

SEQUENCES AT THE INTERFACE OF THE FIFTH IMMUNOGLOBULIN DOMAIN AND FIRST FIBRONECTIN TYPE III REPEAT OF THE NEURAL CELL ADHESION MOLECULE ARE CRITICAL FOR ITS POLYSIALYLATION

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Running head: Ig5-FN1 interdomain sequences in NCAM polysialylation

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Polysialic acid is an anti-adhesive glycan that modifies a select group of mammalian proteins. The primary substrate of the polysialyltransferases (polySTs) is the neural cell adhesion molecule, NCAM. Polysialic acid negatively regulates cell adhesion, is required for proper brain development, and is expressed in specific areas of the adult brain where it promotes on-going cell migration and synaptic plasticity. The first fibronectin type III repeat (FN1) of NCAM is required for polysialylation of the N-glycans on the adjacent immunoglobulin-like domain (Ig5), and acidic residues on the surface of FN1 play a role in polyST recognition. Recent work demonstrated that the FN1 domain from the unpolysialylated olfactory cell adhesion molecule, OCAM, was able to partially replace NCAM FN1 (Foley et al. (2010) J. Biol. Chem. 285, 35056-35067). Here we demonstrate that individually replacing three identical regions shared by NCAM and OCAM FN1, Pro⁵⁰⁰-Ser⁵⁰¹-Ser⁵⁰²-Pro⁵⁰³ (PSSP), Gly⁵²⁶-Gly⁵²⁷-Val⁵²⁸-Pro⁵²⁹-Ile⁵³⁰ (GGVPI), and Asn⁵⁸⁰-Gly⁵⁸¹-Lys⁵⁸²-Gly⁵⁸³ (NGKG), dramatically reduces NCAM polysialylation. In addition, we show that the polyST, ST8SiaIV/PST, specifically binds NCAM, and that this binding requires the FN1 domain. Replacing the FN1 PSSP sequences and the acidic patch residues decreases NCAM-polyST binding, while replacing the GGVPI and NGKG sequences has no effect. The location of GGVPI and NGKG in loops that flank the Ig5-FN1 linker, and the proximity of PSSP to this linker, suggest that GGVPI and NGKG sequences may be critical for stabilizing the Ig5-FN1 linker, while PSSP may play a dual role maintaining the Ig5-FN1 interface and a polyST recognition site.

The neural cell adhesion molecule (NCAM)¹ is a member of the immunoglobulin superfamily of proteins (1). It engages in both heterophilic and homophilic interactions that allow cell-cell adhesion and signal transduction (reviewed in 2,3). NCAM is also the primary substrate for the two polysialyltransferases (polySTs) ST8SiaIV/PST (PST) and ST8SiaII/STX (4-8). These two enzymes are capable of attaching long chains of α 2,8-linked sialic acid residues to N-linked glycans in the fifth immunoglobulin-like domain (Ig5) of NCAM (9). These long chains of negatively charged sialic acid attenuate the interactions of NCAM and other cell surface adhesion molecules (10-11), and this is critical in developing embryos and neonates for proper neuronal cell migration and differentiation, and brain development (reviewed in 12,13).

Mice null for NCAM show mild defects such as a reduced olfactory bulb size, decreased mossy fiber fasciculation, and deficits in spatial learning (14). In contrast, mice lacking the two polySTs show severe defects in brain architecture, hydrocephaly, and most die within 4 weeks of birth (15). A triple knockout of NCAM and the polySTs rescues the lethal phenotype, indicating that polysialic acid is needed to prevent early and inappropriate cell adhesion and differentiation (15). In mammals, polyST expression decreases soon after birth, and in the adult animal, NCAM is mostly unpolysialylated except in a few specific areas of the brain that require on-going cell migration and synaptic plasticity, such as the hippocampus, hypothalamus, and olfactory bulb (13, 17-20). In addition, polysialic acid is aberrantly expressed in several pediatric and adult

cancers, such as neuroblastoma, small and non-small cell lung carcinoma, and Wilms' tumor (21-26). Polysialic acid re-expression in cancer cells has been suggested to increase tumor invasiveness and to promote tumor growth by down-regulating NCAM signaling that activates tumor suppression pathways (27).

Polysialic acid is expressed on a very small subset of mammalian proteins with NCAM being the major substrate for the polySTs. Polysialic acid is found on the O-glycans of dendritic cell neuropilin-2 (28) and the scavenger receptor, CD-36, in milk (29). It is also found on the N-glycans of the α subunit of the voltage dependent sodium channel (30), and a small population of the synaptic cell adhesion molecule, SynCAM 1, expressed in NG2 glia cells (31). In addition, the two polySTs are capable of autopolysialylation (32, 33). This limited number of polyST substrates, as well as the observation that polysialic acid is added preferentially to glycans linked to NCAM as compared to free glycans (34, 35), led to the hypothesis that polysialylation is a protein specific modification event requiring an initial protein-protein interaction between polyST and substrate.

There are three known NCAM isoforms. NCAM180 and NCAM140 are transmembrane proteins that vary in the length of their cytoplasmic tail (tail), while NCAM120 associates with the membrane via a glycosyl-phosphatidylinositol anchor (36). The extracellular portion of all three NCAM isoforms is constant and is composed of five Ig domains and two fibronectin type III repeats. Although NCAM contains six consensus sites for N-linked glycosylation, the majority of polysialic acid is added to glycans attached to Asn⁴⁴⁹ and Asn⁴⁷⁸, the fifth and sixth N-glycosylation sites located on Ig5 (37). Previous work from our laboratory has shown that the first fibronectin type III repeat (FN1) of NCAM is required for the polysialylation of the N-glycans on the adjacent Ig5 domain (38). A truncated NCAM protein consisting of just Ig5-FN1-transmembrane (TM)-tail (NCAM4, Fig. 1) can be polysialylated, but proteins containing only Ig5-TM-tail or lacking the FN1 domain (Δ FN1, Fig. 1) are not (38, 39). In addition a truncated NCAM protein consisting of FN1-FN2-TM-tail

(NCAM7, Fig. 1) is weakly polysialylated on FN1 O-glycans. These results suggest that the minimal domains required for NCAM N-glycan polysialylation are Ig5-FN1, and suggest a model in which the recognition and binding of the FN1 domain by the polyST positions it to polysialylate N-glycans on Ig5, while in the absence of Ig5, the polySTs are able to bind FN1 and polysialylate O-glycans on FN1 (38-40). To investigate this model, a closer examination of NCAM FN1 was required.

Fibronectin type III repeats are present in up to 2% of all human proteins (41), yet very few proteins are polysialylated, implying that NCAM FN1 is intrinsically unique or, in the context of NCAM, it allows for specific protein interaction with the polySTs. Molecular modeling and structural analysis of NCAM FN1 showed two novel features not seen in other fibronectin type III repeats, a surface acidic patch comprised of Asp⁵⁰⁶, Asp⁵²⁰, Glu⁵²¹, and Glu⁵²³ and an α -helix linking strands 4 and 5 of the FN1 β sandwich (39, 40). Two other unique FN1 sequences, Pro⁵¹⁰-Tyr⁵¹¹-Ser⁵¹² (PYS) and Gln⁵¹⁶-Val⁵¹⁷-Gln⁵¹⁸ (QVQ), were also identified (42). The role of all four sequences in NCAM polysialylation was analyzed by mutagenesis and the creation of chimeric proteins. While the PYS sequence was shown to be required for the maintenance of a conformation of the FN1 domain that enhanced the biosynthesis of sialylated O-glycans and promoted O-glycan polysialylation, the acidic surface patch, α -helix, and a QVQ sequence were shown to play roles in polyST recognition and positioning (39, 40, 42).

We found that when either the FN1 α -helix or QVQ sequence of NCAM were replaced with alanines, the polySTs continued to recognize the mutant proteins, but added polysialic acid to O-glycans on FN1 rather than N-glycans on Ig5 (39, 42), suggesting that these sequences play a role in positioning the polySTs for N-glycan polysialylation. The importance of the FN1 acidic patch varies in N-glycan and O-glycan polysialylation. Replacing the acidic patch residues with alanine led to a dramatic decrease in, or even eliminated, the O-glycan polysialylation of the truncated NCAM7 protein (FN1-FN2-TM-tail) (42, see also Fig. 1 and Fig. 4C). In contrast, replacing these residues with alanine only slightly

decreased NCAM N-glycan polysialylation in WT NCAM (39, see Fig. 4B). However, replacing these residues with arginine dramatically decreased NCAM N-glycan polysialylation, and suggested that additional FN1 residues are likely to function together with the acidic patch to allow the polySTs to modify Ig5 N-glycans (39, see also Fig. 4B).

In the course of investigating the requirements for NCAM polysialylation we found that the FN1 domain from the unpolysialylated olfactory cell adhesion molecule, OCAM, could partially replace the FN1 domain of NCAM to allow N-glycan polysialylation (42). This result lead us to consider the possibility that sequences common to NCAM and OCAM FN1 domains are critical for polyST recognition and polysialylation. In this study we evaluate three identical regions shared by NCAM and OCAM FN1 domains and their role in NCAM polysialylation and polyST binding. General requirements for polyST-NCAM binding are also investigated. Our results show that all three regions are critical for NCAM polysialylation and suggest that two sequences, which form loops flanking the Ig5-FN1 linker, function to stabilize this region, while the a third sequence, which is adjacent to the Ig5-FN1 junction, may play a dual role maintaining both the Ig5-FN1 interface and a polyST binding surface.

EXPERIMENTAL PROCEDURES

Tissue culture reagents, oligonucleotides, restriction enzymes, PCR supermix, and anti-V5 epitope tag antibody were purchased from Invitrogen. Anti-myc epitope tag antibody was purchased from Abcam. The cDNA for human NCAM140 was a gift from Dr. Nancy Kedersha (Brigham and Women's Hospital, Boston, MA). The cDNA for human ST8Sia IV/PST was obtained from Dr. Minoru Fukuda (Sandford Burnham Medical Research Institute, La Jolla, CA). The QuikChangeTM 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. Peptide N-glycosidase F (PNGaseF) and T4 DNA ligase were obtained from New England Biolabs. Precision Plus

ProteinTM Standard was purchased from Bio-Rad. Nitrocellulose membranes were purchased from Schleicher and Schuell. Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Jackson ImmunoResearch. Supersignal West Pico chemiluminescence reagent was obtained from Pierce. Other chemicals and reagents were purchased from Sigma and Fisher Scientific.

Mutagenesis of NCAM and NCAM7 - NCAM and NCAM7 mutants were constructed using the Stratagene QuikChangeTM site-directed mutagenesis kit. DNA sequencing performed by the DNA Sequencing Facility of the Research Resources Center at the University of Illinois at Chicago confirmed the mutations. All primers used are listed in Supplemental Table 1.

Construction of soluble PST-myc- Soluble PST (sPST-myc) lacking the cytosolic tail and TM region was generated in multiple steps. First, the canine pre-pro-insulin signal peptide was amplified from a previously generated soluble NCAM-V5 construct (38) using primers A and B in Supplementary Table 1. This DNA was digested with Hind III and Eco RV and ligated into pcDNA 3.1 /Myc-HisB vector with a stop codon inserted before the 6His tag to generate an expression vector including the signal peptide at the amino terminus and myc tag at the carboxy terminus, but no 6His tag. The coding sequences for the ST8SiaIV/PST tail and TM were excised from the wild type ST8SiaIV/PST using primers C and D in Supplementary Table 1. This fragment was digested with EcoRV and XbaI and ligated into the modified signal peptide/myc tag vector.

Transfection of Cos-1 cells with NCAM and PST cDNAs- Cos-1 cells maintained in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), were plated on 100-mm tissue culture plates and grown at 37°C, 5% CO₂, until 50-70% confluent. Cells were transfected using 30 µl Lipofectin in 3 ml Opti-MEM and 10 µg each of V5-tagged NCAM or NCAM mutant and PST-myc cDNA, according to the manufacturer's protocol. The NCAM cDNAs were cloned upstream of the V5 epitope tag in the pcDNA3.1 V5/HisB vector. PST cDNA was cloned upstream of the myc tag in the pcDNA3.1

Myc/HisB vector. A stop codon was inserted before the 6His coding sequence in the pcDNA3.1 Myc/HisB vector. Cells were incubated with transfection mixture for 6 h and then 7 ml DMEM, 10% FBS, was added to bring the mixture to a final volume of 10 ml.

Immunoprecipitation of NCAM proteins- Sixteen hours post-transfection, cells were washed with 10 ml phosphate-buffered saline (PBS) and lysed in 1 ml 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 pre-cleared with 50 μ l protein-A-Sepharose beads (50% suspension in PBS) for 1 h at 4°C. NCAM proteins were immunoprecipitated with 3 μ l anti-V5 epitope tag antibody for 2 h at 4°C, followed by incubation for 1 h with 50 μ l protein-A-Sepharose beads (50% suspension in PBS). Beads were washed four times with immunoprecipitation buffer and once with immunoprecipitation buffer containing 1% SDS. Samples were then resuspended in 50 μ l 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 protein expression levels, an 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.

Co-immunoprecipitation of NCAM and NCAM mutants with PST- Lec 2 CHO cells maintained in F12 medium supplemented with 10% FBS, were co-transfected with 10 μ g V5-tagged NCAM or NCAM mutant cDNA and 10 μ g of PST-myc or empty pcDNA3.1 Myc/HisB vector. Following a 6 h incubation, 7 ml of F12 media was added to each plate and the cells were allowed to grow overnight. The following day, the cell media was removed and cells were washed with 10 ml of PBS. One milliliter of co-immunoprecipitation buffer (50 mM Hepes, 100 mM NaCl, 1% Triton X-100, pH 7.2) was added and the cells were scraped off the plates using a sterile cell scraper. The cells were incubated for 15 min on ice to allow lysis and then pelleted. PST-myc was then

immunoprecipitated from cell lysates using 2.5 μ l anti-myc tag antibody. After 2 h of rotation at 4°C, 50 μ l of protein A-Sepharose (50% suspension in PBS) was added to each sample and rotated at 4°C for 1 h. The immune complexes bound to protein A-Sepharose beads were pelleted and washed four times with 1 ml cold co-immunoprecipitation buffer. Samples were resuspended in Laemmli sample buffer containing 5% β -mercaptoethanol, and heated to 100°C for 10 min. Precipitated proteins were separated on a 5% stacking/7.5% resolving SDS polyacrylamide gel and then subjected to immunoblotting as described below. To evaluate relative NCAM protein expression levels, a 100 μ l aliquot of cell lysate was removed prior to immunoprecipitation and an equal volume of Laemmli sample buffer, 5% β -mercaptoethanol, was added. Samples were heated at 100°C for 10 min, separated by SDS polyacrylamide gel electrophoresis, as described above, and subjected to immunoblotting, as described below. To quantify changes in polyST-NCAM mutants we used NIH ImageJ software and compared the ratio of precipitated to load control for the mutants versus wild type NCAM (set to 100%). In the event that non-specific binding was detected, this was subtracted from the final value (Relative co-precipitation).

Co-immunoprecipitation of soluble NCAM7 with soluble PST- A soluble form of NCAM7-V5 (sNCAM7-V5), lacking the tail and TM regions, was constructed previously (38). The construction of sPST-myc is described above. Lec 2 CHO cells, maintained in F12 medium supplemented with 10% FBS, were co-transfected with 10 μ g sNCAM-V5 cDNA and 10 μ g of sPST-myc or the empty pcDNA3.1 Myc/HisB vector. Following a 6 h incubation, the transfection media was removed, 4 ml of F12 media was added to each plate, and the cells were allowed to grow overnight. The next day, cell media was collected, and debris removed by centrifugation. PST-myc was immunoprecipitated from the cell media using 5 μ l anti-myc tag antibody. Samples were rotated at 4°C for 2 h, followed by rotation for 1.5 h with 75 μ l protein A-Sepharose beads (50% suspension in PBS). The samples were processed for and submitted to SDS polyacrylamide gel

electrophoresis as described above, and then subjected to immunoblotting as described below.

Glycosidase treatment of NCAM proteins- To evaluate the type of glycans (N-linked or O-linked) that are polysialylated on the various NCAM proteins, COS-1 cell transfections were performed in duplicate, and after immunoprecipitation and washing, identical samples were resuspended in 77 μ l H₂O, 10 μ l Nonidet P-40 (NP-40), 10 μ l G7 buffer (0.5 M sodium phosphate, pH 7.5), with or without 3 μ l PNGaseF (500 units/ μ l), and incubated with rotation at 37°C overnight. Fifty microliters of Laemmli sample buffer containing 5% β -mercaptoethanol was added to each sample, which were heated at 65°C for 10 min. Proteins were separated on a 3% stacking/5% resolving SDS polyacrylamide gel, followed by immunoblotting with the OL28 anti-polysialic acid antibody, as described below.

Immunoblot analysis of NCAM proteins- Following SDS PAGE, 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 polysialic acid, membranes were incubated overnight with a 1:100 dilution of the OL28 anti-polysialic acid antibody (43) 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 for NCAM protein co-immunoprecipitation with PST-myc or relative NCAM protein levels, nitrocellulose membranes were incubated for 2 h or overnight with a 1:5000 dilution of anti-V5 epitope tag antibody (Invitrogen) diluted in blocking buffer, and for 1 h with HRP-conjugated goat anti-mouse IgG, diluted 1:4000 in blocking buffer. Membranes were washed two times with Tris-buffered saline, pH 8.0, 0.1% Tween-20 for 15 min 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

OCAM FN1 can replace NCAM FN1 to allow Ig5 N-glycan polysialylation- Prior work has shown the importance of NCAM FN1 for the polysialylation of the N-glycans found on the adjacent Ig5 domain (38-40, 42). In order to identify additional polyST recognition sequences, we sought to reconstitute polysialylation in an unpolysialylated NCAM chimera that contained a FN1 domain from a related, but unpolysialylated protein. OCAM has the same domain structure as NCAM and their Ig5 and FN1 domains share 47% and 37% identity, respectively (Fig. 1) (44). Despite having Ig5 N-glycans in the same locations as those in NCAM that are polysialylated, and being expressed in a similar spatio-temporal manner as NCAM, OCAM is not polysialylated (44). We expected that an NCAM-OCAM (N-O) chimera possessing the OCAM FN1 domain would not be polysialylated. It was therefore surprising when the OCAM FN1 domain allowed polysialylation of the Ig5 N-glycans in the N-O chimera (42, and Fig. 2). This unexpected result led us to ask whether sequences common to both NCAM and OCAM FN1 domains play a role in polyST recognition.

Sequences common to NCAM and OCAM FN1 are required for NCAM polysialylation- Inspection of the NCAM and OCAM FN1 sequences revealed three common regions of 4-5 amino acids: Pro⁵⁰⁰-Ser⁵⁰¹-Ser⁵⁰²-Pro⁵⁰³ (PSSP), Gly⁵²⁶-Gly⁵²⁷-Val⁵²⁸-Pro⁵²⁹-Ile⁵³⁰ (GGVPI), and Asn⁵⁸⁰-Gly⁵⁸¹-Lys⁵⁸²-Gly⁵⁸³ (NGKG) (Fig. 1). Interestingly, when these common sequences are mapped onto the structure of NCAM Ig5-FN1 (PDB ID 3MTR, 45) as shown in Figure 3A, we find that all were located at or near the Ig-FN1 interface. GGVPI and NGKG comprise the two loops connecting strands 2 and 3, and 6 and 7, of the FN1 β sandwich, respectively. These loops flank the Ig5-FN1 linker region (Fig. 3A, GGVPI in green and NGKG in purple). PSSP is two amino acids away from the Ig5-FN1 junction (Fig. 3A, PSSP in red). We also noticed that the PSSP sequence lies next to the core residues of the acidic patch (Asp⁵²⁰, Glu⁵²¹, and Glu⁵²³) (Fig. 3A, compare location of acidic patch in orange and PSSP in red).

To evaluate the role of these three common regions in NCAM polysialylation, we replaced each sequence and co-expressed the mutant NCAM proteins with PST in COS-1 cells (Fig. 4A). Polysialylation was analyzed by immunoprecipitating the NCAM proteins and subjecting them to immunoblotting with the OL28 anti-polysialic acid antibody. We found that replacing the amino acids in these three sequences with either alanines (Fig. 4A) or glycines (not shown) eliminated NCAM polysialylation. Replacing a fourth region of homology, Lys⁵³²-Tyr⁵³³-Lys⁵³⁴ (KYK), located on the opposite face of the FN1 β sandwich and at a greater distance from the Ig5-FN1 interface, led to little decrease in NCAM polysialylation. The outcome of replacing these common sequences was similar to that observed when we replaced the acidic patch residues with arginine, but contrasted with the maintenance of polysialylation when the acidic patch (AP-AAA), α -helix (Δ helix-AA) and QVQ (QVQ-AAA) sequences were replaced with alanines (Fig. 4B).

One possibility is that the PSSP, GGVPI and NGKG mutant proteins are misfolded and retained in the endoplasmic reticulum by the quality control system of the cell (46). To test this, we localized these mutant proteins using immunofluorescence microscopy and found that each mutant protein trafficked from the endoplasmic reticulum, through the Golgi and to the cell surface like wild type NCAM (Supplemental Fig. 1). Taken together, these results suggest that the PSSP, GGVPI, and NGKG sequences in the FN1 domain are critical for NCAM polysialylation.

The GGVPI and NGKG sequences do not play roles in NCAM7 O-glycan polysialylation- We previously found that inserting three amino acids (Ala-Leu-Asp, Gly-Gly-Gly, or Ala-Ala-Ala) at the Ig5-FN1 junction eliminated Ig5 N-glycan polysialylation of both full length NCAM and the N-O chimera (42). These results suggested that the Ig5-FN1 relationship is critical for Ig5 N-glycan polysialylation and led us to consider the possibility that replacing PSSP, GGVPI, and NGKG may destabilize the Ig5-FN1 interface and alter the Ig5-FN1 interdomain relationship. Alternatively, or in addition, any or all of these

three sequences may form part of a polyST recognition region.

We reasoned that if PSSP, GGVPI and NGKG were important for maintaining the Ig5-FN1 relationship, then replacing these sequences in the truncated NCAM7 protein (FN1-FN2-TM-tail) that lacks the Ig5 domain and is polysialylated on FN1 O-glycans (38), would not impact its polysialylation. Conversely, if they formed part of a polyST recognition site, their replacement may reduce or eliminate NCAM7 polysialylation. We found that replacing GGVPI or NGKG with alanine residues did not reduce NCAM7 polysialylation, while replacing PSSP with alanine residues decreased, but did not eliminate, NCAM7 polysialylation (Fig. 4C). Quantifying the differences in the polysialylation of NCAM7 and its mutants in this experiment, we found that the PSSP mutant was polysialylated to 57% the level of NCAM7, while the GGVPI and NGKG mutants were polysialylated to 102% and 122% of the level of NCAM7. These results contrasted with the complete elimination of NCAM7 polysialylation when the acidic patch was replaced with alanine residues (Fig. 4C, AP-AAA). Additional experiments suggested that a single O-glycan attached to a serine residue at the very N-terminus of NCAM7 possesses the bulk of the polysialic acid on this protein (M. Thompson, unpublished data). With this in mind, replacing NGKG with alanines may have made this glycan more accessible to the polySTs and enhanced polysialylation. More importantly, the observation that replacing PSSP eliminated NCAM N-glycan polysialylation, while only reducing NCAM7 O-glycan polysialylation, suggests that this sequence may play a dual role in NCAM polysialylation. PSSP may not only function to maintain the structure of the Ig5-FN1 linker region in full length NCAM, but also may be important for the integrity of the polyST recognition surface and polyST binding.

The FN1 domain is essential for polyST-NCAM binding- To test the hypothesis that the PSSP sequence plays a role in polyST binding, we first had to establish that the polySTs bind to NCAM and that the FN1 domain is essential for this binding. To do this we took a co-immunoprecipitation approach and analyzed the

binding of PST to NCAM and truncated NCAM proteins that had previously been shown to be polysialylated (38). NCAM proteins tagged with a V5 epitope tag were co-expressed with PST-myc. To avoid the possibility that polysialic acid on NCAM would decrease this interaction, we co-expressed these proteins in Lec2 CHO cells. These cells lack a functional CMP-sialic acid transporter and are incapable of sialylating glycoproteins and glycolipids (47). PST was immunoprecipitated using an anti-myc antibody and co-precipitating NCAM proteins were detected by immunoblotting with an anti-V5 epitope tag antibody as described in the “Experimental Procedures” (Fig. 5, top panels). The relative expression levels of NCAM proteins were determined by immunoblotting one tenth of the total cell lysate with the anti-V5 antibody (Fig. 5, bottom panels). We found that full length NCAM, NCAM3 (Ig5-FN1-FN2-TM-tail) and NCAM4 (Ig5-FN1-TM-tail) were all specifically co-immunoprecipitated with PST-myc (Fig. 1, Fig. 5, left panels, +PST-myc), with little or none of these proteins non-specifically precipitated from cell lysates when PST-myc was not present (Fig. 5, left panels, -PST-myc).

To determine whether Ig5 or the TM region are involved in this interaction, we tested the ability of soluble NCAM7-V5 (sNCAM7) consisting of only FN1-FN2 to bind to a soluble PST-myc (sPST-myc) lacking the TM and tail sequences. Both of these proteins are expressed with the canine pancreatic pre-pro-insulin signal peptide for entry into the secretory pathway, and are secreted into the cell media (38). sPST-myc and sNCAM7-V5 were co-expressed in Lec 2 CHO cells and sPST-myc immunoprecipitated from the cell media (Fig. 5, right panels). We found that sNCAM7 specifically co-immunoprecipitated with sPST-myc (Fig. 5, right panels, +sPST-myc), and we saw little non-specific precipitation in the absence of sPST-myc (Fig. 5, right panels, -sPST-myc). These results demonstrated that neither the Ig5 domain nor membrane association is required for polyST-NCAM interaction. Finally, to determine whether the FN1 domain is necessary for polyST binding, we tested PST-myc binding to the Δ FN1 protein, which lacks the FN1 domain and is not polysialylated (Fig. 1) (39). We found that the

Δ FN1 protein was unable to bind to PST-myc (Fig. 6). This result correlated well with the inability of the polySTs to recognize and polysialylate the Δ FN1 protein (39) and demonstrated the importance of the FN1 domain for polyST binding.

Replacing PSSP and the acidic patch decrease polyST-NCAM binding- To determine whether the FN1 acidic patch, α -helix, or the three sequences common to NCAM and OCAM FN1 identified in this work, play a role in polyST-NCAM interaction, we evaluated the impact of replacing these sequences on polyST-NCAM binding. Mutation of acidic patch residues to either alanine or arginine in full length NCAM led to a decrease in polyST binding to 61-64% of the level of wild type NCAM (Fig. 7, top panels, AP-AAA and AP-RRR). Notably, while this did correlate with the small decrease in polysialylation seen for the AP-AAA mutant, it did not correspond to the large decrease in polysialylation seen for the AP-RRR mutant (Fig. 4B). These results suggest that the acidic patch plays a role in polyST-NCAM binding and that replacing these residues with arginine may affect more than the enzyme-substrate interaction. In contrast, replacing the α -helix with two alanine residues led to a surprising increase in polyST-NCAM binding that could be partially explained by a smaller increase in non-specific binding (Fig. 7, top panels, Δ helix-AA). We currently do not understand why this increased binding is observed in the absence of the FN1 α -helix.

We performed a similar polyST-NCAM binding analysis on the three sequences common to NCAM and OCAM FN1. Replacing GGVPI and NGKG with alanine residues did not substantially alter the ability of NCAM to bind PST (Fig. 7, bottom panels, GGVPI-AAAAA, NGKG-AAAAA). In contrast, a substantial decrease in binding was seen for the PSSP-AAAA mutant, which bound to PST at only 41% of the level observed for wild type NCAM (Fig 7, bottom panels, PSSP-AAAA). These results reaffirm the notion that NGKG and GGVPI may be involved in maintaining the Ig5-FN1 relationship, possibly by stabilizing the linker between the two domains, while the presence of PSSP may also be required for optimal polyST recognition and binding.

*Pro*⁵⁰⁰ and *Pro*⁵⁰³ in the PSSP sequence are critical for NCAM polysialylation- Inspection of the three dimensional structure of the Ig5-FN1 tandem predicts that the PSSP sequence is part of a unique surface formed by three adjacent strands (Fig. 3A). The presence of the two proline residues may be critical to maintain the structure of not only this surface, but also the spacing between the Ig5-FN1 domains. Alternatively, the side chains of the intervening serine residues, and in particular Ser⁵⁰², may form hydrogen bonds that stabilize a polyST-FN1 interaction. To determine which amino acids in the PSSP sequence are critical, we generated two additional mutants, GSSG and PGGP, and analyzed their polysialylation. We found that the PSSP-GSSG mutant, like the PSSP-AAAA mutant (Fig. 4A) and PSSP-GGGG mutant (data not shown), eliminated NCAM polysialylation, while a PSSP-PGGP mutant showed little decrease in NCAM polysialylation (Fig. 8). These results indicate that Pro⁵⁰⁰ and Pro⁵⁰³ maintain a structure that is not only critical for the Ig5-FN1 relationship but also polyST recognition.

DISCUSSION

In this study we have evaluated the effects of replacing three sequences common to both NCAM and OCAM FN1 domains in the polysialylation of NCAM. We found that replacing the PSSP, GGVPI, and NGKG sequences located at or near the Ig5-FN1 interface dramatically reduced and even eliminated the polysialylation of the Ig5 N-glycans in full length NCAM but did not have the same effect on the polysialylation of the FN1 O-glycans of the truncated NCAM7 mutant that lacks the Ig5 domain (Fig. 4). The decrease in NCAM7 polysialylation observed when the PSSP sequences were replaced suggested these might play a role in polyST recognition. Additional analysis of polyST-NCAM binding demonstrated a requirement for the FN1 domain (Fig. 5 and 6) and showed that replacing the FN1 acidic patch residues or the PSSP sequence led to decrease in polyST-NCAM interaction (Fig. 7). Taken together these results suggest that the GGVPI and NGKG sequences are likely to stabilize the Ig5-FN1 linker, while the PSSP sequence may play two roles in maintaining both the structure of the

Ig5-FN1 linker region and a polyST recognition site.

How could replacing the GGVPI and NGKG sequences alter the Ig5-FN1 linker and destabilize the Ig5-FN1 relationship? We used the NCAM Ig5-FN1 crystal structure (PDB ID 3MTR, 45) to search for bonding patterns that might explain the importance of the GGVPI and NGKG loops in stabilizing the Ig5-FN1 linker region and/or Ig5-FN1 relationship. The structure predicts that GGVPI is an unstructured loop containing the acidic patch residues, while NGKG is a classic beta turn. Two hydrogen bonds are predicted to stabilize the NGKG beta turn. These include a hydrogen bond between the side chain carboxyl group of Asn⁵⁸⁰ and the main chain amide of Lys⁵⁸² and a hydrogen bond between the main chain amide group of Asn⁵⁸⁰ and the main chain carbonyl of Gly⁵⁸³ (Fig. 3D). Hydrogen bonds between residues in the GGVPI and NGKG loops and Ig5-FN1 linker region residue Asp⁴⁹⁸ are predicted by the structure and may stabilize the loop-linker relationship, and in turn, the Ig5-FN1 relationship. These include a hydrogen bond between the main chain carbonyl of Asp⁴⁹⁸ and the side chain amide group of Asn⁵⁸⁰ (Fig. 3B), and a hydrogen bond between the main chain carbonyl of Gly⁵²⁶ in GGVPI and the main chain amide of Asp⁴⁹⁸ (Fig. 3C). Finally, a hydrogen bond may form between the amine group in the indole ring of Trp⁴¹⁷ in Ig5 and the main chain carbonyl of Gly⁵⁸¹ in the NGKG turn which may further stabilize and orient Ig5 and FN1 (Fig. 3D).

For the hydrogen bonds formed by solely main chain substituents, the side chains of the participating loop amino acids would not immediately appear to be critical. However it is important to note that for both the GGVPI and NGKG loops, a substantial decrease in NCAM polysialylation was only observed when all residues in these loops were converted to alanine or glycine. Replacing single amino acids (for example, V or P or I in GGVPI) showed little or only partial decreases in polysialylation (J. Cruz and M. Thompson, unpublished). This suggests that the integrity of the loop structures is critical for their stabilizing effects. Converting all loop residues to alanine or glycine may have altered the spacing between the loops and Ig5-FN1 linker

region, and this would be expected to prevent hydrogen bonds between both main chain and side substituents. Alternately, replacing the GGVPI sequences that are found on the same unstructured strand as the acidic patch residues could have altered the presentation of the acidic patch, thereby decreasing polysialylation. However, the fact that we see a decrease in polyST-NCAM binding for the acidic patch mutants, but no alteration in polyST-NCAM binding for the GGVPI-AAAAA mutant argues against this possibility.

The effects of replacing the PSSP sequence on NCAM polysialylation are exclusively linked to Pro⁵⁰⁰ and Pro⁵⁰³ (Fig. 8). While glycines and alanines make a region more flexible, proline residues reduce the allowed tensional angles and make a region more rigid. The negative effects observed on NCAM polysialylation when the PSSP sequence is replaced by an AAAA, GGGG or GSSG stretch, coupled with the proximity of this sequence to the Ig5-FN1 junction (Gln⁴⁹⁶-Ala⁴⁹⁷/Asp⁴⁹⁸-Thr⁴⁹⁹-Pro⁵⁰⁰-Ser⁵⁰¹-Ser⁵⁰²-Pro⁵⁰³), suggest that these changes may have made the Ig5-FN1 junction more flexible and that this is detrimental to the polysialylation of Ig5 N-glycans. Strikingly, this elimination of N-glycan polysialylation is similar to what we observed when three amino acids were inserted at the Ig5-FN1 junction (42). Together, these results lead us to question the rigidity or flexibility of the Ig5-FN1 linker and interdomain relationship.

Previous rotary shadowing cryo-electron microscopy of the NCAM extracellular domain suggested that there is a flexible hinge between domains in the vicinity of the polysialic acid attachment sites (48, 49). Work and models by various groups supported the idea that this flexible hinge might be found either between Ig4 and Ig5 (49) or Ig5 and FN1 (49, 51). However, recent crystal structures of the NCAM FN1-FN2 tandem suggest that the flexible hinge may exist between these two domains (52). Our own crystal structure of the NCAM Ig5-FN1 tandem exhibited an

extended relationship between these two domains, but other analyses suggested that there might be flexibility in the inter-domain relationship (45).

Examination of the Ig5-FN1 structure demonstrates that the acidic patch and α -helix and QVQ sequences of the FN1 domain are not aligned precisely with the two Ig5 glycosylation sites (Asn⁴⁴⁹ (Asn5) and Asn⁴⁷⁸ (Asn6)) that carry polysialic acid (45, Fig. 8). Subsequent analysis of the ability of N-glycans on novel engineered glycosylation sites to be acceptors for polysialic acid, demonstrated that glycans further away from the FN1 domain or closer to the unpolysialylated Asn⁴²³ (Asn4) glycosylation site found on the opposite side of the Ig5 domain, could be polysialylated (45). These results suggested some flexibility in the polysialylation process that could be accounted for by either a flexible Ig5-FN1 relationship or the ability of the polySTs to bind the FN1 domain in different ways to allow them to position themselves for the polysialylation of glycans located at different sites on the Ig5 domain.

Our data in this work suggesting that sequences at or near the Ig5-FN1 junction and interface are critical for NCAM Ig5 N-glycan polysialylation strongly indicate that the Ig5-FN1 relationship is relatively rigid. This again leaves us with the task of further defining the polyST recognition site or sites on the NCAM FN1 domain required for interacting with the polySTs. The impact of replacing the PSSP sequences on polyST-NCAM binding suggest that additional sequences may be on the face of the FN1 domain containing the acidic patch and PSSP sequences. In sum, these results indicate a model of NCAM polysialylation that involves two important factors, the ability of the polyST to recognize and bind NCAM FN1 and the correct relative orientation of NCAM Ig5 and FN1 domains to allow for N-linked polysialylation of Ig5 glycans.

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FOOTNOTES

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¹The abbreviations used are: NCAM, neural cell adhesion molecule; polySTs, polysialyltransferases; Ig, immunoglobulin domain; FN1, first fibronectin type III repeat of NCAM; FN2, second fibronectin type III repeat of NCAM; OCAM, olfactory cell adhesion molecule; TM, transmembrane region; tail, cytosolic tail; DMEM, Dulbecco's modified Eagle's medium; HRP, horseradish peroxidase; AP, acidic patch; PNGase F, peptide *N*-glycosidase F; N-O, NCAM-OCAM chimera; PST, ST8SiaIV/PST; PYS, Pro⁵¹⁰-Tyr⁵¹¹-Ser⁵¹²; QVQ, Gln⁵¹⁶-Val⁵¹⁷-Gln⁵¹⁸; PSSP, Pro⁵⁰⁰-Ser⁵⁰¹-Ser⁵⁰²-Pro⁵⁰³; GGVPI, Gly⁵²⁶-Gly⁵²⁷-Val⁵²⁸-Pro⁵²⁹-Ile⁵³⁰; NGKG, Asn⁵⁸⁰-Gly⁵⁸¹-Lys⁵⁸²-Gly⁵⁸³; KYK, Lys⁵³²-Tyr⁵³³-Lys⁵³⁴; sPST-myc, soluble, myc tagged PST; sNCAM7-V5, soluble, V5-tagged NCAM7.

FIGURE LEGENDS

FIGURE 1. Schematic representation of NCAM, the NCAM-OCAM chimera, and NCAM domain deletion mutants. The extracellular portion of NCAM is comprised of five Ig-like domains and two fibronectin type III repeats. The NCAM isoform that is used in these studies is NCAM140 that also contains a transmembrane region (TM) and cytoplasmic tail (tail). OCAM has the same domain structure as NCAM and similar glycosylation site pattern, but is unpolysialylated (44). Sequences common to NCAM and OCAM FN1 domains are bold and underlined in both FN1 sequences. Acidic patch residues (Asp⁵⁰⁶, Asp⁵²⁰, Glu⁵²¹ and Glu⁵²³) and the QVQ sequence are shown in the NCAM FN1 sequence in italics, while the α -helix is underlined. An NCAM-OCAM (N-O) chimera was created in which OCAM FN1 replaces the NCAM FN1 (N-O Chimera) (42). NCAM domain deletion proteins were previously constructed and analyzed (38,40) and are used in this study. These include NCAM3 consisting of Ig5-FN1-FN2-TM-tail, NCAM4 consisting of Ig5-FN1-TM-tail, NCAM7 consisting of FN1-FN2-TM-tail, and a soluble form of NCAM7 (sNCAM7) containing only FN1-FN2. NCAM, NCAM3, and NCAM4 are predominantly polysialylated on N-linked glycans, while NCAM7 is polysialylated on O-linked glycans (38). The Δ FN1 NCAM mutant is not polysialylated (39).

FIGURE 2. The OCAM FN1 domain can replace the NCAM FN1 domain in the N-O chimera to allow the polysialylation of N-glycans. NCAM and the N-O chimera (N-O) were transiently co-expressed with PST in COS-1 cells for 18 h. Proteins were immunoprecipitated using an anti-V5 epitope tag antibody, subjected to SDS PAGE, and immunoblotted with the OL.28 antibody, as described in “Experimental Methods” (upper panel, Polysialylation, OL.28 Antibody). Aliquots of cell lysates were immunoblotted with the anti-V5 antibody to determine relative protein expression levels (lower panel, Expression, Anti-V5 Antibody). To determine whether the polysialic acid was found on N-linked or O-linked glycans, immunoprecipitates were treated with PNGase F that selectively removes N-linked, but not O-linked, glycans (PNGase +/-).

FIGURE 3. Structure of NCAM Ig5-FN1 and predicted Ig5-FN1 interface interactions. *A*, The crystal structure of the NCAM Ig5-FN1 tandem is shown (PDB ID 3MTR, 45). The three major regions of sequence identity between NCAM FN1 and OCAM FN1, Pro⁵⁰⁰Ser⁵⁰¹Ser⁵⁰²Pro⁵⁰³ (PSSP, red), Gly⁵²⁶Gly⁵²⁷Val⁵²⁸Pro⁵²⁹Ile⁵³⁰ (GGVPI, green) and Asn⁵⁸⁰Gly⁵⁸¹Lys⁵⁸²Gly⁵⁸³ (NGKG, purple) are highlighted on the FN1 sequence. NGKG and GGVPI form loops in the interface between Ig5 and FN1 domains. PSSP is in an unstructured region that connects Ig5 to FN1. Also shown are three features unique to NCAM FN1 that have been shown to be involved in the polysialylation of Ig5 N-glycans (39,40,42). These include the three core amino acids of an acidic surface patch, Asp⁵²⁰, Glu⁵²¹, and Glu⁵²³ (Acidic Patch, orange), an α -helix linking strands 4 and 5 of the β sandwich (α -Helix, yellow), and the Gln⁵¹⁶Val⁵¹⁷Gln⁵¹⁸ sequence (QVQ, cyan). Also shown are the two Asn residues that carry the N-glycans that are polysialylated (ASN5, ASN6 in magenta). *B*, predicted hydrogen bond between the main chain carbonyl of Asp⁴⁹⁸ (Ig5-FN1 linker) and the side chain amide group of Asn⁵⁸⁰ (NGKG). *C*, predicted hydrogen bond between the main chain carbonyl of Gly⁵²⁶ (GGVPI) and the main chain amide of Asp⁴⁹⁸ (Ig5-FN1 linker). *D*, two hydrogen bonds that stabilize the NGKG beta turn: one between the side chain carboxy group of Asn⁵⁸⁰ and the main chain amide group of Lys⁵⁸², and a second between the main chain amide group of Asn⁵⁸⁰ and the main chain carbonyl group of Gly⁵⁸³. *E*, a possible hydrogen bond between the amine group in the indole ring of Trp⁴¹⁷ in the Ig5 domain and the main chain carbonyl group of Gly⁵⁸¹ of NGKG. Bond distances were predicted using the Measurement function of the PyMol software.

FIGURE 4. Replacing sequences common to NCAM and OCAM FN1 domains dramatically reduces NCAM polysialylation. The polysialylation of NCAM and NCAM7 proteins, and mutants with selected sequences replaced, was analyzed by immunoblotting with the OL.28 anti-polysialic acid antibody, following co-expression of NCAM or the mutant NCAM proteins with PST in COS-1 cells (upper panels, Polysialylation, OL.28 Antibody). Relative levels of protein expression were determined by

immunoblotting aliquots of the cell lysates with the anti-V5 epitope tag antibody (lower panels, Expression, Anti-V5 Antibody). *A*, analysis of alanine replacement mutants of the sequences common to NCAM and OCAM FN1 domains (PSSP-AAAA, GGVPI-AAAAA, NGKG-AAAA, and KYK-AAA). *B*, analysis of previously generated mutants in the FN1 acidic patch (AP-AAA, AP-RRR) (39), α helix (Δ helix-AA) (40), and QVQ sequence (QVQ-AAA) (42). *C*, analysis of NCAM7 and alanine replacement mutants of the acidic patch (AP-AAA) and sequences common to NCAM and OCAM FN1 domains.

FIGURE 5. PST-NCAM binding does not require Ig domains or membrane association. Co-immunoprecipitation analysis of the binding of membrane associated PST-myc and soluble PST-myc (sPST-myc) to NCAM and truncated NCAM proteins follow co-expression in COS-1 cells. *Left panels*, PST was immunoprecipitated from cell lysates using an anti-myc antibody, co-immunoprecipitating proteins were detected by immunoblotting with an anti-V5 tag antibody (+PST-myc). Nonspecific binding was evaluated by performing the same analysis on lysates from cells not expressing PST-myc (-PST-myc) (upper right panel). NCAM protein expression was evaluated by immunoblotting one tenth of the cell lysate with anti-V5 antibody (lower left panel). *Right panels*, sPST was immunoprecipitated from cell media using an anti-myc antibody, co-immunoprecipitating sNCAM7 was detected by immunoblotting with an anti-V5 tag antibody (+PST-myc). Nonspecific binding was evaluated by performing the same analysis on media from cells not expressing PST-myc (-PST-myc) (upper right panels). sNCAM7 expression was evaluated by immunoblotting an aliquot of media with anti-V5 antibody (lower right panel).

FIGURE 6. PST-NCAM binding requires the FN1 domain of NCAM. Co-immunoprecipitation analysis of the binding of membrane associated PST-myc to NCAM and the NCAM Δ FN1 mutant. *Top panel*, PST was immunoprecipitated from cell lysates using an anti-myc antibody, co-immunoprecipitating proteins were detected by immunoblotting with an anti-V5 tag antibody (+PST-myc). Nonspecific binding was evaluated by performing the same analysis on lysates from cells not expressing PST-myc (-PST-myc). *Lower panel*, NCAM and Δ FN1 protein expression was evaluated by immunoblotting one tenth of the cell lysate with anti-V5 antibody.

FIGURE 7. NCAM mutants with the acidic patch and PSSP sequences replaced demonstrate reduced binding to PST. *Upper panels of each set*, NCAM and its mutants with sequences of the acidic patch (AP-AAA, AP-RRR), α helix (Δ helix-AA), PSSP (PSSP-AAAA), GGVPI (GGVPI-AAAAA) and NGKG (NGKG-AAAA) replaced were co-expressed with PST in COS-1 cells. PST was immunoprecipitated from cell lysates with an anti-myc tag antibody and co-immunoprecipitating proteins detected by immunoblotting with an anti-V5 epitope tag antibody (+PST-myc). Nonspecific binding was evaluated by performing the same analysis on lysates from cells not expressing PST-myc (-PST-myc). *Lower panels of each set*, NCAM and NCAM mutant protein expression was evaluated by immunoblotting one tenth of the cell lysate with anti-V5 antibody. Changes in the extent of co-precipitation of mutant NCAM proteins were quantified using NIH ImageJ and are shown underneath each series of blots.

FIGURE 8. Pro⁵⁰⁰ and Pro⁵⁰³ in the PSSP sequence are critical for NCAM polysialylation. NCAM and mutants with either the two proline residues or two serine residues of the PSSP sequence replaced (PSSP-GSSG and PSSP-PGGP) were co-expressed with PST-myc in COS-1 cells. NCAM proteins were immunoprecipitated using an anti-V5 epitope tag antibody and immunoblotted with the OL.28 antibody to detect polysialylation (upper panel, Polysialylation, OL.28 Antibody). Aliquots of cell lysates were immunoblotted with the anti-V5 antibody to determine relative protein expression levels (lower panel, Expression, Anti-V5 Antibody).

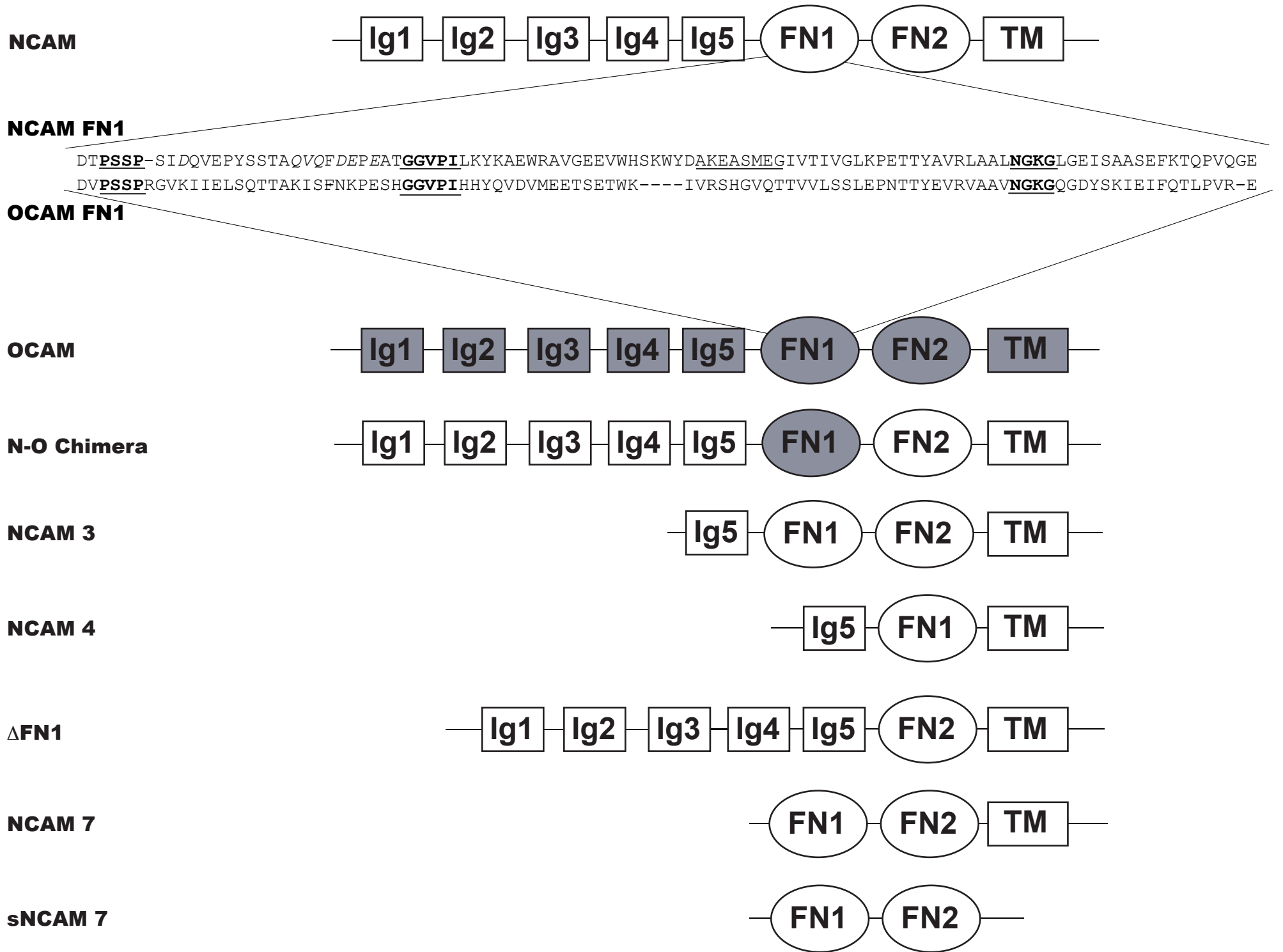


Figure 1

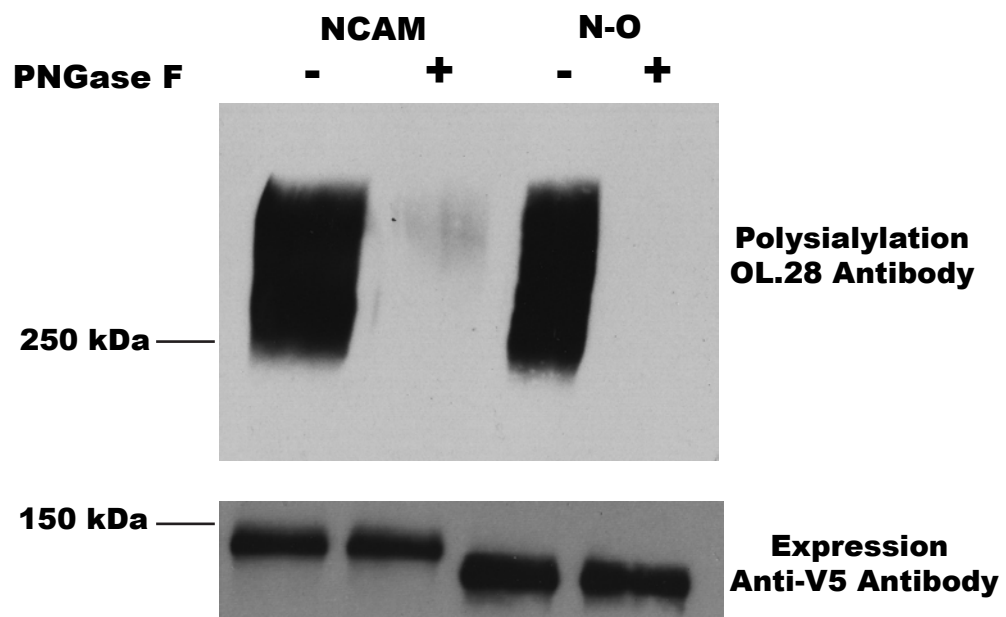


Figure 2

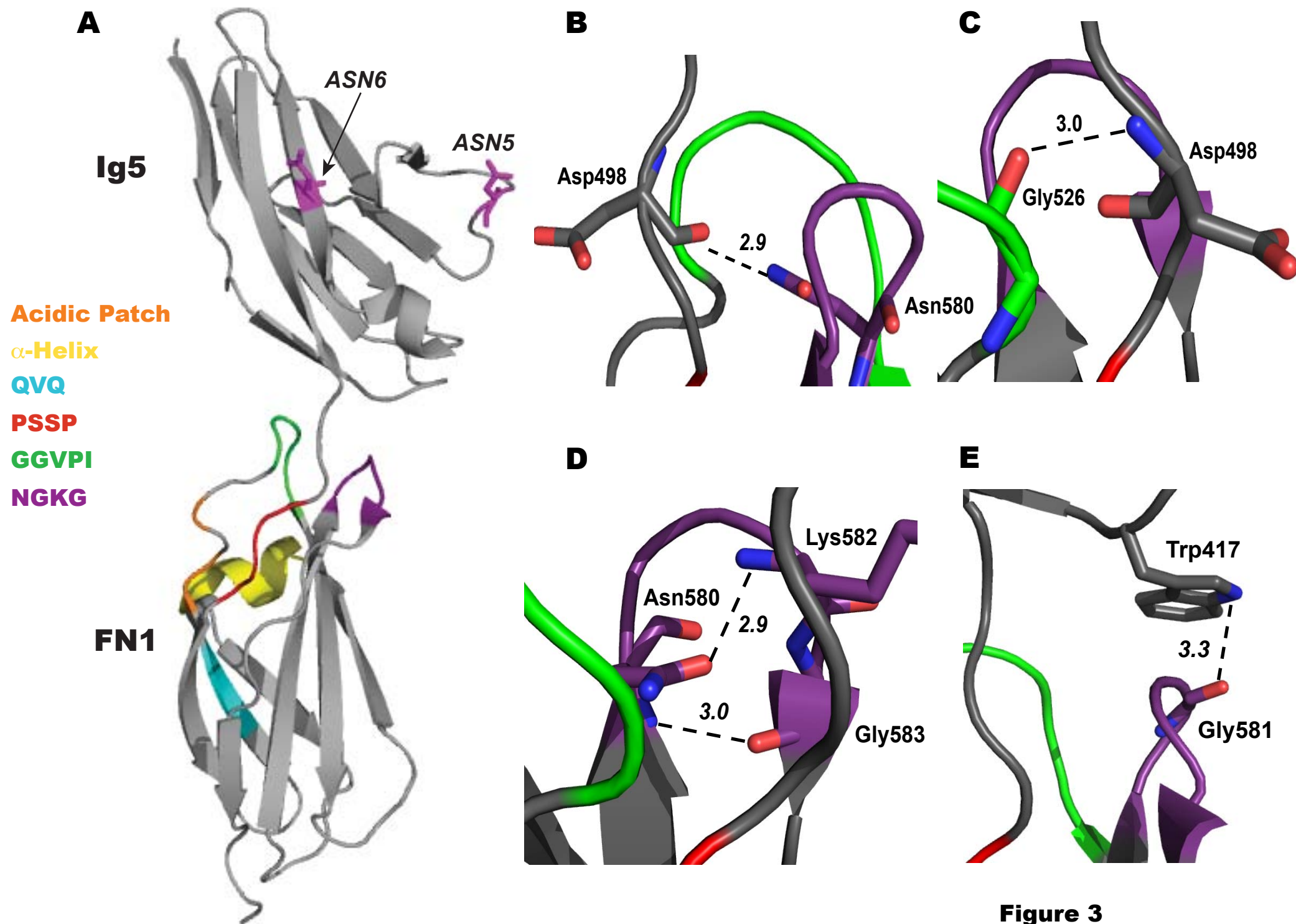
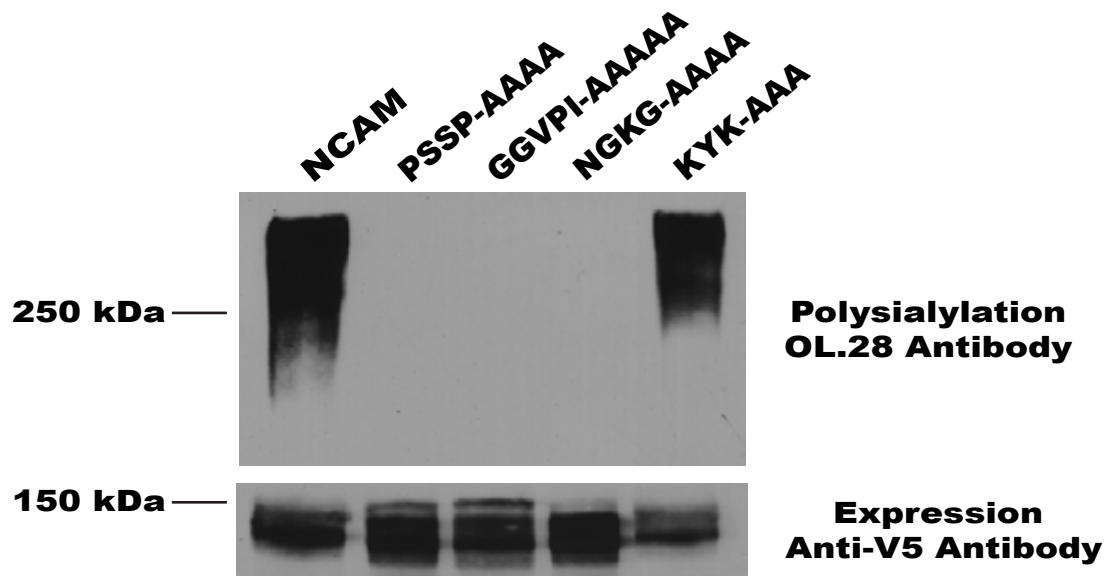
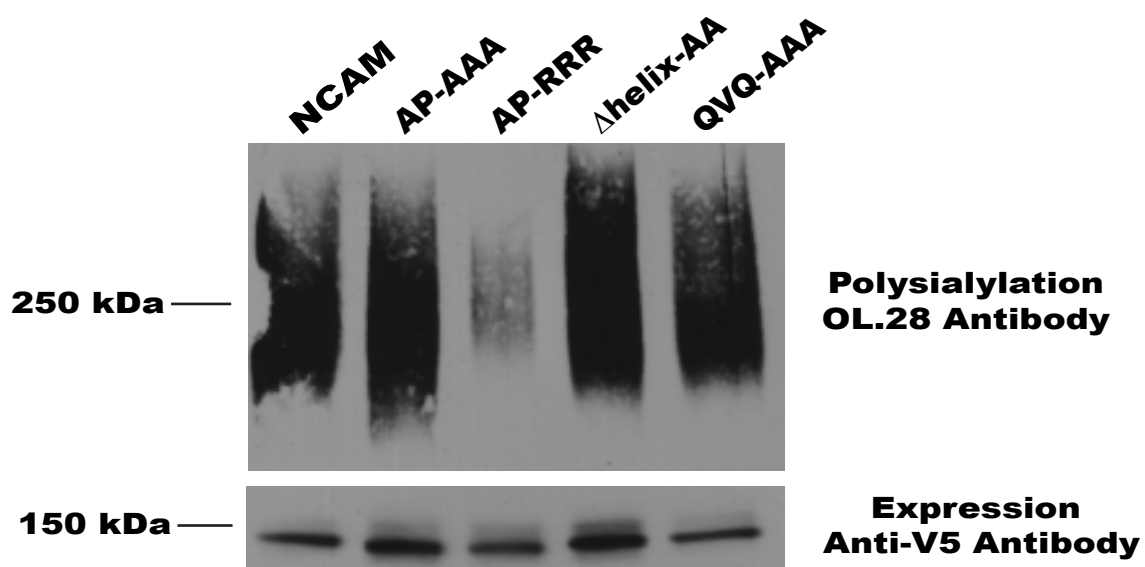


Figure 3

A



B



C

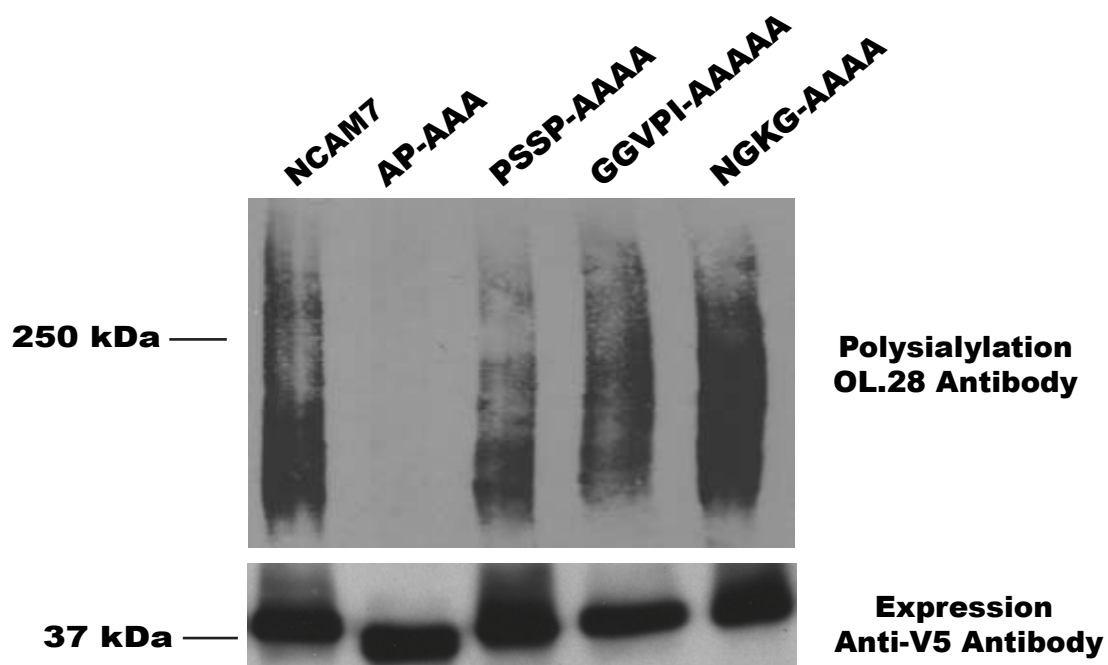


Figure 4

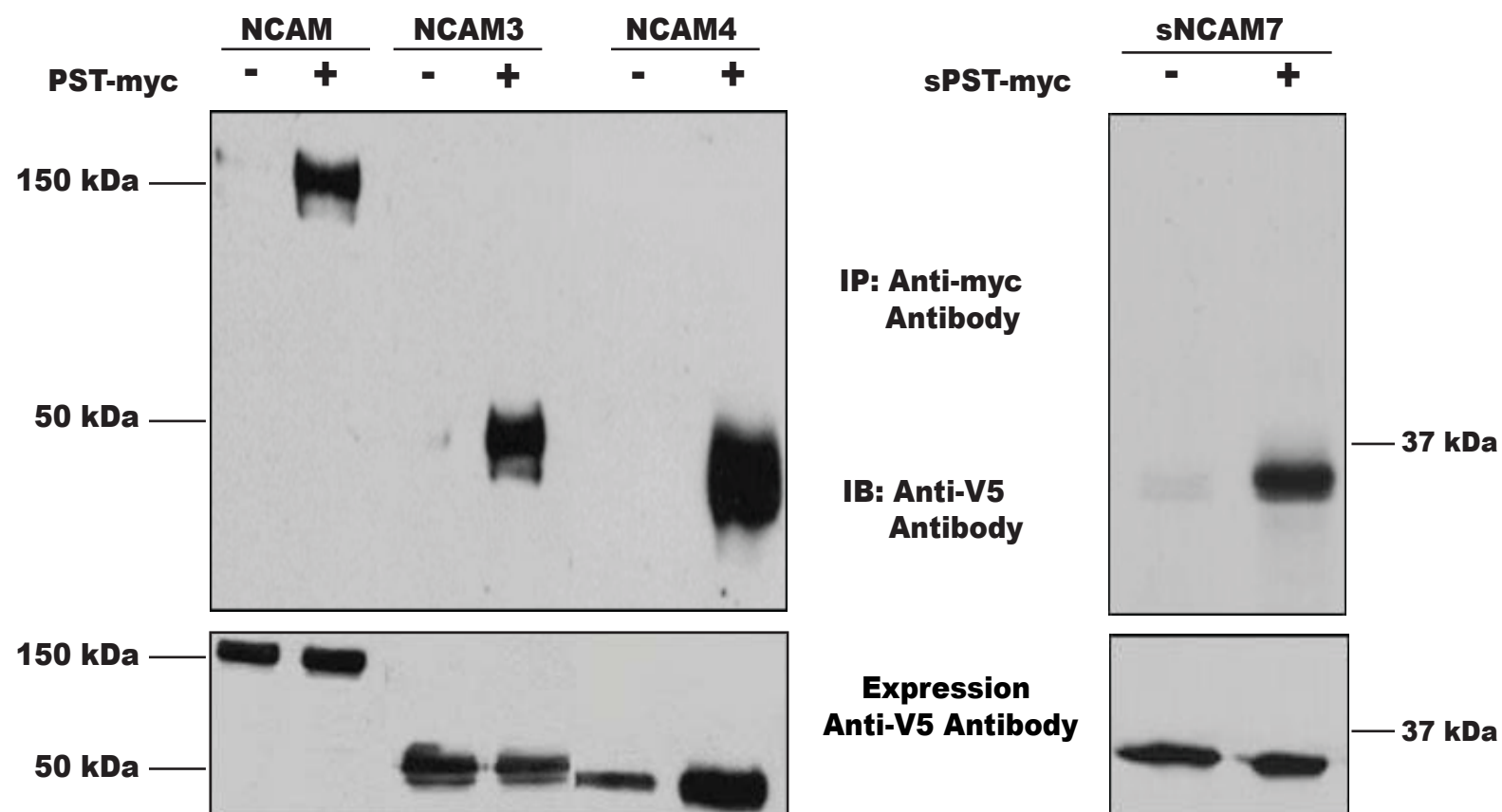


Figure 5

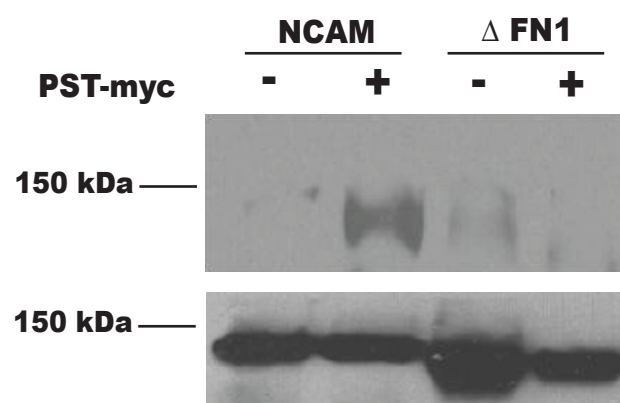


Figure 6

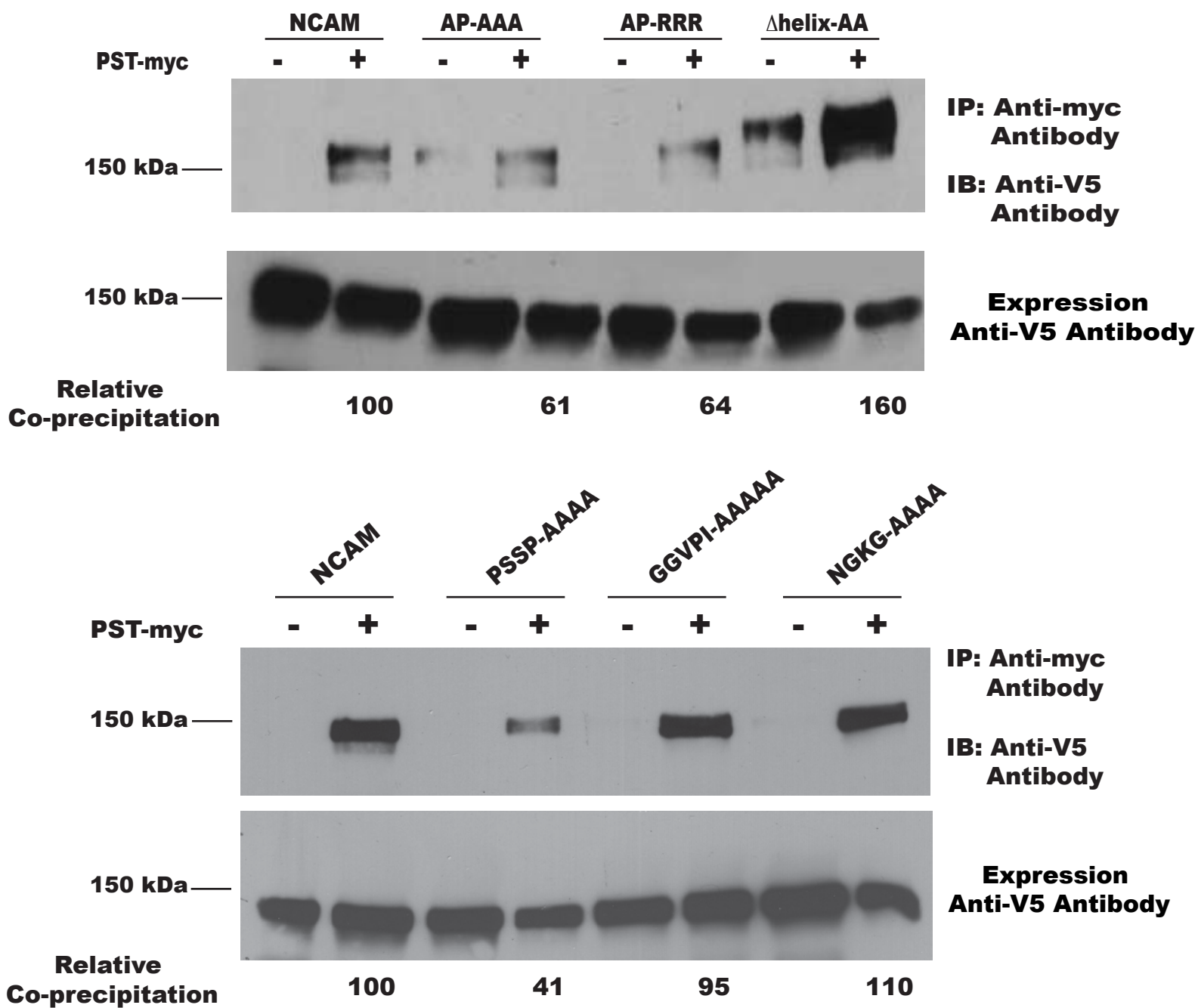


Figure 7

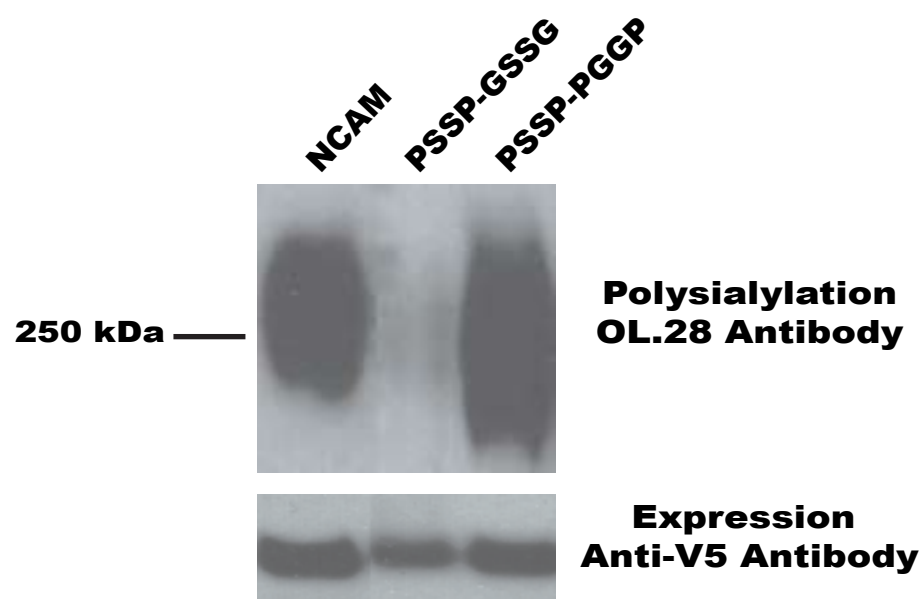


Figure 8