**Differential and Cooperative Polysialylation of the Neural Cell Adhesion Molecule by Two Polysialyltransferases, PST and STX**

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Kiyohiko Angata, Misa Suzuki, and Minoru Fukuda‡

*From the Glycobiology Program, La Jolla Cancer Research Center, The Burnham Institute, La Jolla, California 92037*

PST and STX are polysialyltransferases that form polysialic acid in the neural cell adhesion molecule (NCAM), and these two polysialyltransferases often exist together in the same tissues. To determine the individual and combined roles of PST and STX in polysialic acid synthesis, in the present study we asked if PST and STX differ in the acceptor requirement and if PST and STX act together in polysialylation of NCAM. We first examined whether PST and STX differ in the requirement of sialic acid and core structures of N-glycans attached to NCAM. Polysialic acid was formed well on Lec4 and Lec13 cells, which are defective in N-acetylgalcosaminyltransferase V and GDP-fucose synthesis, respectively, demonstrating that a side chain elongating from GlcNAcβ1-6Manα1-6R and α-1,6-linked fucose are not required. PST and STX were found to add polysialic acid on NCAM-Fc molecules sialylated by α-2,3- or α-2,6-linkage *in vitro*, but not on NCAM-Fc lacking either sialic acid. These results indicate that both PST and STX have relatively broad specificity on N-glycan core structures in NCAM and no remarkable difference exists between PST and STX for the requirement of core structures and sialic acid attached to the N-glycans of NCAM. We then, using various N-glycosylation site mutants of NCAM, discovered that PST strongly prefer the sixth N-glycosylation site, which is the closest to the transmembrane domain, over the fifth site. STX slightly prefer the sixth N-glycosylation site over the fifth N-glycosylation site. The results also demonstrated that polysialic acid synthesized by PST is larger than that synthesized by STX *in vitro*. Moreover, a mixture of PST and STX more efficiently synthesized polysialic acid on NCAM than PST or STX alone. These results suggest that polysialylation of NCAM is influenced by the difference between PST and STX in their preference for N-glycosylation sites on NCAM. The results also suggest that PST and STX form polysialylated NCAM in a synergistic manner.

Polysialic acid is a developmentally regulated carbohydrate composed of a linear homopolymer of α-2,8-linked sialic acid (1). NCAM² is highly polysialylated in embryonic tissues. In contrast, the majority of NCAM in adult tissues lacks this unique glycan, but polysialylated NCAM is present in the olfactory bulb and hippocampus of adult brain, where neural regeneration persists (2, 3). There is increasing evidence that polysialylated NCAM promotes cell migration and enhances neurite outgrowth and branching during development and neural regeneration (2–4). Polysialic acid is thought to modulate the functional properties of NCAM by rendering it less adhesive to itself (homophilic binding) (5, 6) or to other cell surface molecules (heterophilic binding). In the latter case, it has been shown that NCAM engages in interactions with L1 on the same membrane (cis-interaction) (7).

The studies on NCAM knockout mice demonstrated an abnormal formation in the olfactory bulb and hippocampus and a defect in spatial learning and memory (8, 9). By using NCAM knockout mice and endo-N treatment, recent studies have demonstrated that polysialic acid is required for the migration of cells in the subventricular zone of the olfactory bulb (10). Similarly, endo-N treatment of hippocampal cells in organotypic slice cultures was shown to prevent the induction of long term potentiation, presumably by impeding the induction of synaptic plasticity (11). These results, taken together, strongly suggest that polysialylated NCAM plays a critical role during development and neural regeneration.

The cDNAs encoding polysialyltransferases have been cloned, and these enzymes are called PST and STX (12–17). PST (also called STSSia IV) and STX (also called STSSia II) belong to a member of the sialyltransferase gene family which shares the two conserved amino acid sequences, sialyl motif L and S (18). PST and STX are highly homologous to each other and have 59% identity at the amino acid level (13–17).

Consistent with the presumed roles of polysialic acid, it has been shown that polysialic acid synthesized by PST or STX facilitates neurite outgrowth (14, 19). After transfecting HeLa cells with human PST or STX and NCAM cDNAs or NCAM cDNA alone, they were used as the substratum for neurite outgrowth assay. When neurons derived from embryonic chick dorsal root ganglia were grown on these substrata, neurites were much longer and more branched on the substratum cells expressing polysialic acid and NCAM than those on the substratum expressing NCAM alone (14, 19).

By using an *in vitro* assay system, both PST and STX were shown to add polysialic acid to fetuin and soluble chimeric NCAM (20–22). This demonstrates that either PST or STX alone can form polysialic acid by adding the first α-2,8-linked sialic acid to α-2,3-linked sialic acid in an acceptor, followed by the multiple addition of α-2,8-linked sialic acid residues. In this context, PST and STX thus appear to share common enzymatic properties. In contrast to this, it has been suggested that the synthesis of polysialic acid attached to α-2,6-linked sialic acid in mucin-type O-glycans requires an “initiase” α-2,8-sialyltransferase (23). Multiple residues of α-2,8-linked sialic acid are added by an “elongase” α-2,8-sialyltransferase possibly on a...
preformed α-2,8-linked sialic acid. However, this conclusion was mainly based on the structural determination of poly- and oligosaccharide acid present in different stages of trout egg development and the initiative was not assayed (23). Kojima et al. (21), on the other hand, reported that PST and STX require α-1,6-linked fucose attached to N-glycans of NCAM and that PST forms polysialic acid on all forms of NCAM while STX does not form polysialic acid on NCAM-120, suggesting a difference in the acceptor specificity for two polysialyltransfereases (24).

Recent studies indicate that both PST and STX are highly expressed in early stages of mouse or rat development and that this amount is at a maximum before birth (25–28). In mouse, 10 days after birth, STX is dramatically decreased while PST is moderately decreased during development (25, 27). On the other hand, both PST and STX are present in many of the cells of adult brain and prompted us to examine how PST and STX differ in the polysialylation process. However, it is not clear why two similar enzymes differ in polysialylation at different functions, but it is not clear why two similar enzymes are expressed in common regions of the brain. These results prompted us to examine how PST and STX differ in the polysialylation of NCAM, and how two enzymes act together on NCAM.

In the present study, we first examined the requirement in the core structure and sialic acid residue attached to N-glycans for polysialylation by PST and STX. We found that α-1,6-linked fucose is not necessary for the synthesis of polysialic acid, but α-2,3- or α-2,6-linked sialic acid must be present. We then found that PST and STX differ in polysialylation at different N-glycosylation sites of NCAM. Finally, we demonstrated that the actions of PST and STX on NCAM are not mutually exclusive, but instead cooperative.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**Human PST cDNA encoding the full-length PST in pCDNA, pcDNA-PST and pcDNA-A-PST harboring cDNA encoding a soluble PST chimeric with protein A was constructed as described previously (14). The cDNA encoding the fifth Ig domain of human NCAM was excised from NCAM-Fc, HeLa-NCAM-Fc, and pcDNAI-A-Fc, were selected. The permutation of the catalytic domain of STX was digested by BglII digestion. The released fragment is at a maximum before birth (25–28). In mouse, 10 days after the mixture was chased by 24 h. The released polysialic acid was then incubated with HeLa cells, and the sample was incubated with [3H]glucosamine for 24 h as described previously (32).

**Isolation and Characterization of [3H]Glucosamine-labeled N-Glycans from NCAM**—NCAM-Fc bound to protein A-agarose (Pierce) was used to affinity-purify recombinant STX. After digestion with R.110 cm) of Sephadex G-50 (superfine, Amersham Pharmacia Biotech) equilibrated with 0.1 M NH4HCO3. The column was eluted at 6 mlh and fractions (1.4 ml/fraction) were collected. Aliquots of each fraction were taken for the determination of radioactivity and those fractions containing N-glycans were pooled, lyophilized, and desalted by Sephadex G-25 (Amersham Pharmacia Biotech) gel filtration eluted with water. The desalted N-glycans were sequentially digested with NANase II and NANase III (Glyko). NANase II digests α-2,3-linked and α-2,6-linked sialic acid on NCAM-120, while NANase III cleaves α-2,8-linked sialic acid in all forms of NCAM-120, 2,6-linked and 2,3-linked sialic acids. After each digestion, the sample was subjected to Sephadex G-50 gel filtration for fractionation then Sephadex G-25 gel filtration to remove salts, as described above.

**Expression of PST and STX in Wild-type and Mutant CHO Cells—**Wild-type CHO, CHO mutant Lee23 (33), Lee4 (34), and Lec13 (35) cells were used. PST and STX were transiently transfected with pcDNA-PST or pcDNAI-STX using LipofetAMINE (Life Technologies, Inc.) as described (14). Lee4, Lee2, and Lec13 cells were shown to be defective in CMP-sialic acid transporter, N-acetylglucosaminyltransferase V, and GDP-d-mannose-4,6-dehydratase, respectively (33–35). Wild-type CHO and Lee2 cells were obtained from American Type Culture Collection while Lee4 and Lec13 cells were kindly provided by Dr. Pamela Stanley, Albert Einstein College of Medicine, New York, NY. Forty-eight h after the transfection, the cells were dispersed into single cells by the cell dissociation solution (Cell & Molecular Technologies, Lavallette, NJ), and then incubated with anti-polysialic acid antibody (12F8) followed by FITC-conjugated goat affinity-purified (Fab)2 fragment specific to rat IgM. The cells were then analyzed by flow cytometry as described (38).

**Construction of a Soluble STX**—pcDNAI-A, which encodes the signal peptide of human colony-stimulating factor and a Ig binding domain of Staphylococcus aureus protein A, was prepared as follows. pMAoA-GD3 (37) was digested by SalI and BamHI, and cDNA encoding the signal peptide-protein A chimera was cloned into XhoI and BamHI sites of pbLueScript II (Stratagene). This cDNA excised by KpnI and BamHI was cloned into pcDNAS (Invitrogen). By BamHI digestion and partial HindIII digestion of this plasmid, pcDNAI-A was excised and cloned into HindIII and BamHI sites of pcDNAI. This vector, pcDNAI-A, is a universal vector to clone a catalytic domain of glyclosyltransferases using BamHI site and 3′-restriction sites such as EcoRI, EcoRV, NotI, XhoI, and XbaI.

STX cDNA was cloned from the human fetal brain mRNA using Superscript II (Life Technologies, Inc.) and the ‘3′-primer, ASTX-3′, as described previously (19). Using the cDNA as a template, the sequence between nucleotides 96 and 1128 was amplified. Upstream and downstream primers used for this polymerase chain reaction were 5′-GGAAGATCTCGGGAATTCGGGAGGCAG-3′ (underlined) and 5′-TGGGAGGGGAACCAGGTGCAGAT-3′ (underlined). The cDNA encoding the fifth Ig domain of human NCAM was excised from pcDNAPST or pcDNAI-STX as described (19). After selection with G418, HeLa cells expressing both polysialic acid and NCAM-Fc were established using immunostaining with anti-polysialic acid antibody, 12F8 (Ref. 30; Pharmingen), or anti-NCAM antibody, CD56 (Becton Dickinson) on permeabilized cells. Similarly, HeLa cells were transfected with pcDNA-PST or pcDNAI-STX as described (19). After selection with G418, HeLa cells expressing both polysialic acid and NCAM-Fc were established using immunostaining with a saponin and immunostaining protocol was as described previously (31). The selected cell lines were metabolically labeled with [3H]glucosamine for 24 h as described previously (32).
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globulin domain were obtained. All constructs were verified by nucleotide sequencing performed as described (19).

Isolation of NCAM-Fc—Once HeLa-NCAM-Fc cells reached 70% confluence, the medium was replaced with serum-free medium, Opti-MEM I (Life Technologies, Inc.) and cultured for 24 h. NCAM-Fc in the spent medium was transferred to protein A-agarose, and was eluted from the resin by a gentle Ag/Ab elution buffer (Pierce) (36). The eluted material was desalted by repeated concentration and addition of 20 mM Tris-HCl, pH 7.4 containing 0.05% Tween 20 using Microcon 30 (Amicon). Similarly, COS-1 cells were transiently transfected with pIG-NCAM-Fc or pGIG harboring mutated NCAM-Fc using LipofectAMINE as described above. NCAM-Fc was purified in the same procedure as described above. The amount of NCAM-Fc was measured using BCA protein assay (Pierce) and bovine serum albumin as a standard. The concentration of each mutated NCAM-Fc was adjusted after measuring the amount by Western blotting using peroxidase-conjugated goat IgG specific to the Fc protein of human IgG (Cappel) and ECL kit (Amersham Pharmacia Biotech).

Purification of a Soluble Form of PST and STX—pcDNAI-A-PST and pcDNAI-A-STX were separately transfected into HeLa cells with pSVneo using LipofectAMINE as described (19). After selection with G418, clonal cell lines expressing these chimeric proteins were selected following permeabilization of cells and staining with FITC-conjugated human IgG, which reacts with the protein A portion of the chimeric protein. These cell lines were cultured in macrophage-SFM medium (Life Technologies, Inc.) for 24 h and the chimeric enzymes secreted into the culture medium was adsorbed to IgG-Sepharose 6FF (Amersham Pharmacia Biotech) after titrating the pH of the spent medium to 8.0. The resin was collected by centrifugation and washed as described (20, 21) except the following modification. The resin was finally suspended in an equal volume of a serum free medium of macrophage-SFM and used as the enzyme solution.

Sialyltransferase Assays and Product Characterization—The enzyme activity was measured as described previously (20, 21). The substrate solution contained 2 μg of NCAM-Fc dissolved in 50 μl of 0.1 M sodium cacodylate buffer, pH 6.0, containing 5 mM MnCl2 and 2 mM CaCl2, 1% Triton CF-54, 2.4 nmol (0.7 μCi) of CMP-[14C]NeuNAc. To this substrate solution, 50 μl of the enzyme solution prepared above was added and the reaction mixture was incubated at 37 °C for various times. At the end of incubation, the reaction mixture was centrifuged and the supernatant was recovered. To 20 μl of this supernatant, 20 μl of the sample buffer for SDS-polyacrylamide gel electrophoresis (2× concentration) was added, heated, and subjected to electrophoresis followed by fluorography using the same conditions as described (20). The amount of incorporated [14C]NeuNAc into NCAM was measured on each fluorograph by NIH Image version 1.61.

The product, NCAM-Fc, was digested with Pronase (Calbiochem) in 400 μl of 0.1 M Tris-HCl, pH 8.0 containing 1 mM CaCl2, as described (32). The digestion was stopped by phenol extraction and glycopeptides in the aqueous phase, after washing with chloroform, were fractionated by Mono-Q HPLC using a procedure modified from the one reported (40) by Mono-Q HR 5/5 (0.5 × 5.0 cm) column (Amersham Pharmacia Biotech) equilibrated with the solvent A, 50 mM Tris-HCl, pH 7.5, and then eluted with two step linear gradient elution using the solvent B, 1 M NaCl in 50 mM Tris-HCl, pH 7.5, at room temperature. The elution was carried out at a flow rate of 1 ml/min from 0% to 30% of the solvent B in 20 min and then to 55% of the solvent B in the next 75 min using a Gilson 306 HPLC apparatus. The sample was co-injected with partial hydrolysates of colominic acid to determine the degree of polysialic acid. Sialic acid oligomers and polymers were detected at the adsorption at A234, while the NCAM N-glycans were detected by determining the radioactivity of the effluent by scintillation counting.

Polymers and oligomers of polysialic acid were prepared by hydrolysis of colominic acid (Sigma) at the concentration of 10 mg/ml in 0.2 M sodium acetate buffer, pH 4.8, at 37 °C for 48 h as described previously (41). The acid hydrolysis was terminated by the addition of 2 M Tris-HCl, pH 8.0.

Sialylation of NCAM-Fc by α2,3-Sialyltransferase and α2,6-Sialyltransferase—NCAM-Fc was purified from Lec2 cells, which are defective of sialylation in Golgi apparatus (33), as described above. NCAM-Fc was recultured in Lec2 cells at 8 million cell/ml in the sialylation medium (ST3Gal III) (42) or α2,3-sialyltransferase (ST3Gal D) (43) and CMP-[14C]NeuNAc in 100 μl of the same reaction mixture described above for PST and STX. ST6Gal I and ST3Gal III were purchased from Boehringer Mannheim and Calbiochem, respectively. The product was digested with NANase I (Glyko), which specifically cleaves α2,3-linked sialic acid, or NANase II, which cleaves α2,3-linked and α2,6-linked sialic acids (Glyko) to ascertain the enzymatic reactions.

After 24 h of incubation, the sample was desalted using Microcon 30, as described above for isolation of NCAM-Fc. The sample was then divided into aliquots, where 1.46 nmol (0.5 μCi) of CMP-[14C]NeuNAc and non-radioactive 1.6 nmol of CMP-NeuNAc was added and dried in a vacuum evaporator. To this, PST or STX enzyme suspension was added under the same conditions as described above.

RESULTS

Polysialylation of NCAM in Vivo—Polysialyltransferases were shown to add α-2,8-sialic acid in vitro to N-glycans attached to NCAM (21). It has not been shown, however, if polysialyltransferases require an α-2,8-linked sialic acid residue as a substrate, which is attached to α-2,3-linked sialic acid present in N-glycans. To determine this, HeLa cells stably transfected with CDNA encoding PST or STX and CDNA encoding NCAM-Fc were metabolically labeled in the presence of [3H]glucosamine. As shown in Fig. 1A, both PST and STX directed the synthesis of polysialic acid in HeLa cells.

N-Glycans, derived from the parent HeLa cells, were then released from [3H]glucosamine-labeled NCAM-Fc. As shown in Fig. 1B, N-glycans from the NCAM-Fc released sialic acid after NANase II digestion, which cleaves α-2,3- and α-2,6-linked sialic acid (b). The remaining N-glycans (shown by bar in b), however, hardly released sialic acid after NANase III treatment which also cleaves α-2,6-linked sialic acid (c). In contrast, polysialylated N-glycans released negligible amounts of sialic acid after NANase II digestion (e and h) while a large amount of sialic acid was released by NANase III digestion (f and i). These results indicate that α-2,8-linked sialic acid was not detected in NCAM synthesized in HeLa cells in the absence of PST or STX. The results thus strongly suggest that PST and STX directly added α-2,8-linked sialic acid on α-2,3- and possibly α-2,6-linked sialic acid present in N-glycans of NCAM.

Requirement of Polysialylation in Vivo—Previously, the carbohydrate structures of N-glycans were elucidated for polysialylated NCAMs derived from embryonic chick brains (44). These results demonstrate that the core portion of these N-glycans contains a branch elongating from Galβ1→4GlcNAcβ1→6Manα1→R and α-1,6-fucose linked to the innermost N-acetylglucosamine attached to asparagine. It was also reported that α-1,6-linked fucose in NCAM is essential for the synthesis of polysialic acid by PST or STX (21). In order to determine whether or not these structures are crucial for polysialic acid synthesis in NCAM, wild-type CHO, CHO mutant Lec2, Lec4, and Lec13 cells, which endogenously express NCAM and PST-1, hamster counterparts of STX (13) were used. The mutant cell lines, Lec2, Lec4, and Lec13 are known to be defective in CMP-sialic acid transporter, N-acetylglucosaminyltransferase V, and GDP-fucose synthesis, respectively (33–35). The absence of the Galβ1→4GlcNAcβ1→6Manα1→R side chain in Lec4 cells was confirmed by the absence of L-Fucose staining, which was shown to bind to this side chain (45). The absence of fucose in Lec13 cells was confirmed by the absence of Pisum sativum agglutinin binding or anti-H antibody binding after transfecting α-1,2-fucosyltransferase (46). As shown in Fig. 2, polysialic acid was detected on the cell surface of CHO, Lec4, and Lec13, but not on Lec2. The results clearly indicate that PST-1 expressed in these cell lines can direct polysialic acid synthesis on core glycans of NCAM, which lack a side chain elongating from Galβ1→4GlcNAcβ1→6Manα1→R or α-1,6-linked fucose. Transfection of PST or STX enhanced polysialic acid expression on CHO, Lec2, and Lec13, whereas transfection of Lec2 cells showed no effect, as expected (Fig. 2). CHO cells were shown to synthesize α-2,3-linked sialic acid but not α-2,6-linked sialic acid (47, 48). These combined results thus indicate that polysialic acid is formed on α-2,3-linked sialic acid in N-glycans in these cells.

It is not clear why Lec4 and Lec13 cells express more endoglycosidase than do CHO cells (Fig. 2). However, no difference in polysialic acid formation was detected when...
NCAM-Fc isolated from these cells were incubated with the soluble form of PST or STX (data not shown).

Synthesis of Polysialic Acid in Vitro by Polysialyltransferase—The above results indicated that both PST and STX add multiple residues of α-2,8-linked sialic acid in vitro to α-2,3-linked sialic acid in NCAM N-glycans. We then asked if PST and STX can add multiple residues of α-2,8-linked sialic acid in vitro to α-2,3- or α-2,6-linked sialic acid in NCAM N-glycans. First, NCAM-Fc was isolated from HeLa cells and incubated with the enzyme preparation from mock transfected HeLa cells (Fig. 3A, lane 1). As shown in Fig. 1B, this sample contained only α-2,3- or α-2,6-linked sialic acid in N-glycans. Incubation of this NCAM-Fc sample with PST or STX in vitro resulted in the formation of polysialic acid (Fig. 3A, lane 2 (PST) and lane 3 (STX)). To determine if PST and STX can form polysialic acid on α-2,3-linked sialic acid, NCAM-Fc isolated from Lec2 cells was sialylated with ST6Gal I (Fig. 3B, lane 5) and then incubated with PST or STX. α-2,6-sialylated NCAM-Fc also served as acceptors for these polysialyltransferases (Fig. 3B, lanes 7 and 8). The addition of α-2,6-linked sialic acid to NCAM-Fc was confirmed by its resistance to NANase I treatment (Fig. 3B, lane 6), but susceptible to NANase II treatment (data not shown). NCAM-Fc isolated from Lec2 cells did not serve as acceptors unless it was first sialylated with α-2,3- or α-2,6-sialyltransferase (data not shown).

It is noteworthy that α-2,3-linked sialic acid is apparently a better acceptor than α-2,6-linked sialic acid (Fig. 3B, compare lanes 2 and 3 with lanes 7 and 8). Nevertheless, the results clearly indicate that PST and STX can add polysialic acid on a single sialic acid attached to NCAM N-glycans through α-2,3- or α-2,6-linkage.

Addition of Polysialic Acid in Different N-Glycosylation Sites of NCAM—The next question we asked was if PST and STX differ in preferential N-glycosylation sites in NCAM for polysialic acid formation. As shown previously (49), polysialic acid is attached to the last three N-glycosylation sites present in the fifth Ig domain close to the transmembrane domain. This study was, however, carried out using the cell lysates, and those results were probably obtained for a mixture of PST and STX.

We first generated mutant NCAM where these N-glycosylation sites are individually abolished (Fig. 4A). In addition, mu-
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Fig. 2. Flow cytometric analysis of wild-type CHO, mutant CHO Lec2, Lec4, and Lec13 cells before and after transfection with PST or STX. Various CHO cell lines were transiently transfected with pcDNAI-A. Lane 1–4, NCAM-Fc prepared from HeLa cells shown in Fig. 1A but without labeling by [3H]glucosamine and the soluble form of PST (lane 2) or STX (lane 3). Lane 1 represents a control experiment with beads obtained from the cultured medium of HeLa cells transfected with pcDNAI-A. B, NCAM-Fc prepared from Lec2 cells was incubated with ST3Gal III (lanes 1–4) or ST6Gal I (lanes 5–8) and CMP-[14C]NeuNAc. Lanes 2 and 7 represent the products by PST, and lanes 3 and 8 represent the products by STX. Lanes 1 and 5 are the α-2,3- or α-2,6-sialylated NCAM-Fc. Lanes 4 and 6 represent the products after NANAse I treatment of the samples shown in lanes 1 and 5, respectively. The samples were subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography. Experiments A and B were run separately.

Fig. 3. Incorporation of sialic acids to NCAM by a soluble form of PST or STX. A, NCAM-Fc prepared from HeLa cells shown in Fig. 1A but without labeling by [3H]glucosamine and the soluble form of PST (lane 2) or STX (lane 3). Lane 1 represents a control experiment with beads obtained from the cultured medium of HeLa cells transfected with pcDNAI-A. B, NCAM-Fc prepared from Lec2 cells was incubated with ST3Gal III (lanes 1–4) or ST6Gal I (lanes 5–8) and CMP-[14C]NeuNAc. Lanes 2 and 7 represent the products by PST, and lanes 3 and 8 represent the products by STX. Lanes 1 and 5 are the α-2,3- or α-2,6-sialylated NCAM-Fc. Lanes 4 and 6 represent the products after NANAse I treatment of the samples shown in lanes 1 and 5, respectively. The samples were subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography. Experiments A and B were run separately.

The tabulation of incorporated sialic acid revealed additional features in polysialylation (Table I). When the amount of individually incorporated sialic acid by PST at the fifth and sixth sites was added (thus C plus D), it was only 80% of the incorporation on the wild-type NCAM-Fc. In contrast, PST added almost 100% of polysialic acid when both the fifth and sixth N-glycosylation sites were available (G in Table I). These results suggest that there is a cooperative action of PST in two N-glycosylation sites and the addition of polysialic acid in the sixth site may facilitate the addition of polysialic acid to the fifth site or vice versa. Such a synergistic effect was not obvious for polysialylation by STX (Table I).

This difference in polysialylation efficiency toward different N-glycosylation sites was also evident when the degree of polysialylation was measured for shorter incubation times. As shown in Fig. 5, STX added polysialic acid on the fifth site as quickly as on the sixth site. The ratio of polysialic acid formation between the fifth and sixth sites after 15 min incubation was as high as that observed after 60 min of incubation. In contrast, polysialylation of the fifth site by PST after 15 min of incubation was as low as that after 60 min PST incubation compared with polysialylation at the sixth site (Fig. 5). These results confirmed the above conclusion that PST prefers more the sixth over the fifth site than does STX.

PST Forms Larger Polysialic Acid than STX—Figs. 3 and 4 also demonstrated that NCAM-Fc polysialylated by PST in vitro was always larger than that synthesized by STX, regardless of whether the fifth or sixth N-glycosylation site was polysialylated. Since this difference could be due to the amount of polysialic acid or the size of polysialic acid, glycopeptides from

4d, D, F, and G) and at the fifth N-glycosylation site (Fig. 4d, C and E). A band of ~400 kDa most likely represents a dimer of NCAM-Fc, judging from its molecular weight. These results indicate that PST adds polysialic acid much more preferentially to the sixth N-glycosylation site than does STX.
IgG, respectively. domain 1–5, fibronectin type III-like domain and Fc region of human were used to detect NCAM electrophoresis. Peroxidase-conjugated goat anti-human IgG antibodies E–G NCAM a using the soluble form of PST or STX. tirely mutated (Fig. 6, lane 3). These results strongly suggest that PST and STX added polysialic acid in the same NCAM-Fc molecule. Second, the amount of polysialic acid formed was more than the total of individually formed polysialic acid. As shown in lanes 2 and 4 of Fig. 7, PST and STX with a half amount produced approximately 55% and 15% polysialic acid compared with a full amount of PST shown in lane 1. When a mixture of PST and STX was used (lane 3), however, the amount of polysialic acid formed was much more than 70%, which would be expected from a simple addition of two separate reactions (lanes 2 and 4). This experiment was repeated three times, and this synergistic effect was always observed. The results strongly suggest that the co-existence of PST and STX can form polysialic acid on NCAM in a synergistic manner rather than a competitive one.

**DISCUSSION**

Since the cDNAs encoding two polysialyltransferases, PST and STX, were cloned, several studies have been carried out to determine if any difference exists between these two enzymes in their role in polysialic acid synthesis. In particular, the expression profile of PST and STX during development and adult tissues suggest that PST and STX are expressed differentially but overlapping in some specific tissues (19, 25–28). To elucidate the functional difference between PST and STX, in the present study, we first examined the enzymatic activity of these enzymes toward different core glycans between PST and STX, in the present study, we first examined the enzymatic activity of these enzymes toward different core glycans.

NCAM-Fc were released by Pronase and then subjected to Mono-Q anion exchange chromatography. As shown in Fig. 6 (A and B), N-glycans synthesized by PST after 20 h of incubation exhibited similar sialic acid contents at the fifth and sixth sites, ranging from 23 to 60 sialic acids with a peak of 48 or 49 sialic acid residues. In contrast, the total residues of sialic acid synthesized by STX at the fifth N-glycan site were found to be 3–50 with a peak at 10 residues (Fig. 6C). Polysialic acid synthesized at the sixth N-glycosylation site by STX was found to contain 8–50 sialic acid residues with a peak at 19 residues (Fig. 6D).

These results indicate that the size of polysialic acid synthesized by STX is smaller than that synthesized by PST even when the fifth N-glycosylation site is utilized.

On the other hand, the amount of polysialic acid synthesized by STX was comparable to that synthesized by PST, judging from the densitometric analysis of polysialylated NCAM-Fc shown in Figs. 4 and 5. These results combined strongly suggest that STX adds shorter polysialic acid at the fifth N-glycosylation site more efficiently than PST, which adds longer polysialic acid at the same site.
with α-2,6-linked sialic acid can be utilized as acceptors for PST (22). The present study extended this finding by demonstrating that STX also can add polysialic acid on α-2,6-sialylated N-glycans of NCAM, but not on unsialylated NCAM. The structural analysis of N-glycans derived from NCAM showed that only α-2,3-linked sialic acid is present in chicken and rat embryonic brains (1, 44). It is possible that those cells synthesizing NCAM contain only α-2,3-sialyltransferase or that α-2,3-sialylation preferentially takes place in NCAM synthesized by those cells.

The present results obtained on CHO mutant cell lines demonstrated that α-1,6-fucose and a side chain elongating from

|       | WT   | A    | B    | C    | D    | E    | F    | G    |
|-------|------|------|------|------|------|------|------|------|
| PST   | 100  | 3.0 ± 1.3* | 1.7 ± 1.2 | 20.1 ± 5.0 | 60.1 ± 4.9 | 30.9 ± 8.7 | 71.2 ± 7.9 | 101.7 ± 3.7 |
| STX   | 100  | 3.1 ± 2.3 | 3.7 ± 2.8 | 42.4 ± 2.9 | 60.7 ± 4.8 | 48.2 ± 5.9 | 72.0 ± 4.0 | 97.4 ± 5.7 |

* Mean and standard error of six different experiments are shown.

FIG. 5. Time course of sialic acid incorporation into NCAM-Fc mutants B, C, and D. NCAM-Fc mutants B, C, and D shown in Fig. 4a were incubated with the soluble form of PST or STX in the indicated times and analyzed as shown in Fig. 4 (c and d).

FIG. 6. Mono-Q anion exchange chromatography of polysialylated products. NCAM-Fc mutant C and D were incubated with CMP-[14C]NeuNAc and the soluble form of PST or STX for 20 h, and the glycopeptides obtained were separated by Mono-Q anion exchange chromatography. A and C, products from NCAM-Fc mutant C by PST (A) or STX (C). B and D, products from NCAM-Fc mutant D by PST (B) or STX (D). E, the elution profile of sialic acid oligomers and polymers, prepared from colominic acid, under the same conditions in A–D. F, the relationship between the degree of polymerization of sialic acid versus the retention time.
Synergistic Actions of Polysialyltransferases PST and STX

The present study demonstrated that both PST and STX modestly adds polysialic acid at the fifth N-glycosylation site and adds it most preferentially at the sixth site, although PST was also shown to add polysialic acid preferentially to the sixth site. Although STX was mainly utilized on the side chains other than that derived from Galβ1→4GlcANεβ1→6Manα1→R. Moreover, our preliminary studies indicated that inhibition of sulfation by sodium chlorate had an effect on polysialylation of NCAM by PST and STX in vitro and in vivo.\(^2\) The present results, however, do not exclude the possibility that tri- or tetraantennary N-glycans are still better acceptors than biantennary N-glycans for PST and STX. Further studies are significant to address these questions by kinetic studies of the actions of PST and STX on defined acceptors with various core structures.

The present study also demonstrated that PST modestly adds polysialic acid at the fifth N-glycosylation site and adds it most preferentially at the sixth site. Although STX was also shown to add polysialic acid preferentially to the sixth site, it utilizes the fifth site more than does PST (Figs. 4 and 5). These results are generally in agreement with the results obtained previously, showing that the sixth site is most preferentially utilized by polysialyltransferases present in the F11 rat dorsal root ganglion/mouse neuroblastoma cell line (49). One of the striking results obtained in the present study is that the absence of N-glycans in one of three N-glycosylation sites apparently renders the polysialylation by PST at the other sites inefficient. In particular, the total amount of polysialic acid formed at the fifth and sixth sites (102%, in Fig. 4c and Table I) was much more than the addition of polysialic acid formed when only the fifth site (60%) or sixth site (20%) was available. This increased polysialylation upon simultaneous utilization of two sites is less obvious when polysialylation by STX was measured (Table I). These results suggest that cooperative polysialylation by PST apparently occurs when more than one N-glycosylation site is available for polysialylation. However, it should also be considered that N-glycans of each site may affect tertiary conformation of the fifth Ig domain. It has been reported that STX does not add polysialic acid on NCAM-120 (24). It is possible that the projection of NCAM may be important for polysialylation by STX since NCAM120 is linked by a glycosylphosphatidylinositol anchor to the lipid bilayer (39), while the rest of NCAM members contains the transmembrane domain.

Although the crystal structure of the fifth Ig domain of NCAM has not been reported, molecular modeling of immunoglobulin-like domains of NCAM has been reported. According to this study, the fifth and sixth N-glycosylation sites, which contain polysialic acid in the fifth Ig domain, are located on the surface of the molecule (53), which is engaged in NCAM intermolecular interactions. It is thus likely that polysialic acid attenuates homophilic interactions of two opposing NCAMs between the fifth and first Ig domains (54) or between the third Ig domain in two opposing NCAM molecules, which play a major role in NCAM intermolecular interactions (55) (see Fig. 4a for NCAM structure). It is suggested that the former effect is due to the addition of polysialic acid in the fifth Ig domain while the latter is due to a partial cover-up of the third Ig domain by polysialic acid extended from the fifth Ig domain. Further studies will be significant to determine which is the dominant factor.

Consistent with previous reports (19, 21, 24), polysialic acid formed by PST was larger than that formed by STX in vitro. The polysialic acid formed by PST and STX in the sixth N-glycosylation site was found to consist of about 30–60 sialic acid residues and 8–50 residues, respectively (Fig. 6). This size of polysialic acid is consistent with the previous report demonstrating that as many as 55 sialic acid residues were present in polysialic acid isolated from human neuroblastoma cells (40). The present study also demonstrated that both PST and STX together add polysialic acid to NCAM in a cooperative manner (Fig. 7). It is very likely that the addition of polysialic acid at the sixth N-glycosylation site by PST facilitates polysialylation at the fifth site by STX, or vice versa, considering that STX efficiently adds polysialic acid at the fifth N-glycosylation site. Such a cooperative polysialylation may be the reason why both PST and STX are necessary in places such as the hippocampus where extensive polysialylation is required even in adult. The mossy fibers in the hippocampus originate from the dentate gyrus, which expresses both PST and STX and contains much more polysialic acid than CA2 region expressing STX alone (19, 28). It will be of significance to determine if PST forms larger polysialic acid on NCAM than STX in vivo and if PST and STX function in a cooperative manner in neural cells as well.

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\(^2\) K. Angata, M. Suzuki, and M. Fukuda, unpublished results.
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