Enzymatic activity of a developmentally regulated member of the sialyltransferase family (STX): evidence for α2,8-sialyltransferase activity toward N-linked oligosaccharides

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Abstract **We have detected sialyltransferase activity of recombinant mouse STX, which was cloned from rat brain as a new member of the sialyltransferase family, but sialyltransferase activity of which had not been detected previously [Livingston and Paulson, J. Biol. Chem. (1993) 268, 11504–11507]. The activity of mouse STX was specific toward sialylated glycoproteins. *N*-Glycanase treatment and linkage-specific sialidase treatment of glycoproteins revealed that STX transfers sialic acids through α2,8-linkages to only N-linked oligosaccharides of glycoproteins. However, polymerase activity for polysialic acid synthesis was not detected for this sialyltransferase. Since this α2,8-sialyltransferase gene is highly restricted in fetal and newborn brain, it may be involved in the polysialylation of glycoproteins, especially of N-CAM.**

Key words: Sialyltransferase; Glycoprotein; N-linked oligosaccharide; Glycosyltransfease gene; Polysialic acid; Neural cell adhesion molecule

1. Introduction

Sialic acids are key determinants of carbohydrate structures involved in a variety of biological processes, such as cell–cell interaction and cell differentiation [1–3]. The enzymes catalyzing sialic acid transfer to acceptor carbohydrate chains, namely sialyltransferases, have been suggested to number at least 10–12, since sialylated oligosacchride structures vary considerably in both the linkage of the sialic acid and the acceptor oligosaccharides to which sialic acids are attached.

In recent years, several sialyltransferase genes have been cloned [4–11]. Comparison of the cloned sialyltransferases has revealed two highly conserved regions in their active domains, named sialyl motifs L and S [11, 12]. Based on sialyl motif L, Livingston and Paulson cloned the cDNA of a new member of the sialyltransferase family from rat, which was named STX, by a PCR-based approach [12]. The rat STX gene coded for a protein that contains the complete sialyl motifs and is predicted to have a type II membrane topology characteristic of glycosyltransferases, like the other sialyltransferase genes so far cloned. However, they did not detect activity of the enzyme. This gene was reported only to be expressed in newborn brain, but not in other tissues including adult brain, suggesting that the expression of rat STX is highly regulated during brain development.

We cloned the mouse STX gene from 3-day-old mouse brain cDNA, and the activity of the mouse STX was examined using the protein A-fused enzyme. In this paper, we describe the sialyltransferase activity of mouse STX. STX is most probably an α2,8-sialyltransferase which is specific for sialylated N-linked oligosaccharides.

2. Materials and methods

2.1. Materials

Unless otherwise indicated, the materials used in this study were essentially the same as previous papers [8–11]. Lactosylceramide, GM3, GD3, GD1a, GD1b, and GT1b were purchased from Sigma; GQ1b and paragloboside were from IATRON. α2,3- and α2,6-sialylparagloboside were kind gifts from Dr. Iwamori, Tokyo University. Fetuin, asialofetuin, α1-acid glycoprotein, ovomucoid, and bovine submaxillary mucin were from Sigma. Asialo-α1-acid glycoprotein and asialoovomucoid were prepared by mild acid hydrolysis of glycoproteins (0.02 N HCl, 80°C, 1 h).

2.2. PCR cloning of the mouse STX

PCR was performed using primers (5′-primer, TGAAGAATAAGCATTTCCAGACTTGTGCCATCGTGGGCAA; 3′-primer, CAATGGGAAGGGCCAGAAGCCATA) derived from the rat STX sequence [12]. Total RNA from mouse fetal brain was used as a template to synthesize cDNA. Amplification was carried out with 35 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 1 min. The 0.5-kb PCR products were blunt-ended, kinated, and then subcloned into the *Sma*I site of pUC119. Subclones were characterized by sequencing. Random-primed 3-day-old mouse brain cDNA was ligated with *Eco*RI-digested λZAPII (Stratagene). The resultant library was packaged using a Stratagene GigapackII packaging extract and plated on *Escherichia coli* XL-1Blue (Stratagene). Approximately 10^6 plaques were screened with the 0.5-kb PCR fragment, and the cDNA encoding mouse STX was cloned by the standard molecular cloning techniques described by Maniatis et al. [13].

2.3. Construction and purification of STX fused with protein A

A truncated form of STX, lacking the first 33 amino acids of the open reading frame, was prepared by PCR amplification with a 5′-primer containing an in-frame *Xho*I site (5′-AGAAGAATCTCGAGTTCTGGAGGCAGAGGTACAATC-3′) and a 3′-primer containing an *Xho*I site (5′-GAGCCACTCGAGTCCCATGGGCTGTCCTCCTTA-3′). The fusion vector, pcDSA, was constructed by inserting a protein A IgG binding domain, which was prepared by PCR amplification from the pAMoA vector [7], into the *Eco*RI site of the pcDS vector [8]. The amplified DNA fragment was subcloned into the *Xho*I site of pcDSA to yield pcDSA-STX, resulting in fusion of STX to the IgM signal sequence and protein A. COS-7 cells were transfected with pcDSA-STX (10 μg) by the DEAE-dextran method and cultured for 16 h in Dulbecco's modified Eagle medium supplemented with 2% fetal bovine serum. The medium was then replaced with serum-free medium (Mac-

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Abbreviations: N-CAM, neural cell adhesion molecule; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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rophage-SFM, Gibco BRL) and the cells were cultured for another 32 h. After 48 h transfection, the culture medium was collected and the protein A-mouse STX expressed in the medium was adsorbed to IgG-Sepharose (15 μl of resin per 10 ml of culture medium) at 4°C for 16 h. The resin was collected by centrifugation, washed three times with phosphate-buffered saline, suspended in 50 μl (final volume) of Dulbecco's modified Eagle medium without fetal bovine serum, and used as the soluble enzyme.

2.4. Northern blot analysis

5 μg of total RNA was fractionated on a denaturing formaldehyde-agarose gel (1%) and then transferred to a nylon membrane (Nytran, Schleicher & Schuell). A 1157-bp PCR fragment, the full-length STX amplified using synthetic oligonucleotide primers (5′-GCACTCGAG-CCCACCATGCAGCTGCAGTT-3′ and 5′-GAGCCACTCGAGTC-CCATGGGCTGTCCTCCTTA-3′), was radiolabeled and used as a probe.

2.5. Sialyltransferase assays and product characterization

The enzyme activity was measured in the presence of 0.1 M sodium cacodylate buffer (pH 6.0), 10 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5% Triton CF-54, 34 μM CMP-[^{14}C]NeuAc (0.4 μCi), 1 mg/ml acceptor substrate, and 2 μl enzyme preparation in a total volume of 10 μl, as described in the previous papers [10,11]. After 6 h incubation at 37°C, the reaction was terminated by the addition of SDS-PAGE loading buffer (10 μl), and the incubation mixtures were directly subjected to SDS-PAGE (for glycoprotein acceptors). For glycolipid acceptors, the incubation mixtures were applied on a C-18 column, wich was washed with water. The glycolipids were eluted from the column with methanol, dried, and then subjected to chromatography on an HPTLC plate with a solvent system of chloroform/methanol/0.02% $CaCl_2$ (55:45:10), as described previously [10]. Acceptor substrates were visualized by staining with Coomassie brilliant blue (for glycoproteins) or by the orcinol/H_2SO_4 method (for glycolipids). The radioactive materials in glycoproteins or glycolipids were visualized with a BAS2000 radio image analyzer (Fuji Film) and the radioactivity incorporated into acceptor glycoproteins was counted.

For linkage analysis of sialic acids, α1-acid glycoprotein sialylated with the enzyme was precipitated with 70% ethanol, washed three times with 70% ethanol, dissolved in water, and then digested with linkage-specific recombinant sialidase, NANAase I (specific for α2,3-linked sialic acids), NANAase II (specific for α2,3- and α2,6-linked sialic acids), orNANAase III (specific for α2,3-, α2,6-, and α2,8-linked sialic acids) (FACE, Glyko Inc.) at 37°C for 2 h.

For preparation of de-sialylated or de-*N*-glycosylated glycoproteins, glycoproteins (100 μg) were digested with NANAase I, NANAase II, or NANAase III in a total volume of 20 μl for 2 h at 37°C, or with *N*-glycanase (1.5 U; Genzyme) in a total volume of 20 μl at 37°C for 20 h. After inactivation of the enzyme by boiling for 3 min, the resulting de-sialylated or de-*N*-glycosylated glycoproteins were used as acceptors.

3. Results

The cDNA of mouse STX (mSTX) was cloned by a PCR-based approach to obtain a cDNA fragment encoding the conserved sialyl motifs, followed by screening of a cDNA library from mouse brain. Comparison with the rat STX sequence showed that there was 97.2% identity at the nucleotide sequence level in the predicted coding region and 99.2% conservation at the amino acid sequence level. The mRNA size and distribution of the mSTX gene were determined by Northern blot analysis (Fig. 1). Among the various tissues examined, a major mRNA of approximately 5.0 kb was detected in fetal and newborn brain, similar to that seen in rat newborn brain [12] and human fetal brain [14]. Weak expression of the same size transcript was observed in testis and lung.

Livingston and Paulson did not detect the activity of rat STX using several glycolipids, oligosaccharides, and asialoglycoproteins [12]. We also could not detect the activity of soluble

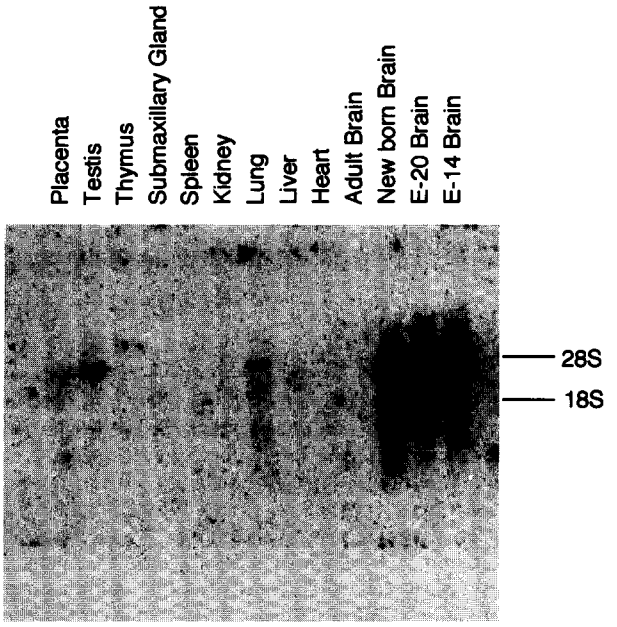

Fig. 1. Northern blot analysis of the mouse STX gene. Total RNAs (5 μg) were prepared from 14 and 20 p.c. mouse embryos (E-14 and E-20), 3-day-postnatal (newborn), and 7-week-old (adult) mouse brains, and adult mouse tissues. The hybridization probe was the 1.2-kb *Xho*I fragment of a cDNA insert of pCD-STX. 28S and 18S indicated the positions of mouse ribosomal RNAs.

protein A-fused mSTX as well as full-length mSTX using the following glycolipid acceptors: lactosylceramide, paragloboside, GM3, GD3, GD1a, GD1b, GT1b, GQ1b, α2,3-sialylparagloboside, and α2,6-sialylparagloboside. When α1-acid glycoprotein was used as an acceptor, we detected activity of soluble mSTX as well as full length mSTX (Fig. 2A). No activity toward α1-acid glycoprotein was observed in the culture medium or cell lysate obtained from cells transfected with the vector alone. Thus, mSTX exhibits sialyltransferase activity. As shown in Fig. 2B, sialylated glycoproteins such as α1-acid glycoprotein, fetuin, and ovomucoid served as acceptors for mSTX. However, human transferrin, one of sialylated glycoproteins, did not serve as an acceptor (data not shown). On the other hand, mSTX did not exhibit activity toward asialo-glycoproteins at all. The broad radioactive bands between mucin and fetuin are derived from the enzyme preparation because they were also observed for the reaction mixture without any substrate glycoproteins (Fig. 2B, lane 8). The linkages of the incorporated sialic acids were analyzed by digestion of [^{14}C]sialylated α1-acid glycoprotein using linkage-specific sialidases. The incorporated [^{14}C]sialic acids were completely resistant to treatment with α2,3-specific or α2,6- and α2,3-specific sialidase, but almost completely disappeared on treatment with α2,3-, α2,6-, and α2,8-specific sialidase, strongly suggesting that the sialic acids incorporated by mSTX were linked to terminal sialic acids through α2,8-linkages (Fig. 3A). The activity of STX toward de-sialylated glycoproteins on treatment with α2,3-specific sialidase was greatly reduced as compared with that toward non-treated glycoproteins, but was not completely abolished, whereas when glycoproteins were treated with α2,3-and α2,6-specific or α2,3-, α2,6-, and α2,8-specific sialidase, the activity was completely abolished (Fig. 3B). Therefore STX may exhibit α2,8-sialyltransferase activity to-

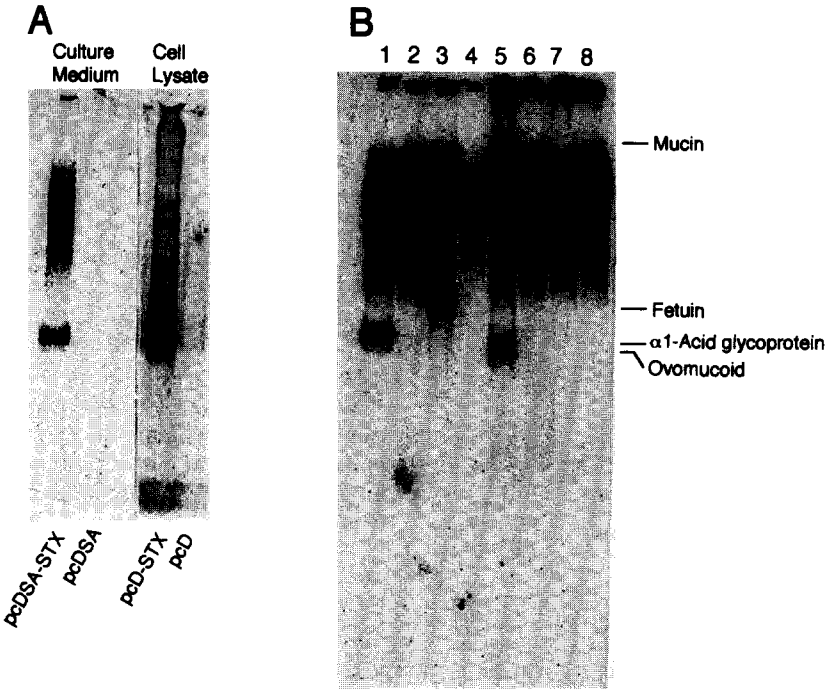

Fig. 2. Incorporation of sialic acids into various glycoproteins by mouse STX. (A) α1-acid glycoprotein was incubated with the culture medium of COS-7 cells transfected with pcDSA-STX or pcDSA (Mock), as described in section 2, and then the reaction mixtures were analyzed by SDS-PAGE. In addition, full-length mouse STX was expressed in COS-7 cells (pcD-STX) and sialyltransferase activity was measured with α1-acid glycoprotein using cell lysate as the enzyme. It should be noted that both culture medium (protein A-fused soluble STX) and cell lysate (full-length STX) of cells transfected with only STX gene showed clear sialic acid incorporation into α1-acid glycoprotein. (B) Various glycoproteins were incubated with the protein A-fused mouse STX enzyme preparation. Lane 1, α1-acid glycoprotein; lane 2, asialo-α1-acid glycoprotein; lane 3, fetuin; lane 4, asialofetuin; lane 5, ovomucoid; lane 6, asialo-ovomucoid; lane 7, bovine submaxillary mucin; lane 8, no acceptor substrate glycoprotein. It should be noted that aisalo-glycoproteins did not serve as acceptors at all.

ward both α2,3- and α2,6-linked sialic acids. As shown in Fig. 2B, bovine mucin, which contains only O-linked oligosaccharides, did not serve as an acceptor for mSTX. To determine whether the sialic acids are incorporated into N-linked oligosaccharides or O-linked oligosaccharides of glycoproteins, [^{14}C]sialylated glycoproteins were digested with *N*-glycanase. The sialic acids incorporated into fetuin, which contains both N-linked and O-linked oligosaccharides as well as those incor-

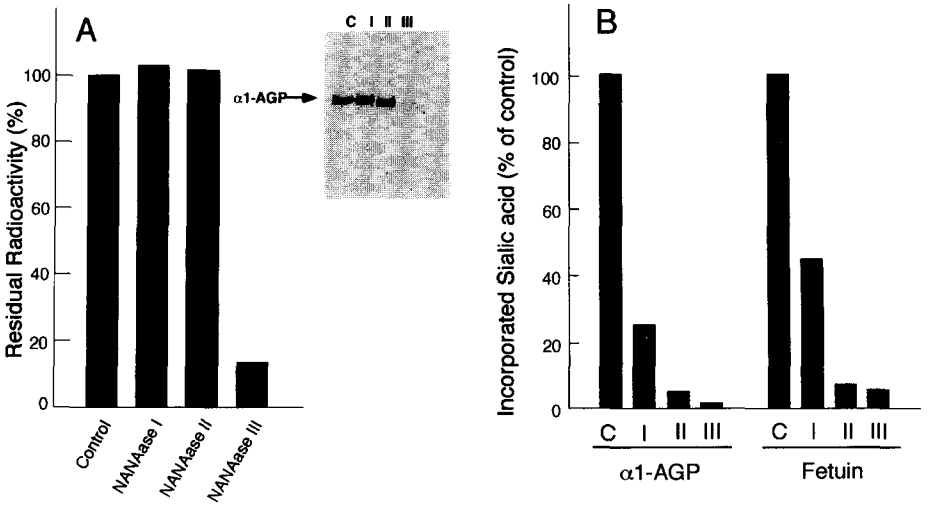

Fig. 3. Effect of linkage-specific sialidase treatment of substrate glycoproteins on incorporated sialic acids and on activity of STX. (A) Linkage analysis of incorporated sialic acids: α1-acid glycoprotein was sialylated with mouse STX and the sialylated glycoprotein was digested with α2,3-specific sialidase (NANAase I), α2,3- and α2,6-specific sialidase (NANAase II), or α2,3-, α2,6-, and α2,8-specific sialidase (NANAase III). The resulting glycoproteins were subjected to SDS-PAGE and radioactivity at the position of α1-acid glycoprotein was counted. The SDS-PAGE pattern is shown in the *inset*. (B) Effect of sialidase treatment of glycporteins for STX activity: fetuin or α1-acid glycoprotein (α1-AGP) was digested with NANAase I, II and III, respectively, and the resulting de-sialylated glycoproteins were incubated with protein A-fused soluble mouse STX, and subjected to SDS-PAGE, and then the radioactivity incorporated into de-sialylated glycoprotein was visualized and quantified with a BAS2000 radio image analyzer. (C) I, II and III indicate treatment with no enzyme, NANAase I, NANAase II, and NANAase III, respectively.

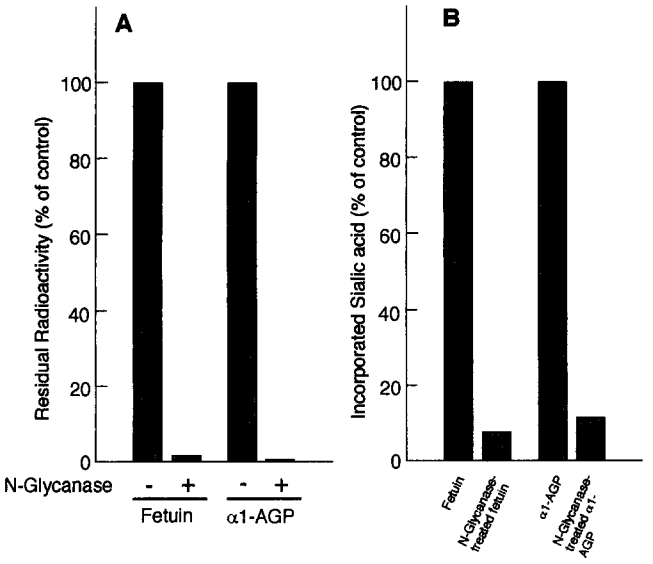

Fig. 4. Effect of *N*-glycanase treatment of substrate glycoproteins for incorporated sialic acids and on activity of STX. (A) Glycoproteins were sialylated with mouse STX and then digested with *N*-glycanase (1.5 U) at 37°C for 36 h. (B) Glycoproteins were first digested with *N*-glycanase. Then the resulting de-*N*-glycosylated glycoproteins were incubated with mouse STX and CMP-[^{14}C]NeuAc, and the incorporated sialic acids were visualized and counted. It should be noted that [^{14}C]sialic acids incorporated into fetuin was sensitive by *N*-glycanase treatment and that de-*N*-glycosylated fetuin did not serve as an acceptor for mouse STX.

porated into α1-acid glycoprotein which contains only N-linked oligosaccharides, were completely released from the proteins (Fig. 4A). In addition, *N*-glycanase-treated fetuin did not serve as an acceptor (Fig. 4B). Therefore, mSTX exhibited activity only toward N-linked oligosaccharides.

4. Discussion

The following results strongly indicate that mouse STX is a novel α2,8-sialyltransferase specific for sialylated N-linked oligosaccharides of glycoproteins: (i) STX only exhibited activity toward sialylated glycoproteins, but not toward asialoglycoproteins or all glycolipids tested including gangliosides; (ii) sialic acids incorporated into glycoproteins are sensitive to *N*-glycanase treatment; (iii) sialic acids incorporated into glycoproteins are resistant to treatment with α2,3-specific and α2,3- and α2,6-specific sialidases, but are completely released on α2,3-, α2,6-, and α2,8-specific sialidase treatment. Since sialylparagloboside (Neuacα2,3/6Galβ1,4GlcNAcβ1,3-Galβ1,-4Glcβ1,1Cer) did not serve as an acceptor for mouse STX, STX should require not only the Neuα2,3/6Galβ1,4GlcNAc sequence but more complex structure containing the Neuα2,3/6Galβ1,4GlcNAc sequence, such as complex-type oligosaccharides of *N*-glycans, for α2,8-sialyltransfer. However, at this time, it is not clear which type of complex N-linked oligosaccharide is a suitable acceptor for STX. Relevant studies are now in progress.

The expression of the mouse STX gene as well as the rat and human STX genes is clearly regulated according to the tissue type and developmental stage [12,14]. The mouse STX gene is expressed only in fetal and newborn brain, but not in adult brain, suggesting that STX expression is closely related to brain development. It is well known that N-CAM acquires a unique polysialic acid substituent on its N-linked oligosaccharides, in the fetal and newborn stages, and polysialic acid chains disappear from N-CAM in adult brain [15,16]. α2,8-linked sialic acid residues in mammalian glycoproteins have only been found in the polysialic acid chains of several glycoproteins, including N-CAM. Based on these facts and the expression pattern of the gene, STX is probably involved in the biosynthesis of the polysialic acid chains of N-CAM. However, STX itself does not exhibit sialic acid-polymerase activity, because colomic acid did not serve as an acceptor for mouse STX. Thus, STX may act as an initiator for the biosynthesis of polysialic acid chains in fetal and newborn N-CAM.

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