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# The Polysialyltransferases Interact with Sequences in Two Domains of the Neural Cell Adhesion Molecule to Allow Its Polysialylation*[S]

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**Background:** Recognition of the protein substrate is the first step in polysialylation of its glycans.
**Results:** Residues in the OCAM Ig5 domain are non-permissive for its polysialylation.
**Conclusion:** The polysialyltransferases interact with residues in both the Ig5 and FN1 domains of NCAM to allow its polysialylation.
**Significance:** A two-domain polysialyltransferase recognition site may be required for all polysialylated proteins.

**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 the polysialylated *N*-glycans 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)[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 upregulated 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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[3] The abbreviations used are: polySia, polysialic acid; polyST, polysialyltransferase; FN1 and FN2, first and second fibronectin type III repeat of NCAM, respectively.

(21), α-subunit of the voltage-dependent sodium channel (22), neuropilin-2 (NRP-2) (23), SynCAM 1 (24), and the polySTs themselves (autopolysialylation) (25, 26). Overwhelming evidence from our laboratory indicates that polysialylation is a protein-specific glycosylation event in which the polySTs initially recognize protein determinants on their glycoprotein substrates prior to modifying substrate glycans (27–30). This protein specificity was first suggested by the observation that *N*-glycans attached to NCAM are far more efficiently polysialylated *in vitro* than free glycans enzymatically released from NCAM (31, 32).

NCAM is the major carrier of polySia. There are three main isoforms of NCAM, which arise from alternative splicing of a single gene. NCAM-140 and NCAM-180 are type I transmembrane proteins that differ in the length of their cytosolic tail, whereas NCAM-120 is linked to the membrane via a glycosylphosphatidylinositol anchor (33). The three isoforms share a common extracellular structure consisting of five immunoglobulin (Ig) domains and two fibronectin type III repeats (FN1 and FN2). The sites of polysialylation are found on the fifth and sixth *N*-glycosylation sites, located on Ig5 (ASN5 and ASN6) (34). We have previously demonstrated that FN1 is required for the polysialylation of ASN5 and ASN6 (27, 29). In fact, deletion of the FN1 domain prevents both polyST-NCAM interaction and NCAM polysialylation (30). Furthermore, we have identified specific residues in NCAM FN1, including a key surface acidic patch, that have roles in polyST recognition and/or positioning and binding (28–30, 35).

The olfactory cell adhesion molecule, OCAM, is mainly expressed in neural tissue such as the brain, olfactory epithelium, and the retina (36, 37). Like NCAM, OCAM has been shown to form homophilic trans interactions (37). Mouse knock-out studies have suggested a role for OCAM in intraglomerular compartmental organization (38). Recently, it has been demonstrated that OCAM is highly expressed in androgen-dependent prostrate cancer cell lines and estrogen-dependent breast cancer cell lines, whereas it is expressed only at low levels in normal prostrate cell lines (39). There are two isoforms of OCAM, a transmembrane and glycosylphosphatidylinositol-anchored form, and they both share the same extracellular domain as NCAM, with five Ig domains and two fibronectin type III repeats (37, 40). Interestingly, OCAM Ig5 has consensus *N*-glycosylation sites at the same positions as ASN5 and ASN6 of NCAM (ASN6 and ASN7 in OCAM). Although the expression pattern of OCAM can overlap with that of NCAM, OCAM is not polysialylated (37). Recently, we generated a chimeric protein in which the FN1 domain of NCAM was replaced with that of OCAM (35). We anticipated that this chimera would not be polysialylated and that we would be able to reconstitute polysialylation by inserting specific NCAM FN1 residues. Surprisingly, ST8SiaIV/PST could polysialylate the NCAM-OCAM chimera, indicating that OCAM FN1 is recognized by the polySTs, enabling them to polysialylate glycans located on NCAM Ig5. The polysialylation of the chimera was not as efficient as NCAM polysialylation, suggesting that although ST8SiaIV/PST can engage OCAM FN1, recognition/binding is not optimal (35). These results suggested that other factors either within FN1 itself or adjacent domains of NCAM could have a role in stabilizing polyST recognition and binding.

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 polyST-substrate binding. These results suggest that the polySTs 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 QuikChange™ 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′-AAGCTTGTCCTGAACATGAGCCTCCTCC-3′ and 5′-TCTAGATGCCTTTATGTCATCTTCTTTAGACTGG-3′. These primers specifically introduced a HindIII and XbaI 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 XbaI. After gel purification, the OCAM PCR product was ligated into the expression vector. A frameshift mutation, introduced during cloning

near the XbaI site, was corrected by mutagenesis using the following primers: 5′-GATGACATAAAGGCAGGTCTAGAGGGCCCGC-3′ and 5′-GCGGGCCCTCTAGACCTGCCTTTATGTCATC-3′. To generate the chimeric proteins, BamHI and XbaI restriction sites, flanking the Ig5, FN1, or Ig5-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′-GGATCCGATGTCCCCTCTAGTCCCCATG-3′/5′-TCTAGAGGCTCACGGACTGGCAGTGTC-3′, 5′-GGATCCTATGCCCCAAAGCTACAGGGC-3′/5′-TCTAGAGCTGCTTGAACAAGGATGAATTC-3′, 5′-GGATCCGACACCCCCTCTTCACCATCC-3′/5′-TCTAGAGCTTCCCCTTGGACTGGCTGCGTC-3′, and 5′-GGATCCTATGCCCCAAAGCTACAGGGC-3′/5′-TCTAGAGCTTCCCCTTGGACTGGCTGCGTC-3′. The PCR products were cut with BamHI and XbaI and ligated in frame into NCAM lacking FN1, OCAM lacking Ig5, OCAM lacking FN1, and OCAM lacking Ig5-FN1, to generate NCAM-OCAM FN1, OCAM-NCAM Ig5, OCAM-NCAM FN1, and OCAM-NCAM Ig5-FN1, respectively. The BamHI and XbaI restriction sites flanking the Ig5 or FN1 domains were removed from all chimeras by site-directed mutagenesis.

*NCAM and OCAM Mutagenesis*—Mutagenesis reactions were performed using the Stratagene QuikChange™ site-directed mutagenesis kit according to the manufacturer's protocol. The primers used are listed in supplemental Table 1. Mutations were confirmed by DNA sequencing performed by the DNA Sequencing Facility of the Research Resources Center at the University of Illinois (Chicago, IL).

*Transfection of COS-1 Cells for Immunofluorescence Localization*—COS-1 cells maintained in DMEM, 10% FBS were plated on 12-mm glass coverslips in 24-well plates and grown at 37 °C in 5% $CO_2$. Cells in each well were transfected using 3 $\mu$l of Lipofectin and 0.5 $\mu$g of NCAM, OCAM, or chimeric protein cDNA in 300 $\mu$l of Opti-MEM I, according to the manufacturer's protocol. After 6 h, 1 ml of DMEM, 10% FBS was added to each well. Cells were incubated at 37 °C in 5% $CO_2$ overnight.

*Analysis of NCAM, OCAM, and Chimeric Protein Localization by Indirect Immunofluorescence Microscopy*—COS-1 cells grown on glass coverslips were transfected as described above. Eighteen hours post-transfection, cells were washed twice with 1 ml of phosphate-buffered saline (PBS) and then fixed and permeabilized with 1 ml of ice-cold methanol to visualize both internal structures and the cell surface. Then 1 ml of blocking buffer (5% normal goat serum in PBS) was added for 1 h at room temperature. Cells were incubated with a 1:250 dilution of anti-V5 epitope tag antibody in blocking buffer for 1 h, washed with PBS four times for 5 min, and then incubated with a 1:100 dilution of FITC-conjugated goat anti-mouse IgG secondary antibody in blocking buffer for 45 min. After washing with 1 ml of PBS four times for 5 min, coverslips were rinsed in distilled $H_2O$ and mounted on glass slides using mounting medium (15% (w/v) Vinol 205 polyvinyl alcohol, 33% (w/v) glycerol, 0.1% azide in PBS, pH 8.5). Cells were visualized using a Nikon Axiophot microscope equipped with epifluorescence illumination and a ×60 oil immersion Plan Apochromat objective. Pictures were taken using a SPOT RT color digital camera and processed using SPOT RT software version 3.5.1 (Diagnostic Instruments Inc., Sterling Heights, MI).

*Transfection of COS-1 Cells for Immunoprecipitation and Immunoblotting*—COS-1 cells maintained in DMEM, 10% FBS were plated on 100-mm tissue culture plates and grown at 37 °C in 5% $CO_2$ until 80–90% confluent. Cells were transfected using either 30 $\mu$l of Lipofectin, 10 $\mu$g of both V5-tagged NCAM/OCAM/chimera and ST8SiaIV/PST-Myc cDNA in 3 ml of Opti-MEM I, or alternatively with 30 $\mu$l of Lipofectamine 2000, 7.5 $\mu$g of both V5-tagged NCAM/OCAM/chimera and ST8SiaIV/PST-Myc cDNA in 3 ml of Opti-MEM I, according to the manufacturer's protocol. The NCAM, OCAM, and chimeric cDNAs were cloned into the pcDNA3.1 V5/HisB vector. ST8SiaIV/PST cDNA was cloned upstream of the Myc tag in the pcDNA3.1 Myc/HisB vector, in which a stop codon was placed before the $His_6$ coding sequence. After a 6-h incubation, the transfection mixture was removed, and 10 ml of DMEM, 10% FBS was added to each plate.

*Immunoprecipitation of NCAM, OCAM, and Chimeric Proteins*—Eighteen hours post-transfection, cells were washed with 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). Lysates were precleared with 50 $\mu$l of protein A-Sepharose beads (50% suspension in PBS) for 1 h at 4 °C. NCAM, OCAM, or chimeric proteins were immunoprecipitated with 3 $\mu$l of anti-V5 epitope tag antibody for 2 h at 4 °C, followed by incubation for 1 h with 50 $\mu$l 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 $\mu$l of Laemmli sample buffer containing 5% $\beta$-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-$\mu$l aliquot of cell lysate was removed prior to immunoprecipitation, and an equal volume of Laemmli sample buffer, 5% $\beta$-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 $\mu$l 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 $\mu$l 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,

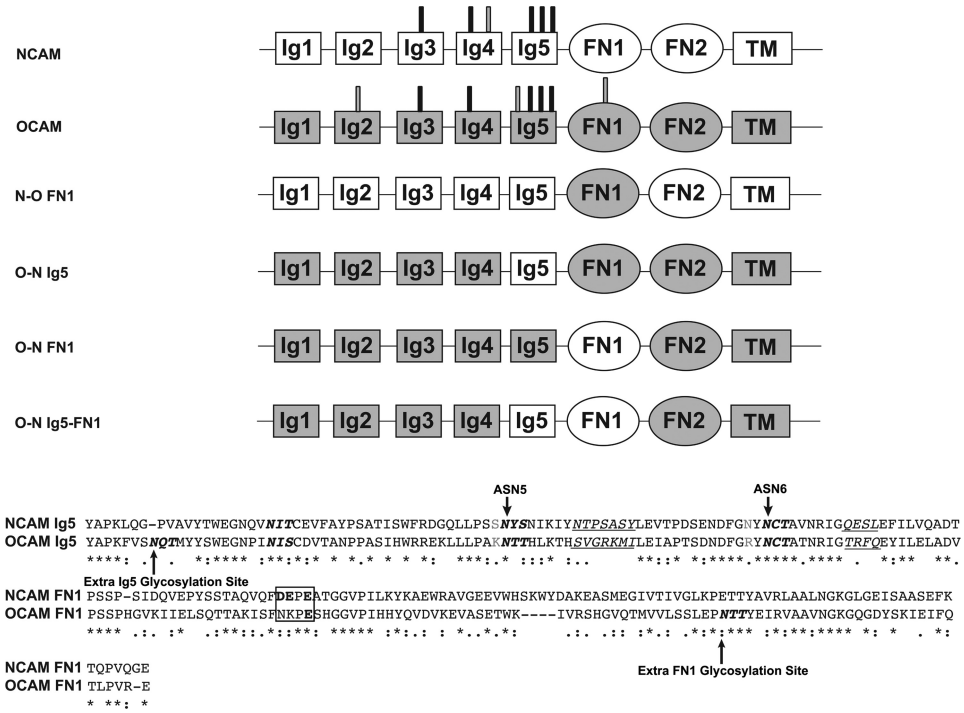


FIGURE 1. **Sequence comparison and schematic of NCAM, OCAM, and chimeric proteins used in this study.** *Top*, schematics of the domain arrangement of NCAM (*white*), OCAM (*gray*), and the chimeric proteins made and analyzed in this study. The *N*-linked glycosylation sites that are conserved between NCAM and OCAM are indicated with *black bars*, and those that are not conserved are indicated by *gray bars*. The FN1 domain of NCAM was replaced with OCAM FN1 to generate N-O FN1. The Ig5, FN1, or Ig5-FN1 domains of OCAM were replaced with the NCAM Ig5, FN1, or Ig5-FN1 domains to generate O-N Ig5, O-N FN1, or O-N Ig5-FN1, respectively. *TM*, transmembrane domain. *Bottom*, comparison of NCAM and OCAM Ig5-FN1 sequences. *Asterisks below* the sequences indicate identity, *double dots* indicate strong homology, and *single dots* indicate weak homology. Consensus *N*-linked glycosylation sites within the Ig5 and FN1 sequences are in *boldface italic type*, with NCAM ASN5 and ASN6 and the extra OCAM *N*-glycosylation sites labeled. Non-conserved residues adjacent to ASN5 and ASN6 in NCAM Ig5 and the analogous residues in OCAM Ig5 are shown in *gray*. Stretches of nonconserved Ig5 amino acids analyzed in this study are *italicized* and *underlined*. The three core acidic path residues of NCAM FN1 (**DE**P**E**; Asp$^{520}$, Glu$^{521}$, and Glu$^{523}$) and the analogous residues in OCAM FN1 (NKP**E**; Asn$^{517}$, Lys$^{518}$, Pro$^{519}$, and Glu$^{520}$) are *boxed*, and the acidic patch residues are in *boldface type*. Note that mouse OCAM was used in this study with Glu at position 520, whereas the structure shown in Fig. 7 is of human OCAM with an Asp at position 520.

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%

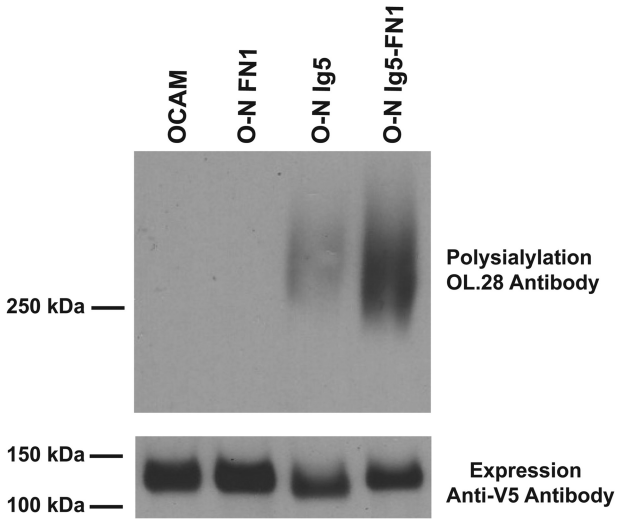


FIGURE 2. **The Ig5 domain of OCAM does not support polysialylation.** *Top*, OCAM or chimeric proteins containing NCAM domains (O-N FN1, O-N Ig5, and O-N Ig5-FN1) were co-expressed with ST8SiaIV/PST-Myc in COS-1 cells. OCAM proteins were immunoprecipitated from cell lysates, and polysialylation was determined by immunoblotting with the anti-polySia antibody, OL.28, as described under "Experimental Procedures." *Bottom*, relative protein expression levels were determined by immunoblotting cell lysate aliquots with anti-V5 epitope tag antibody.

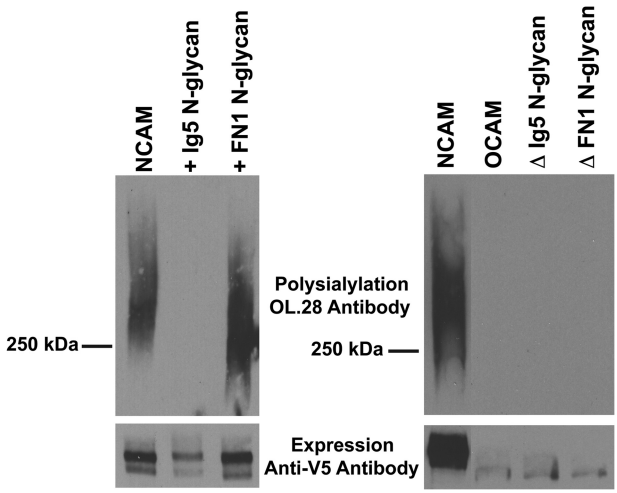


FIGURE 3. **The additional *N*-linked glycosylation site in OCAM Ig5 prevents polysialylation when inserted into NCAM Ig5, but removing this site from OCAM Ig5 is not sufficient to allow OCAM polysialylation.** *Top panels*, COS-1 cells were co-transfected with V5-tagged wild type or mutated NCAM or OCAM and ST8SiaIV/PST-Myc. NCAM, OCAM, or mutant proteins were immunoprecipitated from cell lysates using anti-V5 epitope tag antibody, and polysialylation was measured using the anti-polySia antibody, OL.28. The polysialylation of NCAM mutants with additional OCAM *N*-glycosylation sites inserted is shown on the *left*, and the polysialylation of OCAM mutants with these *N*-glycosylation sites removed is shown on the *right*. *Bottom panels*, relative protein expression levels were determined by immunoblotting cell lysate aliquots with anti-V5 epitope tag antibody.

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 (supplemental Fig. 1*A*). 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 $Gly^{410}$-$Pro^{411}$-$Val^{412}$ was mutated to Asn-Gln-Thr (+Ig5 *N*-glycan), and $Glu^{569}$-$Thr^{570}$-$Thr^{571}$ 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 (supplemental Fig. 1*B*).

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. 6*A* (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 Gln$^{409}$-Gly$^{410}$-Pro$^{411}$-Val$^{412}$-Ala$^{413}$-Val$^{414}$ (QGPVAV) of NCAM and Val$^{404}$-Ser$^{405}$-Asn$^{406}$-Gln$^{407}$-Thr$^{408}$-Met$^{409}$-Tyr$^{410}$ (VSNQTMY) of OCAM were not analyzed because the OCAM sequence contains the additional *N*-linked glycosylation site analyzed earlier (Fig. 3). The NCAM sequence Asn$^{457}$-Thr$^{458}$-Pro$^{459}$-Ser$^{460}$-Ala$^{461}$-Ser$^{462}$-Tyr$^{463}$ (NTPSASY), located in a loop between strands D and E, was replaced with Ser-Val-Gly-Arg-Lys-Met-Ile (SVGRKMI). Likewise, Gln$^{487}$-Glu$^{488}$-Ser$^{489}$-Leu$^{490}$ (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, *QESL-TRFQ*). 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 (Ser$^{448}$) is found adjacent to ASN5 (Asn$^{449}$), whereas in OCAM, the analogous residue is a lysine (Lys$^{444}$ adjacent to Asn$^{445}$, which is ASN6 in OCAM). In NCAM, an asparagine residue (Asn$^{476}$) is positioned two residues away from ASN6 (Asn$^{478}$), and in OCAM the analogous residue is an arginine (Arg$^{472}$ near Asn$^{474}$, which is ASN7 in OCAM). We found that replacing Ser$^{448}$ with lysine and Asn$^{476}$ with arginine individually or together in NCAM substantially reduced or eliminated NCAM polysialylation (Fig. 4*A*) 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. 4*A*). 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. 4*A*, *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 Asn$^{476}$ and Ser$^{448}$ 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. Ser$^{448}$ and Asn$^{476}$ 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 Ser$^{448}$ and Asn$^{476}$ 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. ST8SiaIV/PST-Myc and V5-tagged NCAM or NCAM mutants were co-expressed in Lec 2 CHO cells that lack a functional CMP-Sia transporter and consequently do not (poly)sialylate proteins (41). The polyST was immunoprecipitated using anti-Myc antibodies and co-precipitating NCAM proteins detected by immunoblotting with the anti-V5 epitope tag antibody (Fig. 4*B*, *top*). The relative expression levels of

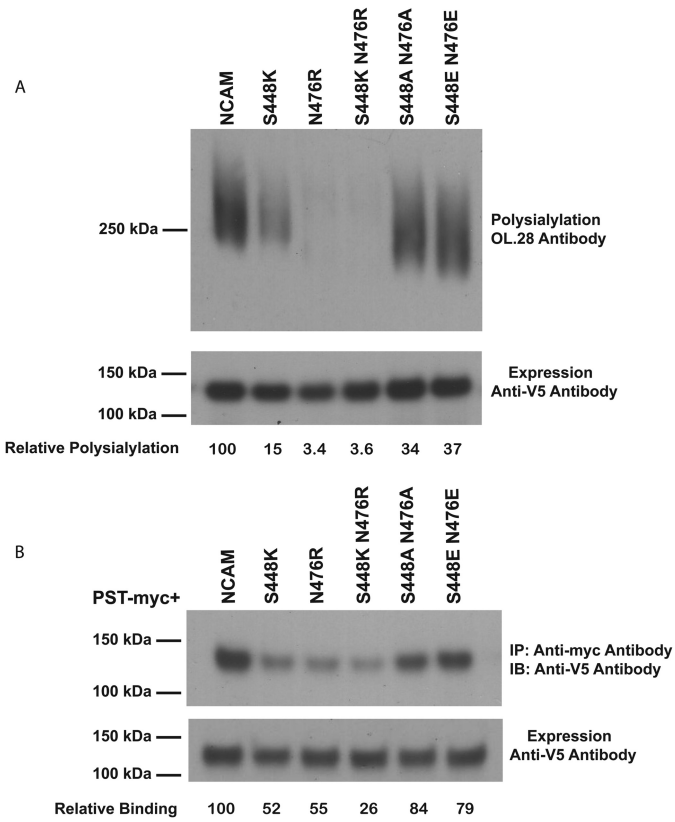


FIGURE 4. **Inserting the basic residues found adjacent to Ig5 glycosylation sites in OCAM into analogous positions in NCAM Ig5 reduces or eliminates NCAM polysialylation and polyST-NCAM interactions.** *A*, *top*, wild type NCAM or mutated NCAM proteins with Ser$^{448}$ and Asn$^{476}$ replaced with Lys or Arg (as found in OCAM Ig5), alanine, or glutamic acid residues were co-expressed with ST8SiaIV/PST-Myc in COS-1 cells. NCAM proteins were immunoprecipitated from cell lysates, and polysialylation was determined by immunoblotting with the OL.28 anti-polySia antibody. *A*, *bottom*, relative protein expression levels were determined by immunoblotting cell lysate aliquots with an anti-V5 epitope tag antibody. *B*, *top*, Lec 2 CHO cells were co-transfected with V5-tagged NCAM or its S448/N476 mutants and ST8SiaIV/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. 4*B*, *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. 4*B*). 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. 4*B*). 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. 5*A*). 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. 5*B*), 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, $Lys^{444}$ and $Arg^{472}$, 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. 6*A*). 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 ($Arg^{472}$ in OCAM, $Asn^{476}$ 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 $\beta$-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. 6*A*, *O-N Ig5*). Thus, whereas $Lys^{444}$ and $Arg^{472}$ 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 $Asp^{520}$,

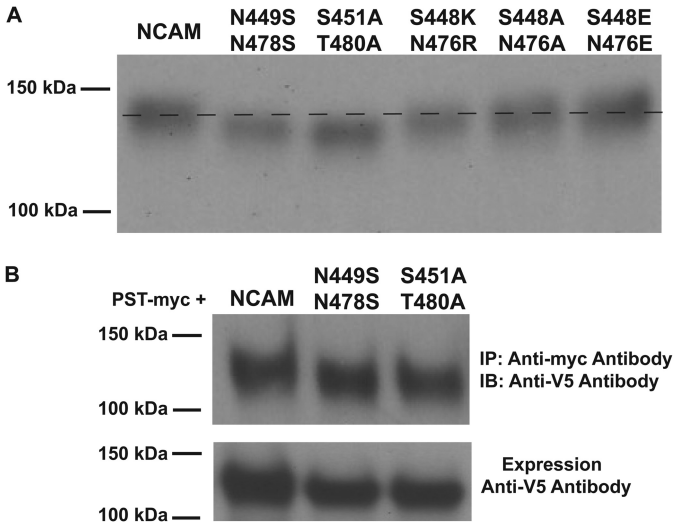


FIGURE 5. **NCAM $Ser^{448}$ and $Asn^{476}$ mutants do not compromise the glycosylation of ASN5 and ASN6, and these *N*-glycans are not required for polyST-NCAM interactions.** *A*, the molecular masses of S448K/N476R, S448A/N476A, and S448E/N476E mutants were compared with those of NCAM glycosylation mutants in which ASN5 ($Asn^{449}$) and ASN6 ($Asn^{478}$) were replaced with serine residues (N449S/N478S), or the third position of each of these glycosylation sites was replaced with alanine (S451A/T480A). Following expression in COS-1 cells, cell lysates (100 $\mu$l, or 10% of total cell lysate) were separated on a 4–15% gradient gel, and proteins were detected by immunoblotting with an anti-V5 epitope tag antibody. *B*, *top*, the ability of NCAM and NCAM glycosylation mutants, N449S/N478S and S451A/T480A, that lack *N*-glycans on ASN5 and ASN6 to bind to the polyST ST8SiaIV/PST-Myc was compared by co-immunoprecipitation (*IP*). NCAM glycosylation mutants (V5-tagged) were co-expressed with ST8SiaIV/PST-Myc in COS-1 cells, the polyST was immunoprecipitated using an anti-Myc antibody, and co-precipitating NCAM proteins were detected by immunoblotting (*IB*) with an anti-V5 epitope tag antibody. *B*, *bottom*, relative protein expression levels were determined by immunoblotting cell lysate aliquots with anti-V5 epitope tag antibody.

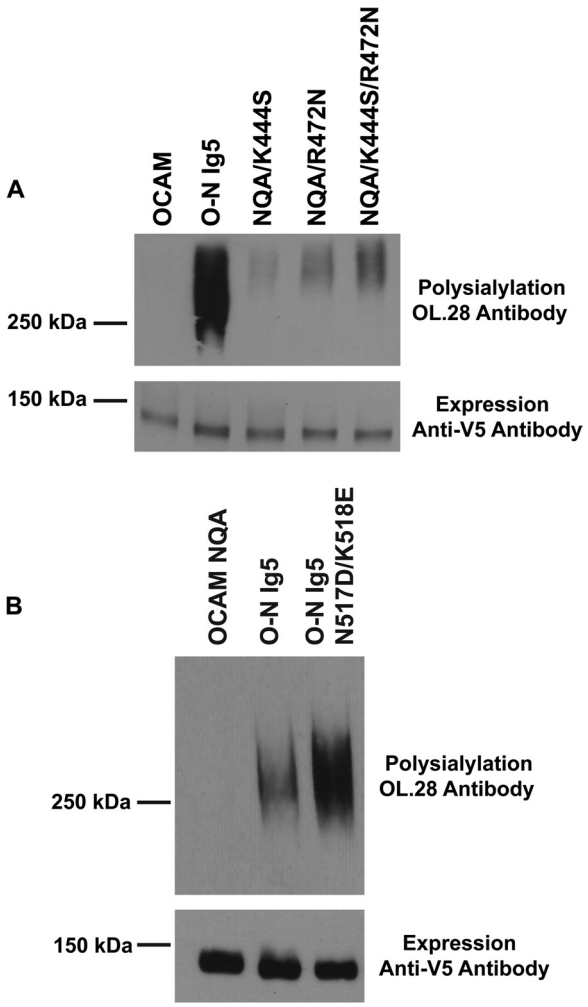


FIGURE 6. **Replacing OCAM Ig5 Lys$^{444}$ and Arg$^{472}$ with NCAM residues in the absence of the extra OCAM Ig5 *N*-glycan allows only low level OCAM polysialylation, whereas creating the core FN1 acidic patch in the O-N Ig5 chimera substantially enhances this protein's polysialylation.** *A*, *top*, to determine whether the extra *N*-glycan in OCAM Ig5 plus the two basic residues (Lys$^{444}$ and Arg$^{472}$) adjacent to OCAM *N*-glycosylation sites in this domain serve to block OCAM polysialylation, the polysialylation of OCAM with K444S, R472N, or both mutations in the absence of the extra OCAM Ig5 *N*-glycosylation site (third position mutant, NQA) was determined by OL.28 immunoblotting following expression in COS-1 cells with ST8SiaIV/PST-Myc and immunoprecipitation of OCAM proteins using the anti-V5 epitope tag antibody. The polysialylations of wild type OCAM and the O-N Ig5 chimera are shown for comparison. *A*, *bottom*, the relative expression levels of wild type and mutant OCAM proteins were determined by immunoblotting cell lysate aliquots with an anti-V5 epitope tag antibody. *B*, *top*, the polysialylations of OCAM, the O-N Ig5 chimera, and this chimera plus the N517D/K518E mutant that recreates the core FN1 acidic patch were compared by expressing these proteins with ST8SiaIV/PST in COS-1 cells and evaluating polysialylation by immunoblotting immunoprecipitated OCAM or O-N chimeric proteins with the OL.28 anti-polySia antibody. *B*, *bottom*, relative protein expression levels were determined by immunoblotting cell lysate aliquots with an anti-V5 epitope tag antibody.

OCAM has Asn$^{517}$, and in place of Glu$^{521}$, 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. 6*B*, *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. 6*B*). 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. *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 *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 *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 *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

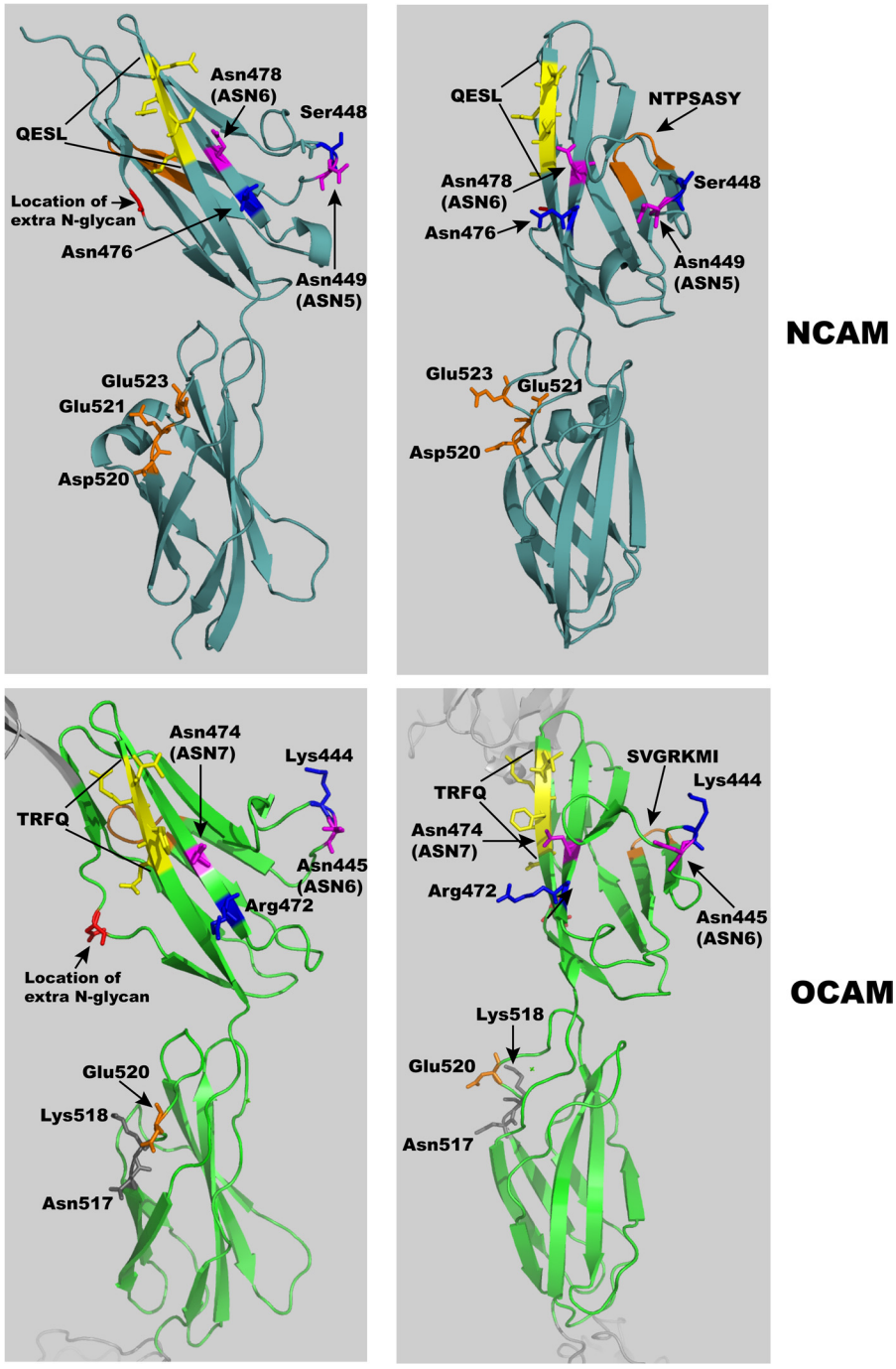


FIGURE 7. **Comparison of key residues in NCAM and OCAM Ig5-FN1 tandems.** Shown are two views of the structures of the human NCAM (Protein Data Bank entry 3MTR) and human OCAM (Protein Data Bank entry 2JLL) Ig5-FN1 tandems. Indicated are common glycosylation sites in *pink*: NCAM Asn$^{449}$ (ASN5) and Asn$^{478}$ (ASN6), which carry the polysialylated *N*-glycans, and the analogous Asn$^{445}$ (ASN6) and Asn$^{474}$ (ASN7) in OCAM. Residues near these glycosylation sites in OCAM (Lys$^{444}$ and Arg$^{472}$) prohibit NCAM polysialylation when used to replace analogous residues in NCAM (Ser$^{448}$ and Asn$^{476}$) (residues in *blue*). *Orange residues* in the two FN1 domains represent the core acidic patch residues for NCAM (Asp$^{520}$, Glu$^{521}$, and Glu$^{523}$) and an analogous acidic residue, Glu$^{520}$, in mouse OCAM. Note that mouse OCAM was used in this study with Glu at position 520, whereas the structure shown is of human OCAM with an Asp at position 520. The two OCAM FN1 non-acidic residues, Asn$^{517}$ and Lys$^{518}$, replaced in Fig. 6 are shown in *gray*. We have labeled the OCAM structure according to the mouse amino acid sequence. Shown in the *right panels* only, replacing a sequence on the strand adjacent to that carrying ASN6 in NCAM (QESL; *yellow*) with sequences from OCAM (TRFQ; *yellow*) leads to a decrease in polysialylation. Notably, a large phenylalanine residue from OCAM (Phe$^{485}$ in TRFQ) replaces a smaller serine residue (Ser$^{489}$ in QESL). Arg$^{472}$, Lys$^{444}$, and Phe$^{485}$ protrude from the surface of OCAM Ig5 and may serve to block polyST access and decrease or prevent OCAM polysialylation. Replacing a second non-conserved sequence (NTPSASY) in NCAM Ig5 with analogous residues from OCAM (SVGRKMI) leads to a smaller decrease in NCAM polysialylation (both sequences shown in *orange*). Shown in the *left panels* only, the location of the extra glycosylation site in OCAM Ig5 and its location when inserted into NCAM Ig5 are indicated (*red*).

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

sequences outside the FN1 acidic patch may be mediating recognition but also demonstrated that polyST-substrate binding *per se* did not guarantee polysialylation. In fact, we see just this in our analysis of the NCAM Ig5 $Ser^{448}$ and $Asn^{476}$ 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. 4*B*). It is likely that the intact FN1 binding site accounts for the residual binding and that the polyST-FN1 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 polySTs, albeit weakly without the FN1 sequences.

Taken in aggregate, these observations suggest a model in which the polyST first engages 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 polyST stem-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 polyST substrates have similar requirements for polyST recognition.

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