A developmentally regulated member of the sialyltransferase family (ST8Sia II, STX) is a polysialic acid synthase

Naoya Kojima, Yukiko Yoshida, Shuichi Tsuji*

Molecular Glycobiology, Frontier Research Program, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan

Received 25 July 1995; revised version received 17 August 1995

Abstract **We found polysialic acid synthase activity of ST8Sia II (STX) in vitro and in vivo. Previously, we showed that mouse ST8Sia II exhibits α2,3-sialylated *N*-glycan α2,8-sialyltransferase activity, but the polysialic acid synthase activity of ST8Sia II was not detected at that time [Kojima, N. et al. (1995) FEBS Lett. 360, 1–4]. When fetuin was [^{14}C]sialylated with ST8Sia II and then its N-linked oligosaccharides were analyzed, a part of the N-linked oligosaccharides was eluted in the void volume from a Sephadex G-50 column, and was eluted from the DEAE-Toyopearl column at almost the same salt concentration as that where colomic acid was eluted. In addition, a series of ^{14}C-labeled oligo-sialic acids were obtained from the oligosaccharides on partial mild acid hydrolysis. These results indicated that a part of N-linked oligosaccharides of fetuin were polysialylated with ST8Sia II. Transfection of ST8Sia II gene into several cell lines including NIH3T3 led to the expression of polysialic acids on the cell surface. Thus, ST8Sia II can directly synthesize polysialic acid chains on α2,3-sialylated N-linked oligosaccharides of glycoproteins without any initiator sialyltransferase.**

Key words: Polysialic acid synthase; Polysialyltransferase; Sialyltransferase; Neural cell adhesion molecule

1. Introduction

Oligo- and poly-sialic acids are ubiquitous in the oligosaccharides of glycoconjugates in a wide variety of animals [1]. It has been reported that Siaα2,8Sia-sequences are associated with only two mammalian proteins, the neural cell adhesion molecule (N-CAM) [2–4] and the α-subunit of the voltage-gated sodium channel in rat brain [5]. Recently, we cloned three similar but different types of sialyltransferase gene (ST8Sia II, III and IV) from mouse cDNAs. By transfecting these clones into COS cells, we identified their activities as those of the *N*-glycan α2,8-sialyltransferases [7–9]. Among them, mouse ST8Sia II, which is a homologue of a rat developmentally regulated sialyltransferase (STX) reported by Livingston and Paulson [6], exhibited *N*-glycan α2,8-sialyltransferase activity [7], but the polysialic acid (PSA) synthase activity was not detected at that time. More recently, we detected α2,3-sialylated *N*-glycan α2,8-PSA synthase activity of mouse ST8Sia IV, which is a homologue of hamster polysialyltransferase I reported previously [9,10]. Strikingly, the predicted amino acid sequence of ST8Sia II exhibits high identity to that of ST8Sia IV (56.0%), suggesting that the enzymatic activity of ST8Sia II may be similar to that of ST8Sia IV. Here we report the PSA synthase activity of mouse ST8Sia II (STX) in vitro and in vivo.

Corresponding author. Fax: (81) (48) 462-4692.
E-mail: stsuji@postman.riken.go.jp

Abbreviations: ST8Sia II, STX, reported as a developmentally regulated member of the sialyltransferase family by Livingston and Paulson [6]; N-CAM, neural cell adhesion molecule; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; DP, degree of polymerization. PSA, polysialic acid.

2. Materials and methods

2.1. Materials

Unless otherwise indicated, the materials used in this study were essentially the same as in the previous studies [7,8]. Fetuin was from Sigma. Protein A-Sepharose and Sephadex G-50 superfine were from Pharmacia, and DEAE-Toyopearl 650M from Toso. Di-, tri-, tetra-, and hexa-sialic acids, which are products of partial hydrolysis of colomic acid, were gifts from Nihon Gaishi Co. The polyclonal anti-polysialic acid antibody, H.46, which was established by Allen et al. [11], and the monoclonal anti-polysialic acid antibody, mAb 735, which was established by Frosch et al. [12], were kindly provided by Prof. Inoue, Tokyo Univ., Tokyo, Japan, and Dr. R. Gerady-Schahn, Inst. für Med. Mikrobiologie, Hanover, Germany, respectively. Lipofectamine and NANase II (specific for α2,3- and α2,6-linked sialic acids) were from Gibco BRL and Glyko Inc., respectively.

2.2. Sialyltransferase assays and preparation of oligosaccharides

The vector plasmid designated as pcDSA-O1, consisting of cDNAs encoding an IgM signal peptide sequence, a protein A IgG binding domain and the truncated soluble form of ST8Sia II, was prepared as described previously [7]. COS-7 cells were transiently transfected with 10 μg each of the plasmid, using the DEAE-dextran procedure, and cultured, and then the enzyme was prepared from the culture medium as previously described [7]. Fetuin (1 mg) was sialylated in the presence of 0.1 M sodium cacodylate buffer (pH 6.0), 10 mM $MgCl_2$, 2 mM $CaCl_2$, 100 μM CMP-[^{14}C]NeuAc (2.5 μCi), and 50 μl enzyme preparation, in a total volume of 200 μl [7]. After incubation at 37°C for 24 h, the reaction mixture was centrifuged and the supernatant was collected. To remove excess CMP-[^{14}C]NeuAc, the protein was precipitated with 70% ethanol and dried. [^{14}C]Sialylated oligosaccharides were prepared from [^{14}C]sialylated fetuin by hydrazinolysis (gas phase, 110°C for 1 h, Hydoraclub C-206, Honen) followed by *N*-acetylation, and then subjected to chromatography on a Sephadex G-50 column (1 × 120 cm) equilibrated with 50 mM ammonium acetate (pH 5.2). Fractions of 1 ml were collected and the radioactivity in an aliquot of each (100 μl) was counted with a scintillation counter. The radioactive oligosaccharides were pooled, dried, and then subjected to chromatography on a DEAE-Toyopearl 650M column (1 × 7 cm) with a linear gradient of ammonium acetate (5–200 mM or 5–500 mM). Fractions of 1 ml were collected. The terminal α2,3- and α2,6-linked sialic acids of the oligosaccharides were removed by treatment with α2,3- and α2,6-specific sialidase (NANase II; 0.5 U/ml) for 37°C for 16 h, and then the sialidase-treated oligosaccharides were subjected to chromatography on a Sephadex G-50 column and then a Toyopearl 650M column.

2.3. Transfection of the sialyltransferase gene into cells

The DNA containing the full open reading frame of ST8Sia II was ligated into the cloning site of the mammalian expression vector, pRc/CMV. Murine fibroblast NIH3T3 cells, murine neuroblastoma Neuro2a cells, and murine melanoma B16 BL6 cells were maintained and grown in DMEM supplemented with 10% FCS on 3 cm diameter dishes (Falcon). Plasmid DNA (2 μg) of pRc/CMV or pRc/CMV-ST8Sia II was transfected into the cells by means of lipofectamine

SSDI 0014-5793(95)01024-6

according to the manufacturer's instructions. After culturing for 72 h in DMEM supplemented with 10% FCS, the cells were fixed with 1% paraformaldehyde and stained with the anti-polysialic acid antibodies, H.46 (1:100 dilution) and mAb 735 (5 μg/ml) [11,12] followed by staining with anti-horse IgM and the mixture of anti-mouse IgG and anti-mouse IgM, respectively, and then analyzed by fluorescent microscopy and subjected to flow cytometry using FACSFlow (Becton Dickinson). In another case, protein A-fused, soluble ST8Sia II was transiently expressed in NIH3T3 cells by transfection with plasmid pcDSA-O1 (encoding soluble ST8Sia II), followed by staining with H.46.

3. Results

To clarify the PSA synthase activity of ST8Sia II (STX) in vitro, fetuin was [^{14}C]sialylated with protein A-fused soluble ST8Sia II (product of plasmid pcDSA-O1), the N-linked oligosaccharides were prepared by hydrazinolysis and separated on a Sephadex G-50 column. Fig. 1A shows the gel filtration pattern of the sialylated N-linked oligosaccharides. Most of the radioactivity was retained on the G-50 column, but a part of it was eluted in the void volume of the column (Peak A). The amount of the Peak A material was very small for fetuin sialylated for 6 h, but dramatically increased on 24 h incubation. On the other hand, the Peak B material was mostly synthesized within 6 h, the amount reaching a plateau after 10 h incubation (data not shown). These results suggested that the Peak A material was synthesized from the Peak B one by ST8Sia II itself. Each peak was pooled, dried, and then subjected to ion-exchange chromatography on a DEAE-Toyopearl column. The major oligosaccharides in Peak B were eluted between the positions where tri-sialic acid and tetra-sialic acids were eluted (Fig. 1B). The Peak B material eluted from the DEAE-Toyopearl column was then treated with α2,3- and α2,6-linked specific sialidase (NANase II). During such treatment, terminal α2,3- and α2,6-linked sialic acids on the oligosaccharides are eliminated, but α2,8-sialic acid residues incorporated by the enzyme are resistant. The molecular weight of material in Peak B after removal of α2,3- and α2,6-linked sialic acids was slightly smaller than that before the treatment with NANase II, however, free sialic acids were not released during the treatment, indicating that the incorporated sialic acids were most probably α2,8-linkages and only part of the blanches of *N*-oligosaccharides was α2,8-sialylated (Fig. 1C). The NANase II-treated Peak B material was then subjected to ion-exchange chromatography on the same DEAE-Toyopearl column (Fig. 1B). Peak B after treatment with NANase II was eluted at the positions of di- and tri-sialic acids, suggesting that the oligosaccharides possessed one Siaα2,8Sia residue or Siaα2,8Siaα2,8Sia residue.

ST8Sia II can synthesize the materials that are eluted in the void volume from the Sephadex G-50 column, as shown in

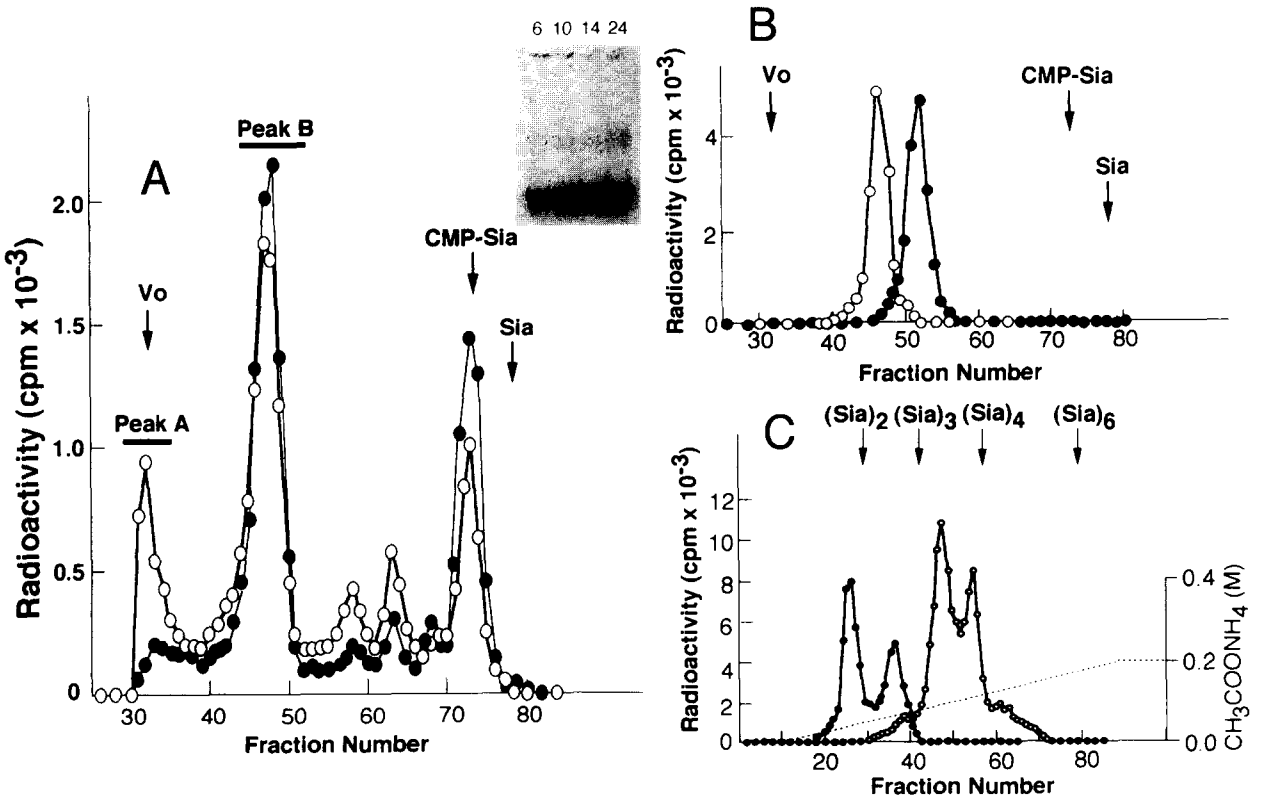

Fig. 1. Chromatography of N-linked oligosaccharides derived from sialylated fetuin and effect of NANase II. (A) *Inset:* SDS-PAGE pattern of fetuin sialylated with ST8Sia II for 6, 10, 14 and 24 h, respectively. The sialylated fetuin (1 mg) incubated for 6 h (●) or 24 h (○) in the *inset* was then subjected to hydrazinolysis, followed by acetylation. The resulting N-linked oligosaccharides were subjected to chromatography on a Sephadex G-50 column (1 × 120 cm) equilibrated with 50 mM ammonium acetate (pH 5.2). One ml fractions were collected and 100 μl aliquots of which were counted. (B and C) The Peak B material was subjected to re-chromatography on the same column (B) or on a DEAE-Toyopearl column, with a linear gradient of 5–200 mM ammonium acetate, before (○) and after (●) treatment with NANase II for 16 h at 37°C. Arrows under $(Sia)_2$, $(Sia)_3$, $(Sia)_4$ and $(Sia)_6$ indicate the elution positions of di-, tri-, tetra and hexa sialic acids, respectively.

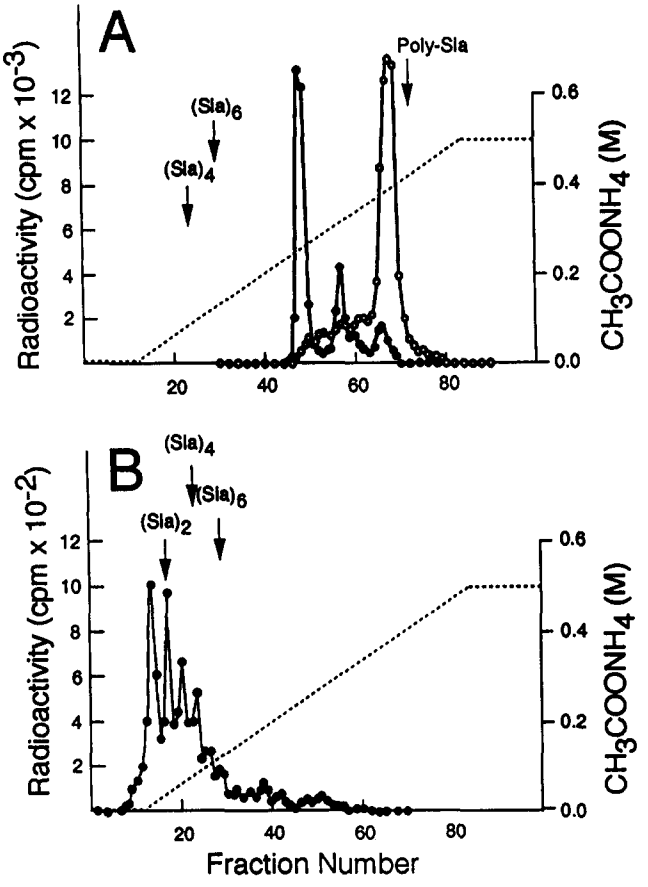

Fig. 2. Presence of polysialic acid chains in N-linked oligosaccharides. (A) The Peak A material in Fig. 1A was subjected to chromatography on a DEAE-Toyopearl column with a linear gradient of 5–500 mM ammonium acetate, before (○) and after (●) treatment of NANase II. (B) The Peak A material after treatment with NANase II was hydrolyzed with 50 mM ammonium acetate buffer (pH 5.4) at 80°C for 3 h. The hydrolysate was diluted with water and then applied to a DEAE-Toyopearl column as described above.

Fig. 1A. When the Peak A material was applied on a DEAE-Toyopearl column (Fig. 2A), it was eluted from the column at the salt concentration of about 400 mM, where is higher than the elution position of hexa-sialic acids and almost the same as that where colomic acid was eluted. After treatment with NANase II, the elution position of Peak A material from the column was at a relatively lower salt concentration than that before sialidase treatment, but still higher than that of hexa-sialic acid and no free sialic acids were observed. To determine whether these materials contain polysialic acids or not, they were partially hydrolyzed with mild acid (pH 5.4, 80°C, for 3 h) and then applied on a DEAE-column [13]. A series of radio-active oligo-sialic acids (DP 1–10) was released from Peak A on hydrolysis of the materials (Fig. 2B). These results strongly indicated that the materials in Peak A derived from sialylated fetuin contain polysialic acid chains, and ST8Sia II exhibits polysialyltransferase activity toward *N*-glycosylated glycoproteins, at least in vitro.

In view of the polysialic acid expression on NIH3T3 cells on transfection of the hamster polysialyltransferase-1 gene [10], we tried to transfect the mouse gene into several cell lines for polysialylation of the proteins in vivo. NIH3T3 cells were transiently transfected with the plasmid, pcDSA-O1 (encoding soluble ST8Sia II). Since the pcDSA-O1 derived enzyme have been used for determination of in vitro PSA synthase activity, the active enzyme should be expressed in the cells by transfection of the plasmid. The transfection of pcDSA-O1 led to the expression of H.46-reactive polysialic acids on cell surface (Fig. 3A). When the full-length ST8Sia II gene was transfected into NIH 3T3, Neuro2a or B16 BL6 cells, which express N-CAM (identified by anti-N-CAM monoclonal antibody, H.28), polysialic acids were strongly expressed on all three cell lines (Fig. 3B and C). These results indicate that ST8Sia II is able to synthesize polysialic acid chains in vivo.

4. Discussion

The following results strongly indicated that mouse ST8Sia II also exhibits PSA synthase activity in vitro and in vivo, in addition to previously reported *N*-glycan α2,8-sialyltransferase activity [7]: (i) a part of [^{14}C]N-linked oligosaccharides derived from sialylated fetuin with ST8Sia II was eluted in the void volume from the Sephadex G-50 column; (ii) the materials eluted in the void volume from the column were eluted from the DEAE-Toyopearl column at almost the same salt concentration as that where colomic acid was eluted; (iii) a series of ^{14}C-labeled oligo-sialic acids was obtained on partial mild acid hydrolysis of the N-linked oligosaccharides; (iv) the transfection of cDNAs encoding ST8Sia II into three different cells led to strong expression of polysialic acids on the cell surface. Thus, ST8Sia II is able to directly synthesize polysialic acid chains on α2,3-sialylated N-linked oligosaccharides of glycoproteins without any initiator sialyltransferase. It should be noted that polysialylation by ST8Sia II as well as IV [9] occurred not only for specific glycoproteins such as N-CAM but also for other glycoproteins such as fetuin, at least in vitro. Therefore, the specific polysialylation of the glycoproteins should depend on the structures of the acceptor N-linked oligosaccharides and the enzyme expression.

PSA synthase was first cloned from a mutant cell line of Chinese hamster ovary cells [10], and its activity was demonstrated using the corresponding soluble enzyme (ST8Sia IV) cloned from mouse [9]. It is clear that ST8Sia II (STX) is a second type of PSA synthase. Although the genes of both ST8Sia II and IV are expressed in the brain, it is not clear why two types of PSA synthases are expressed in the same tissue. Polysialic acid chains have been shown to exist in N-CAM [2–4] and the α_subunit of the voltage-gated sodium channel in rat brain [5]. Therefore, the two different PSA synthases may be responsible for the polysialylation of the two specific glycoproteins, respectively. An alternative possibility is that the two PSA synthases may be involved in the polysialylation of the embryonic and the postponal forms of N-CAM, respectively, because the expression of polysialic acids on N-CAM is developmentally regulated [2–4]. Comparison of the expression and the activities of ST8Sia II and IV, and structural analysis of the polysialylated N-linked oligosaccharides of several glycoproteins including N-CAM using the two cloned PSA synthases is required to answer the above questions. Studies along these lines are currently in progress.

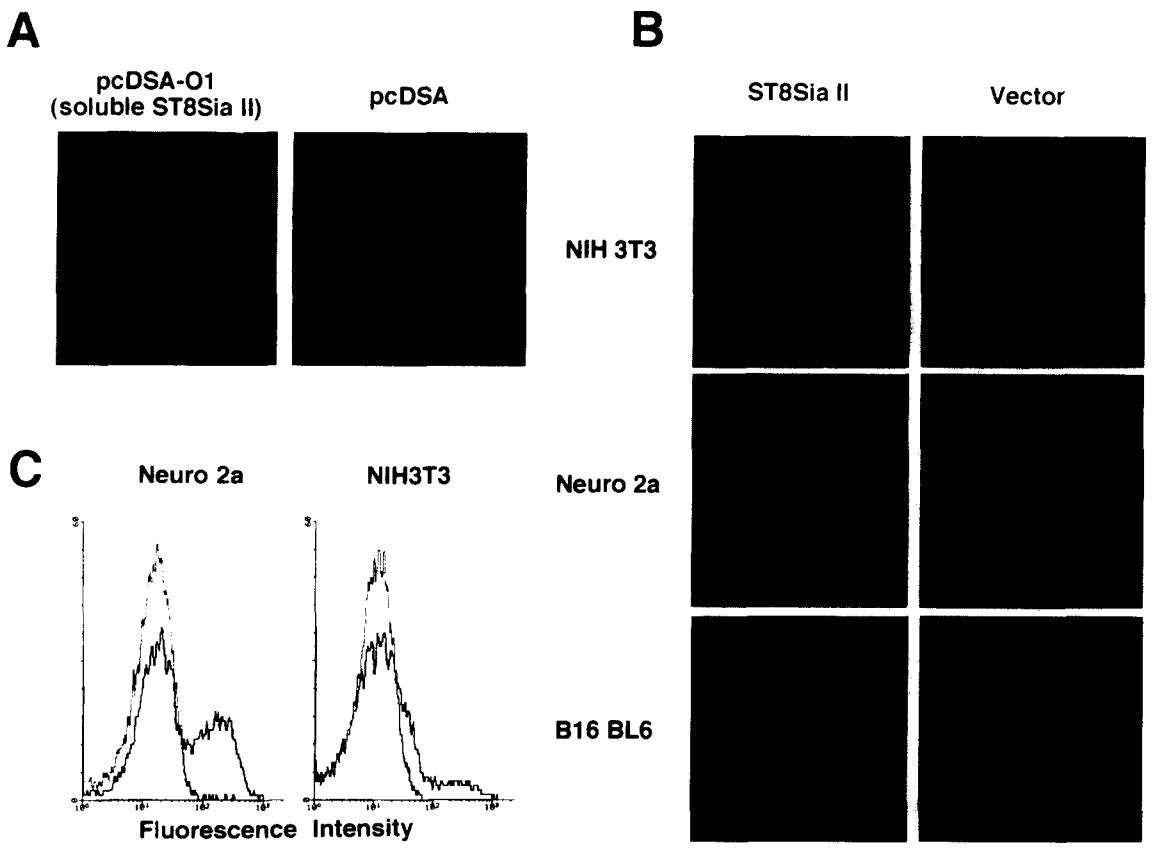

Fig. 3. ST8Sia II induces polysialic acid expression. (A) The plasmid (2 μg), pcDSA-O1, was transiently transfected into NIH3T3 cells by means of lipofectamine. After 72 h incubation, the cells were stained with H.46, as described in section 2. (B) The plasmid DNA (2 μg) of pRc/CMV or pRc/CMV-ST8Sia II was transfected into the mouse cell lines, NIH 3T3, Neuro2a and B16 BL6 and then cells were stained with the anti-polysialic acid polyclonal antibody, H.46. (C) Flow cytometric analysis of Neuro2a and NIH 3T3 cells transfected with pRc/CMV (open peak) or pRc/CMV-ST8Sia II (shaded peak). After 72 h of transfection, the cells were harvested and stained with mAb 735.

Acknowledgements: This work was supported by the following grants: Grants-in-Aid for Scientific Research on Priority Areas, Nos. 07273274, 07279249 and 05274103 (to S.T.), for Scientific Research (c), No. 07680860 (to S.T.), and for Encouragement of Young Scientists, No. 06770042 (to N.K.), from the Ministry of Education of Japan. We are grateful to Dr. Y. Inoue (Tokyo University) and Dr. R. Gerady-Schahn (Inst. für Med. Mikrobiologie) for providing us with the anti-polysialic acid antibodies, H.46 and mAb 735, respectively. We are also grateful to Y. Tachida for her technical assistance.

References

[1] Troy, F.A. (1992) Glycobiology 2, 5–23.
[2] Edelman, G.M. (1985) Annu. Rev. Biochem. 54, 135–169.
[3] Cunningham, B.A., Hemperly, J.J., Murray, B.A., Prediger, E.A., Brackenbury, R. and Edelman, G.M. (1987) Science 236, 799–806.
[4] Rutishauser, U., Acheson, A., Hall, A.K., Mann, D.M. and Sunshine, J. (1988) Science 240, 53–57.
[5] Zuber, C., Lackie, P.M., Catterall, W.A. and Roth, J. (1992) J. Biol. Chem. 267, 9965–9971.
[6] Livingston, B.D. and Paulson, J.C. (1993) J. Biol. Chem. 268, 11504–11507.
[7] Kojima, N., Yoshida, Y., Kurosawa, N., Lee, Y.-C. and Tsuji, S. (1995) FEBS Lett. 360, 1–4.
[8] Yoshida, Y., Kojima, N., Kurosawa. N., Hamamoto, T. and Tsuji, S. (1995) J. Biol. Chem. 270, 14628–14633.
[9] Yoshida, Y., Kojima, N. and Tsuji, S. (1995) J. Biochem. (Tokyo) 118, 658–664.
[10] Eckhardt, M., Muhlenhoff, M., Beethe, A., Koopmann, J., Frosch, M. and Gerady-Schahn, R. (1995) Nature 373, 715–718.
[11] Allen, P.Z., Globe, M., Schneerson R. and Robbins, J.B. (1982) J. Clin. Microbiol. 15, 324–329.
[12] Frosch, M., Görgen, I., Boulnois, G.J., Timmis, K.N. and Bitter-Suermann, D. (1985) Proc. Natl. Acad. Sci. USA 82, 1194–1198.
[13] Kitazume, S., Kitajima, K., Inoue, S. and Inoue, Y. (1992) Anal. Biochem. 202, 25–34.