The Polysialyltransferases Interact with Sequences in Two Domains of the Neural Cell Adhesion Molecule to Allow Its Polysialylation*\textsuperscript{1,2}

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The neural cell adhesion molecule (NCAM) is the major substrate for the polysialyltransferases (polySTs), ST8SiaII/STX and ST8SiaIV/PST. The polysialylation of NCAM N-glycans decreases cell adhesion and alters signaling. Previous work demonstrated that the first fibronectin type III repeat (FN1) of NCAM is required for polyST recognition and the polysialylation of the N-glycans on the adjacent Ig5 domain. In this work, we highlight the importance of an FN1 acidic patch in polyST recognition and also reveal that the polySTs are required to interact with sequences in the Ig5 domain for polysialylation to occur. We find that features of the Ig5 domain of the olfactory cell adhesion molecule (OCAM) are responsible for its lack of polysialylation. Specifically, two basic OCAM Ig5 residues (Lys and Arg) found near asparagines equivalent to those carrying polysialylation in NCAM substantially decrease or eliminate polysialylation when used to replace the smaller and more neutral residues (Ser and Asn) in analogous positions in NCAM Ig5. This decrease in polysialylation does not reflect altered glycosylation but instead is correlated with a decrease in polyST-NCAM binding. In addition, inserting non-conserved OCAM sequences into NCAM Ig5, including an “extra” N-glycosylation site, decreases or completely blocks NCAM polysialylation. Taken together, these results indicate that the polySTs not only recognize an acidic patch in the FN1 domain of NCAM but also must contact sequences in the Ig5 domain for polysialylation of Ig5 N-glycans to occur.

Proteins that co-translationally enter the endoplasmic reticulum and progress through the secretory pathway often become glycosylated on N- or O-glycans. Glycosylation allows proper protein folding and stability and can confer distinct functional properties to a protein (1, 2). For example, the homophilic interactions of the neural cell adhesion molecule (NCAM) mediate cell-cell interactions, and the presence of long chains of polysialic acid (polySia)\textsuperscript{3} on NCAM disrupts these and other interactions to promote cell migration and signaling (3–6).

NCAM is heavily polysialylated during embryonic development and early post-natal growth (7). Mouse knock-out studies demonstrated that polysialylation is absolutely required to down-regulate the adhesive properties of NCAM during nervous system development (8). In adults, polysialylated NCAM is restricted to specific regions of the brain, such as the hippocampus and olfactory bulb, where it has roles in synaptic plasticity and general cell migration (5). Importantly, re-expression of polysialylated NCAM is associated with the growth and invasiveness of several cancers, including neuroblastoma, small cell lung carcinoma, and Wilms tumor (9–13). PolySia is also up-regulated during neuronal regeneration, where it serves to promote the extension and repair of damaged neurons (reviewed in Ref. 5). Conversely, decreased expression of polysialylated NCAM is associated with certain neuropsychiatric disorders, including schizophrenia (14). Studies by Sato, Kitajima, and colleagues (15–17) suggest why this might be the case. They were the first to demonstrate that polySia binds neurotrophic factors, neurotransmitters, and growth factors, and their work suggests that the presence of polySia can modulate signaling by these molecules. In addition, these investigators showed that a single point mutation in the polysialyltransferase ST8SiaII/STX in schizophrenic patients leads to a decrease in enzyme activity and the polymerization of shorter polySia chains on NCAM (15). This change in polySia chain length decreases its ability to bind brain-derived neurotrophic factor and dopamine, two factors whose action is impaired in psychiatric disorders.

The polysialyltransferases (polySTs), ST8SiaII/STX and ST8SiaIV/PST, are responsible for the synthesis of polySia chains on the termini of N- or O-glycans on a very small number of mammalian proteins (7, 18–20). The only polyST substrates identified to date are NCAM, CD36 receptor in human milk

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\textsuperscript{3} The abbreviations used are: polySia, polysialic acid; polyST, polysialyltransferase; FN1 and FN2, first and second fibronectin type III repeat of NCAM, respectively.
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In this study, we performed domain swap experiments and compared the sequences and structures of NCAM and OCAM Ig5 domains to evaluate the factors preventing OCAM polysialylation and, conversely, the requirements for NCAM polysialylation. We found that replacing the OCAM Ig5 domain with NCAM Ig5 allows polysialylation of an OCAM-NCAM chimera, suggesting that aspects of OCAM Ig5 were preventing its polysialylation. To this end, we found that an “extra” N-glycan located within OCAM Ig5 did prevent the polysialylation of NCAM when present, but it is not the only factor preventing OCAM polysialylation. We also found that two large basic residues located near OCAM ASN6 and ASN7, which are replaced by smaller more neutral residues in NCAM Ig5, function to block polysialylation when inserted into NCAM Ig5. The presence of these large basic residues in NCAM does not alter the glycosylation pattern of Ig5 but instead serves to decrease polysialyl-substrate binding. These results suggest that the polysialyltransferases make contact with residues in both the FN1 and Ig5 domains in the process of Ig5 N-glycan polysialylation.

**EXPERIMENTAL PROCEDURES**

Tissue culture materials, including Dulbecco’s modified Eagle’s medium (DMEM), Opti-MEM I, Lipofectin, Lipofectamine 2000, and fetal bovine serum (FBS) were purchased from Invitrogen. Oligonucleotides, restriction enzymes, PCR supermix, and anti-V5 epitope tag antibody were also obtained from Invitrogen. The cDNA for human NCAM-140 was a gift from Dr. Nancy Kedersha (Brigham and Women’s Hospital, Boston, MA). The cDNA for mouse OCAM was kindly provided by Dr Yoshihiro Yoshihara (RIKEN Brain Science Institute, Wako, Saitama, Japan). The cDNA for human ST8SiaIV/PST was obtained from Dr. Minoru Fukuda (Burnham Institute, La Jolla, CA). The QuickChange™ site-directed mutagenesis kit and Pfu DNA polymerase were purchased from Stratagene. DNA purification kits were purchased from Qiagen. Protein A-Sepharose was purchased from GE Healthcare. T4 DNA ligase was obtained from New England Biolabs. Precision Plus Protein™ standard was purchased from Bio-Rad. Nitrocellulose membranes were purchased from Schleicher & Schuell. Horseradish peroxidase (HRP)-conjugated and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were obtained from Jackson Laboratories. Supersignal West Pico chemiluminescence reagent was obtained from Pierce. Other chemicals and reagents were purchased from Sigma and Fisher.

**Construction of V5-tagged OCAM and Chimeric Proteins—**

The full-length mouse OCAM sequence was PCR-amplified using PCR supermix and the following primers: 5’-AACGTT-GTCCCTGAAACATGAGCCTCTC-3’ and 5’-TCTAGAT-GCCCTTATGTTCATCCTTTTAGACTGG-3’. These primers specifically introduced a HindIII and Xbal site at the 5’- and 3’-ends of the amplified OCAM sequence, respectively. The OCAM PCR product and empty pcDNA3.1 V5/HisB expression vector were digested with HindIII and Xbal. After gel purification, the OCAM PCR product was ligated into the expression vector. A frameshift mutation, introduced during cloning.
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near the XbaI site, was corrected by mutagenesis using the following primers: 5’-GATGACATAAAGCGAGTCTAGAG-GGCCGCG-3’ and 5’-GGCCGCCCTCTAGACCTGCTTTTATGTAC-3’. To generate the chimeric proteins, BamHI and XbaI restriction sites, flanking the Ig5 or FN1 domains were inserted into the full-length OCAM or NCAM cDNAs by site-directed mutagenesis, and the domains were subsequently removed by restriction enzyme digestion. The OCAM FN1 domain, NCAM Ig5 domain, NCAM FN1 domain, or NCAM Ig5-FN1 domain was PCR-amplified using the following primers that inserted BamHI and XbaI sites at the 5’- and 3’-ends of the cDNAs, respectively: 5’-GGATCCGGATGTCCCTCTAGTCCCCATG-3’, 5’-GGATCCGAGCTGCCTGCGTC-3’, 5’-TCTAGAGCTGCTTGAACAAGGATGATTCG-3’, 5’-GGATCCGACACCCCCCTCTCACCATCC-3’, 5’-ATTC-3’ and 5’-TCTAGAGGCTCACGG-3’. PCR products were cut with BamHI and XbaI and ligated in frame.

TCTAGAGCTTCCCCTTGGACTGGCTGCGTC-3

immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS). Lysates were precleared with 50 μl of protein A-Sepharose beads (50% suspension in PBS) for 1 h at 4°C. NCAM, OCAM, or chimeric proteins were immunoprecipitated with 3 μl of anti-V5 epitope tag antibody for 2 h at 4°C, followed by incubation for 1 h with 50 μl of protein A-Sepharose beads. Beads were washed four 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, heated at 65°C for 10 min, and separated on a 3% stacking, 5% resolving SDS-polyacrylamide gel. To evaluate relative NCAM, OCAM, and chimeric protein expression levels, a 100-μl aliquot of cell lysate was removed prior to immunoprecipitation, and an equal volume of Laemmli sample buffer, 5% β-mercaptoethanol was added. Samples were boiled at 100°C for 10 min and separated on a 5% stacking, 7.5% resolving SDS-polyacrylamide gel. Note that for each lane in Figs. 2–6, expression analysis reflects the protein present in 100 μl or 10% of the cell lysate, and the detected polySia or co-immunoprecipitated protein reflects that present on or binding to the immunoprecipitated protein from 900 μl or 90% of the sample.

Immunoblot Analysis of Expression and PolySialylation of NCAM, OCAM, and Chimeric Proteins—Following gel electrophoresis, proteins were transferred to a nitrocellulose membrane at 500 mA overnight. Membranes were blocked for 1 h at room temperature in blocking buffer (5% nonfat dry milk in Tris-buffered saline, pH 8.0, 0.1% Tween 20). To detect polySia, membranes were incubated overnight with a 1:200–1:5000 dilution of OL.28 anti-polySia antibody in 2% nonfat dry milk in Tris-buffered saline, pH 8.0, and for 1 h with HRP-conjugated goat anti-mouse IgM, diluted 1:4000 in blocking buffer. To test relative NCAM, OCAM, and NCAM-OCAM expression levels,
membranes were incubated for 2 h or overnight with a 1:5000 dilution of anti-V5 epitope tag antibody diluted in blocking buffer and for 1 h with HRP-conjugated goat anti-mouse IgG, diluted 1:4000 in blocking buffer. Membranes were washed with Tris-buffered saline, pH 8.0, 0.1% Tween 20 for 15 min two times before and four times after secondary antibody incubation. Immunoblots were developed using the SuperSignal West Pico chemiluminescence kit and BioExpress Blue Ultra Autorad film.

RESULTS

Our previous work demonstrated that the FN1 domain of NCAM is required for polyST recognition and the polysialylation of N-glycans on the adjacent Ig5 domain (27, 29, 30). Subsequent studies showed that the polySTs can bind to the NCAM FN1 domain, and we hypothesize that this binding is not only required for the protein specificity of polysialylation but may also facilitate the polymerization of the polySia chain on NCAM Ig5 N-glycans (30). NCAM and OCAM share 45% amino acid identity and the same extracellular domain structure, and the Ig5 domain of OCAM has consensus N-glycosylation sites in positions equivalent to those that are polysialylated on NCAM Ig5 (37). Despite these similarities, OCAM is not polysialylated (37). Surprisingly, we demonstrated that when the FN1 domain of NCAM is replaced with that of OCAM (N-O FN1; see Fig. 1), this adhesion molecule chimera is polysialylated, suggesting that OCAM FN1 allows polyST recognition (35). Why then is OCAM not polysialylated? In this study, we tested the hypothesis that both the Ig5 and FN1 domains of NCAM are involved in polyST recognition and that features of the OCAM Ig5 domain prevent it from participating in polyST recognition.

The OCAM Ig5 Domain Is Not Permissive for PolyST Recognition and Substrate Polysialylation—To evaluate whether OCAM Ig5 is preventing the recognition and/or polysialylation of OCAM, we swapped NCAM and OCAM Ig5 and FN1 domains to create three chimeric OCAM proteins containing NCAM FN1 (O-N FN1), NCAM Ig5 (O-N Ig5), and NCAM Ig5-FN1 (O-N Ig5-FN1) (Fig. 1). To assess polysialylation of OCAM and these OCAM-NCAM chimeras, we co-expressed each protein with the polyST, ST8SiaIV/PST, in COS-1 cells. OCAM and the OCAM-NCAM chimeric proteins were immunoprecipitated from cell lysates, separated by SDS-PAGE, and polysialylation was evaluated by immunoblotting with the anti-polySia antibody, OL.28 (Fig. 2, Polysialylation, OL.28 Antibody). Relative protein expression levels were also determined by immunoblotting an aliquot of each cell lysate with an anti-V5 epitope tag antibody (Fig. 2, Expression, Anti-V5 Antibody). As expected, OCAM was not polysialylated by ST8SiaIV/PST, and neither was the O-N FN1 chimera containing NCAM FN1 (Fig. 2, OCAM and O-N FN1). However, replacing OCAM Ig5 with NCAM Ig5 in the O-N Ig5 chimera allowed a 19%
increase in polysialylation over that of OCAM, and replacing the Ig5-FN1 tandem of OCAM with that of NCAM allowed a 55% increase in polysialylation over that of OCAM (Fig. 2, O-N Ig5 and O-N Ig5-FN1). Analysis of the subcellular localization of these proteins by immunofluorescence microscopy following expression in COS-1 cells demonstrated that OCAM and each OCAM-NCAM chimera was found on the cell surface, suggesting that each was folded properly and efficiently exported out of the endoplasmic reticulum, through the Golgi, and to the plasma membrane (supplementary Fig. 1A). These results demonstrate that the OCAM Ig5 domain is prohibiting OCAM polysialylation and that neither the Ig5 nor the FN1 domains of OCAM are optimal for polyST recognition and polysialylation.

An Additional N-Linked Glycan Present in OCAM Ig5 Blocks Polysialylation of NCAM When Its Attachment Site Is Placed in NCAM Ig5, but Eliminating It Does Not Allow OCAM Polysialylation—OCAM has additional consensus N-linked glycosylation sites in both the Ig5 and FN1 domains (Fig. 1). We wanted to test the effect of inserting or removing these glycosylation sites in the context of NCAM or OCAM, respectively. To insert an additional glycosylation site into NCAM Ig5 or FN1, the sequence Gly410-Pro411-Val412 was mutated to Asn-Gln-Thr (+Ig5 N-glycan), and Glu569-Thr570-Thr571 was mutated to Asn-Thr-Thr (+ FN1 N-glycan), respectively. To remove the additional glycosylation sites from OCAM Ig5 or FN1, the mutations N406Q (ΔIg5 N-glycan) and N562Q (ΔFN1 N-glycan) were introduced, respectively. To verify that these glycosylation mutants folded properly, we evaluated their subcellular localization by immunofluorescence microscopy following expression in COS-1 cells. All mutant proteins trafficked normally to the cell surface, similar to wild type NCAM and OCAM, suggesting that no gross misfolding of the mutant proteins occurred when glycosylation sites were removed or added (supplementary Fig. 1B).

Next, we compared the polysialylation of the glycosylation mutants with that of NCAM and OCAM by co-expressing these proteins with ST8SiaIV/PST in COS-1 cells and analyzing polysialylation by immunoblotting with the OL.28 anti-polySia antibody, as described above (Fig. 3). Inserting the additional N-glycosylation site into NCAM FN1 led to an observed downward shift in the molecular mass of the mutant protein, suggesting that fewer or shorter polySia chains were synthesized on some molecules (Fig. 3, left panel, + FN1 N-glycan). In contrast, the presence of an additional glycosylation site within the NCAM Ig5 domain completely eliminated polysialylation (Fig. 3, left, + Ig5 N-glycan). This suggested that the presence of this N-glycan on OCAM Ig5 could be the major factor preventing OCAM polysialylation. However, when we eliminated the extra consensus glycosylation sites from either the Ig5 or FN1 domains of OCAM, we did not observe polysialylation of the mutant OCAM proteins using our immunoblotting analysis (Fig. 3, right, ΔIg5 N-glycan and ΔFN1 N-glycan). Because the expression level of OCAM was low relative to that of NCAM, we were concerned that we might not have detected very low level polysialylation of the OCAM mutants. To address this possibility, we evaluated the polysialylation of the OCAM mutants by immunofluorescence microscopy analysis of single cells, but still no polysialylation was detected (data not shown). These data, as well as the data shown in Fig. 6A (NQA mutants) suggest that the presence of an extra Ig5 N-glycosylation site is not a major deterrent to OCAM polysialylation.

Comparison of NCAM and OCAM Ig5 Sequences and Structures Reveals Non-conserved Residues That Influence Polysialylation—To determine what residues within OCAM Ig5 may be responsible for preventing polysialylation, we first compared the sequences of the NCAM and OCAM Ig5
domains. Three stretches of non-conserved amino acids were identified (Figs. 1 and 7). The sequences Gln409-Gly410-Pro411-Val412-Ala413-Val414 (QGPVAV) of NCAM and Val404-Ser405-Asn406-Gln407-Thr408-Met409-Tyr410 (VSNQMTY) of OCAM were not analyzed because the OCAM sequence contains the additional N-linked glycosylation site analyzed earlier (Fig. 3). The NCAM sequence Asn457-Thr458-Pro459-Ser460-Ala461-Ser462-Tyr463 (NTPSASY), located in a loop between strands D and E, was replaced with Ser-Val-Gly-Arg-Lys-Met-Ile (SVGRKMI). Likewise, Gln487-Glu488-Ser489-Leu490 (QESL) of NCAM, located on strand G, was replaced with the OCAM sequence Thr-Arg-Phe-Gln (TRFQ). Both replacements led to a downward shift in the molecular mass of the polysialylated protein, suggesting shorter or fewer polySia chains added to the mutant proteins (supplemental Fig. 2). In addition, the insertion of TRFQ led to a more obvious decrease in the intensity of NCAM OL.28 staining, suggesting an overall decrease in polysialylation (supplemental Fig. 2). These results indicate that these sequences in OCAM Ig5 are not completely prohibitory for polyST recognition and substrate polysialylation but could suggest that the analogous sequences in NCAM may contribute to polyST recognition.

**Residues Adjacent to ASN5 and ASN6 in NCAM Are Critical for PolyST Recognition and NCAM Polysialylation**—Further comparison of the NCAM and OCAM sequences and structures revealed two residues near ASN6 and ASN7 (analogous to ASN5 and ASN6 in NCAM) that are vastly different in NCAM and OCAM (see Figs. 1 and 7). In NCAM, a serine residue (Ser448) is found adjacent to ASN5 (Asn449), whereas in OCAM, the analogous residue is a lysine (Lys444 adjacent to Asn445), which is ASN6 in OCAM. In NCAM, an asparagine residue (Asn476) is positioned two residues away from ASN6 (Asn478), and in OCAM the analogous residue is an arginine (Arg472 near Asn474, which is ASN7 in OCAM). We found that replacing Ser448 with lysine and Asn476 with arginine individually or together in NCAM substantially reduced or eliminated NCAM polysialylation (Fig. 4A) without altering trafficking of these proteins through the secretory pathway and their expression on the cell surface (supplemental Fig. 3). The NCAM S448K mutant exhibited substantially reduced polysialylation (15% of wild type NCAM), whereas the polysialylation of the NCAM N476R mutant and the S448K/N476R double mutant was barely detectable (3.4 and 3.6% of wild type NCAM, respectively) (Fig. 4A). However, replacing these residues with alanine or glutamate residues reduced polysialylation to 34 and 37% of that of wild type NCAM and led to NCAM proteins that migrate with lower molecular masses and appear to possess fewer or shorter polySia chains (Fig. 4A, S448A/N476A and S448E/N476E). These results suggest that the presence of these basic residues in OCAM prevents the polysialylation of N-glycans found on nearby asparagine residues. In addition, the decrease in NCAM polysialylation observed when Asn476 and Ser448 are replaced by either alanine or glutamic acid suggests that these residues are part of a larger polyST interaction site on Ig5.

**Placement of Large, Basic Residues Near the Asparagine Residues That Carry the Polysialylated N-Glycans Decreases PolyST-NCAM Interaction**—How could these residues have such a dramatic impact on substrate polysialylation? One possibility is that after an initial interaction with the FN1 domain, the polySTs need to engage Ig5 sequences to position themselves properly to polysialylate the glycans on ASN5 and ASN6. Ser448 and Asn476 may be critical for this polyST-Ig5 interaction, and the large positively charged residues found in these positions in OCAM Ig5 could block an interaction mediated by these residues as well as other nearby sequences. To evaluate the role of Ser448 and Asn476 in polyST-NCAM interaction and the impact of replacing these residues with the large basic residues found in OCAM Ig5, we took a co-immunoprecipitation approach. ST8SialIV/PST-Myc and V5-tagged NCAM or NCAM mutants were co-expressed in Lec 2 CHO cells that lack CMP-Sia transporter and consequently do not (poly)sialylate proteins (41). The polyST was immunoprecipitated with the anti-V5 epitope tag antibody. A, top, Lec 2 CHO cells were co-transfected with V5-tagged NCAM or its S448A/N476R mutants and ST8SialIV/PST-Myc. The polyST was immunoprecipitated (IP) from cell lysates using an anti-Myc tag antibody, and co-precipitating NCAM proteins were detected by immunoblotting (IB) with an anti-V5 epitope tag antibody. A, bottom, the relative expression levels of wild type and mutant NCAM proteins were determined by immunoblotting cell lysate aliquots with an anti-V5 epitope tag antibody. B, top, Lec 2 CHO cells were co-transfected with ST8SialIV/PST-Myc and its 448/476 mutants and ST8SialIV/ PST-Myc. The polyST was immunoprecipitated (IP) from cell lysates using an anti-Myc tag antibody, and co-precipitating NCAM proteins were detected by immunoblotting (IB) with an anti-V5 epitope tag antibody. B, bottom, the relative expression levels of wild type and mutant NCAM proteins were determined by immunoblotting cell lysate aliquots with an anti-V5 epitope tag antibody. Relative polysialylation (top) and relative binding (bottom) was determined by densitometry.
NCAM and the NCAM mutants were also assessed by immunoblotting cell lysates with the anti-V5 antibody (Fig. 4B, bottom). In multiple experiments, we found that binding of the S448K and N476R mutants to ST8SiaIV/PST was reduced to 52–55% of that of wild type NCAM, and the binding of the S448K/N476R double mutant was reduced to 26% of that of wild type NCAM (Fig. 4B). Interestingly, the S448A/N476A and S448E/N476E double mutants that exhibited less reduction in polysialylation also exhibited somewhat reduced polyST-NCAM binding (79–84% of wild type NCAM) (Fig. 4B). These results suggest that replacing just one of these two amino acids with a large basic residue reduces both polyST-NCAM interaction and NCAM polysialylation and suggests that the presence of large, basic amino acids at these positions in OCAM is likely to play a major role in blocking OCAM polysialylation.

The Observed Decreases in Polysialylation or PolyST Binding of S448K and N476R Mutant Proteins Cannot Be Explained by Changes in Ig5 Glycosylation—The observed alteration in NCAM polysialylation and polyST-NCAM binding in Ig5 single and double mutants was particularly intriguing because we have previously shown that the FN1 domain is absolutely necessary for polyST-NCAM recognition and that deleting this domain eliminates NCAM polysialylation and polyST-NCAM binding (29, 30). One possibility is that the presence of basic residues close to the glycan attachment sites may have blocked glycosylation of those asparagines. This would have an obvious deleterious effect on overall polysialylation but also could alter polyST-NCAM binding if the NCAM Ig5 N-glycans participate in this binding along with NCAM FN1. We tested the impact of the S448K and N476R mutations on NCAM glycosylation by comparing the molecular masses of wild type NCAM and the S448K, N476R, and S448K/N476R mutants and the analogous alanine and glutamine replacements with two NCAM proteins in which we have intentionally eliminated the consensus glycosylation sites at ASN5 and ASN6 by either directly replacing the asparagine with a serine (N449S/N478S) or altering the third position of the Asn-X-Thr/Ser consensus sequence to an alanine (S451A/T480A). SDS-PAGE analysis using 4–15% gradient gels suggested that the presence of basic residues adjacent or near to the asparagine residues in the consensus glycosylation sites does not prevent glycosylation of ASN5 and ASN6 (Fig. 5A). We also analyzed the role of the ASN5 and ASN6 N-glycans in polyST-NCAM binding using our co-immunoprecipitation assay described above. NCAM proteins lacking the two Ig5 N-glycans bound as well to ST8SiaIV/PST as did wild type NCAM (Fig. 5B), suggesting that the Ig5 N-glycans do not play a substantial role in NCAM-polyST interactions.

Creating the Core FN1 Acidic Patch in the O-N Ig5 Chimera Enhances Its Polysialylation—To determine whether we could reconstitute polysialylation in OCAM lacking the non-permissive Ig5 N-glycan (OCAM NQA), we replaced the non-permissive Ig5 residues, Lys444 and Arg477, with serine and asparagine, respectively, and evaluated the ability of the resulting mutant proteins to be polysialylated by ST8SiaIV/PST. We found that these changes allowed only a small amount of OCAM polysialylation (Fig. 6A). Specifically, the NQA/K444S mutant demonstrated an 8.5% increase in polysialylation, whereas the NQA/R472N mutant demonstrated a 21.8% increase in polysialylation, and the two replacements together (NQA/K444S/R472N) exhibited a 28.5% increase in polysialylation over wild type OCAM. As with the reciprocal NCAM mutants, the nature of the amino acid near ASN6 (Arg477 in OCAM, Asn476 in NCAM) had the greatest impact. In addition, because structural studies by Kulahin et al. (42) suggested that the linker between OCAM Ig5 and FN1 domains may be very flexible, and our work suggested that a more rigid linker could be important for NCAM polysialylation (30, 43), we also evaluated the impact of replacing the last β-strand of OCAM Ig5 that includes the linker region with that of NCAM (data not shown). We found that replacing this linker alone did not lead to OCAM polysialylation, and replacing it in the presence of the NQA/K444S/R472N mutation led to only a slight increase in the polysialylation of this protein. These results contrasted with the high level of polysialylation observed for the O-N Ig5 chimera that contained the full NCAM Ig5 domain (Fig. 6A, O-N Ig5). Thus, whereas Lys444 and Arg477 in OCAM Ig5 are non-permissive for polysialylation when placed in analogous locations in NCAM Ig5, other factors in the OCAM Ig5 domain are also blocking polysialylation.

Previous work suggested that the OCAM FN1 domain only partially replaced the NCAM FN1 domain because the polysialylation of the N-O FN1 chimera was about 50% that of wild type NCAM (35). Notably, the OCAM FN1 domain is missing two of the three core acidic patch residues. In place of Asp526,
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OCAM has Asn\(^{517}\), and in place of Glu\(^{221}\), OCAM has Lys\(^{518}\). Using the O-N Ig5 chimera, we replaced these residues to recreate the core acidic patch in OCAM FN1 (O-N Ig5 N517D/K518E) to determine whether this would improve its polysialylation. We found that the presence of the core acidic patch substantially improved the polysialylation of the O-N Ig5 chimera by 66% (Fig. 6B, O-N Ig5 N517D/K518E). These results highlighted the importance of the FN1 acidic patch in polyST recognition and substrate polysialylation and suggest that this region is key for polyST recognition and NCAM polysialylation.

DISCUSSION

Earlier work from our laboratory demonstrated that the polySTs recognize an acidic patch in the NCAM FN1 domain and hinted that sequences outside the FN1 domain were involved in this process as well (see below). The evidence presented here indicates that the polySTs need to engage with sequences in both the FN1 and Ig5 domains of NCAM prior to polysialylation of Ig5 N-glycans. Work by Foley et al. (35) demonstrated that OCAM FN1 was able to partially replace NCAM FN1 to allow the polysialylation of an NCAM-OCAM chimera to \(~50%\) of that observed for wild type NCAM. In this work, we found that simply reconstituting the core residues of the FN1 acidic patch in an OCAM chimera that contains the NCAM Ig5 domain (O-N Ig5) by introducing two acidic residues increased the polysialylation of this chimera by over 2-fold, or 66%, again highlighting the importance of the FN1 acidic patch in polyST recognition and NCAM polysialylation (Fig. 6B). Also in this work, we show that OCAM Ig5 is non-permissive for polyST recognition and polysialylation and identify features of OCAM Ig5 that prohibit polysialylation. Specifically, we have identified two large basic residues present in OCAM Ig5 that substantially decrease NCAM interaction with ST8SiaIV/PST and its subsequent polysialylation when inserted into analogous positions in NCAM Ig5 (Fig. 4). Inserting an extra Ig5 N-glycan and non-conserved amino acids from OCAM Ig5 into NCAM Ig5 also decreases NCAM polysialylation to varying degrees. The finding that features in the Ig5 domain influence NCAM-polyST interaction and substrate polysialylation indicates that the polySTs are required to make contact with sequences in both the FN1 and Ig5 domain of NCAM for polysialylation to occur.

To determine what features of OCAM Ig5 were blocking its polysialylation, we compared NCAM and OCAM Ig5 domains. Analysis of the OCAM sequence reveals eight potential N-linked glycosylation sites, including two that are equivalent to ASN5 and ASN6 that carry the polysialylated N-glycans on NCAM and two that are not present in NCAM (Fig. 1). We found that inserting an extra OCAM Ig5 glycosylation site into NCAM Ig5 eliminated NCAM polysialylation, whereas the presence of an extra glycosylation site in the FN1 domain appeared to decrease the number or length of the polySia chains added, resulting in a polysialylated NCAM that migrated with a lower molecular mass (Fig. 3). Although we cannot be sure that the engineered glycosylation sites do in fact become glycosylated, analysis of the load control bands reveals a small upward shift in the NCAM mutant with the extra Ig5 glycan. Therefore, at least in the context of NCAM Ig5, an additional N-glycan located in the unstructured region between Ig5 strands A and A’ has a highly negative effect on polysialylation. Consequently, we anticipated that removing this glycan from OCAM Ig5 would allow some OCAM polysialylation. However, mutation of the extra glycosylation site in the OCAM sequence had no effect (Fig. 3). A recent crystal structure of the human OCAM Ig4-Ig5-FN1-FN2 domains (Protein Data Bank entry 2JLL) revealed GlcNAc residues at only six of the eight potential N-linked glycosylation sites (42). Notably, the only sites where
GlcNAc residues were not observed were the extra N-linked glycosylation sites on both Ig5 and FN1. Therefore, it is possible that these sites are not used on OCAM. Consequently, this might explain why removing them did not lead to detectable OCAM polysialylation. In contrast, in the case of NCAM Ig5, inserting the additional glycosylation site most likely results in the addition of an N-glycan at this site, resulting in a profoundly negative effect on polyST engagement and polysialylation. Analysis of the sequences/structures of the Ig5 domains of both proteins led to the identification of two non-conserved stretches and two basic residues found only in OCAM Ig5, Lys\(^{444}\) and Arg\(^{472}\), positioned near the two potential sites of polysialylation, ASN6 and ASN7. Replacing one stretch of amino acids in NCAM, Gln\(^{487}\)–Glu\(^{488}\)–Ser\(^{489}\)–Leu\(^{490}\) (QESL), with TRFQ from OCAM led to a decrease in the molecular mass of the polysialylated protein and a decrease in OL.28 staining, suggesting that this stretch might be involved in polyST recognition (supplemental Fig. 2 and Fig. 7). However, a much more dramatic effect was observed when the two basic residues found in OCAM Ig5 were used to replace more neutral residues (Ser\(^{448}\) and Asn\(^{476}\)) in NCAM Ig5. This change substantially reduced or nearly eliminated NCAM polysialylation (decrease to 3–15% of wild type NCAM polysialylation) and led to a decrease in NCAM-polyST binding (Figs. 4 and 7). Replacing these residues with neutral alanine or acidic glutamic acid residues had a lesser but notable deleterious effect on NCAM polysialylation (decrease to 34–37% of wild type NCAM polysialylation) and polyST binding. These observations suggested that Ser\(^{448}\) and Asn\(^{476}\) may be part of a larger polyST recognition site on the Ig5 domain, and that the presence of large positively charged residues in these positions is particularly damaging to polyST-Ig5 interaction and subsequent NCAM polysialylation.

We propose that the presence of the large, basic lysine and arginine residues, when placed in NCAM Ig5, obstruct polyST interaction (Fig. 7). With this possibility in mind, we note that the side chains of the basic residues do not exhibit any obvious interactions with other amino acids in the OCAM structure and would not be predicted to if inserted into the NCAM structure. In fact, the side chains of these residues and the analogous residues in NCAM extend away from their respective strands (Fig. 7). Nevertheless, it is tempting to suspect that amino acid changes so close to the glycosylation sites may have prevented the addition of an N-glycan at these sites and that simply eliminating the glycan prevented not only polysialylation but also polyST-NCAM binding. However, our results in Fig. 5 strongly suggest that the presence of these basic residues did not impact NCAM glycosylation and that glycans on ASN5 and ASN6 do not appear to play a role in steady state polyST-NCAM binding as measured by the co-immunoprecipitation assay. Another possibility is that the side chains of these basic amino acids might influence the orientation of the nearby N-glycan. \textit{In silico} modeling suggests that this could be a possibility for Lys\(^{444}\) but not Arg\(^{472}\) in OCAM (44) (data not shown). One could envision that change in the orientation of the N-glycan could obstruct a polyST interaction site on NCAM Ig5 and prevent engagement and polysialylation. However, this would be very difficult to verify.

It is interesting to note that many of the changes made to NCAM Ig5 that did impact its polysialylation are found on the same face of the Ig5-FN1 tandem as ASN6 (Fig. 7). These include the Asn\(^{476}\) to arginine change two residues away from ASN6 and the QESL to TRFQ change that is found on the adjacent strand. In addition, the non-permissive N-glycan attachment site is found on the unstructured region adjacent to the strand containing the QESL/TRFQ sequence (Fig. 7). Early work by Fukuda and colleagues (45) using an \textit{in vitro} approach suggested that ST8SiaIV/PST strongly prefers the N-glycan on ASN6, whereas ST8SiaII/STX has a slight preference for the N-glycan on ASN6. More recent work by Galuska \textit{et al.} (46) in mouse models selectively expressing either ST8SiaIV/PST or ST8SiaII/STX suggests that both enzymes use the ASN5 and ASN6 glycans equivalently. If our cell culture analysis of ST8SiaIV/PST polysialylation of NCAM reflects the \textit{in vitro} situation more closely, we may be observing the preferential polysialylation of the glycan on ASN6 and a more dramatic decrease in polysialylation when nearby sequences are altered. However, it is clear that replacing Ser\(^{448}\) near ASN5 with Lys has a substantial impact on overall polysialylation and that neither ASN6 nor ASN5 precisely align with the FN1 acidic patch, at least according to our crystal structure (43). How can we explain our observations? One possibility is that the polyST wraps around the Ig5 domain after contacting the FN1 acidic patch and must make contacts with sequences close to both glycosylation sites for a stable interaction to occur. However, we cannot rule out the possibility that the Ig5 domain can twist relative to the FN1 domain so that the FN1 acidic patch, the major polyST interaction site, can sit between the two Ig5 glycosylation sites or align with each independently. If this indeed occurs, then a crystal structure would not adequately represent the dynamic situation. We are currently evaluating NCAM Ig5-FN1 tandem dynamics using NMR methodology to address just this question.

Is our data suggesting a role for the Ig5 domain in polyST engagement consistent with the predominant role of FN1 in polyST recognition suggested by our previous studies? Our earlier work demonstrated a requirement for NCAM FN1 in polyST-NCAM binding and NCAM polysialylation; deleting the FN1 domain blocked both processes (29, 30). An acidic patch on the surface of FN1 was identified and shown to partially mediate NCAM polysialylation. Replacing Asp\(^{520}\), Glu\(^{521}\), and Glu\(^{523}\) of this acidic patch with alanines led to a 30% decrease in NCAM polysialylation, whereas replacing these residues with arginines essentially eliminated polysialylation (29, 30). At the time, we reasoned that the large, negatively charged arginine residues had a general disruptive effect on interactions mediated by nearby FN1 residues outside the acidic patch proper. However, analysis of the acidic patch residues in NCAM7, a truncated NCAM protein consisting of FN1, FN2, the transmembrane region, and cytoplasmic tail, which is polysialylated on O-glycans in the FN1 domain, demonstrated that replacing the acidic patch with alanine residues in this protein essentially eliminated its polysialylation (35). This difference in the impact of the acidic patch alanine replacements on polysialylation of full-length NCAM versus NCAM7 was an initial indication that the polySTs may recognize sequences outside...
the FN1-FN2 region in full-length NCAM, and in the FN1 domain, the acidic patch is the major recognition site. Interestingly, later work evaluating the binding of the NCAM acidic patch mutants to the polySTs demonstrated a similar 30% decrease in binding when either alanines or arginines replaced the acidic patch residues (30). This again suggested that other
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sequences outside the FN1 acidic patch may be mediating recognition but also demonstrated that polysialyltransferase binding per se did not guarantee polysialylation. In fact, we see just this in our analysis of the NCAM Ig5 Ser418 and Asn476 mutants in this study. The polysialylation of the S448K/N476R mutant is decreased to ~3% that of wild type, but its binding to ST8SiaIV/PST is 26% that of wild type (Fig. 4B). It is likely that the intact FN1 binding site accounts for the residual binding and that the polysialyltransferase interaction is not sufficient for effective Ig5 N-glycan polysialylation. Finally, in some very early studies, we found that a truncated NCAM lacking the FN1-FN2 region but containing all five Ig domains was very weakly polysialylated (27). At the time, we believed that the overexpression of the enzyme and substrate may have led to this polysialylation, but now in light of our current results, this may have been our very first indication that the Ig5 sequences engage the polysialyltransferases, albeit weakly without the FN1 sequences.

Taken in aggregate, these observations suggest a model in which the polysialyltransferases in the NCAM FN1 domain via its acidic patch and then makes specific contacts with Ig5 sequences to polysialylate the N-glycans on either ASN5 or ASN6. Interestingly, results from our laboratory suggest that residues in a polybasic region at the polysialyltransferase catalytic domain border are likely to be involved in the initial FN1 recognition event (47, 48). In the case of OCAM, the presence of large basic residues and other non-conserved sequences in Ig5 decrease or block the secondary contacts with the Ig5 domain that are essential for the proper positioning of the enzyme and polysialylation. Additional studies are under way to verify this model and determine whether other polysialyltransferase substrates have similar requirements for polysialyltransferase recognition.

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