSA after the mouse ST8Sia II gene was transfected into Neuro2a cells. Very interestingly, only NCAM-180 and NCAM-140, but not other proteins including NCAM-120, were polysialylated in the PSA-positive Neuro2a cells, although the in vitro reaction caused the polysialylation of other membrane proteins. This result coincided with the observation that PSA was found only in NCAM-140 and NCAM-180 in chick embryonic brain (20, 25). Thus, mouse ST8Sia II specifically synthesizes PSA chains on the specific NCAM isoforms, especially in vivo. Transient transfection of the ST8Sia II/STX gene as well as the ST8Sia IV/PST-1 gene caused PSA expression on NCAM-negative cell lines such as COS-7 (15, 26), but the expression of PSA associated with other proteins may be due to over-expression of the PSA synthase genes.

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with C. lampas α-fucosidase, which can cleave α1,6-linked fucose, greatly reduced the polysialylation of NCAM-Fc. These results suggested that the core α1,6-linked fucose attached to GlcNAc and/or the triantennary structure of N-glycans are the signal structures for the polysialylation of the N-glycans by ST8Sia II. Recently, it was shown that PSA-carrying N-glycans isolated from chicken and bovine fetal brains were composed mainly of triantennary N-glycans, most of which were α1,6-fucosylated and sulfated (28). Further studies are required to determine the antennary structures, sulfation, and fucosylation of the N-glycans as to the substrate preference of ST8Sia II during PSA synthesis.

In addition to the core α1,6-linked fucose and the antennary structures, we showed in this report that the protein portion was required for polysialylation by ST8Sia II, although ST8Sia II exhibited activity toward free N-glycans and synthesized oligo and single 2,8-sialylated N-glycans. Furthermore, PSA was not synthesized on oligo and single 2,8-sialylated NCAM-Fc or fetuin. These results suggest that ST8Sia II is first arrested on the peptide chains of the substrate glycoproteins, then forms an enzyme-substrate glycoprotein complex, and then transfers the first α2,8-sialic acid residues to the

\[ \text{α2,3-linked sialic acids of N-glycans (initiation)} \]
\[ \text{followed by addition of multiple α2,8-sialic acid residues to the resulting Siaα2,8Siaα2,3Galβ-R structure (polymerization), yielding PSA.} \]

If the enzyme-glycoprotein complex cannot be formed or is unstable, the enzyme is released from the substrate after N-glycans have been partly α2,8-sialylated (abortive initiation), and such a partial α2,8-sialylated N-glycan may not be polysialylated further. A hypothetical model is shown in Fig. 8. This synthetic model is similar to the initiation of the RNA polymerization by RNA polymerase, in which an abortive initiation process is often observed (29). In this model the contact of ST8Sia II with the glycoproteins and the stability of the enzyme-substrate glycoprotein complex may be important. α1,6-Linked fucose attached to the core GlcNAc and/or the triantennary structures of N-glycans may be important for the interaction between protein and enzyme or the stability of the complex. Since the PSA chains synthesized on glycopeptides were immature and much shorter than those on glycoprotein, not only the peptide sequences around the asparagine residues to which N-glycans are attached but also the protein moiety are required for full-length PSA synthesis. In addition, the stability of the complex must also be affected by the protein structure. Recently, it was reported that the conformational structure of NCAM is important for PSA synthesis on NCAM, and the fourth and fifth immunoglobulin domains and the first fibronectin-type III domain would facilitate the recognition of NCAM by the polysialylation enzyme (30). Furthermore, NCAM-120, a glycosyl phosphatidylinositol-anchored form of NCAM, which contains potential polysialylation sites, is not polysialylated in ST8Sia II-transfected Neuro2a cells. Therefore, the specific conformations of NCAM isoforms stabilize much more strongly the complexes between the specific isoforms of NCAM and ST8Sia II than those between other proteins and ST8Sia II. This model may explain why NCAM is specifically polysialylated in vivo, although other proteins are also polysialylated in vitro.

In conclusion, ST8Sia II as a single enzyme specifically transfers all α2,8-linked sialic acids in PSA chains on the α2,3-linked sialic acids of the N-glycans of NCAM. However, ST8Sia II and IV can add a single or oligo-α2,8-sialic acid to a
wide variety of α2,3-linked sialic acid-containing glycoconjugates, i.e. glycoproteins and free oligosaccharides in vitro, indicating that ST8Sia II and IV essentially act as α2,8-sialyl-transferases toward α2,3-linked sialic acids. As shown in this report, some additional characteristics of substrate glycoproteins, i.e. the protein conformation, the presence of α1,6-linked fucose attached to core GlcNAc of N-glycans, and the triantennary structures of N-glycans, are required as the signals for PSA synthesis. It was recently demonstrated that PSA expression in ciliary ganglion neurons of chick embryo was regulated by PSA synthase level (23). However, it has been shown that the alterations in the extracellular polypeptide structure of NCAM caused by alternative splicing affected the polysialylation of NCAM (31–33). Therefore, the structural change of NCAM caused by alternative splicing affected the polysialylation in ciliary ganglion neurons of chick embryo was regulated PSA synthase (ST8Sia II) is first arrested on the polypeptide portion of NCAM, and the stable enzyme-substrate complex is formed. Then, multiple α2,8-linked sialic acid residues are transferred by the enzyme on a Siaα2,3Galβ1,4GlcNAc sequence (C) of N-glycans from CMP-Sia, yielding PSA. In this process, α1,6-linked fucose attached to core GlcNAc of N-glycans (D), the triantennary structures of N-glycans, and the protein structure as well as the Siaα2,3Galβ1,4GlcNAc sequence must be recognized by the enzyme (upper scheme). If N-glycans and/or the protein structure change, the enzyme-glycoprotein complex may be very unstable. Therefore, the enzyme only recognizes the Siaα2,3Galβ1,4GlcNAc sequence and only transfers single or oligo-α2,8-sialic acid residues to the N-glycans (lower scheme).

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