Biosynthesis and Expression of Polysialic Acid on the Neural Cell Adhesion Molecule Is Predominantly Directed by ST8Sia II/STX during in Vitro Neuronal Differentiation*

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We have recently reported that ST8Sia II/STX as well as ST8Sia IV/PST-1 is a neural cell adhesion molecule (NCAM)-specific polysialic acid (PSA) synthase (Kojima, N., Tachida, Y., Yoshida, Y., and Tsuji, S. (1996) J. Biol. Chem. 271, 19457-19463). To investigate which of two PSA synthase (ST8Sia II and IV) are involved in the biosynthesis of PSA associated with NCAM, the expressions of PSA, PSA synthase activity, and the genes of two PSA synthases during in vitro neuronal differentiation of mouse embryonal carcinoma P19 cells were determined. PSA was not expressed on undifferentiated cells (day 0) or cell aggregates (days 1-3) induced with retinoic acid. Expression of PSA began after cell aggregates had been dissociated and re-plated on a dish (day 4) and increased up to day 7. The expression of the mouse ST8Sia II gene was negligible in both undifferentiated and aggregated cells, it beginning at day 4, then dramatically increasing, and reaching the maximum level at days 6-7. On the other hand, transcription of the ST8Sia IV gene remained at a very low level throughout the entire period, a significant increase in its expression during differentiation not being observed. PSA synthase activity was not detected in undifferentiated or aggregated P19 cells, it increasing in parallel with ST8Sia II gene expression during differentiation. In addition, the cells at day 7 were stained with an anti-mouse ST8Sia II antiserum. Similar up-regulation of the ST8Sia II gene were observed during the differentiation of rat MNS-8 cells, which were derived from E-12 rat neuroepithelium of the neural tube and shown to differentiate into neurons. These results indicate that ST8Sia II predominantly directs PSA expression during neuronal differentiation rather than ST8Sia IV.

Polysialic acid (PSA) is an unusual carbohydrate, which is mainly associated with the neural cell adhesion molecule (NCAM), and modulates the homophilic adhesive properties of NCAM (1). The expression of PSA on NCAM is highly regulated during embryonic development, and the attenuation of cell interactions is important in the pathfinding and target innervation of axons and migration of neuronal cells in the brain (2–5). Although the properties and functions of PSA on NCAM were investigated in detail, knowledge of the mechanism underlying PSA expression remained limited. It was shown recently, using chick embryo brain and diIary ganglion neurons, that the level of PSA is probably regulated by the level of PSA synthetic enzyme(s) (6, 7). We cloned two α,2,8-sialyltransferases named ST8Sia II and IV from mouse, both of which can synthesize PSA on the α2,3-linked sialic acids of N-glycans without any initiator α,2,8-sialyltransferase (8–10). Mouse ST8Sia II exhibits 99.2% identity to rat STX and 56.0% identity to ST8Sia IV. On the other hand, mouse ST8Sia IV and hamster PST-1 exhibit 99.2% identity (11).

PST-1 has been shown to be involved in the biosynthesis of PSA associated with NCAM (11). On the other hand, there was no evidence of whether or not ST8Sia II/STX specifically synthesizes PSA on NCAM in vivo as well as in vitro, because PSA was synthesized on some glycoproteins, such as fetuin in vitro (9), and the transfection of the human STX gene into NCAM-negative cells also caused the expression of PSA on the cell surface (12). However, we recently demonstrated that ST8Sia II directly transferred all α2,8-sialic acid residues on the α2,3-linked sialic acids of N-glycans of specific NCAM isoforms to yield PSA-NCAM and that the polysialylation did not require any initiator α,2,8-sialyltransferase, but did depend on the carbohydrate and protein structures of NCAM (13). These results indicate that two distinct enzymes (ST8Sia II and IV) are potentially involved in the biosynthesis of PSA associated with NCAM in mammalian cells and tissues.

Northern blot analysis of mouse tissues indicated that expression of the ST8Sia II gene was restricted to the brain and was well regulated during brain development, like the expression of PSA (8), while the ST8Sia IV gene was only weakly expressed in the mouse brain (10). Developmentally regulated expression of the ST8Sia II/STX gene has been observed in not only mouse brain but also in human and rat brains (14, 15). Other groups demonstrated that the PST-1 gene is expressed much more abundantly in newborn than adult brain (11, 16). There is no doubt that both ST8Sia II and IV are expressed in the same tissue, i.e. the brain, at the same developmental stage (e.g. postnatal day 1). Therefore, it is important to identify the PSA synthase(s) responsible for the biosynthesis of PSA during neuraminidase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid.
neural differentiation and development to understand the reg-
ulation of PSA expression.

Mouse embryonal carcinoma P19 cells are multipotential
stem cells, which differentiate into a variety of cell types,
including neurons, and therefore are used as model cells for
differentiation. In addition, P19 cells have been shown to ex-
press PSA during differentiation (17). In this study, we ex-
amined the expression of two PSA synthase genes as well as PSA
expression and the activity of PSA synthase during the neural
differentiation of P19 cells induced with retinoic acid. During
neuronal differentiation of P19 cells, only the ST8Sia II/STX
gene was up-regulated in parallel with the expression of PSA
and PSA synthase activity.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—The anti-mouse NCAM monoclonal anti-
body (mAb), H.28 (rat IgG1), was obtained from Immunotech, the anti-
PSA mAb, 735 (mouse IgG), (18) was kindly provided by Dr. R. Gerady-
Schahn, Institut für Medizinische. Mikrobiologie, Hanover, Germany
and the anti-microtubule-associated protein 2 (MAP-2) mAb, HM-2,
was from Sigma. Anti-mouse ST8Sia II and anti-mouse ST8Sia IV
rabbit antisera were prepared using bacterially expressed mouse
ST8Sia II and IV, respectively. Endo-neuraminidase (endo N) purified
from bacteriophage K1F, which only cleaves PSA, was kindly provided
by Dr. F. A. Troy, University of California, Davis, CA (19). Poly-D-lysine,
prepared from bacteriophage K1F, which only cleaves PSA, was kindly provided
rabbit antisera were prepared using bacterially expressed mouse
ST8Sia II and IV, respectively. The amount of amplified cDNAs were calculated from

the respective standard curves.

anti-PSA mAb, 735, followed by treatment with fluorescein-conjugated
anti-mouse IgG. For MAP-2 staining, the fixed cells were further fixed
in 95% ethanol, 5% acetic acid at −20 °C for 2 min, washed with PBS,
and then treated with the anti-MAP-2 antibody.

For immunoblot analysis, the cells were sonicated on ice in an
extraction buffer (20 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 150 mM
NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), incubated for
15 min on ice, and centrifuged at 15,000 × g for 15 min and then the
protein concentration in the supernatant was measured by the BCA
method (Pierce). The lysate (100 μg of protein) was immunoprecipitated
with the anti-NCAM mAb, H. 28, and protein G-Sepharose, followed by
SDS-PAGE on a 5% gel (24). The proteins were then transferred
using Zeta-Probe (Bio-Rad) filter paper. The filter was blocked for 60 min
with PBS containing non-fat dry milk and then incubated with the anti-PSA
mAb, 735, overnight at 4 °C, followed by incubation with horseradish
peroxidase-conjugated anti-mouse IgG. The filter was visualized using
Konica Immunostaining HRP-1000 (Konica).

PSA Synthase Assay—The cells were sonicated for 5 s in 25 mM MES,
(pH 6.0, and centrifuged at 3000 × g for 10 min, and then the superna-
tant was centrifuged at 100,000 × g for 30 min. The precipitate was
suspended in 25 mM MES, pH 6.0, and used as the enzyme after the
protein concentration had been measured. The PSA synthase activity
was measured in a reaction mixture containing 0.1 mM CMP-[3H]Sia
(0.25 μCi), 10 mM MgCl2, 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
to the supernatant. The NCAM-Fc was then divided into two, one-half
being treated with endo N, followed by SDS-PAGE. The radioactivity
incorporated into NCAM-Fc was visualized with a BAS2000 image
analyzer (Fuji Film) and counted. PSA synthase activity was estimated as
the difference in radioactivity between before and after treatment
with endo N (13).

RESULTS

PSA Was Expressed during the Neuronal Differentiation of P19 Cells—Neuronal differentiation was induced by aggregating
P19 cells with 1 μM retinoic acid for 3 days and then the
cells were dissociated and plated in the absence of the inducing
agent. The differentiation of P19 cells at days 0, 3, 4, and 6 is shown in Fig. 1. Neuronal differentiation was indicated by
staining with anti-MAP-2. The expression of MAP-2 was neg-
ligible at day 0 and on aggregation of the cells (day 3). At day
4, P19 aggregates were attached to the surface of the dishes.
At this time, some of the cells expressed MAP-2. At day 6, exten-
sive networks of developing axons were stained strongly with
the anti-MAP-2 mAb and the anti-phosphorylated neurofil-
ament mAb, indicating that the cells had differentiated into
neurons. During the differentiation (up to day 7), the cells did

Fig. 1. Expression of PSA on the cell surface during the dif-
ferentiation of P19 cells. P19 cells were induced to differentiate into
neuronal cells with retinoic acid as described under “Experimental
Procedures.” The cells were fixed with 1% paraformaldehyde and
stained with the anti-PSA mAb, 735, or the anti-MAP-2 mAb, HM-2.
PSA as well as MAP-2 was expressed after cell aggregates had been
dissociated and replated onto dishes (days 4 and 7).
ST8Sia II Directs PSA Expression

Fig. 2. Expression of PSA-NCAM and the ST8Sia II and IV Genes. A, cells at days 0, 3, and 7 were lysed and NCAM was immunoprecipitated with anti-mouse NCAM mAb, H.28, from each lysate (100 μg of protein). The immunoprecipitated materials were then subjected to SDS-PAGE. Western blotting on a Zeta-Probe, immunostaining with anti-PSA mAb, 735, as described under “Experimental Procedures.” D denotes Me2SO-treated cells. B, 1 or 5 μg of poly(A)+ mRNA prepared from cells at days 0, 3, 4, 5, 6, and 7, respectively, was fractionated on a denaturing formaldehyde-agarose gel (1%) and then transferred to a nylon membrane. The full-length mouse ST8Sia II and IV genes were radiolabeled and used as probes. D denotes Me2SO-treated cells. GAPDH, glyceroaldehyde-3-phosphate dehydrogenase. Not shown.

The expression of PSA was first observed at day 4. Almost all the MAP-2-positive neurons expressed PSA on their cell bodies and axons at day 6. On the other hand, PSA as well as MAP-2 was not expressed during the differentiation into muscle cells induced by Me2SO. Therefore, PSA expression was up-regulated during P19 cell differentiation into neurons. To analyze the expression of PSA associated with NCAM, cells at days 0, 3, 4, and 7 were lysed, and the resulting lysates were immunoprecipitated with the anti-NCAM mAb and then subjected to immunoblotting with the anti-PSA mAb. PSA associated with NCAM was slightly expressed on the cells at day 4 and dramatically increased at day 7 (Fig. 2A).

The ST8Sia II Gene but Not the ST8Sia IV Gene Was Up-regulated during Neuronal Differentiation of P19—Two sialyltransferases, both of which exhibit α2,8-sialic acid polymerization activity in vitro and have been shown to be directly involved in PSA synthesis in vivo, have been cloned, namely ST8Sia II/STX and ST8Sia IV/PST-1 (8–11, 13, 15). In order to determine which of the two enzymes is involved in the PSA expression during the neuronal differentiation of P19 cells, mRNAs were prepared from cells at days 0, 3, 4, 5, 6, and 7, and the expression of the ST8Sia II and IV genes was analyzed by Northern blotting and the RT-PCR method. As shown in Fig. 2B, the ST8Sia II and IV genes were both expressed at very low levels at days 0 and 3, at which time the PSA expression was negligible. ST8Sia II transcripts began to appear at day 4, then dramatically increased, and reached a maximum level at days 6–7. Throughout the period of cell differentiation, the transcriptional profile of the ST8Sia II gene was similar to the expression profile of PSA associated with NCAM. The signals for the ST8Sia IV gene were hardly detected if 1 μg of mRNA was used for analysis, but clearly detected when 5 μg of mRNA was applied. The ST8Sia IV transcripts remained at a low level at days 4–7, a significant increase in the gene expression during differentiation not being observed. The lower expression of the ST8Sia II gene than that of the ST8Sia II gene was confirmed by the RT-PCR method (Fig. 3). When primers, which gave almost the same intensities of amplified products for both the ST8Sia II and IV genes, were used, the signals for the ST8Sia IV gene at days 4, 6, and 7 were hardly detected on 26 cycles of PCR, but those for the ST8Sia II gene were clearly observed in an increasing manner. With 30 cycles of PCR, the signals for the ST8Sia IV gene were detected, but no significant increase in the signals between days 0 and 7 was seen.

Expression of PSA Synthase Activity Is Correlated with Expression of the ST8Sia II Gene but Not the ST8Sia IV Gene during P19 Cell Differentiation—The relative gene expression levels, which were normalized as to transcription of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, was subjected to 26 or 30 cycles of PCR. As shown in Fig. 3, the ST8Sia II transcript remained at a low level at days 4–7, a significant increase in the gene expression during differentiation not being observed. The lower expression of the ST8Sia II gene than that of the ST8Sia II gene was confirmed by the RT-PCR method (Fig. 3). When primers, which gave almost the same intensities of amplified products for both the ST8Sia II and IV genes, were used, the signals for the ST8Sia IV gene at days 4, 6, and 7 were hardly detected on 26 cycles of PCR, but those for the ST8Sia II gene were clearly observed in an increasing manner. With 30 cycles of PCR, the signals for the ST8Sia IV gene were detected, but no significant increase in the signals between days 0 and 7 was seen.

Up-regulation of the ST8Sia II Gene during Neuronal Differentiation Was Also Observed in Rat MNS-8 Cells—Similar re-
sultswereobtainedforanotherratcellline,MNS-8,whichwas
established from embryonic rat neuroepithelium by introduc-
ing the mycer fusion gene and was shown to differentiate into
a variety of neural cells including neurons (23). In monolayer
cells, weak expression of rat ST8Sia II was detected on RT-
PCR, but expression of PSA was not detected. In aggregated
cells induced with inducing agents, ST8Sia II gene expression
had disappeared. After the differentiation of MNS-8 cells into
neurons, expression of PSA and up-regulation of the rat ST8Sia
II gene were detected (Fig. 6). During the entire period, expres-
sion of the rat ST8Sia IV gene was not observed, even with the
RT-PCR method. It should be noted that the rat ST8Sia IV
gene was detected in mRNAs prepared from postnatal day 1 rat
brain under the same RT-PCR conditions.

DISCUSSION
The present study showed that the gene expression of one of
the NCAM-specific PSA synthases, ST8Sia II/STX, was well
correlated with expression of PSA and PSA synthase activity of
the cells in timing and amounts during the neuronal differen-
tiation of mouse P19 and rat MNS-8 cells, as in vitro models for
neuronal differentiation. On the other hand, such correlation
between the expression of ST8Sia IV gene and PSA expression

Results were obtained for another rat cell line, MNS-8, which
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between the expression of ST8Sia IV gene and PSA expression
was not observed during the neuronal differentiation of these
cells.

We have shown recently that both recombinant ST8Sia II
and IV can specifically synthesize PSA on the recombinant
NCAM (13), indicating that the two enzymes could potentially
be involved in the biosynthesis and expression of PSA associ-
ated with NCAM in mammalian cells, particularly neuronal
cells. P19 cells are a line of multipotential stem cells derived
from a mouse teratocarcinoma, and many studies have shown
that induced differentiation of embryonal carcinoma cells in
vitro closely resembles events occurring during mammalian
embryogenesis. When P19 cells were induced to differentiate
with retinoic acid, the differentiated cells expressed MAP-2,
indicating they had differentiated into neuronal cells. At the
time of MAP-2 expression (days 4–7), ST8Sia II gene expres-
sion dramatically increased, at which time PSA expression and
PSA synthase in the cells activity increased. In contrast, ex-
pression of the ST8Sia IV gene in differentiated P19 cells
remained at the very low level, which was almost the same
level as that in nondifferentiated P19 cells. In addition, expres-
sion of ST8Sia II was determined by the immunostaining with
anti-ST8Sia II rabbit antiserum (upper panel), or anti-ST8Sia IV rabbit
antiserum (lower panel) (each, 1:500 dilution), followed by with fluores-
cell-conjugated anti-rabbit IgG.

![Fig. 4. Changes in polysialic acid synthase activity and gene
expression. A, membrane fractions were prepared from cells by soni-
cation of cells followed by centrifugation at 100,000 × g, and the
polysialic acid synthase activity in the membrane fractions was ana-
yzed. Enzymatic assays were carried out as described under "Experi-
mental Procedures." SE, three batches of cells in duplicate, B, the
relative expression of the ST8Sia II (closed triangles) and IV (open
triangles) genes, based on expression of the glyceraldehyde-3-phos-
phate dehydrogenase (GAPDH) gene, was calculated from the radioac-
tivity on Northern blot analysis, as shown in Fig. 2B. The data indicate
the averages for two batches of cells. C, cells at day 7 were fixed, treated
with 0.1% Nonidet P-40 in PBS for 15 min, and then stained with the
anti-ST8Sia II rabbit antiserum (upper panel), or anti-ST8Sia IV rabbit
antiserum (lower panel) (each, 1:500 dilution), followed by with fluores-
cell-conjugated anti-rabbit IgG.](http://www.jbc.org/)

![Fig. 5. Effect of cations on PSA synthase activity in differen-
tiated P19 cells. PSA synthase activity of cells at day 6 was deter-
mined in a reaction mixture containing 0.1 mM CMP-[14C]Sia (0.25
μCi), 25 mM MES, pH 6.0, 0.5 μg of NCAM-Fc, and 50 μg of membrane
protein, with or without cations at the concentration of 10 mM. The
recombinant ST8Sia II and IV were prepared as described in previous
papers (8–10), and the enzymatic assay was carried out under the same
conditions as above. A, recombinant ST8Sia II; B, recombinant ST8Sia
IV; C, membrane fractions of differentiated P19 cells.](http://www.jbc.org/)
Fig. 6. Expression of PSA and the PSA synthase genes in rat MNS-8 cells. A, MNS-8 cells were induced to differentiate into neural cells as described under “Experimental Procedures” and then stained with the anti-PSA mAb, 735. Left panel, undifferentiated cells; right panel, differentiated cells. B, mRNAs were prepared from MNS-8 cells at the monolayer (day 0, M), aggregate (A), and differentiation (D) stages and then RT-PCR (30 cycles) was carried out using specific primers for mouse ST8Sia II and IV as described under “Experimental Procedures.” As a control, mRNA of postnatal day 1 rat brain (B) was prepared and RT-PCR was carried out using the same specific primers.

directed by the expression of ST8Sia II rather than ST8Sia IV. On the other hand, when the cells were induced to differentiate into muscle cells with Me2SO (22), the ST8Sia II gene as well as the ST8Sia V gene was not up-regulated, the expression remaining at a very low level.

We showed previously that expression of the ST8Sia II gene was observed at 14 embryonic days (E-14) in mouse brain, reaching a maximum at E-20, then decreasing, and almost completely disappearing by 10 postnatal days, while expression of the ST8Sia IV gene was very low throughout the development of the mouse brain (8, 10). In addition, the mouse ST8Sia IV gene was expressed in lung and heart rather than brain (10). Similar ST8Sia II/STX gene expression in a developmentally regulated manner was observed in rat and human (14, 15). On the other hand, other groups demonstrated that the expression of the ST8Sia IV/PST-1 gene in hamster and human was greater in embryonic than adult brain, like PSA expression, and therefore PST-1 was responsible for the biosynthesis of PSA associated with NCAM in the brain (11, 16). The results that expression of the ST8Sia II gene, but not the ST8Sia IV gene, increased in parallel with the expression of PSA and PSA synthase activity during the in vitro neuronal differentiation of mouse P19 are consistent with our previous findings in mouse brain (8–10).

Since the embryonal carcinoma cell differentiation is not exactly the same as the embryogenesis, we examined rat MNS-8 cells as the other model. MNS-8 cells are derived from the E-12 rat neuroepithelium of the neural tube (23), from which the three types of neural cells in mammalian central nervous systems, i.e. neurons, astrocytes, and oligodendrocytes, originate (25). In the case of MNS-8 cells, only the ST8Sia II gene was expressed in differentiated cells, in which PSA was expressed, while expression of the ST8Sia IV gene was negligible in differentiated and PSA-expressing cells, as indicated by the RT-PCR method. Taken together with the results obtained from P19 and MNS-8 cells, PSA associated with NCAM are predominantly synthesized by ST8Sia II, not by ST8Sia IV, during the initial stages of neuronal differentiation. To further confirm the involvements of ST8Sia II and IV in the PSA synthesis during neuronal differentiation and brain development, experiments on the effects of blocking of their mRNAs are required. Studies along these lines are currently in progress.

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