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Molecular basis for polysialylation: A novel polybasic polysialyltransferase domain (PSTD) of 32 amino acids unique to the α2,8-polysialyltransferases is essential for polysialylation

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Abstract To determine the molecular basis of eukaryotic polysialylation, the function of a structurally unique polybasic motif of 32 amino acids (pI∼12) in the polysialyltransferases (polySTs), ST8Sia II (STX) and ST8Sia IV (PST) was investigated. This motif, designated the "*polysialyltransferase domain*" (PSTD), is immediately upstream of the sialylmotif S (SM-S). PolyST activity was lost in COS-1 mutants in which the entire PSTD in ST8Sia IV was deleted, or in mutants in which 10 and 15 amino acids in either the N- or C- terminus of PSTD were deleted. Site-directed mutagenesis showed that Ile_{275}, Lys_{276} and Arg_{277} in the C-terminus of PSTD in ST8Sia IV, which is contiguous with the N-terminus of sialylmotif-S, were essential for polysialylation. Arg_{252} in the N-terminus segment of the PSTD was also required, as was the overall positive charge. Thus, multiple domains in the polySTs can influence their activity. Immunofluorescent microscopy showed that the mutated proteins were folded correctly, based on their Golgi localization. The structural distinctness of the conserved PSTD in the polySTs, and its absence in the mono- oligoSTs, suggests that it is a "polymerization domain" that distinguishes a polyST from a monosialyltransferases. We postulate that the electrostatic interaction between the polybasic PSTD and the polyanionic polySia chains may function to tether nascent polySia chains to the enzyme, thus facilitating the processive addition of new Sia residues to the non-reducing end of the growing chain. In accord with this hypothesis, the polyanion heparin was shown to inhibit recombinant human ST8Sia II and ST8Sia IV at 10 μM.

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Abbreviations

Sia	sialic acid
polySia	polysialic acids
N-CAM	neural cell adhesion molecules
DP	degree of polymerization
ST8Sia II (STX)	ST8 α-*N*-acetylneuraminide α-2,8-sialyltransferase II
ST8Sia IV (PST)	ST8 α-*N*-acetylneuraminide α-2,8-sialyltransferase IV
PolySTs	polysialyltransferases
Endo-N	Endo-*N*-acylneuraminidase

Introduction

In mammalian cells, the α2-8-linked polysialic acid (polySia) glycotope is an oncodevelopmental, tumor-associated antigen that plays a key role in modulating cell-cell interactions, principally during embryonic development, neural plasticity and tumor metastasis [reviewed in 1,2]. The major carrier protein of polySia is the neural cell adhesion molecule (N-CAM), in which polySia extends tri- and tetraantennary N-linked glycan chains [3–5]. Polysialyltransferases (polySTs) are members of the gene family of sialyltransferases that catalyze synthesis of polySia chains by transferring multiple Sia residues from the donor substrate, CMP-Neu5Ac, to N- and O-linked oligosaccharides on acceptor glycoprotein [6–10]. During polysialylation, the growing polySia chains appear to remain bound to the polyST, a feature in common with processive enzymes,

e.g. DNA polymerases. The processive mechanism of polysialylation is in contrast to the distributive mechanism of monosialylation wherein the monosialyltransferases (monoSTs) catalyze the transfer of single Sia residue to their acceptor substrate before release [10]. A comparison between the enzymatic properties of the membrane-bound α2,8-polySTs and the monoST activities in 14-day old embryonic chick brain revealed a number of distinct differences [10].

Prior to molecular cloning of the eukaryotic polySTs in 1995 [11–14], extensive biochemical studies in the preceding decade led to identification in fetal rat brain of the first mammalian α2,8-polyST [6]. This activity was responsible for polysialylation of N-CAM. These studies were made possible by the earlier development in our laboratory of prokaryotic-derived probes that specifically recognized α2-8-linked polySia chains, and which allowed the temporal expression of polySia in developing neural tissue to be determined [15]. Subsequent studies using these probes led to the discovery that extended polySia chains (DP > 55 Sia residues) were expressed on N-CAM in human neuroblastoma cells [16]. Following the development of a new strategy to determine the DP of polySia chains on N-CAM that avoided acid hydrolysis prior to chromatographic profiling, sub-populations of chains extending up to DP$\sim$400 have now been shown to decorate N-CAM [17].

The two key mammalian polySTs that control polySia synthesis are designated ST8Sia II (STX) and ST8Sia IV (PST). Both enzymes have been cloned, sequenced and shown to be responsible for the polysialylation of N-CAM, the major carrier protein of polySia in vertebrates [11–14]. Both enzymes also share $\sim$59% identity at the amino acid level, and their catalytic domains are located in the lumen of the Golgi complex [18]. The amino acid sequence of the human ST8Sia IV is 97% homologous with the hamster and mouse and codes for a protein with a predicted molecular mass of 41.2 kD [12]. Conserved disulfide bonds in the mono- and polySTs have been described [reviewed in 19]. Chemical modification studies using the thiol-directed alkylating reagents, *N*-ethylmaleimide and iodoacetamide showed that at least one cysteinyl residue in the embryonic chick brain polyST was critical for polysialylation, but was of lesser importance for monosialylation catalyzed by the α2,3-, α2,6-, and α2,8- monoSTs [10]. This finding led to the hypothesis that a sulfhydryl residue may be involved as a "reactive thiol" in the initiation of polySia chain synthesis [20].

Both mono- and polySTs share at least four conserved amino acid motifs, sialylmotif L (long; SM-L), sialylmotif S (short; SM-S), sialylmotif VS (very short; SM-VS) and motif III consisting of 48, 23, 6 and 4 amino acids, respectively [21–25]. Studies on the monoST, ST6Gal I, led to the suggestion that SM-S was involved in the binding of CMP-Neu5Ac, the common donor substrate for all the sialyltransferases, whereas SM-L was postulated to participate in binding both CMP-Neu5Ac and acceptor glycoproteins [22,23]. While substitution of His residues (His_{348} in ST8Sia II and His_{331} in ST8Sia IV) in SM-VS eliminated polyST activity [9], the function of SM-VS in catalysis remains unknown. More recently, a possible fourth sialylmotif unique to the ST8Sia gene family was identified by computational analysis [26]. This 46 amino acid sequence, KTxxxTxNPSx(33)PAF, was postulated to be a linkage-specific sequence motif. But, since no direct biochemical or mutational analyses were carried out, it remained to be determined experimentally the possible role of this sequence in substrate recognition or N-CAM polysialylation.

To further identify protein domains within the polyST that are required for N-CAM polysialylation, Angata *et al*. constructed chimeric enzymes using the "catalytic domain" of ST8Sia IV with the corresponding segments of ST8Sia II and ST8Sia III [27]. While ST8Sia III has some modest homology with ST8Sia II and ST8Sia IV, it lacks the PSTD and cannot polysialylated N-CAM, although it may oligosialylate itself. The catalytic domain of ST8Sia IV was defined as comprizing amino acid residues 62 to 356 in the protein, and was identified initially on the basis of deletion analysis. These studies revealed that multiple protein domains within the polySTs appeared to be required for N-CAM polysialylation, and that these domains were distinct from those required for N-CAM recognition. This latter finding confirms the earlier conclusions reached by Colley *et al.* [28,29].

In contrast to the monoSTs, we discovered that both ST8Sia II and ST8Sia IV contain a structurally unique polybasic motif (calculated pI $\sim$12) of 32 amino acids immediately upstream and contiguous with SM-S [10]. We have now designated this domain as the "polysialyltransferase domain" (PSTD), since it is a structural motif unique to ST8Sia II and ST8Sia IV, and which distinguishes the polySTs from the mono- oligoSTs. Organization of the PSTD within ST8Sia IV is shown in the top of Fig. 1.

Discovery of PSTD in ST8Sia II and ST8Sia IV led us to hypothesize that the polybasic motif may be important for the processive synthesis of polySia to take place by tethering nascent polySia chains to the polySTs during the addition of new sialyl residues to the non-reducing termini of the growing chains [10]. To test experimentally the potential functional importance of the PSTD in polysialylation, we have determined the mono- and polyST activities in a series of mutants obtained by deletion and site-directed substitutions within the PSTD in ST8Sia IV. The results reported herein show that the PSTD is an essential motif required for polysialylation. Furthermore, the C-terminal Ile_{275}, Lys_{276} and Arg_{277} residues located at the juncture between the PSTD and SM-S are key residues of critical importance for polymer synthesis, as is the overall positive charge of the domain. Neither the monoSTs or the putative oligoST, ST8Sia III, contain

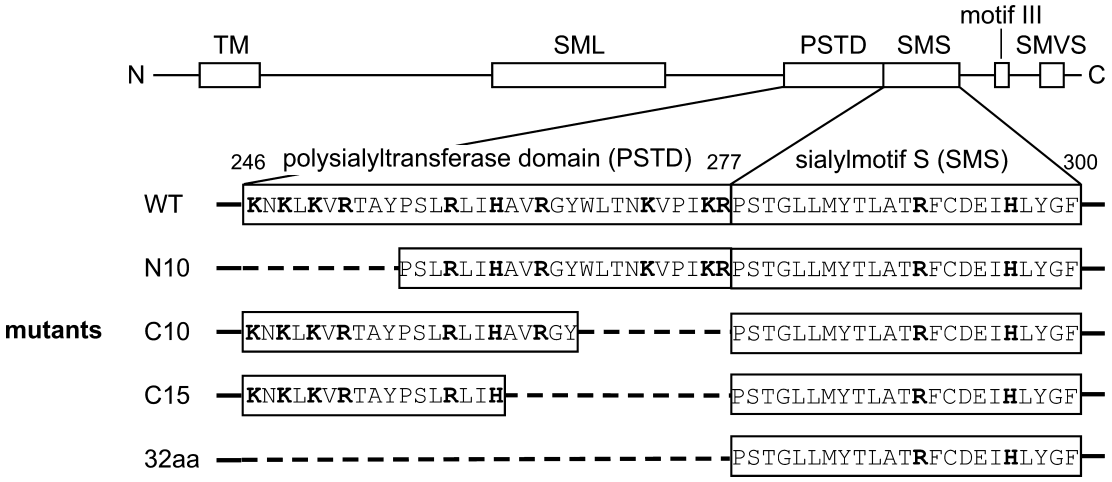

Fig. 1 Protein domains in the polySTs showing the polybasic PSTD (top panel) and the deleted sequences in the ST8Sia IV deletion mutants (bottom panels). The top panel represents the complete protein domain structure of the polySTs showing the relevant domains. TM, transmembrane domain (aa 8–25); SM-L, sialylmotif-L (aa 139–186); PSTD, polysialyltransferase domain (aa 246–277); SM-S, sialylmotif-S (aa 278–300); motif III (aa 315–318) SM-VS, sialylmotif-VS (aa 331–336). The lower panels show the amino acid sequences in wild-type (WT) PSTD and SM-S and in the deletion mutants (N10, C10, C15 and the 32 mer deletion). The dashed lines indicate the amino acid region deleted in each mutant.

the conserved PSTD, nor do they contain the conserved Ile, Lys and Arg residues immediately upstream of SM-S.

Materials and methods

Materials

Expression plasmids for a full length construct of ST8Sia IV (pcDNA3.1-PSTfV5His), a soluble form of this construct fused with either V5 and 6× His epitope tags at the C-terminus (pcDNA3.1-PSTsV5His) and a soluble (mouse) homologue of FcN-CAM were kindly provided by Dr. K. J. Colley, University of Illinois, Chicago, IL. Heparin, with an average molecular mass of 3,000 Da and other chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO). CMP-[^{14}C]-Neu5Ac (Specific activity 249 mCi/mmol; 80 μM) was obtained from New England Life Science Products, Inc. (Boston, MA) The restriction enzyme, pcDNA3.1/V5HisB, plasmid and mouse anti-V5 antibody were obtained from Invitrogen (Carlsbad, CA). Alexa 488-conjugated goat anti-mouse IgG was obtained from Molecular Probes (Eugene, OR). The bacteriophage-induced endo-*N*-acylneuraminidasewas (Endo-N) used in these studies was originally isolated and purified in our laboratory, as described previously [30].

Construction of deletion and amino acid substituted mutants in ST8Sia IV

For the 32 amino acid deletion mutant and the N10, C10 and C15 deletion mutants of ST8Sia IV, forward and reverse oligonucleotide primers were constructed that contained both lateral side sequences (28 or 30 nucleotide each) in the region to be deleted (Table 1) Two DNA fragments flanking either side of the deleted sequences were amplified from pcDNA3.1-PSTfV5His as template by using these primers with corresponding upstream (T7) or downstream (SP6) primers. Five PCR cycles were carried out to allow the purified fragments to act as templates and primers to be extended to a full-length DNA coding deletion mutant, followed by amplification using T7 and SP6 primers. The amplified fragments were digested with *Hind* III and *Xba* I and ligated into the expression vector, pcDNA3.1/V5HisB (Invitrogen), through the same site.

For single and multiple point substitutions, the mutants were constructed following the manufacture's instruction of QuikChange^{TM} Site-directed Mutagenesis Kit (Stratagene). The sets of oligonucleotide PCR primers for generating the substitution mutants are also described in Table 1.

Expression of wild-type and ST8Sia IV mutants in COS-1 Cells

Wild-type ST8Sia IV and its mutants were transiently expressed in COS-1 cells using Qiagen's Superfect reagent. Cells were maintained in DMEM with 10% fetal bovine serum (FBS) and grown to approximately 40–80% confluency on the day of transfection. Superfect (150 μl) and 25 μg of plasmid DNA in MEM were used for transfection in 150-mm tissue culture plates. Superfect (7.5 μl) and 1.5 μg of plasmid DNA in MEM were used for transfection in 12-well tissue culture plates. Cells were harvested 48 h post transfection and frozen as pellets at –80°C. ST8Sia IV activity was

Table 1 PCR primers used for construction of the deleted and amino acid substituted mutants in ST8Sia IV

mutants	mutagenesis primers	forward reverse
N10	5'-CACGTGGAGTGGG TTAATGCATTAATCCTTCCGTCATTGAGACTTATTCATGCTGT-3'	
	5'-TCTGACAGCATGA ATAAGTCTCAATGACGGAAGGATTAATGCATTAACCCACTCCA-3'	
C10	5'-TTGAGACTTATTC ATGCTGTCAGAGGTTACCCCAGCACAGGTCTTCTCATGTATAC-3'	
	5'-AAGTGTATACATG AGAAGACCTGTGCTGGGGTAACCTCTGACAGCATGAATAAGTC-3'	
C15	5'-ACTGCCTATCCGT CATTGAGACTTATTCATCCCAGCACAGGTCTTCTCATGTATAC-3'	
	5'-AAGTGTATACATG AGAAGACCTGTGCTGGGATGAATAAGTCTCAATGACGGATAGG-3'	
32aa	5'-CACGTGGAGTGGG TTAATGCATTAATCCTTCCCAGCACAGGTCTTCTCATGTATACACTT-3'	
	5'-AAGTGTATACATG AGAAGACCTGTGCTGGGAAGGATTAATGCATTAACCCACTCCACGTG-3'	
K250Q	5'-CCTTAAGAATAAA CTG**C**AAGTGCGAACTGCC-3'	
	5'-GGCAGTTCGCACT T**G**CAGTTTATTCTTAAGG-3'	
R252L	5'-AAACTGAAAGTGC **T**AACTGCCTATCCG-3'	
	5'-CGGATAGGCAGTT **A**GCACTTTCAGTTT-3'	
H262A	5'-CATTGAGACTTAT T**GC**TGCTGTCAGAGGT-3'	
	5'-ACCTCTGACAGCA **GC**AATAAGTCTCAATG-3'	
K272I	5'-TGGCTGACCAACA **T**AGTTCCTATCAAA-3'	
	5'-TTTGATAGGAACT **A**TGTTGGTCAGCCA-3'	
I275K	5'-CCAACAAAGTTCC TA**A**GAAAAGACCCAGCACAGG-3'	
	5'-CCTGTGCTGGGTC TTTTC**T**TAGGAACTTTGTTGG-3'	
K276Q	5'-CAAAGTTCCTATC **C**AAAGACCCAGCACAGG-3'	
	5'-CCTGTGCTGGGTC TTT**G**GATAGGAACTTTG-3'	
R277I	5'-GTTCCTATCAAAA **T**ACCCAGCACAGGT-3'	
	5'-ACCTGTGCTGGGT **A**TTTTGATAGGAAC-3'	
KK	5'-AATGCATTAATCC TT**C**AGAAT**C**AACTGAAAGTGCGAACTGCCTAT-3'	
	5'-ATAGGCAGTTCGC ACTTTCAGTT**G**ATTCT**G**AAGGATTAATGCATT-3'	
KKKR	5'-ATCCTT**C**AGAAT**C** AACTG**C**AAGTGC**T**AACTGCCTATCCGTCATTGAGA-3'	
	5'-TCTCAATGACGGA TAGGCAGTT**A**GCACTT**G**CAGTT**G**ATTCT**G**AAGGAT-3'	
KR	5'-ACCAACAAAGTTC CTATC**C**AAA**T**ACCCAGCACAGGTCTTCTCATG-3'	
	5'-CATGAGAAGACCT GTGCTGGGT**A**TTT**G**GATAGGAACTTTGTTGGT-3'	
KKR	5'-TACTGGCTGACCA AC**C**AAGTTCCTATC**C**AAA**T**A-3'	
	5'-T**A**TTT**G**GATAGGA ACTT**G**GTTGGTCAGCCAGTA-3'	
KR2RK	5'-ACCAACAAAGTTC CTATCA**G**AA**A**ACCCAGCACAGGTCTTCTCATG-3'	
	5'-CATGAGAAGACCT GTGCTGGGT**T**TT**C**TGATAGGAACTTTGTTGGT-3'	

Table shows the oligonucleotide primer sets used for construction of the deletion and amino acid substitution mutants in ST8Sia IV. For the single and multiple amino acid substitutions, the substituted nucleotides are indicated in bold.

determined in membranes isolated from approximately 10^7 COS-1 cells essentially as described previously [10].

PolyST activity in membranes isolated from wild-type and mutated ST8Sia IV in COS-1 cells

The polyST assay was carried out using methods previously described for measuring this activity in embryonic chick brain membranes [10,31]. Briefly, frozen cell pellets were re-suspended in lysis buffer containing 50 mM MES buffer (pH 6.1), 10% glycerol, 10 μg/ml aprotinin, 4 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM PMSF and the cells disrupted in a dounce homogenizer. The homogenate was centrifuged at 1,000 × g for 10 min at 4°C to remove nuclei. The supernatant containing the membrane fraction was centrifuged at 150,000 × g for 30 min. Membrane pellets were re-suspended in the lysis buffer. The protein concentration was measured by Pierce's BCA™ protein assay kit. The expression level of the wild-type and mutants was confirmed by Western blot analysis of the membrane of fraction (50 mg protein) using anti-V5 antibody (Invitrogen). Approximately 200 μg of COS-1 membranes in a final volume of 200 μl were used to measure the polyST activity at 33°C. Each polyST incubation mixture contained 50 mM MES buffer (pH 6.1), 10% glycerol, 1 mM DTT, 10 mM $MnCl_2$ and 200 μM CMP-[^{14}C]-Neu5Ac (0.05 μCi/nmol). Thirty μl of the incubation mixture were spotted onto Whatman 3 MM paper at zero time and after 1 h and 2 h. The remaining 100 μl of the incubation mixture were divided into 50 μl aliquots and one was treated

with Endo-N for 1 h at 37°C. The other aliquot served as the Endo-N minus control and was incubated without Endo-N. After Endo-N digestion, 30 μl of each incubation mixture were spotted on Whatman 3 MM paper and subjected to descending paper chromatography [10]. In this assay, sialyl oligomers released from N-CAM by Endo-N migrate away from the origin, whereas sialylated N-CAM containing sialyl residues incorporated by the polyST, and any monoST activity, are chromatographically immobile and remain at the origin. The origins were cut out and the radioactivity in each was quantitatively determined by liquid scintillation counting. PolyST activity was determined by subtracting the amount of radioactivity remaining after Endo-N digestion from the Endo-N minus control. The latter represents the total amount of Sia that was incorportated by both the mono- and polySTs. Radioactivity remaining after Endo-N treatment represents the amount of [^{14}C]Sia that was incorporated by the monoSTs [10].

Immunofluorescence localization of wild-type and mutated ST8Sia IV proteins in COS-1 cells

COS-1 cells were plated on glass cover slips in 12-well plates and transfected with either the wild-type ST8Sia IV or the deletion and amino acid substitution mutants. Nineteen h post transfection, the cells were fixed at –20°C with methanol and blocked with 5% goat serum in PBS. ST8Sia IV was detected in these cells with anti-V5 antibody diluted 1:500, and Alexa 488 goat-anti-mouse IgG secondary antibody (Molecular Probes) diluted 1:1,000. Cover slips were mounted on glass slides using Vectashield (Molecular Probes). Cells were visualized and photographed using a Nikon Eclipse 800 microscope equipped with a digital camera.

Construction of a soluble form of ST8Sia II and ST8Sia IV with V5 and 6× his tags at the N-terminus

A DNA fragment encoding for a truncated form of ST8Sia IV, and lacking the N-terminal 46 amino acid sequence, was amplified by PCR using pcDNA3.1-PSTfV5His as a template, and the following primers: 5′-GCGATATCGGCCCTCGAGTCACTTGTCAATAGCTCTGA-3′ and 5′-CGTCTAGATTATTGCTTTACACACTTTCCTG-3′. The amplified DNA fragment was subcloned into pGEM-T Easy vector (Promega). The inserted fragment was removed by digestion with *Xba* I and *Eco*R V, and ligated into pcDNA3.1-PSTsV5His through the same site (pcDNA3.1-PSTs). The DNA fragment coding for V5 and 6× His epitope tags was amplified by PCR using pcDNA3.1 plasmid as a template and the following primers: 5′-TGGATATCATGGGTAAGCCTATCCCTAACCC-3′ and 5′-TGCTCGAGATGGTGATGGTGATGATGAC-3′. The DNA fragment coding V5 and 6× His epitope tags subcloned into pGEM-T Easy vector was removed by digestion with *Xho* I and *Eco*R V, and ligated into pcDNA3.1-ST8Sia IVs through the same site (pcDNA3.1-V5HisPSTs).

Construction of an expression plasmid of a soluble form of ST8Sia II with N-terminal V5 and His tags (pcDNA3.1-V5HisSTXs) was carried out as described recently [17].

Heparin inhibition of human ST8Sia II and ST8Sia IV: Reconstituted in vitro PolyST assay using soluble constructs of ST8Sia II and ST8Sia IV

Purification of the soluble constructs of ST8Sia II and ST8Sia IV used in the reconstituted *in vitro* polyST assay to test for heparin inhibition was carried out as previously described [17]. Purified ST8Sia II and ST8Sia IV (∼5–10 μg protein) were incubated with CMP-[^{14}C]-Neu5Ac (0.05 μCi/nmol) and FcN-CAM (0.6 mg protein) in 10 μl of 100 mM MES buffer (pH 6.1) containing 10 mM $MnCl_2$ at 37°C for 1 h. For the heparin inhibition studies, 10 μM of heparin (3 kDa) was added to the above incubation mixtures. PolyST activity was quantitatively determined by liquid scintillation counting, as described above.

Results and discussion

Identification of the polysialyltransferase domain (PSTD): Unique primary sequences with high content of basic amino acids are highly conserved in the polysialyltransferases

In previous studies we reported that a unique polybasic 32 amino acid sequence with a calculated pI∼12 was located immediately upstream of SM-S in the polySTs [10]. Alignment analyses for this domain within the sialyltransferases gene family is shown in Table 2.

As shown, the PSTD is well conserved between ST8Sia II and ST8Sia IV (81%), but is not present in ST8Sia I, III, V or VI, or in any of the monoSTs. There are conserved Val, Pro, and Trp residues in all six members of the ST8Sia family, however, but their relevance to polysialylation is not known. Of particularly note in the ST8Sia family, in contrast to the ST3Gal I-VI, the ST6GalNAc I-VI and the ST6Gal I and II families are two distinct structural features. First, with the exception of ST8Sia VI, they all have Lys-Arg residues at their C-terminus, which is at the juncture of the PSTD with the N-terminus of SM-S. As described below, these two residues and the adjacent Ile, are essential for polysialylation. Second, ST8Sia II and IV, the only two members of the family known to catalyze polysialylation, are rich in basic amino acids, containing 11 and 10 residues, respectively. This gives rise to their calculated pI values >12. While ST8Sia I has 7 basic amino acids (pI 12.24), this G_{D3}/G_{T3}

Table 2 Comparative alignment analysis and properties of the poly-basic 32 amino acid sequence in the PSTD of ST8Sia II and ST8Sia IV compared with the corresponding sequences in other members of the ST8Sia gene family (Panel A). Panel B compares the 32 amino acid sequences within the ST3Gal, ST6Gal and ST6GalNAc family members with the ST8Sia gene family.

A

ST8 family	amino acid residue #	32 amino acid sequence upstream of SM-S	basic amino acid	sialylation	accepter substrates
ST8Sia II	261-292	KHHVNVRTAYPSLRLLHAVRGYWLTNKVHIKR	11	poly-	NCAM (*N*-glycan)
ST8Sia IV	246-277	KNKLKVRTAYPSLRLIHAVRGYWLTNKVPIKR	10	poly-	NCAM (*N*-glycan)
ST8Sia I	241-272	DVGANQTVLFANP--NFLRSIGKFWKSRGIHAKR	7	mono-oligo-	ganglioside
ST8Sia III	267-298	HRGQLKVQLAWPGNIMQHVNR-YWKNKHLSPKR	10	mono-oligo-	ganglioside, glycoprotein, sialyloligosaccharide
ST8Sia V	267-298	DFESPQAVYYFHP--QYLVNVSRYWLSLGVRAKR	5	mono-	ganglioside
ST8Sia VI	289-320	ESKARQKVLFFHP--KYLKDLALFWRTKGVTAYR	9	mono-	glycoprotein (*O*-glycan)

B

ST family	amino acid residue #	32 amino acid sequence upstream of SM-S	basic/acidic amino acid	predicted pI	accession number
ST3Gal I	235-266	AKIRVKQDKILIYHPAFIKYVFDNWLQGHGRY	8/2	10.27	Q11201
ST3Gal II	245-276	SFLRVDKEKVQIYNPAFFKYIHDRWTEHHGRY	9/4	9.69	Q16842
ST3Gal III	268-299	EIRILNPYFIQEAAFTLIGLPFNNGLMGRGNI	2/2	7.08	Q11203
ST3Gal IV	227-258	KQIRILNPFFMEIAADKLLSLPMQQPRKIKQK	7/2	11.07	Q11206
ST3Gal V	251-282	RILNPVIIKETAFDILQYSEPQSRFWGRDKNV	5/4	9.37	Q9UNP4
ST3Gal VI	225-256	QIRILDPFIIRTAAYELLHFPKVFPKNQKPKH	8/2	10.61	AAH23312
ST6Gal I	289-320	PNQPFYILKPQMPWELWDILQEISPEEIQPNP	1/5	3.61	P15907
ST6Gal II	391-421	PNQPFYILHPKFIWQLWDIIQENTKEKIQPNP	4/3	7.39	AAH08680
ST6GalNAc I	487-518	RYLLLHPDFLRYMKNRFLRSKTLDGAHWRIYR	10/2	11.36	Q9NSC7
ST6GalNAc II	271-302	KFKLLHPDFISYLTERFLKSKLINTHFGDLYM	7/3	9.72	AAH40455
ST6GalNAc III	184-215	QIYVTTEKRMSYCDGVFKKETGKDRVQSGSYL	6/4	9.21	AAH59363
ST6GalNAc IV	180-211	QVYTFTERMMAYCDQIFQDETGKNRRQSGSFL	4/4	6.49	AAH36705
ST6GalNAc V	200-231	KAFMITRHKMLQFDELFKQETGKDRKISNTWL	8/4	10.33	AAH01201
ST6GalNAc VI	211-241	EAYAVSPGRMRQFDDLFRGETGKDREKSHSWL	7/6	7.75	AAH06564
ST8Sia I	241-272	DVGANQTVLFANPNFLRSIGKFWKSRGIHAKR	7/1	12.24	Q92185
ST8Sia II	261-292	KHHVNVRTAYPSLRLLHAVRGYWLTNKVHIKR	11/0	12.08	Q92186
ST8Sia III	267-298	HRGQLKVQLAWPGNIMQHVNRYWKNKHLSPKR	10/0	12.25	O43173
ST8Sia IV	246-277	KNKLKVRTAYPSLRLIHAVRGYWLTNKVPIKR	10/0	12.10	Q92187
ST8Sia V	267-298	DFESPQAVYYFHPQYLVNVSRYWLSLGVRAKR	5/2	9.80	O15466
ST8Sia VI	289-320	ESKARQKVLFFHPKYLKDLALFWRTKGVTAYR	9/2	10.80	P61647

In panel A, the alignment analysis of the amino acid sequences between members of the human ST8Sia gene family was carried out using the DNASIS ver. 3 software package and the entire amino acid sequence for each family member. The Swissprot accession numbers for each ST8Sia gene family members were: Q92185 for ST8Sia I; Q92186 for ST8Sia II; O43173 for ST8Sia III; Q92187 for ST8Sia IV; O15466 for ST8Sia V and P61647 for ST8Sia VI. The conserved amino acids in each sialyltransferase are denoted by shading, while the basic residues are shown in bold. The type of sialylation reaction and acceptor substrate for each sialyltransferase is also noted.

Panel B compares the 32 amino acid sequences upstream of SM-S in the ST8Sia gene family with the corresponding segment in the ST3Gal, ST6Gal and ST6GalNAc gene families. The Swissprot accession numbers, predicted pI and the ratio of basic to acidic amino acids in each transferase are also compared. The predicted pI values were calculated using a function of DNASIS ver. 3.0 software (Hitachi Software Engineering Co.).

synthase catalyzes only mono- or disialylation of gangliosides [32, 33]. Angata *et al*. reported that ST8Sia III, which has 10 basic amino acids (pI 12.25) but lacks the conserved PSTD, was capable of synthesizing oligo/polySia chains on *N*-glycans of N-CAM or sialylated oligosaccharide *in vitro* [34]. ST8Sia III also lacks the Ile residue in the triad at the juncture of the PSTD with SM-S. ST8Sia V is interesting because it is the only member of the ST8Sia family

that catalyzes oligosialylation of gangliosides [35], yet its 32 amino acid stretch adjacent to the SM-S contains only 5 basic residues with a pI 9.8. While it contains the Lys_{276} and Arg_{277} residues at the juncture with SM-S, it lacks the Ile residue in the triad that is required for polysialylation in ST8Sia II and ST8Sia IV. A phylogenetic analysis between sialyltransferases reported by Harduin-Lepers *et al*. showed that ST8Sia II, III and IV were grouped in the same phylogenetic family [36]. This finding is also consistent with our postulate that the high content of basic amino acids in this polybasic domain may be related to oligo-polysialylation activity. While recent studies have shown that ST8Sia II and ST8Sia IV can both synthesize polySia chains extending to ∼400 Sia residues (DP∼400) [17], there are no structural studies showing that ST8Sia III can catalyze polysialylation of N-CAM. To test experimentally our hypothesis that the polybasic PSTD in the polySTs was required for polysialylation, we constructed by site-directed mutagenesis a series of deletion, single and multiple amino acid substitutions in human ST8Sia IV and determined the effect of these mutations on polyST activity and intracellular localization.

Deletions in the polybasic PSTD in ST8Sia IV abolished polysialyltransferase activity

To determine if the polybasic PSTD was essential for polysialylation, four deletion mutants in ST8Sia IV were generated (Fig. 1). The N10 and C10 mutants each had ten amino acids deleted from the amino- and carboxyl termini of the PSTD, respectively. The C15 construct had fifteen amino acid residues deleted from its carboxyl terminus, while the 32 amino acid mutant had the entire PSTD deleted. The polyST activity in membranes isolated from wild-type COS-1 cells expressing the recombinant ST8Sia IV was linear for at least 2 h. Based on the transfection efficiency and the specific activity of the expressed ST8Sia IV, approximately 500–750 pmol of Sia were incorporated per mg protein per h in the wild-type control enzyme (Fig. 2A).

Essentially all of this Sia was incorporated into α2-8-linked polySia chains, based on its sensitivity to depolymerization by Endo-N. Endo-N is an enzyme useful for confirming the presence of α2-8-linked oligo- or polySia chains with a DP of ∼5 or more in glycoconjugates [10,31]. In contrast to the wild-type ST8Sia IV, <10 pmol of Sia were incorporated per h in the deletion mutants, showing that the PSTD was required for polysialylation of N-CAM, or for the proper folding of the transferase. The low level of activity shown for the mutants represented background, based on the same level seen in non-transfected (vector only) control cells.

Intracellular localization of ST8Sia IV in deletion mutants

Based on co-transfection of COS-1 cells with green fluorescent protein and an anti-V5 mAb specific for the V-5 tagged ST8Sia IV protein, the expression level of ST8Sia IV in all four deletion mutants described above was shown by Western blotting to be approximately the same as wild-type ST8Sia IV. Immumostaining using the anti-V5 antibody showed that wild-type ST8Sia IV was predominately expressed in the perinuclear regions, consistent with its localization in the Golgi apparatus [8]. In contrast, immunostaining of the deletion mutants showed less perinuclear staining with more diffuse staining throughout the cytoplasm (Fig. 2B). While these results supported the conclusion that the deletion mutants were still capable of exporting some ST8Sia IV to the Golgi apparatus, they also suggested that deletion of all or part of the PSTD may have caused some misfolding of the enzyme leading to the loss in polyST activity [37]. Because of the possible ambiguity that these findings introduced in the interpretation of the essentiality of the PSTD in polysialylation, we carried out site-directed substitutions within the PSTD to determine the importance of specific amino acid residues in ST8Sia IV that are required for polysialylation.

Site-directed substitutions in the polybasic PSTD in ST8Sia IV identified key amino acids essential for polysialylation of N-CAM

To identify key amino acids in the polybasic PSTD in ST8Sia IV that are required for polysialylation of N-CAM, we constructed a series of single amino acid substitutions within the domain by site-directed mutagenesis, as shown in Table 3.

In the single amino acid substitutions, Lys_{250}, R_{252}, H_{262}, K_{272}, I_{275}, K_{276} or R_{277} were substituted with Gln (mutant K250Q), Leu (mutant R252L), Ala (mutant H262A), Ile (mutant K272I), Lys (mutant I275K), Gln (mutant K276Q) or Ile (mutant R277I). Except for the I275K mutant, a basic amino acid was substituted with a non-basic amino acid. In I275 K, an Ile residue was substituted for Lys.

Using membrane fractions from COS-1 cells transfected with the substitution mutants, the level of polyST activity was determined as described under "*Materials and methods.*"

As shown in Fig. 3A, the substitution of Arg_{252}, Arg_{272}, $Ile_{275,}$ or Arg_{277} with Leu, Ile, Lys, or Ile caused a reduction of ∼75% or greater in the level of polyST activity. Particularly striking was the finding that Lys_{272}, Ile_{275}, Ile_{276} and Arg_{277}, all residues within the C-terminal segment of the PSTD, and even Arg_{252}, a residue more distal from the C-terminus, were of key importance for N-CAM polysialylation. These results thus highlighted the structural importance of Lys_{272} and the

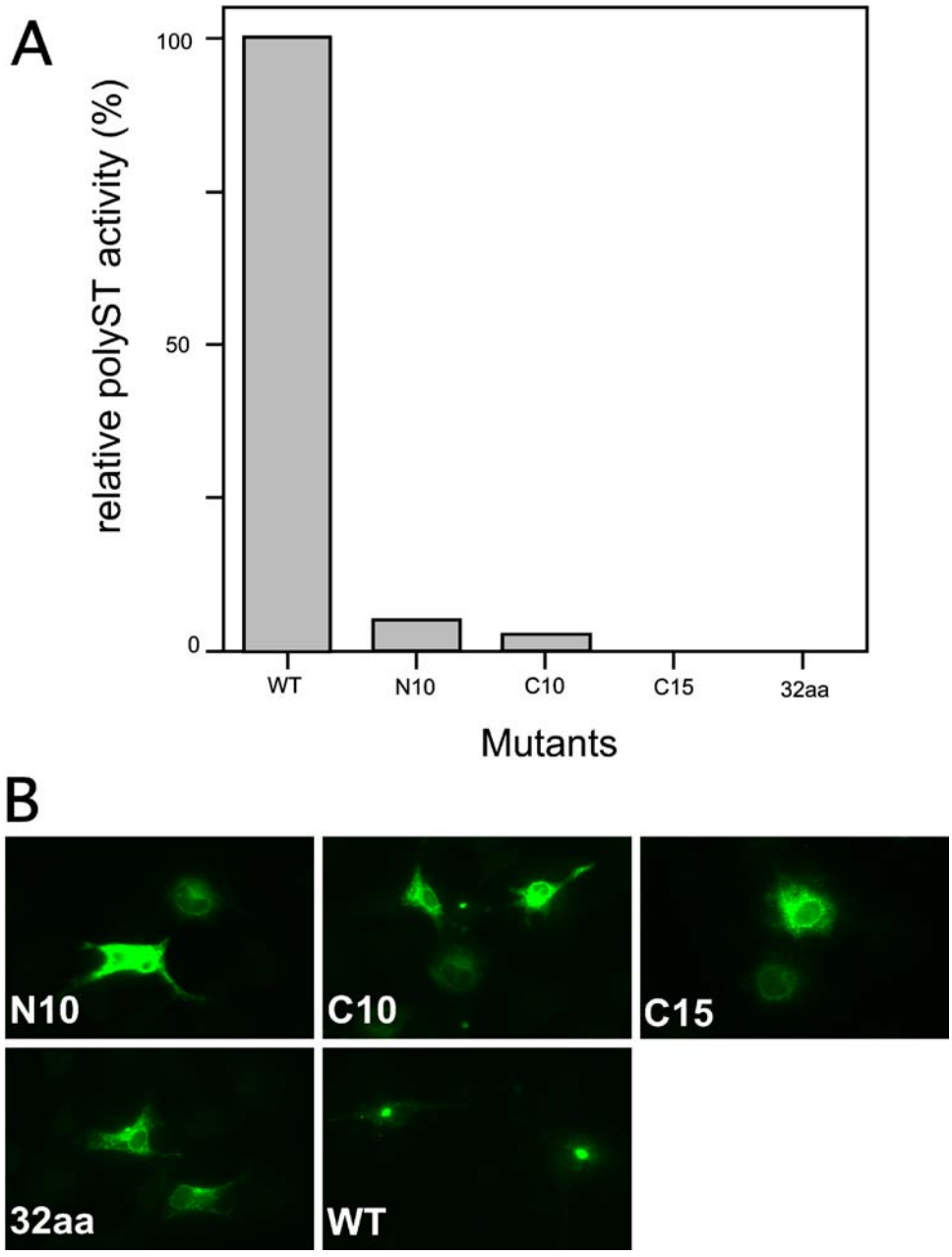

Fig. 2 PolyST activity and intracellular localization of V5 epitope-tagged wild-type and deletion mutants within the PSTD of ST8Sia IV expressed in COS-1 cells. PolyST activity in membranes from COS-1 cells transiently expressing the V5 epitope-tagged wild type or deletion mutants within the PSTD of ST8Sia IV was carried out as described under "*Materials and methods*" (panel A). The average specific activity from three independent experiments was 427 ± 50 pmol/h/mg protein. This represents the 100% "relative polyST activity" value shown on the coordinate. The wild-type monoST activity was 200 ± 40 pmol/h/mg protein. For the immunofluorescence localization studies (panel B), COS-1 cells were permeabilized, fixed, and incubated with the anti-V5 antibody, followed by incubation with Alexa 488-conjugated goat anti-mouse IgG antibody, as described under "*Materials and methods*".

triad, Ile_{275}, Lys_{276} and Arg_{277} in the C-terminal juncture of the PSTD with the N-terminus of SM-S for polyST activity. The Arg_{252} mutant demonstrated further that a basic residue distal to the SM-S juncture was also required for polysialylation. Somewhat surprising was the finding that replacing His_{262} with Ala in the middle of the PSTD reduced activity by ∼40–50% (mutant H262A) and that replacing Lys_{250} with Gln, five residues in from the N-terminus of the PSTD, resulted in ∼25% loss in activity (mutant K25OQ). These results thus demonstrated that multiple amino acid residues in the middle and N-terminus of the PSTD were also essential to insure proper polysialylation of N-CAM. Finally, while the substitution of Ile_{275} to Lys (mutant I275K) increased the basicity of the PSTD, the polysialylation activity was only ∼6% of wild-type. This suggested that the total number of basic amino acids residues in the domain was not the sole or only factor responsible for determining ST8Sia IV activity, as the singly substituted mutants also confirmed.

Immunostaining of the mutated PSTD expressed in COS-1 cells was carried out using the anti-V5 antibody, as described under "*Materials and methods.*" These studies showed that all of the mutants gave a perinuclear staining pattern similar to the wild-type ST8Sia IV (Fig. 3B). This finding confirmed that all of the singly substituted mutated proteins were folded properly so as to exit the ER and localize in the Golgi apparatus. This result is in accord with the finding that the molecular

Table 3 Sequences comparison in the wild-type PSTD of ST8Sia IV with the single or multiple substituted mutants.

mutants	PSTD sequences (246-277)	replaced a.a.
WT	**K**N**K**L**K**V**R**TAYPSL**R**LI**H**AV**R**GYWLTN**K**VPI**KR**	
K250Q	**K**N**K**L[Q]V**R**TAYPSL**R**LI**H**AV**R**GYWLTN**K**VPI**KR**	Lys_{250} >Gln
R252L	**K**N**K**L**K**V[L]TAYPSL**R**LI**H**AV**R**GYWLTN**K**VPI**KR**	Arg_{252} > Leu
H262A	**K**N**K**L**K**V**R**TAYPSL**R**LI[A]AV**R**GYWLTN**K**VPI**KR**	His_{262} > Ala
K272I	**K**N**K**L**K**V**R**TAYPSL**R**LI**H**AV**R**GYWLTN[I]VPI**KR**	Lys_{272} > Ile
I275K	**K**N**K**L**K**V**R**TAYPSL**R**LI**H**AV**R**GYWLTN**K**VP[K]**KR**	Ile_{275} > Lys
K276Q	**K**N**K**L**K**V**R**TAYPSL**R**LI**H**AV**R**GYWLTN**K**VPI[Q]**R**	Lys_{276} > Gln
R277I	**K**N**K**L**K**V**R**TAYPSL**R**LI**H**AV**R**GYWLTN**K**VPI**K**[I]	Arg_{277} > Ile
KK	[Q]N[Q]L**K**V**R**TAYPSL**R**LI**H**AV**R**GYWLTN**K**VPI**KR**	Lys_{246} > Gln, Lys_{248} > Gln
KKKR	[Q]N[Q]L[Q]V[L]TAYPSL**R**LI**H**AV**R**GYWLTN**K**VPI**KR**	Lys_{246} > Gln, Lys_{248} > Gln, Lys_{250} > Gln, Arg_{252} > Leu
KR	**K**N**K**L**K**V**R**TAYPSL**R**LI**H**AV**R**GYWLTN**K**VPI[QI]	Lys_{276} > Gln, Arg_{277} > Ile
KKR	**K**N**K**L**K**V**R**TAYPSL**R**LI**H**AV**R**GYWLTN[Q]VPI[QI]	Lys_{272} > Gln, Lys_{276} > Gln, Arg_{277} > Ile
KR2RK	**K**N**K**L**K**V**R**TAYPSL**R**LI**H**AV**R**GYWLTN**K**VPI[RK]	Lys_{276} > Arg, Arg_{277} > Lys

The basic amino acid residues in wild-type and each mutant is shown in bold. The mutated amino acids in each mutant are indicated by boxed letters.

mass of the mutated proteins, as determined by Western blot analysis, and their level of expression as determined by anti-V5 staining, were essentially identical to wild-type ST8Sia IV (results not shown).

Reverse and multiple amino acid substitutions in the polybasic PSTD in ST8Sia IV confirmed the importance of this domain for N-CAM polysialylation

Further evidence in support of the essentiality of the polybasic PSTD in polysialylation came from reversing amino acid residues in the domain and in multiple amino acid substitutions. To test the effect of these mutations, substitutions were made in ST8Sia IV in which 2–4 basic amino acids at the N- or C-terminus were replaced by non-basic residues. The following substitutions were made: (1) In mutant KK, Lys_{246} and Lys_{248} were both switched to Gln; (2) In mutant KKKR, $Lys_{246,}$ Lys_{248} and Lys_{250} were replaced with Gln and Arg_{252} was replaced with Leu; (3) In mutant KR, Lys_{276} and Arg_{277} were changed to Gln and lle, respectively; (4) In mutant KKR, Lys_{272}, Lys_{276} and Arg_{277} were changed to Ile, Gln and lle, respectively. An interesting mutant in which Arg_{276} and Lys_{277} were switched to Lys_{276} and Arg_{277} was also generated (mutant KR2RK).

As shown in Fig. 4B, all of the above mutants after transfection into COS-1 cells localized to the Golgi apparatus. PolyST activity was quantitatively determined in membranes isolated from these cells as described under "*Materials and methods.*" As shown in Fig. 4A, the KK mutant retained ~80% of the wild-type ST8Sia IV activity. PolyST activity in all of the other mutants, however, was reduced to essentially background level (nontransfected control). These results, and those from the single substituted mutants, clearly shows that Lys_{250}, Arg_{252}, Lys_{272}, Ile_{275}, Lys_{276} and Arg_{277} were key amino acid residues within the PSTD of ST8Sia IV that are required for polysialylation. Of particular note is the substitution of a single basic amino acid at the C-terminus of the PSTD. The single substituted mutants, K276Q and R277I still retained about 30~40% of the activity of wild-type ST8Sia IV. The double substituted mutant, Lys_{276} and Arg_{277} (mutant KR), however had essentially no activity above background. That the switching of these residues in mutant KR2RK also reduced polyST activity suggested that not only is the number of basic amino acid residues in the PSTD important for polysialylation, as in the I275K mutant, but so are their positions within the PSTD. These results thus provide further evidence that multiple amino acid domains within the ST8Sia IV protein are required for N-CAM polysialylation.

Inhibition of human polysialyltransferases by heparin

In a previous study we showed that the polyST activity in embryo chick brain was non-competitively inhibited by heparin with a Ki of ~9 μM [10]. Heparin is an acidic polysaccharide consisting of D-GlcNAc, D-GlcUA and L-IdUA. We

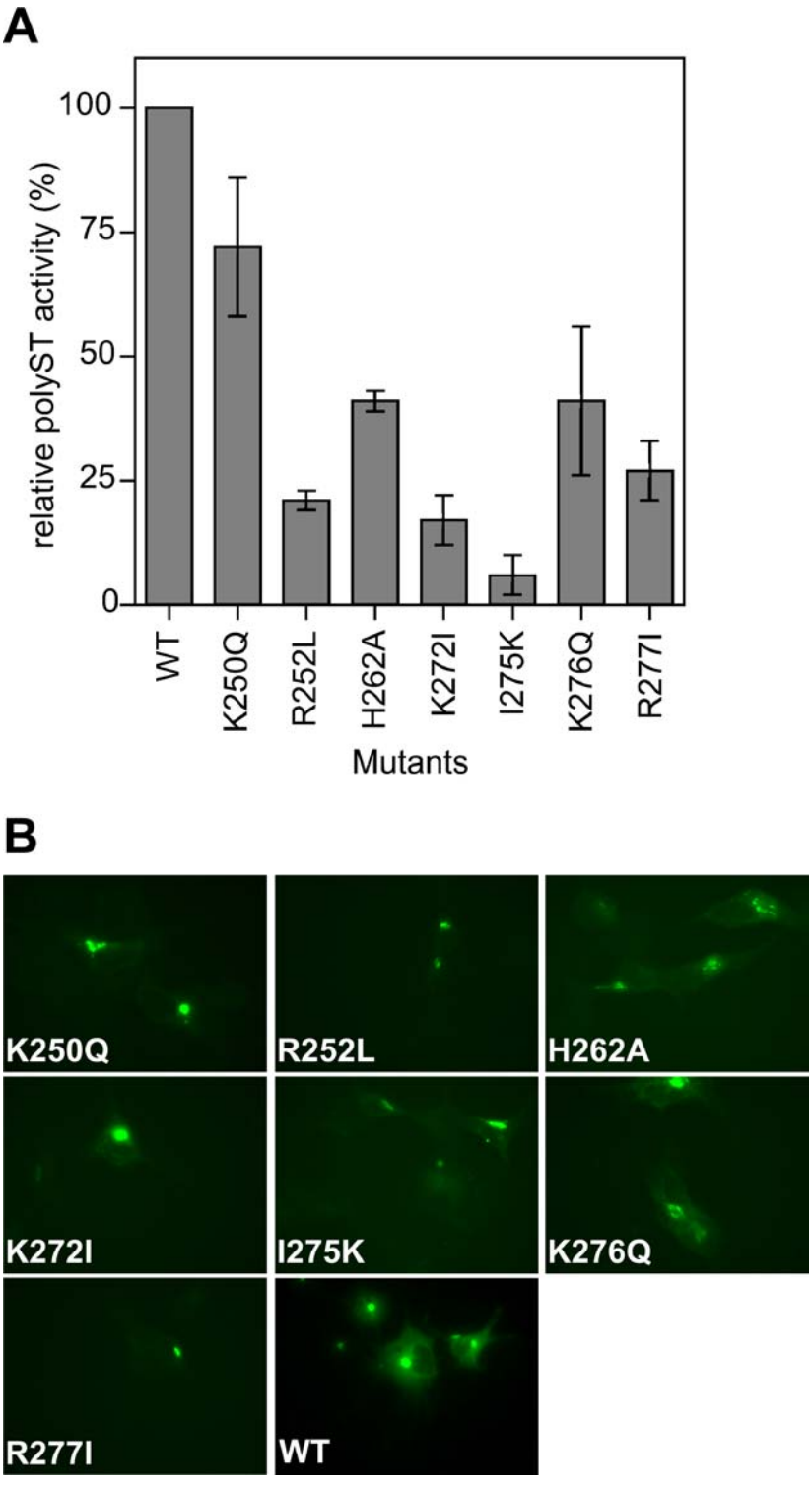

Fig. 3 PolyST activity and Golgi localization of V5 epitope-tagged wild-type and single amino acid substituted mutants within the PSTD of ST8Sia IV in COS-1 cells. PolyST activity in membranes from COS-1 cells transiently expressing the V5 epitope-tagged wild type or the single amino acid substituted mutants within the PSTD of ST8Sia IV was carried out as described under "*Materials and methods*" (panel A). The average specific activity from three independent experiments was 940 pmol/h/mg protein. This represents the 100% "relative polyST activity" value shown on the coordinate. The monoST activity was 280 ± 60 pmol/h/mg protein. The immunofluorescence localization studies (panel B) were carried out as described in the legend to Fig. 2, and under "*Materials and methods*".

also determined that the chick brain polyST activity was inhibited by other polyanions, including chondroitin sulfate, hyaluronic acid and tRNA (unpublished results). This led us to suggest that these polyanions may inhibit polysialylation by binding to the polybasic domain of the polySTs that may be involved in recognizing polySia chains. To confirm that heparin also inhibited the human polySTs, our reconstituted *in vitro* polyST assay was carried out using recombinant ST8Sia II and ST8Sia IV soluble forms transiently expressed in COS-7 cells, as recently described [17].

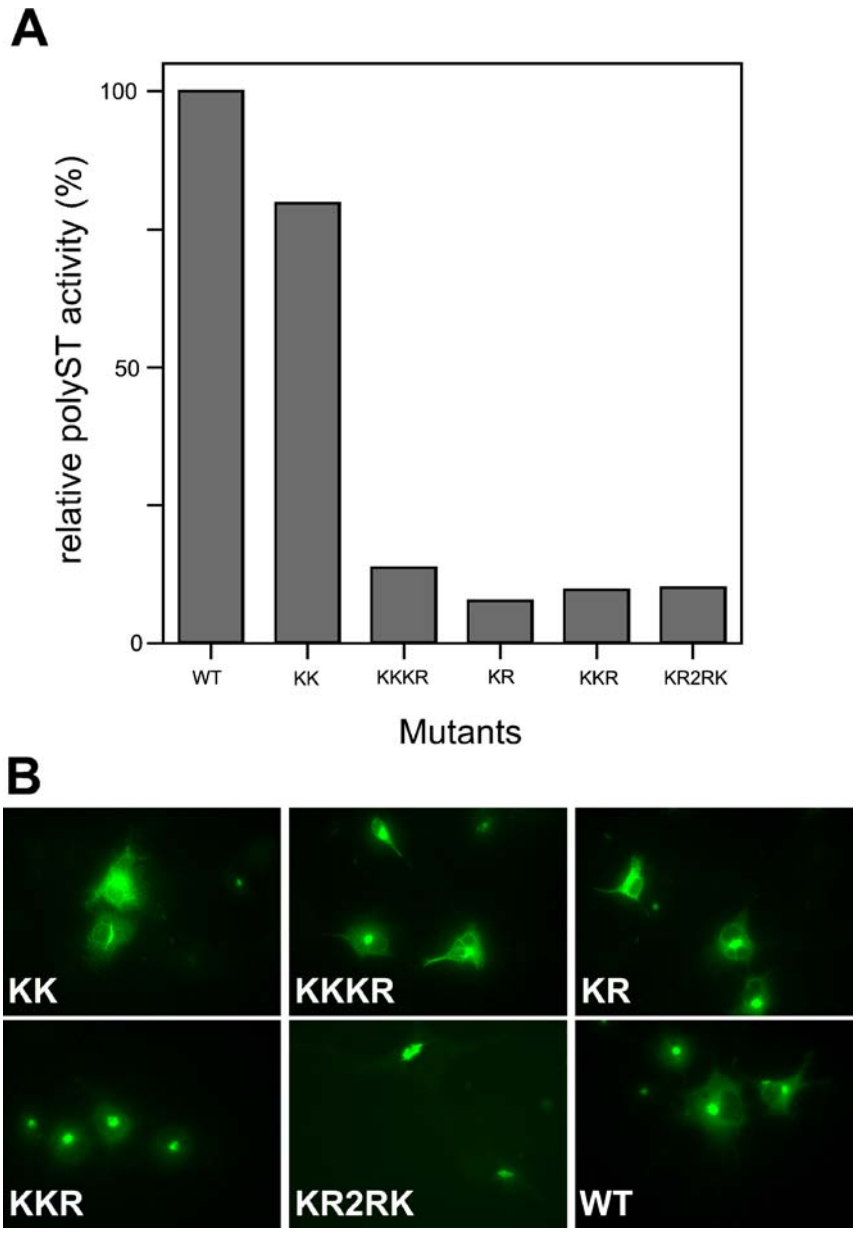

Fig. 4 PolyST activity and Golgi localization of V5 epitope-tagged wild-type and multiple amino acid substituted mutants in the PSTD of ST8Sia IV. PolyST activity in membranes from COS-1 cells transiently expressing the V5 epitope-tagged wild type or multiple amino acid substituted mutants within the PSTD of ST8Sia IV was carried out as described under "Materials and methods" (panel A). The average specific activity from three independent experiments was 940 pmol/h/mg protein. This represents the 100% "relative polyST activity" value shown on the coordinate. The wild-type monoST activity was 280 ± 60 pmol/h/mg protein. The immunofluorescence localization studies (panel B) were carried out as described in the legend to Fig. 2, and under "*Materials and methods*".

As shown in Table 4, polysialylation activities for both ST8Sia II and ST8Sia IV were inhibited ∼90% by 10 μM heparin. These results thus supported our earlier findings and shows that the human ST8Sia II and ST8Sia IV, like their mouse homologues, are extremely sensitive to heparin inhibition. As noted by Varki *et al.*, heparin can inhibit tumor metastasis in animal models and its use to treat human cancers has shown promise [38,39]. While the focus molecule of their interest was on sialylated, fucosylated mucins, it is also relevant to note that polySia is an oncodevelopmental surface anti-adhesive factor whose overexpression has been associated with metastasis in several human cancers [1,2]. It seems possible, therefore, that the beneficial effects of heparin in the treatment of some human cancers could, in part, result from its inhibition of the polySTs that are expressed in highly malignant tumors.

Concluding remarks

Despite recent advances in the study of the α2-8-linked polySTs, there is a paucity of information about the molecular mechanism whereby these enzymes catalyze polySia chain initiation, polymerization and termination. While an α2,8-KDN transferase that terminates elongation by capping α2-8-linked oligo-polySia chains in trout egg polysialoglycoproteins has been described [40], relative few studies have addressed the question of what structural features are present

Table 4 Heparin inhibition of human recombinant ST8Sia II and ST8Sia IV activities.

Heparin	0 μM	10 μM
	Relative activity (%)	
ST8Sia IV	100	7
ST8Sia II	100	8

Reconstituted polyST assays using the recombinant soluble forms of human ST8Sia II and ST8Sia IV were carried out in the presence or absence of 10 μM heparin, as described under "*Materials and methods.*" The specific activities for ST8Sia II and ST8Sia IV were 1800 and 1500 pmol/h/mg protein, respectively. These specific activities represents the 100% "relative activity" values shown in the table.

within the polyST gene family of polymerases that directs or facilitates the processive mechanism of chain polymerization. Thus, an unresolved problem of fundamental importance is what structural features make an α2,8-monoST an α2,8-polyST? Based on earlier gene sequence gazing analysis, we identified a polybasic sequence of 32 amino acids that was unique to ST8Sia II and ST8Sia IV [10]. This sequence, now designated the "*polysialyltransferase domain*" (PSTD), has a calculated pI of ~12, and is located immediately upstream of the SM-S. Based on the polybasic nature of this domain, we proposed earlier that it may function to bind the polyanionic polySia chains. This, it was suggested, may help ensure fidelity of polysialylation by tethering the growing nascent chains to the enzyme during the processive addition of new Sia residues to the non-reducing termini during the polymerization reaction [10]. To test experimentally the role that the PSTD in ST8Sia IV may play in N-CAM polysialylation, we used site-directed mutagenesis to generate a series of deletion mutants in which all or a segment of the 32 mer was deleted. These studies have now shown that complete or partial amino acid deletions within the PSTD completely abolishes polyST activity in ST8Sia IV. Because some ST8Sia IV appeared to remain in the endoplasmic reticulum in the deletion mutants, this created ambiguity in assessing the importance of the PSTD in polysialylation. This ambiguity was clarified by using single and multi-site substitutions within the PSTD. As described herein, immunofluorescence localizations studies showed that all of the substitution mutants were properly folded, based on their ability to traverse the secretory pathway and localize in the Golgi apparatus. PolyST activity measurements in these mutants clearly identified the importance of the PSTD as an essential domain that was obligatorily required for polySia chain synthesis. The substitution mutants also allowed us to identify the essentiality of the Ile_{275}, Lys_{276} Arg_{277} triad in the C-terminus region of ST8Sia IV that was required for polysialylation. This region is at the juncture with the N-terminus of SM-S. The fact that just switching Lys_{276} and Arg_{277} (mutant KR), which did not change the isoelectric point of the PSTD, completely abolished polyST activity attests to the importance of these residues and their location within the PSTD for activity. It seems probable that the triad residues in the C-terminus may actually play a critical role in catalysis. Finally, it was unexpected to find that even subtle substitutions in the middle and N-terminal region of the PSTD influenced polysialylation. This finding is in accord with those of Angata et al. who showed using chimeric construct between ST8Sia IV and ST8Sia II and ST8Sia III that polyST activity was not determined by a single domain sequence within the enzyme. The finding that deletion or substitutions within the stem region caused a marked reduction in polysialylation is a finding similar to what we report here. It was also a surprise to discover that heparin inhibited the human ST8Sia II and ST8Sia IV activities at μM concentrations, similar to the heparin inhibition found with the embryonic chick brain polySTs [10]. The polyST "targets" may thus provide a mechanistic explanation for why heparin has shown potential in treating some malignant human cancers [38,39].

Our future studies will test directly the possible tethering function of the PSTD within the polySTs. Domain segments or substitution of single amino acids within the PSTD will also be swapped with the corresponding segments in the 32 mer from an α2,8-mono- oligoSTs to determine if we can convert a monoST into a polyST. Deletion analysis will also be used to determine if we can convert a polyST into a monoST.

Acknowledgments This paper is dedicated to the memory of Professor Yasuo Inoue, a gifted and innovative scholar, and to his commitment to top-quality, creative science. The glycobiology community owes him a great debt, and we shall miss him. Fortunately, his many seminal contributions will live on. We thank Drs. Jean Ye, Sandra Reynoso-Paz and Mi-Sun Won for assistance during the early phase of this study. This research was supported in part by grants from the National Institutes of Health (GM 55703) and the University of California TRDRP (Grant #12RT-0254) to FAT.

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