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Involvement of the α2,8-Polysialyltransferases II/STX and IV/PST in the Biosynthesis of Polysialic Acid Chains on the *O*-Linked Glycoproteins in Rainbow Trout Ovary

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Polysialoglycoprotein (PSGP) in salmonid fish egg is a unique glycoprotein bearing α2,8-linked polysialic acid (polySia) on its *O*-linked glycans. Biosynthesis of the polySia chains is developmentally regulated and only occurs at later stage of oogenesis. Two α2,8-polysialyltransferases (α2,8-polySTs), PST (ST8Sia IV) and STX (ST8Sia II), responsible for the biosynthesis of polySia on *N*-glycans of glycoproteins, are known in mammals. However, nothing has been known about which α2,8-polySTs are involved in the biosynthesis of polySia on *O*-linked glycans in any glycoproteins. We thus sought to identify cDNA encoding the α2,8-polyST involved in polysialylation of PSGP. A clone for PST orthologue, rtPST, and two clones for the STX orthologue, rtSTX-ov and rtSTX-em, were identified in rainbow trout. The deduced amino acid sequence of rtPST shows a high identity (72–77%) to other vertebrate PSTs, while that of rtSTX-ov shows 92% identity with rtSTX-em and a significant identity (63–76%) to other vertebrate STXs. The rtPST exhibited the *in vivo* α2,8-polyST activity, although its *in vitro* activity was low. However, the rtSTXs showed no *in vivo* and very low *in vitro* activities. Interestingly, co-existence of rtPST and rSTX-ov in the reaction mixture synergistically enhanced the α2,8-polyST activity. During oogenesis, rtPST was constantly expressed, while the expression of rtSTX-ov was not increased until polySia chain is abundantly biosynthesized in the later stage. rtSTX-em was not expressed in ovary. These results suggest that the enhanced expression of rtSTX-ov under the co-expression with rtPST may be important for the biosynthesis of polySia on *O*-linked glycans of PSGP.

Key words: cortical alveolus, *O*-linked glycan, oocyte development, polysialic acid, polysialoglycoprotein, polysialyltransferase, rainbow trout.

Sialic acid (Sia) often occurs at non-reducing ends of glycans of glycoproteins and glycolipids (*1, 2*), and is involved in cell–cell, pathogen–host cell, and ligand–receptor interactions (*3–5*). In some cases, Sia residue is linked to each other to form polysialic acid (polySia) chain in prokaryotic and eukaryotic organisms (*6–8*). The α2,8-linked polySia structures occur in capsular polysaccharides of neuroinvasive bacteria (*9, 10*). In eukaryotes, α2,8-polySia chain is present on salmonid egg polysialoglycoprotein (PSGP) (*11*), neural cell adhesion molecule (NCAM) (*12*), sodium channels (*13, 14*), and CD36 (*15*). Thus, polySia is widely distributed in nature from bacteria to human.

PolySia-NCAM is abundantly expressed in vertebrate embryonic brain. NCAM contains five immunoglobulin (Ig)-like domains and two fibronectin type III–like domains in the extracellular domain with six potential *N*-glycosylation sites. The fifth and sixth *N*-glycosylation sites in the fifth Ig-like domain may be modified by polySia chains (*16, 17*). Two polysialyltransferases, PST (ST8Sia IV) and STX (ST8Sia II), are responsible for the biosynthesis of polySia chain on *N*-linked glycan of NCAM (*18–25*). These enzymes share approximately 60 % similarity to each other. Both PST and STX can catalyze the formation of polySia chains on α2,3- or α2,6-sialylated *N*-linked glycans of NCAM *in vivo* and *in vitro*. The comparison of PST-deficient and STX-deficient mice demonstrates that PST but not STX is involved in long term potentiation in CA1 region of hippocampus (*26, 27*). However the functional differences of these enzymes in the formation of polySia chain remains unclear.

In contrast to NCAM with polySia on *N*-glycans, PSGP contains the polySia on *O*-linked glycans. PSGP is a cortical alveolus glycoprotein of fish oocyte. Cortical alveoli are Golgi-derived secretory vesicles and aligned in the peripheral cytoplasm (*28, 29*). On fertilization, PSGP is discharged into perivitelline space and plays roles in fertilization and subsequent early development, such as osmoregulation, transport of calcium to the perivitelline space, blockage of polyspermy, and stimulation of cell migration and growth (*30, 31*). PSGP consists of tandem repeats of tridecaglycopeptide carrying three *O*-linked glycans with polySia chain which is an α2,8-linked polySia with chain length of up to 25 Sia residues (*28, 32, 33*). No *N*-glycan is

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present in PSGP. With regard to the biosynthesis of polySia chain on *O*-linked glycans, nothing has been known about the responsible enzymes in any of polySia-containing *O*-linked glycoproteins. Thus, it is an interesting issue as to which sialyltransferases are responsible for the polySia chain on *O*-glycans. In the present study, we have searched for cDNA clones for rainbow trout orthologues of PST and STX as α2,8-polysialyltransferase (α2,8-polyST) candidates, and identified them as α2,8-polyST that are possibly involved in the biosynthesis of polySia chain on *O*-linked glycoproteins.

MATERIALS AND METHODS

Rainbow Trout—Males and females of adult rainbow trout (*Oncorhynchus mykiss*) were kindly provided by the Shiga Prefectural Samegai Trout Farm. Trout ovaries were excised from females in each month during oogenesis. Various tissues were excised from males and females in May (5 months prior to ovulation) and December (2 months after ovulation). Embryos of rainbow trout were kindly provided by Aichi Fisheries Research Institute. The excised tissues and the embryos were frozen immediately using liquid nitrogen and kept at –80°C until use.

Materials—Lake trout (*Salvelinus namaycush*) which contains α2,8-linked oligo/poly (Neu5Ac), was prepared as reported previously (*34*). Rainbow trout (*O. mykiss*) PSGP and asialo-PSGP was prepared as described previously (*28*). Carbohydrate structures of PSGP are shown in Fig. 11. Oligonucleotide primers were obtained from Rikaken (Nagoya, Japan) and Sigma Genosys Japan (Hokkaido, Japan). DNA polymerases and restriction enzymes were purchased from TaKaRa (Kyoto, Japan). A pPROTA vector was kindly provided by Dr. John Lau (Roswell Park Cancer Inst., USA). COS-1 cells and the expression vectors, pcDNA hPST-V5, pcDNA hSTX-V5, protA hPST-V5 and protA hSTX-V5, were prepared as described previously (*35, 36*). A plasmid, pIG NCAM containing cDNA encoding the soluble human neural cell adhesion molecule fused with Fc region of human IgG1 (NCAM-Fc) was kindly gifted from Dr. Paul Crocker (University of Dundee). Mouse monoclonal antibody 12E3, which recognizes (Neu5Ac)n ($n \geqslant 5$), was kindly gifted from Dr. Tatsunori Seki (Juntendo University School of Medicine). Mouse monoclonal antibody anti-myc was kindly gifted from Dr. Rita Gerardy-Schahn (Medizinische Hochschule Hannover). Mouse monoclonal antibody anti-V5 was purchased from Invitrogen (Carlsbad, CA, USA). Fluorescein isothiocyanate–conjugated goat anti–mouse IgG+IgM was purchased from Seikagaku Co. (Tokyo, Japan). Endo-*N*-acylneuraminidase (Endo-N) was prepared from bacteriophage K1F as described previously (*37*).

Polymerase Chain Reaction (PCR) Amplification and Screening of cDNA Clone Encoding Rainbow Trout α2,8-PolyST—Total RNA was isolated from rainbow trout ovary by TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). The first strand cDNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) with random primers. Based on the nucleotide sequences for sialyl motif L and sialyl motif S of human PST (accession number L41680), mouse PST (accession number X86000), hamster PST (accession number Z46801), human STX (accession number U33551 and U82762) and frog STX (accession number AB007468), degenerated primers were designed. PCR was performed with a sense primer, 5′-ACBTGTGCHRTYGTKGGMAAYTC-3′, and an antisense primer, 5′-AADCKDGTSGCMAGDGTRTACAT-3′. The symbols for polynucleotides follow Nomenclature Committee of the International Union of Biochemistry (NC-IUB) recommendation. The cDNA fragment was amplified through 30 cycles of a step program (94°C, 1 min; 48°C, 1 min; 72°C, 2 min). The amplified products were subcloned and sequenced by deoxynucleotide chain termination method. A homology search using BLAST search program was carried out.

Poly(A)$^+$ RNA was purified with oligo(dT)-cellulose (Invitrogen). In order to remove contaminants, poly(A)$^+$ RNA was purified with oligotex-dT30<super> (TaKaRa). Double strand cDNA was synthesized, and then ligated into Lambda ZAP II Predigested *Eco*RI/CIAP-Treated Vector (Stratagene). Approximately 500,000 plaques were screened with amplified cDNA probe labeled with 32P. Six positive clones were obtained and sequenced as described above. Those clones had the same sequence, and designated pRT-PST1-1.

5′-Rapid amplification of cDNA ends (5′RACE) for rainbow trout PST (rtPST) was carried out as described previously (*38*). The cDNA containing open reading frame of rtPST was amplified with a sense primer, 5′-GAAGTCATGGTAATGCGCGC-3′ (accession number AB094402, nucleotides 1–20), and an antisense primer, 5′-AGTGTATGTCACGAACTGGC-3′ (accession number AB094402, nucleotides 1214–1233), utilizing a first strand cDNA of rainbow trout spleen and ovary as a template. The amplified cDNA was subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced.

Polymerase Chain Reaction (PCR) for Cloning of cDNA Encoding Rainbow Trout ST8Sia II (rtSTX)—The cDNA fragment homologous to ST8Sia II (STX) was amplified with rtPST-specific primer, 5′-TGTCAGCCAGTACCCTCTCA-3′ (accession number AB094402, nucleotides 906–925) in amplification of 5′-end of rtPST cDNA. Amplification of 5′- and 3′-end of rainbow trout STX (rtSTX) cDNA was carried out as described previously (*38*).

Tissue Distribution of rtPST and rtSTX in Rainbow Trout Tissues—Twenty micrograms of total RNA from tissues excised in December for rtPST and 2 μg of poly(A)$^+$ RNA from tissues excised in May for rtSTX were separated on a 1.0% agarose gel containing 5% formaldehyde, and then transferred to Hybond N$^+$ (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Digoxigenin (DIG)-labeled full-length rtPST cRNA, rtSTX cRNA (accession number AB262976, nucleotides 634–1254), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cRNA (accession number AF027130, nucleotides 25–1062) were used as probes. Hybridization and detection of DIG-labeled probes were carried out as described previously (*39*).

Developmental Changes of mRNA for rtPST and rtSTX during Oogenesis—Total RNAs were prepared from the rainbow trout ovaries of three individuals in each month (April to September) with TRIZOL Reagent. The cDNAs were synthesized, and then used as templates for the following semi-quantitative PCR as described previously (*39*). PCR reactions, 25 cycles for GAPDH, 30 cycles for rtPST and 32 cycles for rtSTX-ov involving a three-step

incubation (94°C, 30 s; 54°C, 1 min; 72°C, 2 min) were carried out. The cycles were set in such a way that the amount of amplified DNA did not reach a plateau. The oligonucleotide primers used were following; rtPST (accession number AB094402, nucleotides 1–585) 5′-GAAGTCATGGTAATGCGCGC-3′ and 5′-CCACATCCGCTGTTGAGAAG-3′; rtSTX-ov (accession number AB262976, nucleotides 582–822) 5′-AGGAGTATGCTGGGGATGTG-3′ and 5′-AAGGCGGTGTGCACATCAAC-3′; and GAPDH (accession number AF027130, nucleotides 25–1062) 5′-CAACCCAATCAACAGCAACT-3′ and 5′-AGCCGGACAGTTACTCCTTA-3′. For Northern blot analysis, 2 μg of poly(A)$^+$ RNA from ovaries were probed with the rtSTX cRNA.

Preparation of Recombinant NCAM Proteins (NCAM-Fc)—COS-1 cells on 90-mm plates (5×10^6 cells) were transiently transfected with 20 μg of pIG NCAM using the Lipofectin Reagent (Invitrogen) and the secreted NCAM-Fc was purified with protein A–Sepharose (GE Healthcare Bio-Sciences corp.), as described previously (*35, 36*).

Expression of Recombinant rtPST and rtSTX and Assay for Enzymatic Activity—For enzymatic characterization, the fusion proteins containing the signal peptide sequence of transin, a collagenase family gene, the IgG binding domain of protein A and the truncated form of sialyltransferases were constructed in mammalian expression vector pPROTA (*40*). The full-length cDNAs of rtPST, rtSTX-em and rtSTX-ov were subcloned into pcDNA4/myc-His B vector (Invitrogen). The resulting plasmids were used as templates of the following amplification. The 3′-primer specific for BGH reverse priming site of pcDNA4/myc-His, 5′-GGCTGAATTCTAGAAGGCACAGTCGAGG-3′ was used for all amplifications. The following 5′-primers for cloned sialyltransferases were used: rtPST (accession number AB094402, nucleotides 181–203) 5′-ATCGAATTCCAAGACGAAAGAAATAACTAGAA-3′; rtSTX-em (accession number AB262977, nucleotides 256–277) 5′-GAATTCAATTGCGAATCTTGGAGGTTCC-3′; and rtSTX-ov (accession number AB262976, nucleotides 97–118) 5′-GAATTCAATTGGGAATATTGGAGGTTCC-3′. The underlines show synthetic *Eco*RI sites. The PCR experiments were carried out as described above and the amplified DNAs were ligated into *Eco*RI site of pPROTA. The resulting constructs for rtPST, rtSTX-em and rtSTX-ov protein designated pPROTA rtPST-myc, pPROTA rtSTX-em-myc and pPROTA rtSTX-ov-myc, respectively. COS-1 cells were transiently transfected with each plasmid as described above. Twelve hours after transfection, the culture medium was exchanged to serum-free medium Macrophage SFM (Invitrogen). The culture medium containing the secreted fusion protein was collected 48 h after transfection, and then the fusion protein was adsorbed to IgG-Sepharose (GE Healthcare Bio-Sciences corp., 20 μl of 50% slurry resin/10 ml of culture medium). The fusion protein–adsorbed resin was used as an enzyme source for α2,8-polyST activity assay. Enzyme activity was measured at 25°C or 37°C for 24 h in 10 μl of reaction mixture containing 4 μl of the recombinant enzyme, 50 mM MES buffer (pH 6.0), 10 mM $MnCl_2$, 10 mM $CaCl_2$, 0.5% Triton CF-54, 100 μM CMP-[^{14}C]Neu5Ac (10.7 kBq), and acceptor substrate. As an acceptor substrate, 200 μg/ml of NCAM-Fc and 51 μg/ml as Gal of lake trout PSGP were used. For enzyme assay using a mixture of recombinant enzymes, the mixture containing either of the culture medium (3 ml each) derived from the cells transfected with pPROTA rtST6GalNAc (*39*), pPROTA rtPST, or pPROTA rtSTX-ov was adjusted to 12 ml with the culture medium from mock transfectant. The mixed recombinant enzymes were adsorbed to IgG-Sepharose (20 μl of 50% slurry resin/12 ml of culture medium). Enzyme activity was measured at 25°C for 24 h using 1.0 mg/ml of asialo-PSGP as an acceptor substrate. An aliquot of reaction mixture was spotted on Whatman 3MM paper, and developed with ethanol/1 M ammonium acetate (pH 7.5) (7:3, v/v) for 30 min. After air-drying, the amount of incorporated [^{14}C]Neu5Ac remaining at the origin was determined by a BAS 2000 imaging analyzer (Fuji Film, Tokyo, Japan). Another aliquot containing fusion protein-adsorbed resin was washed with PBS and was subjected to SDS-PAGE followed by treatment with or without 9 milliunits of Endo-N at 3°C for 20 h in reaction mixture containing 50 mM Tris-HCl (pH 7.5). The amount of fusion protein adsorbed to IgG-Sepharose was analyzed by Western blotting using the antibody against epitope-tag.

Immunofluorescence Microscopy of Culture Cells Expressing rtPST and rtSTX Proteins—The cDNAs encoding open reading frame of rtPST, rtSTX-em and rtSTX-ov were amplified by PCR using the following oligonucleotide primers: 5′-CATTGAATTCGCGAGATGCGTCTCTCAC-3′ and 5′-ATCTCGAGGATTCGCACTTCGAAGTC-3′ for rtPST; 5′-ACGAATTCAGTCATGCAGTTAGAATTACGA-3′ and 5′-GCTCGAGCCTGTTCCTACATCACAAGAGCC-3′ for rtSTX-em and rtSTX-ov. The underlines indicated synthetic *Eco*RI sites or synthetic *Xho*I sites. The amplified cDNAs were subcloned into pcDNA3.1/V5-His B (Invitrogen). The resulting plasmid designated pcDNA rtPST-V5, pcDNA rtSTX-em-V5 and pcDNA rtSTX-ov-V5, respectively. These constructs produce the proteins with V5 epitope tag at carboxyl terminal portion. The cells were seeded on glass coverslips in 6 well plastic plate at 7×10^5 for murine neuroblastoma Neuro2A cell, 5×10^5 for COS-1 cell and 3×10^5 for rainbow trout gonadal tissue-derived RTG-2 cell. The cells were cultured for 24 h in a 5% CO_2 incubator at 37°C for Neuro2A cells and COS-1 cells and at 20°C for RTG-2 cell. For Neuro2A cells and COS-1 cells, 1 μg of expression vector was transiently transfected into the cells using Lipofectamine Reagent (Invitrogen) according to manufacture's instruction. For RTG-2 cell, 2 μg of expression vector was transiently transfected into the cells using FuGENE 6 Transfection Reagent (Roch diagnostics) according to manufacture's instruction. Immunofluorescence was carried out as described previously (*41*).

RESULTS

Isolation of cDNAs for Rainbow Trout α2,8-PolySTs—(i) *Rainbow trout orthologue to mammalian PST:* The cDNA fragment (nucleotides 536–987 in accession number AB094402), which was homologous to mammalian PST, was amplified using rainbow trout ovary cDNA as a template. To obtain the full-length cDNA sequence, a cDNA library of rainbow trout ovary was screened using this fragment as a probe. Six positive clones analyzed had the identical sequence, which was designated pRT PST1-1. The open reading frame of pRT PST1-1 contained

sialyl motifs, but it did not contain the putative transmembrane domain. The cDNA fragment including the putative transmembrane domain was isolated from rainbow trout spleen by 5′RACE. Based on the sequences of this fragment and pRT PST1-1, a cDNA encoding a whole protein with type II membrane topology was amplified using rainbow trout spleen and ovary cDNA as templates. The cloned cDNA thus obtained was designated rtPST (accession number AB094402). The deduced amino acid sequence of rtPST (Fig. 1) shows high homology to human PST (77%, accession number L41680), mouse PST (77%, accession number X86000), chicken PST (76%, accession number AF008194) and zebra fish PST (72%, accession number AJ715545). However, the 5′-upstream sequence from nucleotide 234 of rtPST is different from that of pRT PST1-1. The alignment with mouse PST indicated that the position at nucleotide 234 of rtPST is corresponding to the splice site between exons 1 and 2 of mouse PST. The AG at nucleotides 233–234 of pRT PST1-1 matches with the consensus sequence of 3′-splice site in intron sequences. Therefore, pRT PST1-1 is likely to be a partially spliced premature mRNA or an alternative splicing variant of rtPST. The 5′-partial fragment of pRT PST1-1 could be amplified using rainbow trout ovary cDNA derived from DNase I–treated poly(A)$^+$ RNA (data not shown). Thus, the mRNA for pRT PST1-1 encoding the protein lacking the putative transmembrane domain is actually expressed in ovary.

(ii) *Rainbow trout orthologues to mammalian STX:* Besides the 5′-end of rtPST cDNA fragment, another cDNA fragment similar to mammalian STX was also obtained. The 3′RACE and 5′RACE were performed to obtain full-length cDNA sequence using rainbow trout ovary and embryo cDNA as templates. Two different cDNA sequences, rtSTX-ov (accession number AB262976) and rtSTX-em (accession number AB262977), were obtained from ovary and whole embryo, respectively (Fig. 2). Identity of deduced amino acid sequences between rtSTX-ov and rtSTX-em is 92%, and differences between them largely exist in the stem region. The rtSTX-ov shows high amino acid sequence identity to zebra fish STX (76%, accession number AY055462), fugu STX (74%, accession number AJ715538), human STX (63%, accession number U33551), mouse STX (63%, accession number X83562), rat STX (63%, accession number L13445) and frog STX (63%, accession number AB007468). Like typical Golgi-localized glycosyltransferases, both rtSTX-ov and rtSTX-em show type II membrane topology with the amino terminal short cytosolic tail, the putative transmembrane domain, the stem domain and the catalytic domain containing the sialyl motifs highly conserved among vertebrate sialyltransferases (*42*). It is noted that, as is the case with pRT PST1-1 and rtPST, the rtSTX cDNAs lacking the putative transmembrane and the stem domains were also isolated from ovary, but not from embryo (data not shown). These cDNAs may be partially spliced premature mRNAs or alternative splicing variants of rtSTX.

Expression of Genes for rtPST and rtSTXs in Various Tissues of Rainbow Trout.—(i) rtPST: As shown in Fig. 3, a single mRNA band of approximately 4.9 kb was evident in kidney, spleen and ovary at oocyte maturing stages (December). The 4.9 kb transcript was faintly detectable in heart, brain and intestine. The 4.9 kb transcript was constantly expressed in ovary during oogenesis tested from April to September without additional transcripts (data not shown). RT-PCR was also performed using cDNA prepared from tissues at immature oocyte stages (May). Strong expression of rtPST mRNA was detected in liver, kidney, spleen, and ovary (data not shown). These results indicate that rtPST mRNA was expressed not only in ovary, a tissue of biosynthesis of PSGP, but also in many other tissues of rainbow trout. Notably, irrespective of the presence of two cDNA clones (pRT PST1-1 and rtPST) that could be detected by the same DIG-labeled rtPST cRNA probe, the single transcript was detected on Northern blot analysis. Therefore, the size of two cDNA clones is likely similar.

(ii) rtSTXs: Northern blot analysis of various tissues for rtSTX was performed using the DIG-labeled rtSTX cRNA probe recognizing both rtSTX-ov and rtSTX-em mRNAs. The rtSTX was detected in ovary (2.7, 2.4, 2.1, 1.8 and 1.5 kb) and embryo (2.7 kb) (Fig. 4, A and B). The 1.8 and 1.5 kb transcripts were predominantly expressed in ovary. All these five transcripts were constitutively expressed in ovary. The ratio of the expression level of five transcripts remained unchanged during oogenesis, although 2.7 kb transcript slightly increased in May to July and 1.8 kb transcript decreased in September (Fig. 4C). RT-PCR was performed for the cDNA prepared from tissues at immature oocyte stage (May), showing that rtSTX was also expressed in brain and kidney (data not shown).

Developmental Expression of rtPST mRNA and rtSTX-ov mRNA during Oogenesis.—The expression profiles of mRNAs for rtPST and rtSTX-ov were examined with semi-quantitative RT-PCR (Fig. 5). The expression level of rtPST largely unchanged during oogenesis. In contrast, the expression level of rtSTX-ov was up-regulated in late stages of oogenesis (July, August, and September), when the elongation of polySia chain on *O*-linked glycans of PSGP extensively occurs (*43*). In Northern blot analysis, the expression level of rtSTX-ov mRNA against that of GAPDH mRNA elevated in late stage of oogenesis and reached at the peak in August (Fig. 4C). These results suggest that rtSTX-ov is a key enzyme in the biosynthesis of polySia chain on *O*-linked glycans of PSGP.

Assay for In Vitro Sialyltransferase Activity of rtPST, rtSTX-ov and rtSTX-em—To evaluate the ability of rtPST, rtSTX-ov, and rtSTX-em to elongate polySia chain on the *O*-linked glycan of PSGP, *in vitro* sialyltransferase activity was measured with the soluble recombinant enzymes. Lake trout PSGP, in which the polySia chain consists of only Neu5Ac residues, was used as an acceptor substrate for polySia synthesis on *O*-linked glycans, and NCAM-Fc was used as an acceptor substrate for polySia synthesis on *N*-liked glycans. Incubation temperatures 37°C and 25°C were chosen for the assay of the enzyme activity, due to the body temperature of human and the inhabiting temperatures of rainbow trout, respectively.

As shown in Table 1, the recombinant rtPST showed activity toward both PSGP and NCAM-Fc at 25°C and 37°C. The recombinant hPST showed 7.8 times and 4.4 times higher activity toward PSGP and NCAM than the recombinant rtPST, respectively. For recombinant rtSTX-ov and rtSTX-em, they also showed the activity toward PSGP and NCAM-Fc, although the activities

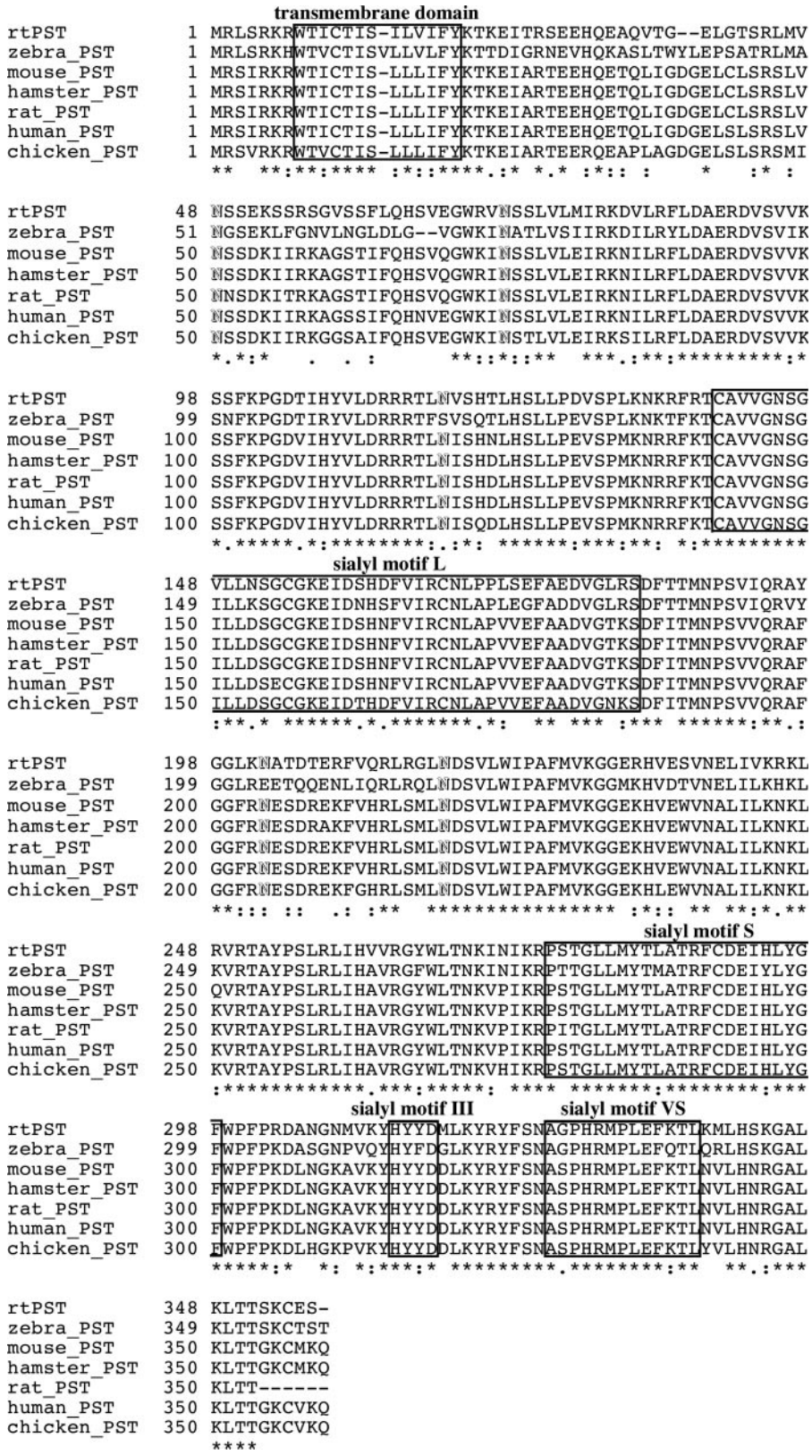

Fig. 1. **Sequence alignment of the deduced amino acid sequence of rainbow trout PST and vertebrate PSTs.** The alignment of the deduced amino acid sequence of rtPST (accession number AB094402) with vertebrate PSTs was generated by ClustalW program. The putative transmembrane domain and sialyl motifs, highly conserved motifs in catalytic domain among vertebrate sialyltransferases, are boxed. The potential *N*-glycosylation sites are shadowed. Asterisks indicate an identical amino acid in the alignment. Colons indicate a position that is highly conserved. Dots indicate a position that is well conserved.

were low or undetectable depending on the incubation temperature. The recombinant rtSTX-ov and rtSTX-em showed about 16 and 48 times lower activity toward NCAM-Fc than hSTX at 37°C. The recombinant rtSTX-ov showed 16 times lower activity toward PSGP than hSTX at 37°C. To confirm if these activity transferred Sia onto polySia chains, Endo-N treatment of the enzyme reaction products was performed. The 82% and 87% of polySia chains

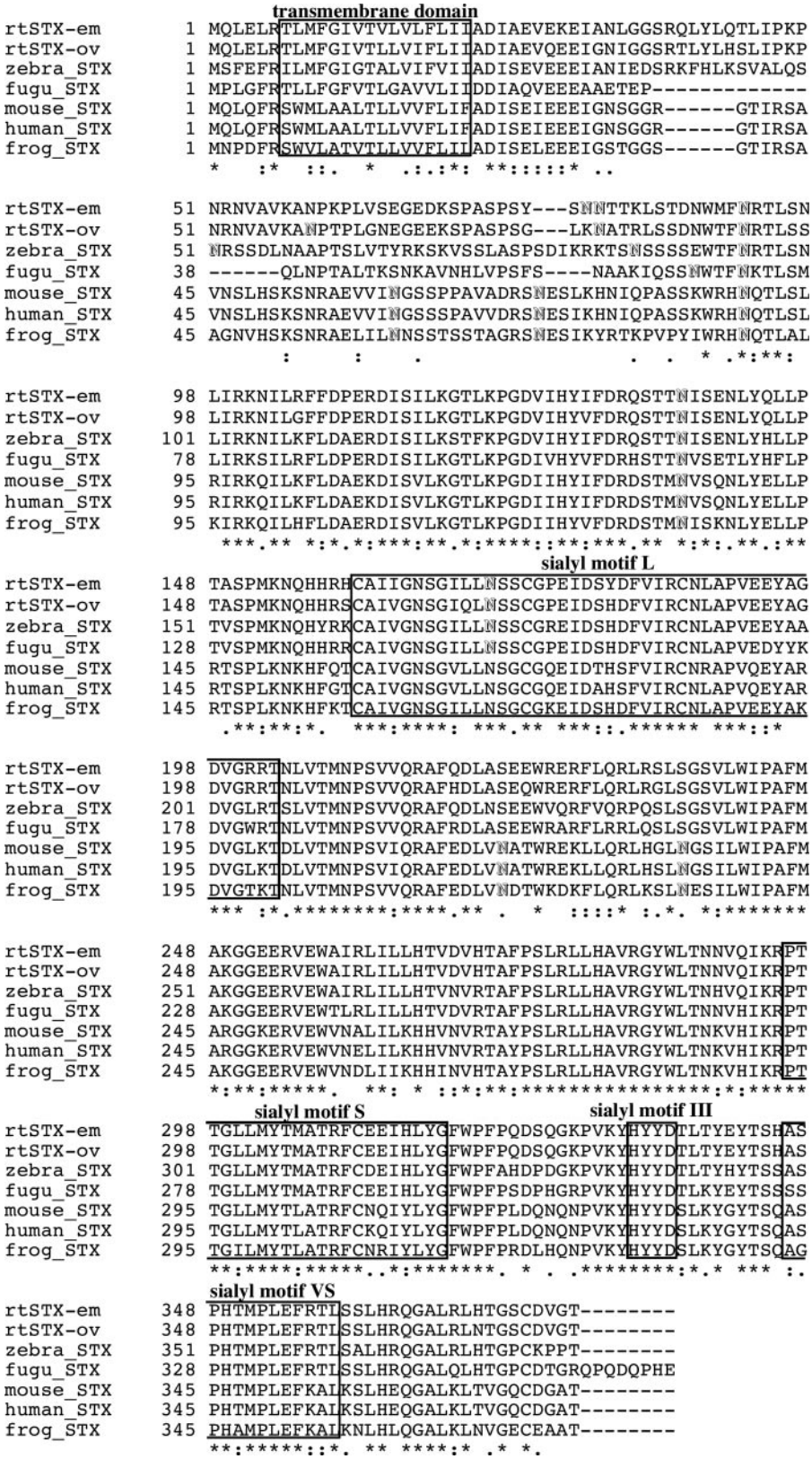

Fig. 2. **Sequence alignment of the deduced amino acid sequence of rainbow trout STX-ov, STX-em, and vertebrate STXs.** The alignment of the deduced amino acid sequence of rtSTX-ov (accession number AB262976) and rtSTX-em (accession number AB262977) with vertebrate STXs was generated by ClustalW program. See also the legend of Fig. 1.

formed by hPST on NCAM-Fc and PSGP, respectively, were sensitive to the Endo-N digestion (data not shown), confirming that hPST elongates the polySia chain on these substrates. However, we could not perform the Endo-N digestion experiments for the products synthesized from NCAM-Fc and PSGP by rtPST, rtSTX-ov, or rtSTX-em, because amounts of the obtained products were not large enough to be subjected to further analyses. In these

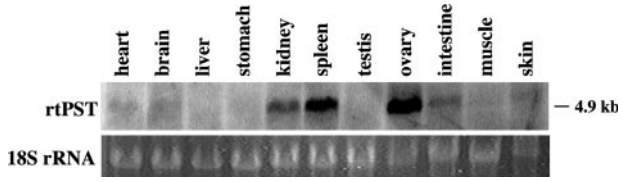

Fig. 3. **Northern blot analysis for the expression of rtPST mRNA in various tissues of rainbow trout.** Total RNAs from various tissues of rainbow trout excised in December were run on a 1.0% agarose gel containing 5% formaldehyde and blotted on a nylon membrane. The membrane was hybridized with DIG-labeled rtPST full-length cRNA as a probe, and visualized as described under "MATERIALS AND METHODS." The same membrane was also reprobed with DIG-labeled GAPDH cRNA (accession number AF027130, nucleotides 25–1062).

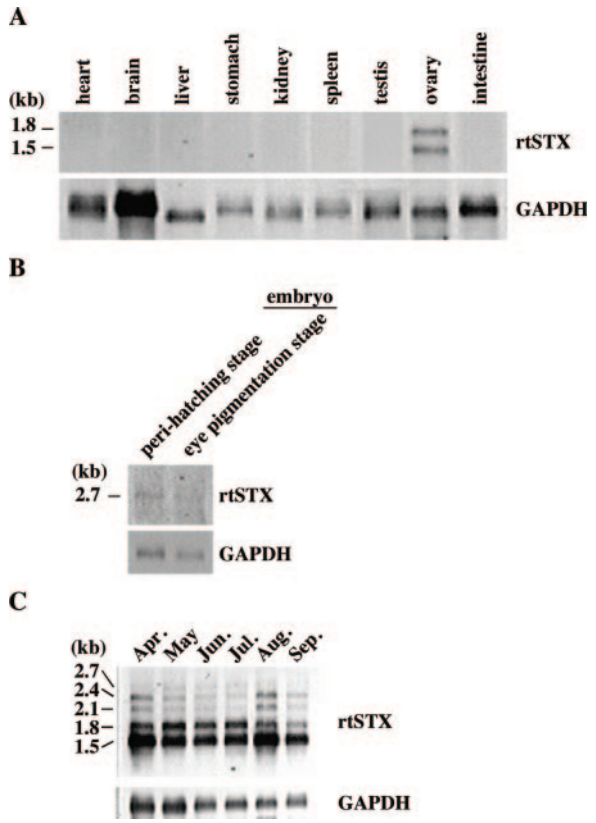

Fig. 4. **Northern blot analysis for the expression of rtSTX mRNA in various tissues of rainbow trout.** Poly(A)$^+$ RNAs were prepared from various tissues of rainbow trout excised in May (A), embryos (B) and ovaries harvested at a month interval from April to September (C), and analyzed for the rtSTXs and GAPDH mRNAs, as described under "MATERIALS AND METHODS."

experiments, the amount of the recombinant enzymes used was much the same between these rainbow trout and human recombinant enzymes, as estimated by Western-blot analyses of the affinity-purified recombinant enzymes with anti–epitope tag antibody (data not shown). The relatively lower activities of rainbow trout enzymes may be due to their heat-unstable nature, compared with those of the human enzymes. Previously, it was reported that rainbow trout sialyltransferases were unstable at 37°C (*43*).

According to the previous reports (*44, 45*), mammalian PST and STX could modify themselves with polySia chain on their own *N*-linked glycans. This process is termed *in vitro* autopolysialylation. We thus investigated if rtPST, rtSTX-ov, and rtSTX-em could catalyze the autopolysialylation. The recombinant rtPST was incubated with radio-labeled CMP-Neu5Ac in the absence of exogenous acceptor substrate, and the radio active products were subjected to SDS-PAGE. As shown in Fig. 6A, no *in vitro* autopolysialylation activity was detected in rtPST, although hPST showed *in vitro* autopolysialylation activity, which was confirmed by the sensitivity of the products to the Endo-N treatment. Approximately 300 kDa smear band of autopolysialylated hPST shifted to approximately 180 kDa band after the Endo-N treatment (Fig. 6A, lanes 1 and 2). In the same way, no *in vitro* autopolysialylation activity was observed for rtSTX-ov or rtSTX-em, irrespective of the incubation temperatures, while hSTX exhibited obvious *in vitro* autopolysialylation activity (Fig. 6B, lane 1). Taken together, rtPST, rtSTX-ov, and rtSTX-em were shown not to exhibit *in vitro* autopolysialylation activity.

Effects of Co-incubation of rtSTX and rtPST on the Polysialylation of PSGP—To examine if rtSTXs are involved in the polysialylation of PSGP, co-incubation of rtPST and rtST6GalNAc with rtSTX-ov was performed using asialo-PSGP as the substrate. We previously showed that asialo-PSGP is converted to monosialo-PSGP by the action of rtST6GalNAc (*43*). Consistent with the results shown in Table 1, rtSTX-ov and rtPST exhibited very low activities, 37 and 26 fmol/h, respectively, when the activity of rtSTX-ov or rtPST was defined as subtraction of the amount of Sia incorporated by rtST6GalNAc (Fig. 7, rtST6GalNAc+) from those of Sia incorporated by combinations of rtSTX-ov or rtPST with rtST6GalNAc (Fig. 7, rtST6GalNAc+/rtSTX-ov+ and rtST6GalNAc+/rtPST+). Notably, co-incubation of the three enzymes, rtPST, rtSTX-ov and rtST6GalNAc resulted in two times higher activity (120 fmol/h) than the sum of each activity (Fig. 7, rtST6GalNAc+/rtSTX-ov+/rtPST+). These results showed that rtPST and rtSTX-ov synergistically enhanced the polysialylation of PSGP. Thus, rtSTX-ov is suggested to be functionally involved in the polysialylation of PSGP under the co-existence with rtPST.

Transfection of NCAM-Bearing Neuro2A Cells with cDNAs for rtPST and rtSTXs—Transient expression of mammalian PST and STX in cell lines of NCAM-positive/polySia-negative phenotype resulted in cell surface expression of polySia chain (*19, 23*). To examine if rtPST, rtSTX-ov, and rtSTX-em could synthesize polySia chain *in vivo*, Neuro2A cells of NCAM-positive/polySia-negative phenotype were transfected with pcDNA rtPST-V5, pcDNA rtSTX-ov-V5 or pcDNA rtSTX-em-V5, all encoding the V5-tagged full-length enzymes. The transfectant Neuro2A cells with hPST or hSTX cDNA showed the cell surface staining with monoclonal antibody 12E3 which recognizes $(Neu5Ac)_n$ ($n \geq 5$) (Fig. 8, A and B). The surface staining with 12E3 was also detected in the rtPST-expressing cells (Fig. 8D). On the other hand, no staining with 12E3 was observed in the transfectant cells with rtSTX-ov or rtSTX-em (Fig. 8, E and F). These results indicate that rtPST exhibits the polysialyltransferase activity *in vivo*, while the rtSTX-ov and rtSTX-em do not.

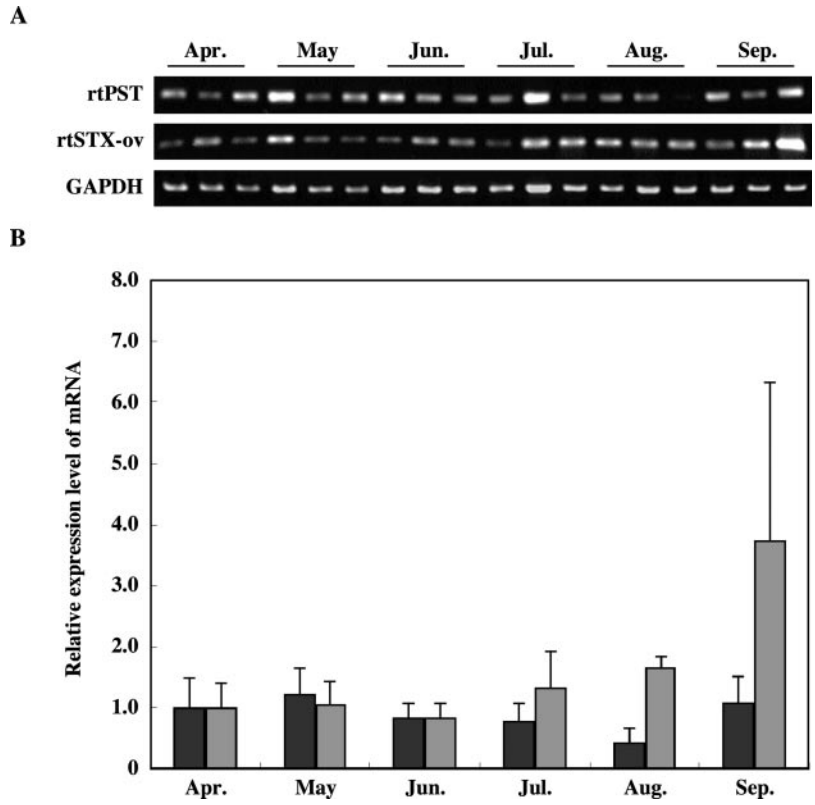

Fig. 5. **RT-PCR Analysis of the rtPST and rtSTX-ov mRNA expression during oogenesis.** A: The cDNA was prepared from ovaries of each of three animals harvested at a month interval from April to September, and used as a template in the subsequent semi-quantitative PCRs. The amplifications were carried out at 25 cycles for GAPDH, 30 cycles for rtPST and 32 cycles for rtSTX-ov. The cycles of PCRs were set in such a way that the amplification of cDNA fragments was not saturated as described in "MATERIALS AND METHODS." The PCR products were analyzed on 1.0% agarose gel containing ethydium bromide. Photographs were taken under UV light. B: To estimate quantity of amplified DNA, densitometric analysis was carried out. Developmental expression of rtPST mRNA (black bar) and rtSTX-ov mRNA (gray bar) normalized against that of GAPDH mRNA in each month during oogenesis. The values are relative normalized ratios when the normalized ratio in April is set to 1.0, and shown as mean ± SEM. The standard error bar indicates deviations between values for three different animals.

Table 1. ***In vitro*** **sialyltransferase activity of the α2,8-polysialyltransferases.**

Substrate	Reaction temperature	Enzyme activity (fmol/h)**				
		rtPST	hPST	rtSTX-ov	rtSTX-em	hSTX
NCAM-Fc	37°C	126	559	28.2	9.21	439
	25°C	91.0	n.d.*	0	0	n.d.
Lake trout PSGP	37°C	21.6	170	20.9	0	343
	25°C	117	n.d.	23.4	24.5	n.d.

*n.d. not determined. **The amount of protein for each enzyme was similar to each other as revealed by immunoblotting using anti-V5.

The internal staining with anti-V5 revealed that rtSTX-ov and rtSTX-em were certainly expressed in the transfected cells (Fig. 8, G and H). Notably, the rtSTX-ov and rtSTX-em were apparently localized in cytoplasm and/or ER of the cells, but not only in the Golgi apparatus. This localization patterns of rtSTX-ov and rtSTX-em were different from the torch-like Golgi localization of hSTX (Fig. 8I).

Transfection of Mammalian COS-1 and Rainbow Trout RTG-2 Cells That Lack NCAM or PSGP Expression with rtPST or rtSTXs cDNAs—The mammalian PST and STX are known to be autopolysialylated and localized in the Golgi as well as on the cell surface, when they are transiently expressed in culture cells, and they are the only polysialylated glycoproteins in COS-1 cell of NCAM negative/polySia negative phenotype (*35*). The transiently expressed rtPST was localized in the Golgi of the transfectant COS-1 cells (Fig. 9, V5/rtPST). The rtPST protein was also immunoreactive with 12E3 (Fig. 9, 12E3/rtPST). These staining patterns showed typical Golgi localization, like autopolysialylated hPST, which was localized in the Golgi in COS-1 cells (Fig. 9, V5/hPST and 12E3/hPST). These results suggest that rtPST has an *in vivo* autopolysialylation activity in COS-1 cells.

The similar transfection experiments were also performed for rtSTX-ov and rtSTX-em. While autopolysialylated hSTX was detected in the Golgi of the transfectant COS-1 cells with hSTX cDNA, autopolysialylated rtSTX-ov and rtSTX-em were not detectable in the transfectant cells with their cDNAs (data not shown), consistent with the case with the transfectant Neuro2A cells (Fig. 8, E and F). To exclude the possibility that rtSTX-ov and rtSTX-em tend to undergo unusual localization in mammalian cells, these enzymes were expressed in rainbow trout RTG-2 cell, which is of NCAM negative/polySia negative phenotype. The internal staining with 12E3 gave no signal in the cells expressing rtSTX-ov or rtSTX-em (data not shown), while autopolysialylated hSTX was detected in the Golgi of the transfectant RTG-2 cell (Fig. 10D). The internal staining patterns of rtSTX-ov and rtSTX-em (Fig. 10, A and B) were different from that of hSTX in the transfectant RTG-2 cell (Fig. 10C), but similar to those in Neuro2A cell (Fig. 8, G and H). These results indicate that rtSTX-ov and rtSTX-em lack the ability of *in vivo* autopolysialylation,

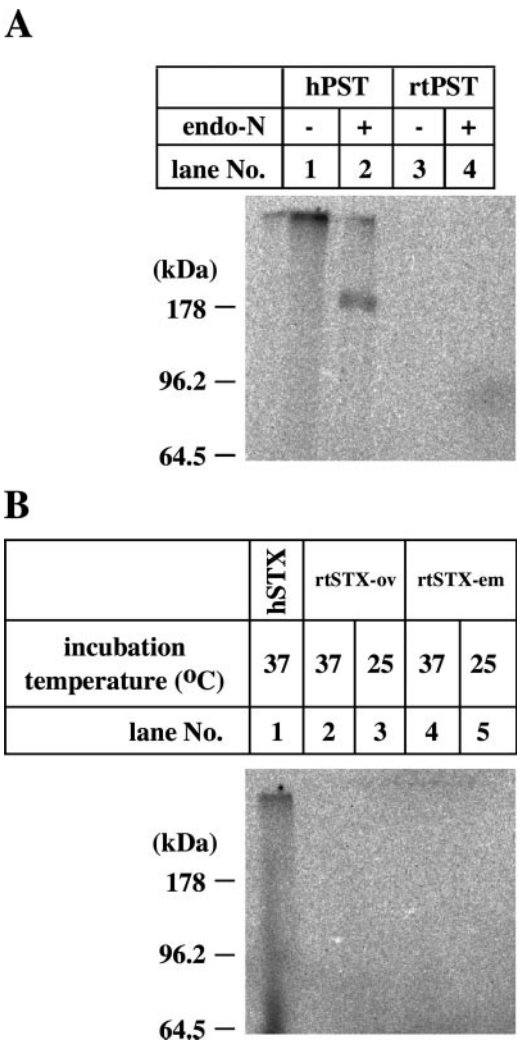

Fig. 6. ***In vitro*** **autopolysialylation activity of rtPST, rtSTX-ov, and rtSTX-em.** The enzyme reactions were carried out at 37°C. For rtPST, rtSTX-ov and rtSTX-em, the reactions were also carried out at 25°C, which is close to the inhabiting temperatures of rainbow trout. An aliquot of the reaction mixture containing recombinant enzyme-adsorbed resin was treated with or without Endo-N. The [^{14}C]Neu5Ac incorporated into recombinant enzyme was detected as described under "MATERIALS AND METHODS". A: rtPST; B: rtSTX-ov and rtSTX-em.

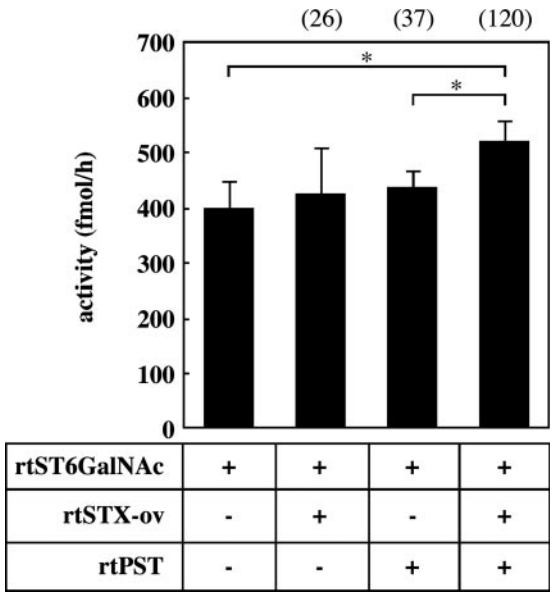

Fig. 7. **Effects of co-incubation of rtPST and rtST6GalNAc with rtSTX-ov on the polysialylation of PSGP.** Asialo-PSGP and CMP-[^{14}C]Neu5Ac were incubated with a combination of either of the same amount of recombinant rtST6GalNAc, rtPST, or rtSTX-ov at 25°C for 24 h. The sialyltransferase activity was determined as describe under "MATERIALS AND METHODS" (n = 3). *p < 0.05. The α2,8-sialyltransferase activity (fmol/h) is defined as subtraction of the amount of incorporated Sia by rtST6GalNAc (rtST6GalNAc+) from those by combinations of rtSTX-ov, rtPST, or both with rtST6GalNAc, and indicated in parentheses.

and that they are localized in the cytoplasm including the Golgi and ER in the rainbow trout cells. No apparent activity and the unusual localization of rtSTX-ov and rtSTX-em may raise questions if they are really enzymes under particular conditions, and if they serve as activator-like proteins that may be related with the expression of polySia through an unknown mechanism.

DISCUSSION

Structural Features of Rainbow Trout PST and STXs— In the present study, we have identified a PST/ST8SiaIV orthologue (rtPST) and two STX/ST8SiaII orthologues (rtSTX-ov and rtSTX-em) in rainbow trout. The deduced amino acid sequence of rtPST shows 72–77% similarity to mammalian, bird, and other fish PSTs (Fig. 1), while that of rtSTX-ov shows 63–76% similarity to mammalian, amphibian and other fish STXs (Fig. 2). This is the first comparison of PSTs and STXs between fish and other animals, and here we describe the points as follows. In both rtPST and rtSTXs, the amino acid residues and sequences that are highly conserved among mammalian orthologues are conserved in fish (Figs. 1 and 2): the sialyl motifs L, S, and VS; the sialyl motif III which situates between the sialyl motifs S and VS, (H/Y)Y(Y/F/W/H)(E/D/Q/G); and the C-terminal region of the sialyl motif S, (I/L)(F/Y) GFWPF; the length of transmembrane region except for zebra fish PST with an additional amino acid; C354 in rtPST and C374 in rtSTXs that form disulfide bond to the second C residue in the sialyl motif L (*46*). The *N*-glycosylation sites in rtPST are well conserved among rainbow trout, mammalian, and bird PSTs, except for zebra fish PST in which the 3rd and the 4th sites (N117 and N202 in rtPST) are missing. On the other hand, the N171 in the sialyl motif L of rtSTX is converted to potential *N*-glycosylation site in fish STXs, while two potential *N*-glycosylation sites (N117 and N202 in rtSTX) between the sialyl motifs L and S in mammals and amphibians are lost in fish. These two Asn residues, corresponding to the *N*-glycosylation sites [N219 and N234 in human STX (*45, 47*)] of mammalian STXs, are autopolysialylation sites, but do not affect the polysialyltransferase activity (see Fig. 7C of Ref. *47*).

Structural differences mostly exist in the stem region. For PST, the stem regions of fish PST (rtPST and zebra fish PST) are shorter by two amino acid residues compared with those of mammalian PSTs. The region corresponding

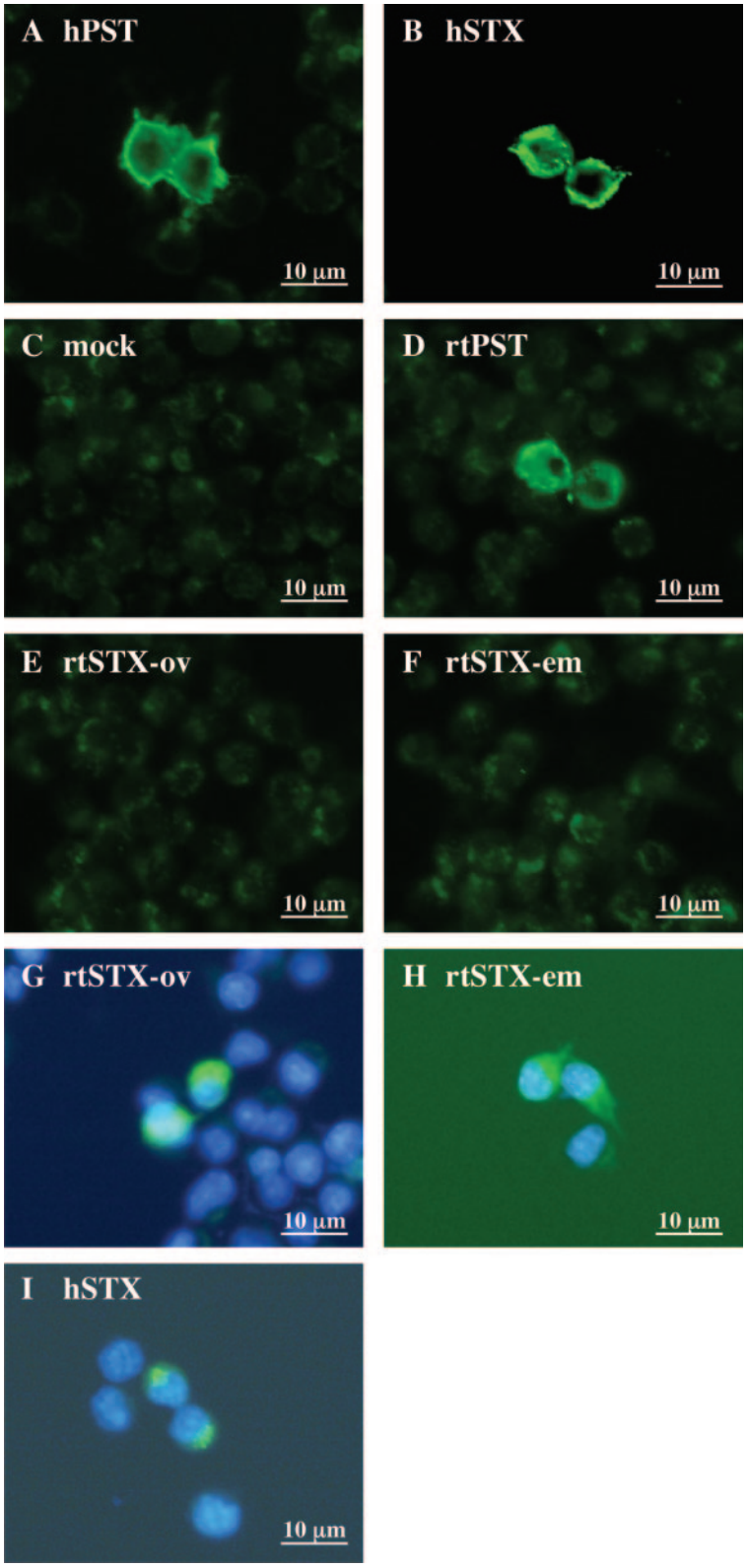

Fig. 8. **Expression of rtPST and rtSTXs in Neuro2A cells of NCAM-positive/polySia-negative phenotype.** The murine neuroblastoma Neuro2A cells were grown on glass coverslips. The Neuro2A cells were transiently transfected with the following plasmids using Lipofectamine: pcDNA hPST-V5 (A), pcDNA hSTX-V5 (B and I), pcDNA3.1/V5-His B (C), pcDNA rtPST-V5 (D), pcDNA rtSTX-ov-V5 (E and G) and pcDNA rtSTX-em-V5 (F and H). The cells were fixed with 4% paraformaldehyde and immunostained with 12E3, anti-polySia antibody (green) (A–F) or permeabilized with methanol and immunostained with anti-V5 antibody (green). Nuclei were stained with DAPI (blue) (G–I).

to amino acids 37–67 of rtPST are well conserved between mammals and birds, but most different between fish species as well as between fish and mammals or birds. For STX, the stem regions of rtSTXs (rtSTX-ov and rtSTX-em), zebra fish STX, and *T. rubripes* STX are 3 and 6 amino acid residues longer and 11 amino acid residues shorter than those of mammalian STXs, respectively. The region corresponding to amino acids 35–88 of rtSTXs is highly

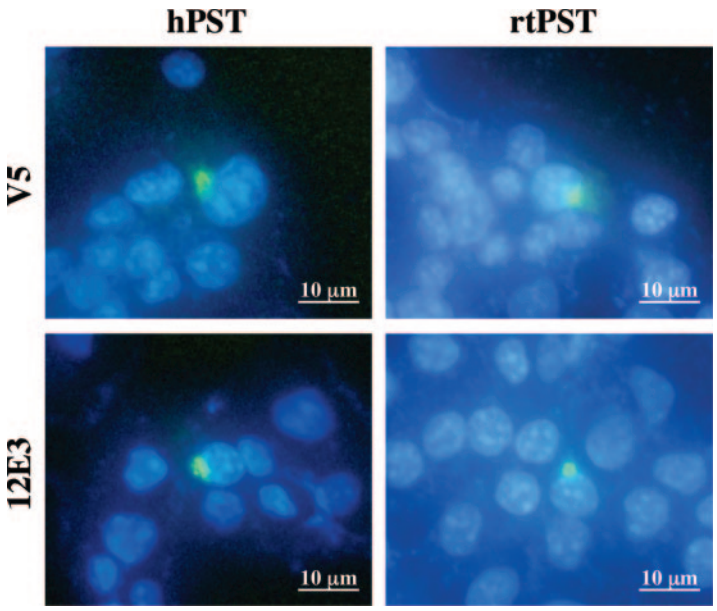

Fig. 9. ***In vivo* autopolysialylation of rtPST in COS-1 cell of NCAM-negative/polySia-negative phenotype.** The COS-1 cells were transfected with pcDNA hPST-V5 and pcDNA rtPST-V5. The cells were fixed and permeabilized with methanol and immunostained with anti-V5 antibody or 12E3, anti-polySia antibody (green). Nuclei were stained with DAPI (blue).

conserved between mammals and amphibians, but not very conserved between fish species, or between fish and mammals or amphibians. rtSTX-ov and rtSTX-em shows 92% identity of amino acid sequence, but the differences reside mostly in the stem region. Biological importance of such structural differences in the stem regions are not yet understood, but it is interesting to note that the amino terminus of the stem region of PST is shown to be involved in recognition of acceptor substrate NCAM (*48*).

α2,8-PolyST Genes in Rainbow Trout—At least two rtSTX genes, *rtSTX-ov* and *rtSTX-em*, exist in rainbow trout. This is the first case with the α2,8-polyST family, but appears to be common feature in teleost sialyltransferase subfamilies. Harduin-Lepers *et al.* have recently pointed out that there are two genes in ST3Gal I, ST3Gal II, ST3Gal III, and ST3Gal V subfamilies in zebra fish, ST3Gal I, ST6GalNAc I/II, ST8Sia III, and ST8Sia VI subfamilies in *T. rubripes*, and ST3Gal I and ST8Sia III subfamilies in *T. nigroviridis*, probably given rise to by gene duplication events (*49*). In rainbow trout, similar gene duplication events also known for lactate dehydrogenase and malate dehydrogenase (*50*). We also observed that a few *N*-acetylneuraminic acid 9-phosphate synthase genes probably given rise to by gene duplication exist in rainbow trout (unpublished data). Phylogenic analysis and genetic mapping of *Hox* clusters suggest two rounds of chromosome duplication before the divergence of ray-finned and lobe-finned fishes, and one more in ray-finned fish before the teleost radiation (*51*). Thus, rtSTX-ov and rtSTX-em genes might be diverged from the common ancestral gene in ray-finned fish before the teleost radiation.

Unique Features of mRNA Expression of Rainbow Trout PST and STXs—The rtPST mRNA is widely expressed in heart, brain, liver, stomach, kidney, spleen, testis, ovary, and embryo, while the rtSTX-ov and rtSTX-em mRNAs are restricted in ovary, kidney, brain in adult, in addition to embryo. This is also the case with the expression of PST and STX in mammals: *i.e.*, PST is widely expressed in heart, placenta, spleen, thymus, small intestine, peripheral blood leukocyte, and some region of adult brain (*52*), while STX is restricted to a few tissues in adult, although abundant in fetal brain (*19, 20, 52*). It should be noted that, in addition to the full-length mRNA for the rtPST gene, the truncated form of mRNA (pRT PST1-1) is abundantly expressed in rainbow trout ovary. The truncated forms may be an alternative splicing variant of rtPST or a premature mRNA of rtPST. The pRT PST1-1 is devoid of the putative transmembrane domain that is conserved among the conventional Golgi-localized vertebrate sialyltransferases. The truncated forms lacking the transmembrane and the stem domains also exist in rtSTX cDNAs from ovary, but not from embryo. This is the common feature in PST and STX from rainbow trout ovary. A cDNA for truncated form without a transmembrane domain also known in ST6GalNAc III from human brain and is not expressed as a soluble enzyme (*53*). Alternative splicing variants lacking the sialyl motif S due to premature stop codons are also known for human ST6GalNAc II (*54*). The function of these alternative splicing variants remains unknown.

Requirement of Co-expression of PST and STX for the PolySia Expression—Tissue expression profiles of rtPST and rtSTXs in rainbow trout lead us to an interesting hypothesis that the expression of rtSTX, in addition to rtPST, is required for the *in vivo* biosynthesis of polySia chains. For example, in ovary where polySia-PSGP is synthesized, both rtPST and rtSTX are expressed, while only rtPST is expressed in liver where no polySia epitope is detected (data not shown). Both rtPST and rtSTX are also expressed in embryos and adult brain of rainbow trout, in which polysialylated NCAM is present as revealed by Western blot analysis using 12E3 combined with Endo-N treatment (data not shown). During oogenesis, the polySia chains are expressed on rainbow trout PSGP from June (4 months prior to ovulation) to October (maturation and ovulation), consistent with the fact that the responsible α2,8-polyST activity is also detected in July and later (*43*). This study shows that the expression of rtSTX-ov starts increasing from the basal level in July and is continuously increased until September during oogenesis,

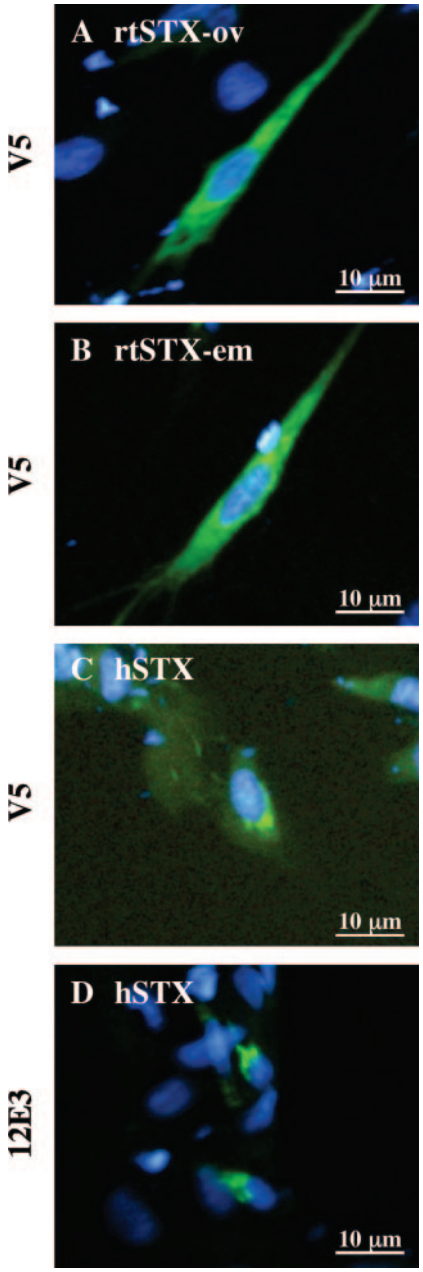

Fig. 10. **Expression of rtPST and rtSTXs in rainbow trout RTG-2 cell of NCAM-negative/polySia-negative phenotype.** The rainbow trout gonadal tissue–derived RTG-2 cells were transfected with pcDNA rtSTX-ov-V5 (A), pcDNA rtSTX-em-V5 (B) and pcDNA hSTX-V5 (C and D). The cells were fixed and permeabilized with methanol and immunostained with anti-V5 antibody (A–C) or 12E3, anti-polySia antibody (D) (green). Nuclei were stained with DAPI (blue).

while the expression of rtPST is largely constant (Fig. 5). This temporal, enhanced expression of rtSTX-ov mRNA is coincided with that of the α2,8-polyST activity and the subsequent increase in polySia chains on PSGP. It is thus suggested that the expression of rtSTX, but not rtPST, is much related to the enhanced expression of polySia chains in rainbow trout PSGP. However, it should be noted that the basal expression levels of rtPST and rtSTX-ov do not result in the polySia expression or α2,8-polyST activities (*43*). This may happen because the enzyme expression is not enough to synthesize the polySia on PSGP, but we will have to know much more about structure–enzyme activity relationship at the molecular level in future studies.

In mammals, STX is abundantly expressed compared with PST during development of central nervous system where polysialylated NCAM is extensively expressed, and the regional and temporal expression of STX are concomitant with those of polySia structure (*55*). Similarly, during neuronal differentiation of mouse teratocarcinoma P19 cells, the developmental expression of STX is correlated with that of polysialylated NCAM and α2,8-polyST activity, while the level of PST expression constantly remains very low (*56*). Taken together, in any polySia-expressing cells and tissues, STX may predominantly direct the elongation of polySia chains on both *O*- and *N*-linked glycans *in vivo*, although the co-expression of PST is also required.

In Vitro and In Vivo Activity of rtPST and rtSTXs—The recombinant rtPST shows *in vitro* activity toward fish egg PSGP and human NCAM, although no *in vitro* autopolysialylation activity is exhibited. The rtPST that is transiently expressed in the culture cells also exhibits *in vivo* NCAM polysialylation as well as *in vivo* autopolysialylation activities. Thus, the rtPST is suggested to catalyze the synthesis of polySia chains on PSGP. On the other hand, both rtSTX-ov and rtSTX-em exhibit very low activity, if any, both *in vitro* and *in vivo*. They do not exhibit *in vivo* autopolysialylation activity even in rainbow trout RTG-2 cells, either. Therefore, it is unclear if the rtSTXs catalyze the synthesis of polySia chains on PSGP in oocyte. Interestingly, the co-incubation of rtPST and rtSTX-ov synergistically enhances the *in vitro* polysialylation of PSGP (Fig. 7). This synergistic effect suggests that rtSTX-ov is functionally involved in the polysialylation of PSGP under the co-existence with rtPST. A similar cooperative action of PST and STX is also reported in mammals (*17*), although it differs in that mammalian STX shows polysialyltransferase activity. Consistent with *in vitro* nature of the synergistic action of rtPST and rtSTX-ov, the massive polysialylation of PSGP occurs concomitantly with the enhanced expression of rtSTX-ov together with the constant expression of rtPST in ovary at oocyte maturing stages (see above). Although further experiments should be done before understanding the mechanism of the synergistic action, it would be interesting to speculate that the rtSTX-ov may function as activator-like proteins for rtPST, rather than polysialyltransferase itself. In this regard, it should be noted that rtSTX-ov and rtSTX-em are expressed not only in the Golgi, but also other organelles like ER, on the transfection of Neuro2A and RTG-2 cells with their cDNAs (Figs. 8 and 10).

Biosynthesis of polySia Chains on O-Linked Glycans of PSGP—During oogenesis, two forms of PSGP are detected (*43*). One is a PSGP molecule containing a disialyl group (diSia-PSGP), and another is a PSGP molecule containing polySia with an increasing length of polySia chain with up to 25 sialic acid residues (polySia-PSGP). The diSia-PSGP is expressed at earlier stages of oogenesis, while polySia-PSGP is expressed only at later stages. For the synthesis of diSia-PSGP, at least two sialyltransferases, α2,6-ST and α2,8-ST, are required, and α2,8-polyST involved in the subsequent elongation of polySia chain from the diSia residue

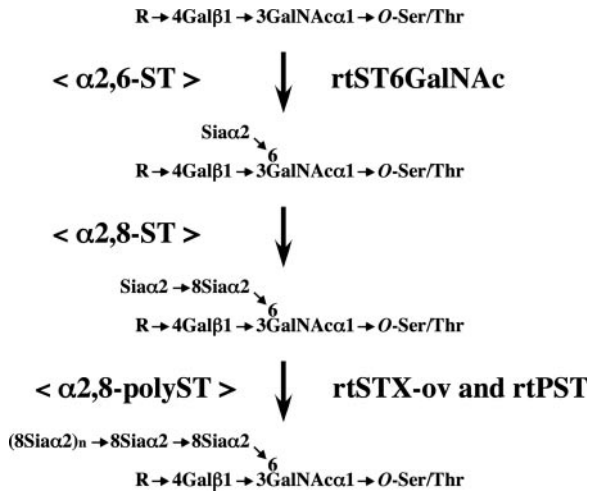

Fig. 11. **Hypothetical pathway for the biosynthesis of polySia on *O*-linked glycans of PSGP.** Biosynthesis of polySia chain on *O*-linked glycans of PSGP slowly proceeds during oogenesis over 6-month period (April to October). At earlier stage of oogenesis (until May), the diSia-PSGP bearing the diSia group predominantly exists. The diSia-PSGP is synthesized by at least two sialyltransferases, α2,6-ST and α2,8-ST. At later stage of oogenesis (from June), α2,8-polyST responsible for the subsequent elongation of polySia chain from the diSia residue synthesizes polySia-PSGP from diSia-PSGP. As the α2,6-ST, rtST6GalNAc (*39*) catalyzes the transfer of the first sialic acid residue to the proximal GalNAc residue. Here we propose that rtPST and rtSTX-ov are cooperatively involved in the biosynthesis of polySia chains as α2,8-polyST. The α2,8-ST has remained to be identified. R = H-, Galβ1-, GalNAcβ1,3Galβ1-, Fucα1,3GalNAcβ1,3Galβ1-, GalNAcβ1,4(Siaα2,3)GalNAcβ1,3Galβ1-. PSGP consists of tandem repeats of the tridecapeptide, DDAT*S*EAAT*GPSG, where the glycans are attached to particular S and T marked by * (*28*).

is required for the synthesis of polySia-PSGP. Because polySia chains are not detected in the *O*-linked glycans of PSGP at earlier stages, the α2,8-ST should be distinct from the α2,8-polyST (*43*). Based on these observations, we hypothesize the biosynthetic pathway of polySia on *O*-linked glycans of PSGP as shown in Fig. 11. We previously identified rtST6GalNAc from rainbow trout ovary as the α2,6-ST which transfer a Sia residue to *O*-6 position of the proximal GalNAc residue (*39*). In the present study, we propose that rtPST and rtSTX-ov are involved in the elongation of polySia chain, based on the enzyme activity as well as the developmental expression of these enzymes. As far as we examined in rainbow trout ovary, the possibility of the presence of an enzyme responsible for the biosynthesis of polySia on PSGP other than rtPST and rtSTX-ov is very low, provided that such enzymes should retain the conserved sialyl motifs (Figs. 1 and 2). As for the α2,8-ST which synthesizes α2,8-diSia structure on *O*-linked glycans, we have not identified it yet. In this regards, ST8Sia III and ST8Sia VI have been recently reported to form the disialyl structure on *O*-linked glycans (*41, 57–59*). We have identified the partial cDNA sequences homologous to ST8Sia III and ST8Sia VI from rainbow trout ovary (accession number AB262974 and AB262975, respectively), and we have demonstrated that these mRNAs are expressed in ovary during oogenesis. Thus, we may hypothesize that ST8Sia III and/or ST8Sia VI are responsible for the synthesis of diSia residues on diSia-PSGP as α2,8-ST. Alternatively, a low level expression of rtPST and rtSTX-ov in early stages of oogenesis may be related with the synthesis of the diSia, but not polySia, on *O*-linked glycans of PSGP, as discussed above.

In contrast, the mammalian α2,8-polySTs, PST and STX, can both catalyze NCAM polysialylation utilizing α2,3- or α2,6-sialyl structure as an acceptor substrate (*60*). This suggests that α2,8-ST might not be prerequisite for the polysialylation on *N*-linked glycans of NCAM. In the present study, for the first time, we show that human PST and STX exhibit α2,8-polyST activity on *O*-liked glycans, although the chain length of the polySia appears not to be so long as on *N*-linked glycans of NCAM (data not shown). Information about NCAM polysialylation have been accumulated including polysialylated *N*-glycan sites of NCAM, essential domain of NCAM for polysialylation by PST and STX, and NCAM recognition region by PST (*16, 17, 48, 61, 62*). However, we do not have enough data if rtPST or rtSTXs recognize a protein part of the endogenous substrate PSGP, just like PST and STX recognize the NCAM domain, or how they recognize core glycan structures different from *N*-linked glycans. Therefore, it is still an important issue if an α2,8-ST undergoes elongation of polySia chains on *O*-linked glycans or if PST and/or STX do it all as discussed above.

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