Sequences Prior to Conserved Catalytic Motifs of Polysialyltransferase ST8Sia IV Are Required for Substrate Recognition*  
Joseph L. Zapater and Karen J. Colley  
From the Department of Biochemistry and Molecular Genetics, University of Illinois, College of Medicine, Chicago, Illinois 60607

Background: The polysialyltransferase, polysialylate, selects a group of proteins.  
Results: Substrate recognition and polysialylation are reduced when basic residues in a noncatalytic region of ST8SiaIV/PST are replaced.  
Conclusion: Specific residues in a polysialyltransferase polybasic region are critical for substrate recognition.  
Significance: Understanding the mechanism of protein-specific polysialylation will allow for the modulation of this process during development and disease.

Polysialic acid on the neural cell adhesion molecule (NCAM) modulates cell-cell adhesion and signaling, is required for proper brain development, and plays roles in neuronal regeneration and the growth and invasiveness of tumor cells. Evidence indicates that NCAM polysialylation is highly protein-specific, requiring an initial polysialyltransferase-NCAM protein-protein interaction. Previous work suggested that a polybasic region located prior to the conserved polysialyltransferase catalytic motifs may be involved in NCAM recognition, but not overall enzyme activity (Foley, D. A., Swartzentruber, K. G., and Colley, K. J. (2009) J. Biol. Chem. 284, 15505–15516). Here, we employ a competition assay to evaluate the role of this region in substrate recognition. We find that truncated, catalytically inactive ST8SiaIV/PST proteins that include the polybasic region, but not those that lack this region, compete with endogenous ST8SiaIV/PST and reduce NCAM polysialylation in SW2 small cell lung carcinoma cells. Replacing two polybasic region residues, Arg82 and Arg93, eliminates the ability of a full-length, catalytically inactive enzyme (PST H331K) to compete with SW2 cell ST8SiaIV/PST and block NCAM polysialylation. Replacing these residues singly or together in ST8SiaIV/PST substantially reduces or eliminates NCAM polysialylation, respectively. In contrast, replacing Arg82, but not Arg93, substantially reduces the ability of ST8SiaIV/PST to polysialylate neuropilin-2 and SynCAM 1, suggesting that Arg82 plays a general role in substrate recognition, whereas Arg93 specifically functions in NCAM recognition. Taken together, our results indicate that the ST8SiaIV/PST polybasic region plays a critical role in substrate recognition and suggest that different combinations of basic residues may mediate the recognition of distinct substrates.

The mammalian polysialyltransferases (polySTs), STX and ST8Sia IV (PST), catalyze the synthesis of linear homopolymers of α2,8-linked sialic acid, called polysialic acid (polySia), on the termini of N- or O-linked glycans found on a small group of glycoproteins (1–4). These glycoprotein substrates include the neural cell adhesion molecule (NCAM) (5), neuropilin-2 (NRP-2) (6), synaptic cell adhesion molecule 1 (SynCAM 1) (7), the α chain of the voltage-sensitive sodium channel (8), a small proportion of the CD36 scavenger receptor found in human milk (9), and the polySTs themselves, which can polysialylate their own N-glycans (autopolysialylation) (10, 11). The limited number of polyST substrates, the observation that the polySTs polysialylate N-glycans attached to NCAM more efficiently than free glycans (12, 13), the absence of any unique features on the modified core glycan structures (14–16), as well as data from our laboratory (17–19), suggest that polysialylation is highly protein-specific and requires an initial protein-protein interaction between the polySTs and their substrates.

The most abundant polysialylated protein is NCAM. NCAM engages in both heterophilic and homophilic interactions that allow for cell-cell adhesion and signal transduction (reviewed in Refs. 20 and 21). NCAM consists of five immunoglobulin-like domains (Ig1–5), two fibronectin type III repeats (FN1 and FN2), a transmembrane region, and a cytosolic tail (NCAM140 and NCAM180) (supplemental Fig. S1) or a glycosyl-phosphatidylinositol anchor (NCAM120) (22). The polySTs add polySia to two N-glycans in the Ig5 domain of NCAM (23). The addition of long negatively charged polySia chains to NCAM glycans allows for the coordination of water molecules and an increase in the hydrodynamic radius of the protein (24). This leads to an increase in intermembrane repulsion (25) and pre-

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1 The abbreviations used are: polyST(s), polysialyltransferase(s); STX, ST8Sia II or sialyltransferase X; PST, ST8Sia IV or polysialyltransferase-1; NCAM, neural cell adhesion molecule; NRP-2, neuropilin-2; SynCAM 1, synaptic cell adhesion molecule 1; Ig1, immunoglobulin-like domain; FN, fibronectin type III repeat of NCAM; polySia, polysialic acid; SML, large sialyl motif; SMS, small sialyl motif; SMVS, very small sialyl motif; PSTD, polysialyltransferase domain; PBR, polybasic region; TRITC, tetramethylrhodamine isothiocyanate.
vents both NCAM-dependent and NCAM-independent cell-cell adhesion (26, 27). In addition, polySia was recently demonstrated to bind and sequester neurotrophic factors and neurotransmitters and this ability could impact signaling and contribute to its various roles during development and in the adult animal (28, 29).

Polysialylated NCAM is critical for neurite outgrowth, axon guidance and pathfinding, synaptic plasticity, and consequently plays roles in nervous system development, learning, memory formation, and neuronal regeneration (reviewed in Refs. 24 and 30–33). PolySia is first expressed on embryonic day 8–8.5 in the mouse, remains expressed throughout prenatal development (33), then disappears from a majority of neural regions 3 weeks after birth (34), coinciding with expression patterns of PST and STX (35). Simultaneous deletion of STX and PST to generate mice lacking all polySia revealed that polySia is critical for brain development (36). These mice exhibit abnormalities in major brain fiber tracts, hydrocephalus, impaired postnatal growth, and died within 3–4 weeks after birth. Subsequent deletion of NCAM reversed these effects, suggesting that without polySia, there is an aberrant gain of NCAM function causing abnormal neuronal migration and differentiation and altered nervous system development (36). In the adult, polySia expression is restricted to distinct regions of the brain, such as the olfactory bulb, hippocampus, and hypothalamus (37–42), where it allows the proper formation of new neuronal tracts. Transient up-regulation of polySia in damaged peripheral neurons is important for their regeneration and myelination (43). Interestingly, aberrantly high polyST and polySia expression has been noted in the late stages of several human cancers, including neuroblastoma, malignant astrocytoma, and small cell lung carcinoma, where it is suggested to enhance metastatic potential and invasiveness (44–49).

The precise mechanism by which the polySTs add polySia to NCAM is not fully understood. Current evidence suggests that the polysialylation of NCAM is highly protein-specific, requiring an initial recognition step between the enzymes and NCAM. We found that the NCAM FN1 domain is necessary for the polysialylation of N-glycans on the adjacent Ig5 domain (17). Additional studies highlighted the importance of an acidic surface patch in polyST recognition and binding (18, 50, 51). These observations led us to ask what complementary sequences of the polySTs mediate NCAM recognition.

The polySTs, PST and STX, are Golgi-localized glycosyltransferases that synthesize polySia chains extending to ~60 sialic acid units in mouse brain and over 100 units in human tumor cells (52, 53). The polySTs are type II membrane proteins with short N-terminal cytoplasmic tails, single pass transmembrane regions, and large C-terminal regions that include a membrane proximal stem region and a catalytic domain (5). Four conserved sequences have been identified in the catalytic domains of all sialyltransferases, including the polySTs (see Fig. 1). The large sialyl motif (SML) is thought to bind to the donor substrate, CMP-sialic acid (54), whereas the small sialyl motif (SMS) is believed to bind to both CMP-sialic acid and the acceptor glycan (55). The role of the very small sialyl motif (SMVS) is still unclear, however, mutation of a conserved histidine residue in this region (His311 in PST, His346 in STX) abolishes PST catalytic activity (56, 57). Motif III is a sequence of four amino acid residues located between the SMS and SMVS that may also play a role in recognizing the acceptor glycans (58).

Angata et al. (59) used chimeric polyST proteins to pinpoint residues critical for NCAM polysialylation. Their work suggested that PST residues 62–127 (prior to SML) and possibly 194–267 (between SML and SMS) are required for NCAM polysialylation and potentially substrate recognition. These results allowed the identification of two conserved polyST regions that are involved in NCAM polysialylation. Troy and colleagues (60) characterized a stretch enriched in basic residues termed the polysialyltransferase domain (PSTD) (residues 246–277 in PST and 261–292 in STX), and found that the overall positive charge of this region was required for NCAM polysialylation. They postulated that the PSTD facilitates processivity of the polysialylation process by interacting with the growing, negatively charged polySia chain (60). However, our analysis of these critical PSTD residues suggested that this region is important for polyST catalytic activity (61). We identified a conserved polybasic region (PBR) we thought might function as the complementary binding region for the FN1 acidic patch (residues 71–105 in PST and 86–120 in STX). Mutation of specific basic residues (Arg82/Arg93 in PST and Arg97/Lys108 in STX) within this region substantially decreased NCAM polysialylation without similarly decreasing enzyme autopolysialylation, suggesting that the PBR is important for recognition of the NCAM substrate and not general catalytic activity (61). Unfortunately, we were unable to consistently detect decreases in the interaction of PST PBR mutants and NCAM in co-immunoprecipitation experiments (data not shown). Consequently, we sought another approach to further evaluate the potential role of the PBR, and specifically Arg82/Arg93, in PST recognition of NCAM.

In this article, we use a competition strategy to evaluate the role of the PBR in PST recognition of NCAM. Our studies indicate that PST sequences between residues 71 and 127 are required for NCAM recognition, and truncated PST proteins containing these sequences bind to NCAM and compete with the endogenous SW2 cell PST to block NCAM polysialylation. We demonstrate that replacing Arg82 and Arg93 with alanine residues in a full-length catalytically inactive PST protein (PST H331K) blocks its ability to compete with endogenous SW2 cell PST to reduce NCAM polysialylation, indicating that these PST residues are essential for NCAM recognition. We also find that the PST R82A/R93A mutant exhibits reduced ability to polysialylate both SynCAM 1 and NRP-2. Interestingly, Arg82 appears to play a greater role in the recognition and polysialylation of these two proteins than does Arg93. This is in contrast to PST recognition of NCAM, where Arg82 and Arg93 seem to play relatively equivalent roles (61). Collectively, our work has identified two residues in a polybasic region prior to the conserved sialyl motifs that are essential for substrate recognition and polysialylation.
EXPERIMENTAL PROCEDURES

Tissue culture media and reagents, including Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Opti-MEM I, Lipofectin, Lipofectamine, and Lipopectamine 2000 were purchased from Invitrogen. The cDNA for full-length human NCAM140 and the SW2 small cell carcinoma cell line were gifts from Dr. Nancy Kedersha (Brigham and Women's Hospital, Boston, MA). The cDNA for full-length human ST8Sia IV/PST was obtained from Dr. Minoru Fukuda (Sanford Burnham Medical Research Institute, La Jolla, CA). The cDNA for full-length human neuropilin-2 was obtained from Dr. Nicholas Stamos (University of Maryland School of Medicine, Baltimore, MD). The cDNA for full-length human SynCAM I was obtained from Dr. Thomas Biederer (Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT). The QuikChange™ site-directed mutagenesis kit and Pfu DNA polymerase were purchased from Stratagene (La Jolla, CA). All oligonucleotides were purchased from Invitrogen. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). DNA purification kits were obtained from Qiagen (Valencia, CA). Protease inhibitors were purchased from Roche Applied Science. Poly-l-lysine microscope coverslips were purchased from ImmunoResearch (West Grove, PA). Precision Plus™ Protein standards were obtained from Bio-Rad. Nitrocellulose membranes were purchased from Schleicher & Schuell. SuperSignal West Pico chemiluminescence reagent was obtained from Pierce. Blue Ultra Autorad film was obtained from BioExpress (Kaysville, UT). All other chemicals and reagents were purchased from Sigma and Fisher.

Creation of Membrane-associated PST Mutants—The ST8Sia IV/PST cDNA was cloned into the EcoRV and XbaI sites of a previously digested pcDNA3.1/Myc-HisB mammalian expression vector containing a carboxyl-terminal Myc epitope tag and a stop codon prior to the His6 tag. The resulting plasmid containing the DNA sequence for PST-Myc served as the template for the creation of all full-length and truncated PST mutants. All mutations were performed using the QuikChange™ site-directed mutagenesis kit and Pfu DNA polymerase according to the manufacturer's instructions, utilizing the oligonucleotide primers listed in supplemental Table S1. PST mutants that are truncated from the carboxyl terminus were created in two steps. First, an XbaI restriction site (5'-TCTAGA-3') was inserted into the PST cDNA immediately downstream of the codon corresponding to the amino acid that will serve as the truncated carboxyl-terminal residue of PST.

Second, this plasmid was digested with XbaI at 37 °C for 16 h, to cleave both the newly created XbaI restriction site as well as an XbaI site located between the carboxyl terminus of the full-length PST DNA sequence and the start of the Myc epitope DNA sequence. Following digestion, the expression plasmid was ligated using T4 DNA ligase at 16 °C for 3 h to create an expression vector containing a truncated Myc-tagged PST mutant. All DNA sequences resulting from mutagenesis and digestion/ligation were checked for accuracy by DNA sequencing performed by the DNA Sequencing Facility at the Research Resources Center at the University of Illinois at Chicago.

Construction of Soluble PST-Myc and Soluble PST140-Myc Mutants—Soluble PST (sPST-myc) lacking the cytosolic tail and transmembrane region was generated as described previously by Thompson et al. (50). Soluble PST140 (sPST140-Myc) was then generated from this vector using mutagenesis and XbaI digestion, as described above.

Competition Studies in SW2 Cells and Confocal Immunofluorescence Microscopy to Evaluate Protein Expression and Changes in Polysialylation—SW2 small cell lung carcinoma cells maintained in DMEM, 10% fetal bovine serum (FBS) were plated on poly-l-lysine-coated coverslips and grown in a 37 °C, 5% CO2 incubator until 50–70% confluent. Cells on each plate were then transfected with 1 μg of PST−, sPST−, or a PST-Myc mutant DNA in 300 μl of Opti-MEM I containing 3 μl of Lipopectamine 2000. Cells were incubated with the transfection mixture for 6 h at 37 °C followed by the addition of 1 ml of DMEM, 10% FBS. After 18 h, cells were washed with phosphate-buffered saline (PBS) and permeabilized using −20 °C methanol to view internal structures as well as the cell membrane. After permeabilization, cells were blocked with immunofluorescence blocking buffer (5% normal goat serum in PBS) for 1 h, then incubated with the following primary antibodies diluted in immunofluorescence blocking buffer for 2 h at room temperature: anti-Myc tag antibody (1:250) to detect expression and localization of PST or a mutant PST, and the OL.28 anti-polySia antibody (1:250) to detect polysialylation. After washing with PBS (4 times), cells were then incubated for 1 h with the following secondary antibodies diluted in blocking buffer: FITC-conjugated goat anti-mouse IgG (1:100) to visualize protein localization, and TRITC-conjugated goat anti-mouse IgM (1:100) to visualize polySia. After washing with PBS (4 times), the coverslips were mounted on glass slides using 20 μl of mounting medium (15% Vinol 205 polyvinyl alcohol (w/v), 33% glycerol (v/v), 0.1% azide, pH 8.5). Cells were visualized with a Zeiss Axiovert 200M inverted confocal microscope using a ×63 oil immersion objective.

Co-immunoprecipitation of NCAM with PST and PST Mutants—COS-1 and Lec2 CHO cells were maintained in DMEM and F-12 media, respectively, supplemented with 10% FBS, were plated onto 100-mm tissue culture plates and grown in a 37 °C, 5% CO2 incubator until 50–70% confluent. Cells were then co-transfected with 10 μg of V5-tagged NCAM cDNA and 10 μg of empty pcDNA3.1/Myc-HisB vector (control), Myc-tagged PST, or Myc-tagged PST mutant cDNA in 3 ml of Opti-MEM I containing 30 μl of Lipofectin (COS-1) or Lipopectamine (Lec2 CHO) transfection reagent. Cells were incubated with the transfection mixture for 6 h at 37 °C. Fol-
following incubation, 7 ml of DMEM (COS-1) or F-12 (Lec2 CHO) media supplemented with 10% PBS was added and the cells were allowed to grow overnight in a 37 °C, 5% CO₂ cell incubator. The following day, cell medium was removed and the cells were washed with 10 ml of PBS. One milliliter of co-immunoprecipitation buffer (50 mM Hepes, 100 mM NaCl, 1% Triton X-100, pH 7.2) was then added and the cells were scraped off the plates using a sterile cell scraper. The cells were incubated on ice for 20 min to allow for lysis, and then pelleted by centrifugation. To immunoprecipitate PST-Myc or a Myc-tagged PST mutant, the resulting supernatant was incubated with 2.5 μl of rabbit polyclonal anti-Myc tag antibody. After 2 h of rotation at 4 °C, 50 μl of protein A-Sepharose beads (50% suspension in PBS) was added to each sample and the samples were rotated for 1 h at 4 °C. The immune complexes bound to Protein A-Sepharose beads were pelleted and washed 4 times with 1 ml of cold PBS. To immunoprecipitate PST-Myc or a Myc-tagged PST mutant cDNA. For controls, one sample of a Myc-tagged PST mutant cDNA. For controls, one sample was transfected with 2 μg each of NCAM-V5 and PST-Myc and 16 μg of empty pcDNA3.1/Myc-HisB vector.

Evaluation of NCAM, Neuropilin-2, and SynCAM 1 Polysialylation by PST and Mutant PST Proteins—Eighteen hours post-transfection, cells were washed with 10 ml of PBS and lysed in 1 ml of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS). The lysates were pre-cleared with 50 μl of protein A-Sepharose beads, and NCAM-V5, neuropilin-2-V5, and SynCAM 1-V5 were immunoprecipitated with 3 μl of anti-V5 tag antibody for 2 h at 4 °C with rotation, followed by incubation with 50 μl of protein-A-Sepharose beads for 1 h at 4 °C. Beads were then washed 4 times with immunoprecipitation buffer and once with immunoprecipitation buffer containing 1% SDS. Samples were then resuspended in 50 μl of Laemmli sample buffer containing 5% β-mercaptoethanol and heated to 100 °C for 10 min. Precipitated proteins were separated on a 5% stacking, 10% resolving SDS-polyacrylamide gel and then subjected to immunoblotsing as described below. To determine the relative expression of NCAM and PST or PST mutant proteins in the initial samples, a 150-μl sample was removed from each lysate prior to co-immunoprecipitation. Fifty microliters of Laemmli sample buffer containing 5% β-mercaptoethanol was added to each sample. Prior to immunoblotting, the samples for determining NCAM expression levels were separated on a 10% resolving gel, whereas those for determining the PST or PST mutant expression levels were separated on a 15% resolving gel.

**Immunoblot Analysis of NCAM Proteins**—Following SDS-PAGE, proteins were transferred to nitrocellulose membranes at 450 mA overnight. Membranes were blocked for 1 h at 4 °C in blocking buffer (5% nonfat dry milk in Tris-buffered saline, pH 8.0, 0.1% Tween 20). To detect NCAM co-immunoprecipitated with a Myc-tagged PST or PST mutant and determine relative NCAM expression levels, nitrocellulose membranes were incubated with anti-V5 epitope tag antibody diluted 1:5000 in blocking buffer containing 5% β-mercaptoethanol and heated for 10 min at 65 °C, and separated on a 3% stacking, 5% resolving SDS-polyacrylamide gel. The relative expression levels of NCAM, neuropilin-2, SynCAM 1, and PST proteins were evaluated as described above.

The polysialylation of NCAM, SynCAM 1, and NRP-2 by PST and mutant PST proteins was evaluated with O.L28 immunoblotting. Briefly, proteins were transferred to a nitrocellulose membrane at 450 mA overnight. Membranes were blocked for 1 h at 4 °C in blocking buffer (5% nonfat dry milk in Tris-buffered saline, pH 8.0, 0.1% Tween 20). Membranes were then incubated overnight at 4 °C with O.L28 anti-polySia antibody diluted 1:150 in 2% nonfat dry milk in Tris-buffered saline, pH 8.0, followed by a 1-h incubation at 4 °C with HRP-conjugated goat anti-mouse IgG antibody diluted 1:4000 in blocking buffer. Membranes were then washed and developed as described above.

**RESULTS**

**Expression of Truncated PST Proteins Blocks NCAM Polysialylation by Endogenous PST in SW2 Cells**—We hypothesize that polysialylation is a highly protein-specific process in which the polySTs must first recognize the substrate protein prior to modifying its glycans. Our data indicate that the polySTs recognize sequences in the NCAM FN1 domain that then allow them to polysialylate two N-glycans in the adjacent Ig5 domain (supplemental Fig. S1). Based on previous studies from our laboratory (61) and Angata et al. (59), we hypothesize that sequences in the membrane proximal region of the polySTs prior to the SML function in the recognition of NCAM. To test this hypothesis we have taken a competition approach using SW2 small cell lung carcinoma cells that contain endogenous PST and polysialylated NCAM. For PST competitors we created a catalytically inactive full-length PST (PST H331K) (57), and a series of catalytically inactive PST mutants truncated prior to the SML (Fig. 1). We expected that the expression of a catalytically inactive PST mutant containing sequences important for NCAM recognition would compete with endogenous, active PST for NCAM recognition and block NCAM polysialylation.

We first examined whether the expression of a truncated PST protein consisting of the first 140 residues of PST (PST140), or a soluble version of PST140 lacking the cytosolic
and transmembrane sequences (sPST140) (Fig. 1), altered NCAM polysialylation in SW2 small cell carcinoma cells. We also expressed a full-length catalytically inactive PST mutant (PST H331K) that would be expected to compete with the endogenous catalytically active SW2 cell PST, and catalytically active, full-length PST and soluble PST (sPST) proteins that would be expected to increase NCAM polysialylation to levels greater than those generated by the endogenous enzyme (Fig. 1). Like wild type PST, the membrane-associated PST mutants were found to localize primarily to the Golgi apparatus in COS-1 cells (supplemental Fig. S2, localization). Likewise, sPST and sPST140 are transported through the Golgi and are efficiently secreted (supplemental Fig. S2, localization, and data not shown). Furthermore, when expressed in COS-1 cells, PST and sPST were autopolsialylated as expected for active enzymes, whereas PST H331K, PST140, and sPST140 that lack critical catalytic sequences were not (supplemental Fig. S2, autopolsialylation).

To determine whether PST H331K, PST140, and sPST140 could compete with endogenous PST and decrease NCAM polysialylation, each protein was individually expressed in SW2 cells, which were then stained with anti-Myc antibody to identify cells expressing the Myc-tagged PST proteins (Fig. 2, localization), and the OL.28 anti-polySia antibody to examine the polysialylation of endogenous NCAM (Fig. 2, polysialylation). We found that SW2 cells expressing exogenous PST or sPST displayed an increase in cell surface NCAM polysialylation and Golgi enzyme autopolsialylation relative to surrounding cells that did not express these proteins (Fig. 2, PST and sPST, arrowheads). Our ability to detect enzyme autopolsialylation only when PST or sPST are highly expressed likely reflects the low expression level of endogenous PST. In contrast, cells express-
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Notably, as with PST140, PST127, PST71, and PST62 were analyzed the impact on NCAM polysialylation as described above. We expressed these truncated PST mutants in SW2 cells and analyzed the impact on NCAM polysialylation as described above. We found that PST127, PST71, and PST62 were mainly Golgi-localized and were not autopolysialylated when expressed in COS-1 cells, as expected (supplemental Fig. S2), but PST105 expression was not detected and this construct was not used for further experimentation (data not shown). We expressed these truncated PST mutants in SW2 cells and analyzed the impact on NCAM polysialylation as described above. Notably, as with PST140, PST127, PST71, and PST62 were detected in fewer SW2 cells than full-length PST or PST H331K suggesting some instability or increased turnover of the shorter proteins in these cells. Nevertheless, we found that cells expressing PST127 exhibited a decrease in NCAM polysialylation similar to that seen for PST140 (Fig. 3, PST140 and PST127, arrowheads). In contrast, cells expressing PST71 or PST62 did not show a decrease in NCAM polysialylation (Fig. 3, PST71 and PST62, arrowheads). Rather, the polysialylation of NCAM seemed unchanged from baseline levels, suggesting that PST71 and PST62 do not have sequences required for NCAM recognition and are unable to compete with endogenous PST to block NCAM polysialylation. Collectively, these results suggest that residues 71–127 of PST are important for NCAM recognition and are unable to compete with endogenous, catalytically active PST. To evaluate this possibility we took a co-immunoprecipitation approach. Following co-expression of V5-tagged NCAM with Myc-tagged wild-type PST, sPST, or one of the four truncated PST mutants in COS-1 cells, the enzymes were immunoprecipitated from the cell lysates with an anti-Myc antibody. Co-immunoprecipitated NCAM-V5 was detected by immunoblotting with anti-V5 anti-
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FIGURE 4. Wild type PST and all PST mutants containing the PBR effectively bind to NCAM, whereas PST71 and PST62 that lack the PBR do not. V5-tagged NCAM was co-expressed with Myc-tagged PST or a PST mutant in COS-1 cells (A) or Lec2 CHO cells (B). After 18 h, cells were lysed and lysates were incubated with anti-Myc antibody to immunoprecipitate (IP) the PST proteins, followed by incubation with protein A-Sepharose beads for 1 h. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting (IB) with anti-V5 antibody to detect co-immunoprecipitated NCAM (CoIP). IP: Anti-Myc antibody, IB: Anti-V5 antibody. To determine the relative expression levels of NCAM and PST proteins, an aliquot of lysate was removed prior to co-immunoprecipitation and subjected to SDS-PAGE and immunoblotting with anti-V5 antibody to detect NCAM (NCAM expression, Anti-V5 antibody) and anti-Myc antibody to detect the PST proteins (Enzyme expression, Anti-Myc antibody).

body as detailed under “Experimental Procedures” (Fig. 4A, upper panel). Relative expression levels of NCAM and PST proteins were determined by immunoblotting an aliquot of cell lysate with anti-V5 and anti-Myc antibodies, respectively (Fig. 4A, middle and lower panels). We did detect some nonspecific binding of NCAM to the protein A-Sepharose beads in the absence of co-expressed enzyme (Fig. 4, A and B, lane 1). However, as expected, NCAM-V5 was co-immunoprecipitated with PST (Fig. 4A, lane 2). In addition, NCAM-V5 was co-immunoprecipitated with sPST, suggesting that the cytosolic tail and transmembrane region of PST are not critical for NCAM binding (Fig. 4A, lane 5). We observed that NCAM-V5 also bound to Myc-tagged, membrane-associated PST H331K, PST140, as well as sPST140 (Fig. 4A, lanes 3, 4, and 6).

A similar co-immunoprecipitation analysis was performed in Lec2 CHO cells that compared the binding of NCAM to PST and the truncated PST proteins (Fig. 4B). Lec2 CHO cells cannot (poly)sialylate glycoconjugates due to the absence of a functional CMP-sialic acid transporter (62), and were used here to eliminate the potential effects of polysia on binding of wild type PST and NCAM. We found that PST140 and PST127 bound to NCAM like full-length PST (Fig. 4B, lanes 2–4), consistent with their ability to block NCAM polysialylation in SW2 cells. In contrast, PST71 and PST62 exhibited a significantly reduced ability to bind to NCAM, even when their somewhat lower level of expression is taken into consideration, and this correlates with the inability of these two truncated PST proteins to block NCAM polysialylation (Fig. 4B, lanes 5 and 6).

Arg$^{82}$ and Arg$^{93}$ in the PST PBR Are Required for Ability of PST H331K to Block NCAM Polysialylation—Previous work from our laboratory suggested that Arg$^{82}$ and Arg$^{93}$ in the PBR of PST are critical for NCAM polysialylation (61). It was postulated that these residues play a role in NCAM recognition because in the presence of a catalytically active PST R82A/R93A mutant, NCAM polysialylation was substantially decreased without an equal decrease in PST autopolsialylation. Because Arg$^{82}$ and Arg$^{93}$ are found within the stretch of PST amino acids (71–127) that our results suggest are important for NCAM recognition, we wondered whether replacing these residues would decrease the ability of the various PST mutants to competitively inhibit NCAM polysialylation in SW2 cells. We found that replacing Arg$^{82}$ and Arg$^{93}$ in PST140 and PST127 compromised their folding leading to some retention in the endoplasmic reticulum (data not shown), and so decided to focus on the impact of replacing these residues on the ability of PST H331K to block SW2 cell NCAM polysialylation. As a control we also evaluated the impact of expressing PST R82A/R93A on SW2 cell NCAM polysialylation. PST H331K proteins with the R82A and R93A mutations introduced individually or together were predominantly localized to the Golgi apparatus like the parent protein, wild type PST, and PST R82A/R93A (supplemental Figs. S2 and S3). Following expression of PST, PST H331K, and their mutants in SW2 cells, the cells were stained with anti-Myc antibody and OL.28 anti-polySia antibody as described above (Fig. 5). As shown in Fig. 2, expressing wild type PST enhanced the polysialylation of NCAM (cell surface) and allowed detection of the autopolsialylated enzyme in the Golgi (Fig. 5). However, cells expressing the PST R82A/R93A mutant retained detectable Golgi enzyme autopolsialylation, but exhibited no enhanced cell surface polysialylation consistent with the inability of the PST R82A/R93A mutant to recognize endogenous SW2 cell NCAM (Fig. 5). Most strikingly, whereas PST H331K significantly decreased NCAM polysialylation in SW2 cells as shown above (Figs. 2 and 5, PST H331K), the introduction of the R82A/R93A mutation appeared to abolish the ability of PST H331K to compete with endogenous enzyme (Fig. 5). The impact of the single arginine replacements on the ability of PST H331K to block NCAM polysialylation was more difficult to discern in these experiments, and led us to take another approach.

To more precisely quantify the contribution of each of these arginine residues to NCAM recognition, we wanted to establish a more sensitive assay to measure changes in NCAM polysialylation. Initially, we attempted to generate SW2 cells stably expressing the various PST constructs, but found that these cells lines were difficult to establish and that the selection procedure actually led to increased polysialylation (data not shown). In lieu of this, we established a triple expression system in COS-1 cells, in which NCAM and PST would be co-expressed with an excess of competitor. We expressed V5-tagged NCAM, Myc-tagged PST, and either a Myc-tagged PST H331K or a PST...
H331K PBR mutant in COS-1 cells at a 1:1:6 ratio. NCAM was precipitated from cell lysates with anti-V5 antibody, and the relative NCAM polysialylation observed in the presence of PST H331K and its mutants in this representative immunoblot. We found that in the presence of PST and no competitor, NCAM was polysialylated (Fig. 6, lanes 1–3), as well as enzyme expression (Fig. 6, bottom panels), were evaluated as described above. As seen previously, replacing Arg^{82} and Arg^{93} with alanine residues decreased the polysialylation of NCAM (Ref. 61 and Fig. 7A, lane 4). This result suggests that both arginine residues are required for NCAM recognition. In contrast, mutation of Arg^{82} to alanine alone, or in combination with Arg^{93}, eliminates NRP-2 and SynCAM 1 polysialylation by PST (Fig. 7, B and C, lanes 1, 2, and 4). However, mutation of Arg^{93} alone to alanine did not reduce NRP-2 or SynCAM 1 polysialylation (Fig. 7, B and C, lane 3). Taken together, these results suggest that the PST PBR functions specifically in the recognition of substrates for polysialylation, and that Arg^{82} in the PST PBR may be required for general substrate recognition, whereas Arg^{93} specifically aids the recognition of NCAM.

**DISCUSSION**

In this study, we sought to identify specific sequences within PST that are required for substrate recognition leading to protein-specific polysialylation. Competition and binding studies demonstrated that PST sequences between residues 71 and 127 are required for NCAM recognition (Figs. 2–4). Previous work
suggested that the PBR of both ST8SialIV/PST and ST8SialII/STX may play a role in substrate recognition, and two PBR residues (Arg$^{82}$ and Arg$^{93}$ in PST) are particularly important for NCAM polysialylation (61). In this study, we found that replacing these residues in the inactive PST H331K protein eliminated its ability to compete with endogenous PST in SW2 cells (Figs. 5 and 6). From this study and analysis of NCAM polysialylation by PST R82A and R93A mutants, it appeared that both residues make substantial contributions to NCAM recognition and polysialylation, with Arg$^{93}$ possibly playing a slightly larger role (Ref. 61 and Figs. 5 and 6). However, analysis of the polysialylation of NRP-2 and SynCAM 1 by PST and its arginine mutants suggests that Arg$^{82}$ plays a predominant role in PST recognition and polysialylation of these substrates (Fig. 7, B and C). These results emphasized the role of Arg$^{82}$ and Arg$^{93}$ in substrate recognition rather than general enzyme activity, and raised the intriguing possibility that different substrates may be recognized by slightly different binding surfaces of the polySTs.

A particularly interesting and repeatable observation was the consistent increase in NRP-2 and SynCAM 1 polysialylation observed when PST Arg$^{93}$ is replaced by alanine (Fig. 7, B and C). One possibility is that the presence of Arg$^{93}$ forms a less optimal binding site for these substrates. If this is the case, then eliminating Arg$^{93}$ could enhance polysialylation of NRP-2 and SynCAM 1 by optimizing their interaction with the polyST. Additional experiments will be performed to determine whether other PBR residues play roles in the recognition and polysialylation of NRP-2 and SynCAM 1.

How can just one or two arginine residues have such an impact on substrate recognition? Researchers studying the propensity of certain amino acids at protein interfaces suggest that these interfaces favor residues that can engage in hydrophobic and electrostatic interactions, including tryptophan, arginine, and tyrosine (reviewed in Ref. 66). Arginine is favored in “hot spots” of binding energy because it is capable of multiple types of favorable interactions, including forming up to five hydrogen bonds and a salt bridge utilizing the positive charge on its guanidinium moiety (67). In fact, an arginine-aspartate salt bridge is found at the interface of the $\alpha$- and $\beta$-subunits of the GABA$_A$ receptor, and removal of this bridge through alanine mutagenesis compromises GABA binding and subunit association (68). Applying these observations to the polyST-NCAM interaction, Arg$^{82}$ and Arg$^{93}$ may promote recognition through the formation of hydrogen bonds and salt bridges with residues in NCAM. Previous results from our lab suggest that it is plausible that salt bridges could be formed between the polySTs and NCAM. Earlier work identified an acidic patch on the surface of NCAM FN1 comprised of several aspartate and glutamate residues (18). Mutating these residues to alanine resulted in reduced NCAM polysialylation (18) and reduced PST binding (50), indicating that these residues may form part of a docking site for the polySTs.

It is tempting to try and model the interactions of these basic residues in PST and the NCAM FN1 domain. However, whereas the NCAM Ig5-FN1 structure has been solved by our group (69), the polyST structures are not known and unlikely to precisely match the structure of the bovine ST3Gal I (70) or the Campylobacter jejuni CST enzyme (71) sialyltransferases that show limited overall homology and little to no homology in the stem regions prior to the SML where the critical arginine residues are located in PST. Secondary structure predictions using PSIPRED (72) or JUFO (73) place Arg$^{82}$ in an $\alpha$-helix and Arg$^{93}$ in an adjacent coil but provide no information as to the orientation of their side chains or the relationship of these two structural motifs, making it difficult to predict how these residues might form salt bridges with acidic residues of the NCAM FN1.
FIGURE 7. The ability of PST to polysialylate NCAM is eliminated when Arg82 and Arg93 are replaced, whereas the ability of PST to polysialylate NRP-2 and SynCAM 1 requires only the presence of Arg82. NCAM-V5 (A), NRP-2-V5 (B), or SynCAM 1-V5 (C) were co-expressed with Myc-tagged PST, PST R82A, PST R93A, or PST R82A/R93A in COS-1 cells. Eighteen hours post-transfection, cells were lysed and substrates were immunoprecipitated using the anti-V5 antibody. Immunoprecipitates (IP) were subjected to SDS-PAGE and immunoblotting (IB) with the OL.28 anti-polySia antibody to analyze the level of substrate polysialylation. Relative protein expression levels were determined by removing an aliquot of cell lysate prior to immunoprecipitation and subjecting it to SDS-PAGE followed by immunoblotting with anti-V5 antibody (NCAM, NRP-2, or SynCAM 1 Expression, Anti-V5 antibody) or anti-Myc antibody (Enzyme Expression, Anti-Myc antibody).
acidic patch that extends along one face of this domain (18). On the other hand, acidic surfaces are predicted or observed for SynCAM 1 and NRP-2 domains that may be recognized by the polySTs. For example, modeling the SynCAM 1 extracellular portion (Ig1-Ig2-Ig3), based on the NCAM Ig1-Ig2-Ig3 structure (74) (PDB code 1QZ1), predicts an acidic surface patch on Ig2 that may allow polyST docking for the polysialylation of a single N-glycan on Ig1.

Attempts to demonstrate a role for Arg82 and Arg93 in direct protein–protein interaction between polyST and NCAM in coinmunoprecipitation experiments were unsuccessful. We were unable to detect a reduction in polyST-NCAM binding when Arg82 and Arg93 are replaced, despite the profound effect this alteration has on NCAM polysialylation. One possibility is that the polySTs can interact with substrates in more than one way, but only when they are aligned properly with the glycans to be modified can substrate polysialylation take place. In support of this idea, we have demonstrated that deleting NCAM FN1 eliminates polyST binding (50), however, replacing NCAM FN1 with other FN1 domains allows binding, but not polysialylation, suggesting that residues in NCAM FN1 allow the precise positioning of enzyme and substrate and that this is critical for substrate polysialylation.3

In this regard it is notable that replacing Arg82 and Arg93 has different effects on the three substrates. These results suggest that the polySTs use different but overlapping sequences to recognize their substrates and position them for polysialylation. Removing the key PST basic residues may redirect substrates to other polyST surfaces that do not allow optimal alignment of the enzyme and substrate, and consequently prevent polysialylation without greatly altering enzyme–substrate binding. Related to this possibility, previous work in our laboratory demonstrated that changes in the linker region between NCAM Ig5 and FN1 are capable of eliminating NCAM polysialylation, and highlight the importance of a defined Ig5-FN1 relationship for the ability of a polyST interacting with the NCAM FN1 domain to access and polysialylate the Ig5 N-glycans (51).

Another possibility is that the polyST PBR residues are involved in an interaction with the growing polySia chain and that this is critical for chain elongation. Troy and colleagues (60) looked for conserved basic residues in the polySTs, and identified the PSTD, based on the idea that these enzymes would likely engage the growing, negatively charged polySia chain using a stretch of positively charged, basic residues to increase the processivity of the process. Indeed, glycosyltransferases that synthesize bacterial capsular polysaccharides frequently have carbohydrate recognition sequences outside the catalytic site that mediate association with the growing polysaccharide chain. For example, the capsular polysaccharide of Streptococcus pneumoniae is synthesized by a processive glycosyltransferase that requires a “primer” of approximately eight monosaccharides that is engaged by a carbohydrate recognition sequence to initiate the processive phase of synthesis (75).

Our results strongly suggest that the PBR residues, and in particular Arg82 and Arg93, are involved in PST-substrate protein–protein recognition, but we cannot rule out a secondary interaction of the PBR sequences with the growing polySia chain. In fact, it seems likely that mammalian polysialylation may be a two-stage, processive process, where the first stage involves an initial protein–protein interaction between polyST and substrate that facilitates the synthesis of short polySia chains, whereas the second stage is mediated by polyST noncatalytic sequences that interact with the growing polySia chain, allowing the enzyme to maintain association with the substrate and polymerize long polySia chains. Whether polysialylation is a two-stage process, and whether both stages involve the polyST PBR is yet to be determined.

In conclusion, our results have shown that sequences within a polybasic region of the polyST, PST, are involved in substrate recognition. In particular, two PST residues within this region, namely Arg82 and Arg93, are specifically required for efficient recognition and polysialylation of NCAM. In contrast, PST Arg82 plays a predominant role in the recognition and polysialylation of NRP-2 and SynCAM 1. Taken together, these results support the premise that polysialylation is highly protein specific and reveal that the polySTs may use overlapping but distinct surfaces to recognize their different substrates.

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