Sequences in the NCAM Ig5-FN1 Tandem Affect Interactions with the Polysialyltransferases

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THESIS

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LIST OF ABBREVIATIONS

Amp	Ampicillin
Asn	Asparagine
Asp	Aspartic Acid
BDNF	Brain Derived Neurotrophic Factor
CDG	Congenital Disorders of Glycosylation
CHO	Chinese Hamster Ovary
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
ER	Endoplasmic Reticulum
ERAD	ER assisted degradation
FBS	Fetal Bovine Serum
FGFR	Fibroblast Growth Factor Receptor
FITC	Fluorescein Isothiocyanate
FN	Fibronectin
GAG	Glycosaminoglycans
GalNAc	N-acetylGalactosamine
GlcNAc	N-acetylGlucosamine
Glu	Glutamine
Gly	Glycine
GPI	Glycosyl Phosphatidylinositol
HNK-1	Human Natural Killer
HRP	Horseradish Peroxidase
Ig	Immunoglobulin

LIST OF ABBREVIATIONS (continued)

Ile	Isoleucine
KDN	2-keto-3-deoxynonic-D-glycero-D-galacto-nonulosonic acid
LB	Lysogeny Broth
LTP	Long Term Potentiation
Lys	Lysine
M6P	Mannose 6 Phosphate
MAP	Membrane Associated Protein
MAPK	Membrane Associated Protein Kinase
mRNA	Messenger Ribonucleic Acid
NCAM	Neural Cell Adhesion Molecule
NMR	Nuclear Magnetic Resonance
NSCLC	Non-small Cell Lung Carcinoma
OCAM	Olfactory Cell Adhesion Molecule
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PKCδ	Protein Kinase C delta
PolySia	Polysialic Acid
PolyST	Polysialyltransferase
Pro	Proline
RNA	Ribonucleic Acid
SCLC's	Small Cell Lung Carcinomas
SDS	Sodium Dodecyl Sulfate
Ser	Serine

LIST OF ABBREVIATIONS (continued)

SML	Large Sialyl Motif
SMS	Small Sialyl Motif
SMVS	Very Small Sialyl Motif
TBS	Tris Buffered Saline
Thr	Threonine
TM	Trans-membrane
Tyr	Tyrosine
Val	Valine
VASE	Variable Alternatively Spliced Exon

Summary

Polysialic acid is an anti-adhesive glycan added to a select group of mammalian proteins. The major carrier of polysialic acid is the neural cell adhesion molecule, NCAM. Polysialylated NCAM plays a crucial role in the developing nervous system of embryos and neonates. In adults its continued expression is critical for learning and memory, synaptic plasticity and neuronal regeneration. Polysialylated NCAM is also aberrantly expressed in several cancer cells. This expression is often correlated with increased tumor invasiveness and tumor growth.

The addition of polysialic acid to NCAM unlike most glycosylation reactions depends on the protein-protein interaction of NCAM and the polysialyltransferases (polySTs), ST8SiaII/STX and ST8SiaIV/PST. These enzymes add polysialic acid chains to N-linked glycans in NCAM's fifth immunoglobulin like domain (Ig5). Previous work from the Colley lab has demonstrated that polysialylation of the Ig5 domain also requires the first fibronectin type III repeat (FN1) domain. The proposed model for this interaction was that the polySTs bound to NCAM FN1, which then positioned them to polysialylate N-linked glycans in Ig5.

Sequences in NCAM FN1 including a surface acidic patch (Asp^{509}, Asp^{520}, Glu^{521}, Glu^{523}), α helix, and two unique sequences, Pro^{510}, Tyr^{511}, Ser^{512}, and Gln^{516}, Val^{517}, Gln^{518} (PYS and QVQ) have been shown to impact the polysialylation of NCAM. Replacement of the acidic patch and to a lesser degree PYS led a decrease in NCAM polysialylation, while replacement of the α helix and QVQ caused a switch from N-linked to O-linked polysialylation.

To better understand the roles of these sequences in NCAM's interaction with the polySTs, I preformed co-immunoprcipitation studies on NCAM and NCAM sequence replacements with the ST8SiaIV/PST protein. I observed that replacement of only the acidic patch region with either alanine or arginine's led to a decrease in the interaction between NCAM and the polyST. Replacement of PYS, QVQ or the α helix did not disturb this interaction.

Next these mutants were examined in a truncated NCAM construct (NCAM 7) consisting of only the first two fibronectin type III repeats (FN1-FN2), that is polysialylated on O-linked glycans in FN1. This revealed that in the absence of the Ig5 domain mutation of the acidic patch led to a much more drastic decrease in NCAM 7 polysialylation, indicating that sequences in Ig5 may play a role in the NCAM-polyST interaction.

Deirdre Foley in collaboration with the Lavie lab obtained the crystal structure of the NCAM Ig5-FN1 tandem. Additionally, it was discovered that the FN1 domain from olfactory cell adhesion molecule, an unpolysialylated homolog of NCAM, could substitute for NCAM FN1 and allow for polysialylation of a chimeric protein. These two findings allowed me to look at sequences in common between NCAM and OCAM FN1 and where they were located in the NCAM structure. I found three regions of identity between the two domains that comprised the sequences; $Pro^{500}Ser^{501}Ser^{502}Pro^{503}$ (*PSSP*), $Gly^{526}Gly^{527}Val^{528}Pro^{529}Ile^{530}$ (*GGVPI*), and $Asn^{580}Gly^{581}Lys^{582}Gly^{583}$ (*NGKG*). All three sequences mapped to the interface of the Ig5-FN1 domain. When each sequence was replaced with alanine residues NCAM polysialylation was eliminated. I examined the interaction of these mutants with the polyST and found that only the PSSP replacement disrupted interaction between the two proteins. This implied that residues in these regions are critical to maintain the proper orientation and spacing of Ig5 and FN1 to allow polysialylation of N-linked glycans in Ig5. Since these residues were not part of the polyST binding region, I then went back and looked at possible effects on interaction by the Ig5 domain.

Using OCAM-NCAM chimera proteins I investigated the effects of individual domains on NCAM-polyST interaction and looked at specific sequences in Ig5 that had been implicated in affecting polysialylation. The results of these studies indicated that while the polyST will bind to just FN1, a tandem of Ig5-FN1 leads to much higher interaction and polysialylation of chimeric proteins. It was also observed that residues Ser^{448} and Asn^{476} in NCAM Ig5 affect how NCAM and the polysialyltransferase interact. When these residues were replaced with the OCAM equivalents, Lys and Arg, the NCAM-polyST interaction and NCAM polysialylation both

decreased dramatically. However replacement of these residues with either alanines or glutamic acid showed a lesser decrease. This indicates that these specific residues may be a factor in why OCAM is not polysialylated and that the analogous residues in NCAM are permissive for interaction with the polyST.

In sum, to further understand the protein specificity of polysialylation, the NCAM-polyST interaction was investigated using mutational analysis and chimeric proteins. Specific sequences in NCAM FN1 and Ig5 were found to play roles in the NCAM-polyST interaction or the relationship of the Ig5-FN1 domain. This work changed the way the NCAM-polyST interaction is envisioned indicating that both Ig5 and FN1, rather than FN1 alone, make contact with the polySTs to allow NCAM recognition and polysialylation.

Chapter I

Introduction

In this work I investigated the interaction between the Neural Cell Adhesion Molecule (NCAM) and the polysialyltransferases (polyST). Though composed of commonly found structural domains the NCAM-polyST interaction is one of only a select few interactions that result in polysialic acid addition. The background and significance of the polysialylation of NCAM is outlined below.

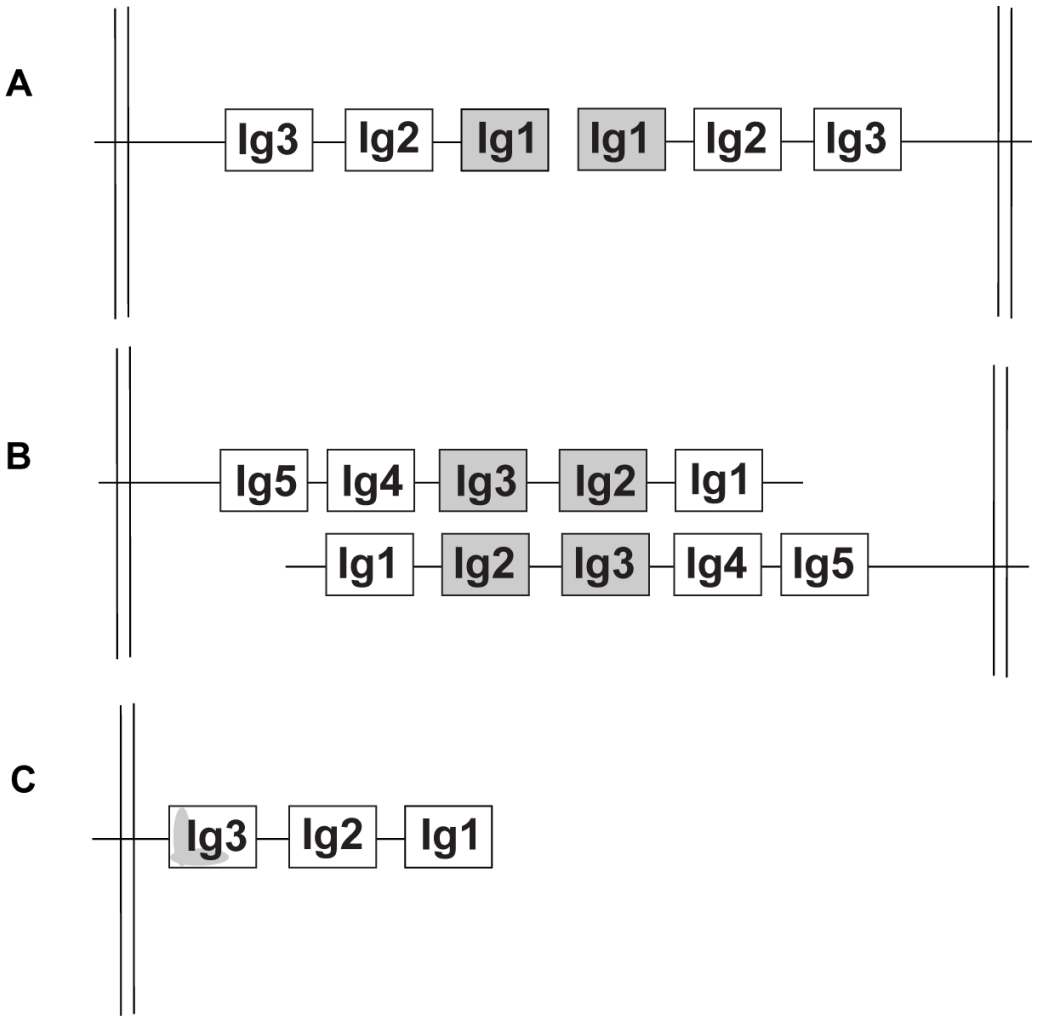
Immunoglobulin Super Family Proteins

Immunoglobulin superfamily proteins are proteins that all contain a structural motif of approximately 100 amino acids, known as the immunoglobulin (Ig) domain, first characterized in the immunoglobulin protein (1). The signature characteristic of this domain is antiparallel β-sheets usually linked by a single disulfide bond to stabilize the structure (review 2 and 3). The Ig-like fold has been found in many other protein domains including the cadherin, fibronectin type III (FN) and cytokine receptor domains (3). These domains often have few conserved amino acids, but nonetheless form similar structures that serve similar functions (3). Often the only conserved residues in a given family of Ig proteins are the cysteine residues that form the disulfide bridge linking the β-sheets and inward facing hydrophobic residues which make up the core of the Ig domain (3).

Ig-like domain containing proteins make up the one of the most populous groups of human proteins with 765 members (4). The functions of proteins in this family include but are not limited to; cell adhesion, cell signaling, cytokine and growth factor receptors and antigen

receptors (3). Ig-like domains are often used in recognition/adhesion proteins found at the cell surface. These proteins tend to have multiple linked Ig-like domains and interact with binding partners or ligands in generalized ways (5) (Figure 1). Unlike the Ig-domains of antibodies, where the recognition and binding region is located in the variable loops linking the β-sheets, the binding and recognition regions in linked Ig-like domains is often found on the β-sheets (5).

Most Ig domain containing proteins pass through the Golgi en route to the cell surface and are subject to varying levels of glycosylation. Glycosylation is a common feature seen in Ig containing proteins and can have a large impact on the function of these proteins. In the next section we take a look at the process of glycosylation in the endoplasmic reticulum (ER) and Golgi.



Modified from figures in Holness et al. (5)

Figure 1. Binding modes of Ig like domains.

A) Head to head binding of terminal Ig-like domains. This interaction can be between heterotypic or homotypic domains.

B) Binding of internal Ig-like domains. This interaction can be heterotypic or homotypic domains. Proteins are aligned in an anti-parallel fashion.

C) Binding sites for small molecule ligands such as growth factors or cytokines are often found on internal Ig-like domains.

Glycosylation

The addition of carbohydrate moieties (glycans) to a protein or lipid is termed glycosylation and is critical for the folding and function of many proteins (6). The addition of glycans to a protein is catalyzed by proteins called glycosyltransferases, found in the cytosol, ER and Golgi. In the process of N-glycosylation, glycans are added to asparagines in a specific consensus sequence, whereas in the process of O-glycosylation, glycans are added to the side chains of serines or threonines. The biosynthesis of N- and O-linked glycans occurs in different ways and these are described below.

N-linked Glycosylation

The process of N-glycosylation occurs in the ER and Golgi. The first step in the addition of glycans to asparagine residues is the *en bloc* transfer of a 14 monosaccharide precursor ($Glc_3Man_9GlcNAc_2$) from dolichol diphosphate to the nitrogen in asparagine side chain as the target protein is translocated across the ER membrane and into the ER lumen. The biosynthesis of this lipid linked precursor oligosaccharide that serves as the donor in this reaction occurs in both the cytosol and ER lumen (see Figure 2). Proteins are N-glycosylated on Asn residues in the consensus sequence Asn-X –Ser/Thr, where X is any amino acid except proline (7). However, not every Asn-X-Ser/Thr site will be glycosylated (8).

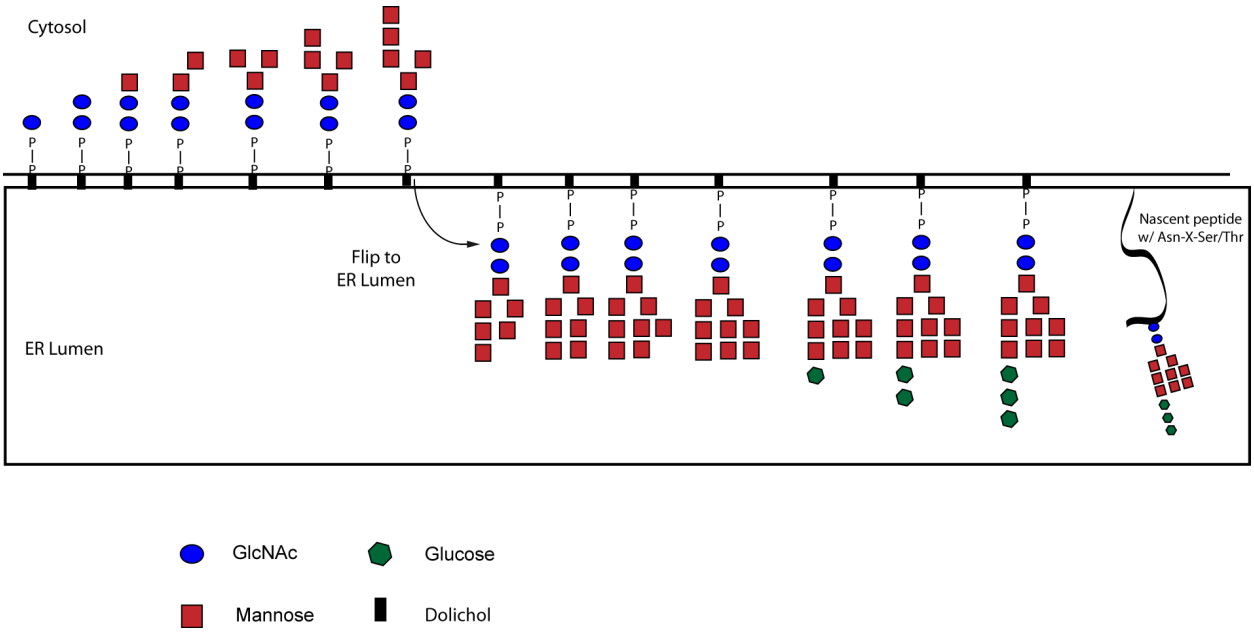


Figure 2. Construction of precursor oligosaccharide on dolichol diphosphate that used as the precursor for N-linked glycosylation. The 14 sugar oligosaccharide is assembled stepwise on dolichol diphosphate. Each sugar addition requires a nucleotide sugar donor and a separate enzyme. The $Man_5GlcNAc_2$-DLO intermediate is flipped by an unknown flippase from the cytosol to the ER lumen where synthesis continues with the addition of four Man and two GlcNAc monosaccharides. Transfer of the oligosaccharide occurs when nascent proteins display the N-linked consensus sequence Asn-X-Ser/Thr, were X is any amino acid but proline.

Figure modified from *Mol Biol Cell* (6)

After the addition of the precursor structure to the growing peptide, the oligosaccharide will be modified further by both glycosidases and glycosyltransferases. The first two enzymes in the processing pathway, glucosidase I and II modify these glycans by removing the terminal glucose residues and also control entry into the ER assisted degradation (ERAD) system that controls for incorrectly folded proteins (Figure 4, glucosidase I and II). In this quality control system, glycans are modified by the two glucosidases to form mono-glucosylated intermediates that are recognized by the ER lectins calnexin and calreticulin (9), and the associated oxidoreductase ERp57 (10). Calnexin and calreticulin are chaperone proteins that assist in protein folding (11) and retain mis-folded proteins in the ER (12). ERp57 interacts with both calnexin and calreticulin to promote disulfide bond formation. (13). If the targeted glycoprotein folds correctly it will be released from interaction with the chaperone proteins after glucosidase II removes the final glucose residue to generate the $Man_9GlcNAc_2$ structure (Figure 4), If it is not folded properly it will be re-glucosylated by a glucosyltransferase that recognizes aspects of misfolded proteins (e. g. exposed hydrophobic regions) and then reengage with the calnexin or calreticulin chaperone to attempt folding again (14). If however repeated folding cycles fail, terminal mannose residues will be removed by ER α-mannosidase I (Figure 4) which then targets the protein to interact with ER degradation enhancing α-mannosidase like proteins (EDEM and EDEM2) or other lectins like OS9, and these interactions ultimately lead to the retro-translocation of the protein to the cytosol for degradation (15-17). In this way N-linked glycosylation acts as a quality control system for incorrect protein folding. If the protein folds correctly it then moves on to the Golgi for further processing.

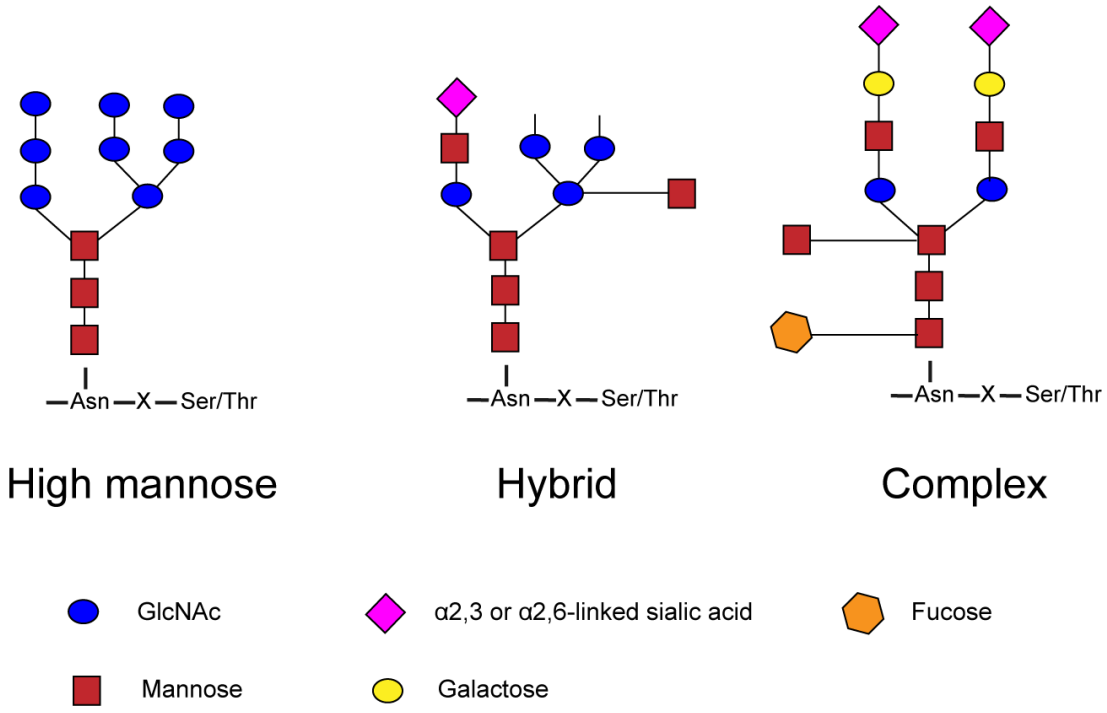


Figure 3. The three forms of N-linked glycans. The three N-glycan forms share a common core pentasaccharide composed of two mannose and three GlcNAc residues. High mannose N-glycans have only mannose branches, while complex type N-glycans have branches containing GlcNAc, galactose and sialic acid. These structures may also have a fucose residue added to a core GlcNAc. Hybrid type N-glycans have both mannose and more complex type branches.

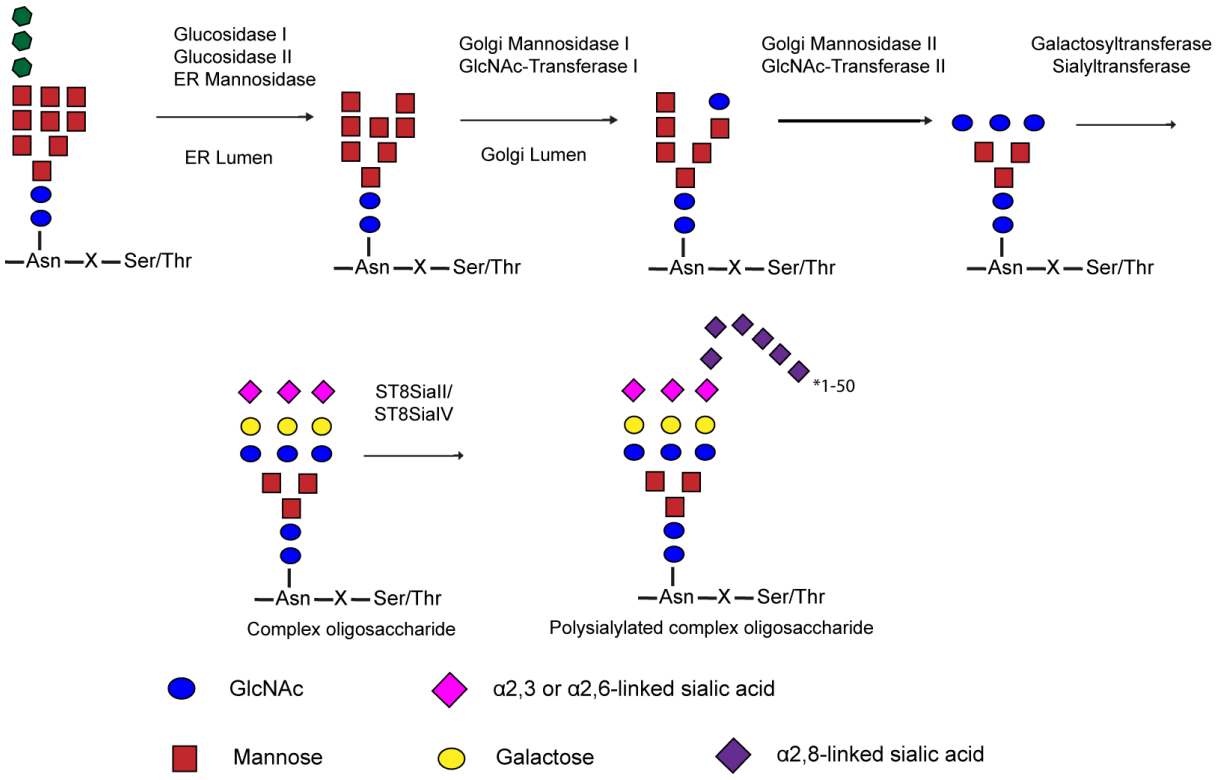


Figure 4. Processing of N-linked glycans begins in the ER and continues as the protein moves through the Golgi. The enzymes that catalyze each addition or removal are indicated. The enzymatic reactions are highly ordered with each enzyme recognizing the product of the previous enzyme as its substrate.

There are three main types of N-linked glycans shown in Figure 3 that are created as the protein moves through the Golgi (see Figure 4). Most of the glycosyltransferases and glycosidases present in the Golgi recognize and modify protein-attached glycan structures that serve as their substrates with no regard to what protein they are attached to. An exception to this is the process of a2, 8-polysialylation which occurs on a small subset of proteins and is believed to be protein-specific and require an initial protein-protein interaction between the polysialyltransferase and substrate. This and other protein-specific glycosylation events will be discussed later in this chapter.

N-linked glycosylation is critical in mammalian development and defects in the biosynthesis of the lipid linked precursor oligosaccharide and in N-glycosylation processing enzymes can lead to Congenital Disorders of Glycosylation (CDG). Table 1 lists some examples of the CDG associated with known enzyme defects (18).

Table I. Examples of congenital disorders of glycosylation and related enzymes.

Defects in Dolichol-diphosphate oligosaccharide synthesis (type I)	**Defects in N-linked glycan processing (type II)**

Enzyme Name	**Function**	**Enzyme name**	**Function**
Phosphomannomutase	Man-6-P→Man-1-P	GlcNAc transferase II	$GN_1M_3GN_2$-Asn→$GN_2M_3GN_2$-Asn
Phosphomannose isomerase	Fru-6-P→Man-6-P	α-glucosidase-I	$G_3M_9GN_2$-Asn→$G_2M_9GN_2$-Asn
α1,3-Glucosyl transferase	M_9GN_2-Dol→$G_1M_9GN_2$-Dol	GDP-Fucose Transporter	Moves GDP-Fuc from cytosol into Golgi
α1,3-Mannosyl transferase	M_5GN_2-Dol→M_6GN_2-Dol	β1, 4-Galactosyl transferase	$GN_{(1-4)}M_3GN_2$-Asn→$G_{(1-4)}GN_{(1-4)}M_3GN_2$-Asn
Man-P-Dol synthase	Dol-P→M-P-Dol		
α1,2-Mannosyl transferase	M_7GN_2-Dol→M_8GN_2-Dol		

Phenotypes of these disorders vary wildly even in patients with the same congenital defect. In general, common pathologies include delays in psychomotor development, hypotonia, feeding problems, seizures, and often impaired vision (19, 20). The importance of N-glycosylation in embryonic development are highlighted by two knockout studies. First, knocking out UDP-GlcNAc: dolichol phosphate N-acetylglucosamine-1-phosphate transferase, the enzyme that catalyzes the first step in the synthesis of the dolichol oligosaccharide precursor, results in embryonic lethality (21). Similarly, the embryonic lethality of deleting the GlcNAc transferase I enzyme, that is the first step in the biosynthesis of complex type N-glycans, demonstrates the importance of this N-glycan type in embryonic development (22).

O-linked Glycosylation

O-linked glycosylation is the addition of glycans to either serine or threonine residues and occurs mainly in the Golgi. Unlike N-linked glycosylation, O-linked glycosylation does not begin with the bulk transfer of a common core oligosaccharide, but instead involves the sequential addition of single monosaccharides (23). Most O-linked glycosylation begins with the addition of GalNAc to the hydroxyl group of Ser or Thr by enzymes in the family of ppGalNAc transferases (24). The resulting sugars are known as mucin-type O-glycans. The initial O-GalNAc structure is termed the Tn antigen, and can be further extended by other glycosyltransferases (25). This form of O-linked glycosylation is often found on mucin like proteins in the digestive and respiratory tracts, where it acts to form protective barriers and provide lubrication (26, 27).

The addition of glycosaminoglycan (GAG) chains to serine residues via the linker tetrasaccharide GlcAβ1–3Galβ1–3Galβ1–4Xy produces proteoglycans (28). Unlike most O-linked glycans the attachment of GAGs is associated with a specific amino acid sequence, Ser-Gly-X-Gly, were X is any amino acid. Proteoglycans such as heparin/heparan sulfate and chondroitin sulfate are often found in the extracellular matrix in connective tissue and joints were they serve a structural role. Proteoglycans are also found on cell surface proteins were they bind and present soluble growth factors (29).

Other forms of O-linked glycosylation including O-glucosyl and O-fucosyl glycans that modify the epidermal growth factor homology regions (EGF modules) of some human proteins are more rare (30). As is an initial O-mannosyl linkage on proteins found in the brain, neurons and skeletal muscle. The most well-known instance of O-mannosylation is found on α-

dystroglycan a extracellular matrix protein found in skeletal muscle. The under glycosylation of this protein, including the lack of extension of the O-mannose structure, prevents proper binding to laminin in the extracellular matrix, and is the cause of one form of congenital muscular dystrophy (30).

Mechanisms Controlling Glycosylation

Although N-linked and O-linked glycosylation begin in different ways, the further processing of the growing glycan chain occurs in a stepwise and controlled manner. Expression levels of enzymes, their compartmentalization in different sub-compartments of the Golgi and competition for common substrates can all impact the expression of glycan structures. The expression of both glycosyltransferases and transporters for nucleotide sugar donors (egUDP-GlcNAc, UDP-Gal, CMP-sialic acid) is regulated at the transcriptional level both developmentally and in a cell type specific manner. Consequently, the types of glycans expressed can be different during development, in the adult animal and during disease (31, 32). Glycosyltransferases and the transporters for nucleotide-sugar donors are localized to specific sub-compartments of the Golgi (cis, medial, and trans Golgi). As the protein and attached glycan progress through these compartments different enzymes and nucleotide sugar donors will be present to modify accessible glycan chains. Thus in a given compartment the availability of both enzymes and nucleotide sugar donors can control the types of glycans made. Further control is exerted by a competition among glycosyltransferases for common acceptor glycan structures (33). The glycosyltransferase that is localized earlier in the secretory pathway and "sees" the glycan substrate first will have the opportunity to modify that glycan (33). Most glycosylation enzymes recognize the terminal monosaccharide or disaccharide of a glycan as a

substrate regardless of the protein it modifies. However, examples of protein-specific glycosylation are becoming more prevalent and will be discussed below.

Protein Specific Glycosylation

One of the best examples of protein specific glycosylation is the synthesis of the mannose 6-phospahte (M6P) recognition marker that is required for targeting enzymes to the lysosome (34). The protein-specificity of M6P biosynthesis was suggested by initial studies that demonstrated that lysozomal enzymes were used more efficiently as a substrate for mannose phosphorylation than non-lysozomal proteins also displaying high mannose N-glycans (35), and free high mannose glycans removed from lysozomal enzymes are not efficiently modified (36). Work done by several groups defined the M6P biosynthetic pathway and confirmed its protein-specificity.

Lysozomal enzymes are N-glycosylated in the usual manner, but their high mannose N-glycans are further specifically modified in a two-step process. First, a UDP-N-acetylglucosamine: lysozomal enzyme N-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase) in the cis-Golgi recognizes the lysozomal enzyme and transfers GlcNAc-1-phosphate to the C6 hydroxyl group of select mannose residues of its high-mannose-type N-glycans. Second, the terminal GlcNAc residues are removed by the 'uncovering enzyme', an N-acetylglucosamine-1 phosphodiesterase in the trans Golgi network, which exposes the M6P residues (34). Studies of lysozomal cathepsins D and L revealed that critical lysine residues near the glycosylation site mediate GlcNAc-phosphotransferase recognition (37).

Another example of protein specific glycosylation comes in the biosynthesis of the GalNAc-4-SO_4 structure that is found on the termini of the N-glycans of pituitary glycoprotein hormones and functions to mediate their clearance from the circulation (38). The addition of β1, 4-linked GalNAc (rather than the typical galactose) to terminal positions on N-glycans is unusual and found only on the common α subunit of pituitary glycoprotein hormones and a few other proteins including carbonic anhydrase VI (38). It was observed early on that the addition of GalNAc to the N-glycans of some glycoproteins was far greater than to others even though both display identical N-glycan structures (39, 40). For carbonic anhydrase VI it was found that a carboxy-terminal 19 amino acid α helix, rich in basic residues, is critical for recognition and modification by a specific GalNAc tranferase (36). A similar recognition marker was initially observed in the common α subunit of the glycoprotein hormones lutropin and thryotropin (41).

The O-mannosylation of a-dystroglycan is also a protein-specific event. The modification of the α-dystroglycan mucin domain with O-linked mannose residues is dependent on a 41 amino acid sequence located N-terminal to the domain. Removing this sequence abrogated the mannosylation (42). Additionally, it was noted that basic residues flank the preferred serine and threonine sites of O-mannosylation in α-dystroglycan (42).

The addition of polysialic acid (polySia) to specific N-glycans of the neural cell adhesion molecule, NCAM, is also a protein specific modification and requires interaction with the polysialyltransferases (polySTs), ST8SiaII/STX and ST8SiaIV/PST. The protein specificity of this modification and the NCAM sequences recognized by the polySTs is the focus of my thesis.

Polysialic Acid

Polysialic acid (polySia) is the formed by the polymerization of sialic acid monomers in α2, 8-linkage on the termini of N- or O-glycans on selected glycoproteins. Sialic acid is the common name for N- or O-substituted derivatives of neuraminic acid. Neuraminic acid is found in bacteria, plants and mammals, and over fifty structural derivatives have been identified in nature (43). The most common derivatives of neuraminic acid found in polySia chains are N-acetylneuraminic acid, N-glycolylneuraminic acid and 2-keto-3-deoxynonic-D-glycero-D-galacto-nonulosonic acid (KDN) (Figure 5) (44).

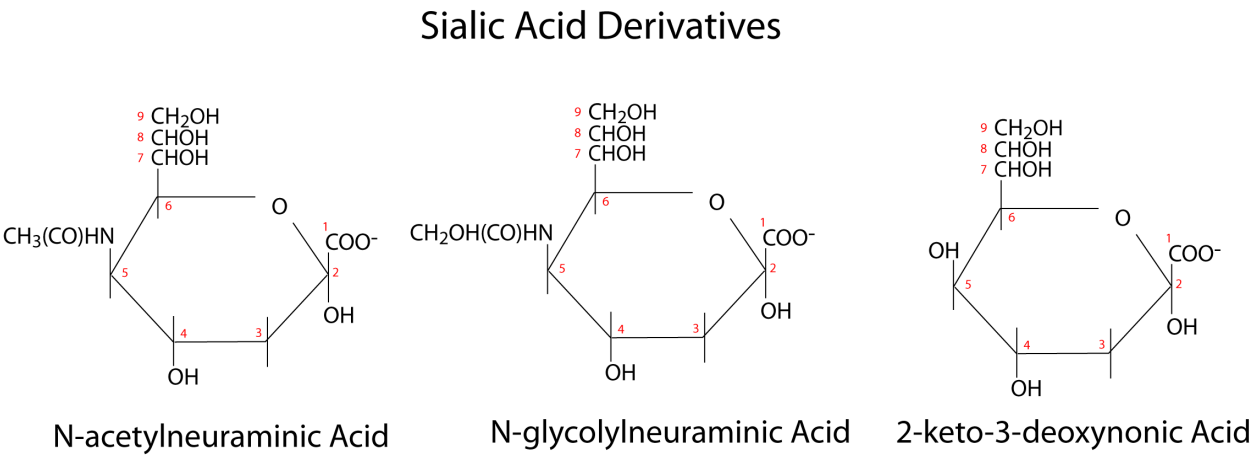


Figure 5. Sialic acid derivatives most commonly found in polySia. Sialic acid is found as over 50 different substituted derivatives. Sialic acid is typically found at the termini of N-glycans and O-glycans, where it imparts a negative charge. The above derivatives are most commonly incorporated into polySia.

NMR studies of bacterial polySia chains show that the polymer adopts an extended helical structure of 9 sialic residues per turn (45, 46). The acidic carboxyl group is required to maintain this conformation as replacement of the carboxyl group with alcohol reduces the number of residues per turn down to two or three (47). Long sialic acid chains often associate into bundles (48). Long chains of sialic acid are unstable under mildly acidic conditions such as in the lysosome or endosome, due to the tendency to undergo intramolecular self-cleavage of glycosidic bonds. Shorter sialic oligosaccharides of two-three residues are more stable (49).

PolySia is found in many different species from bacteria to humans. It is expressed on the capsules of certain bacteria in a form structurally identical to mammalian polySia seen on NCAM (50-52). These bacteria use polySia to help evade immune responses from the host organism and to provide a barrier against serum complement attack (53, 54). Capsular bacteria expressing polySia such as *Neisseria meningitidis* escape immune detection and cause invasive disease (54).

In mammals, polySia exists as homopolymers of α2, 8-linked N-acetylneuraminic acid attached to N or O-linked glycans. The two polysialyltransferases in mammals ST8SiaII/STX and ST8SiaIV/PST catalyze the addition of polySia and are themselves polysialylated (55-58, 66). In addition to NCAM, only a few other proteins are polysialylated by ST8SiaII/STX and ST8SiaIV/PST (Table 2). The polysialylation of NCAM will be the focus of the remainder of this work.

Table II. Mammalian polysialylated proteins

Protein	Protein function	PolySia on N or O-linked glycan	Effect of polySia	References
NCAM	Adhesion molecule	N-linked	Prevents homophilic trans interactions	59
Neuropilin-2	VEGF and semaphorin co-receptor	O-linked	Chemokine binding and cell signaling	60-62
Voltage gated sodium channel α subunit	Sodium intake	N-linked	Decrease excitability of sodium channels	63
CD36 glycoprotein receptor	Ligand binding, cell signaling	O-linked	Unknown/ possibly trapping pathogenic bacteria via sialic acid receptors	64
SynCAM 1	Adhesion molecule	N-linked	Prevents homophilic trans interactions	65
ST8SiaII/ST8SiaIV	Polysialyltransferase	N-linked	unknown	66

NCAM Structure and Function

NCAM is a member of the Immunoglobulin superfamily of proteins, and is expressed in mammalian cells as three major isoforms which arise from alternate splicing of a single gene. The three isoforms of NCAM include NCAM-180, NCAM-140 and NCAM-120 (Figure 6). The three isoforms are identical in their extracellular domains, which is composed of five Ig-like domains and two fibronectin type III repeats (FN1 and FN2). The difference between the NCAM 180 and NCAM140 isoforms is the length of their cytosolic tail. NCAM-120 contains no cytosolic tail or transmembrane region and is linked to the membrane by a glycosyl phosphatidylinositol (GPI) anchor (67). NCAM is most highly expressed in the nervous system, with all three

isoforms present. NCAM-120 is found primarily in glial cells, NCAM-180 in neurons, and NCAM-140 is present in both cell types (68).

Several minor NCAM isoforms are also generated by alternative mRNA splicing. One isoform includes a variable alternatively spliced exon (VASE), in the fourth Ig domain. Another isoform includes the insertion of the muscle-specific domain (MSD) between FN1 and FN2. Inclusion of the VASE exon introduces an additional 10 amino acids and is distinctly expressed in certain tissues during development and in the adult mammal (69, 70). The presence of the VASE exon down regulates NCAM-mediated neurite outgrowth (71). NCAM-120 expressed in skeletal and cardiac muscle includes the four MSD exons. These exons are expressed in varying combinations during muscle development (72-75). This form of NCAM-120 is glycosylated with mucin type O-linked glycans (72, 76). These O-glycans appear to facilitate myoblast fusion during cell adhesion events possibly by stabilizing the structure of NCAM (77).

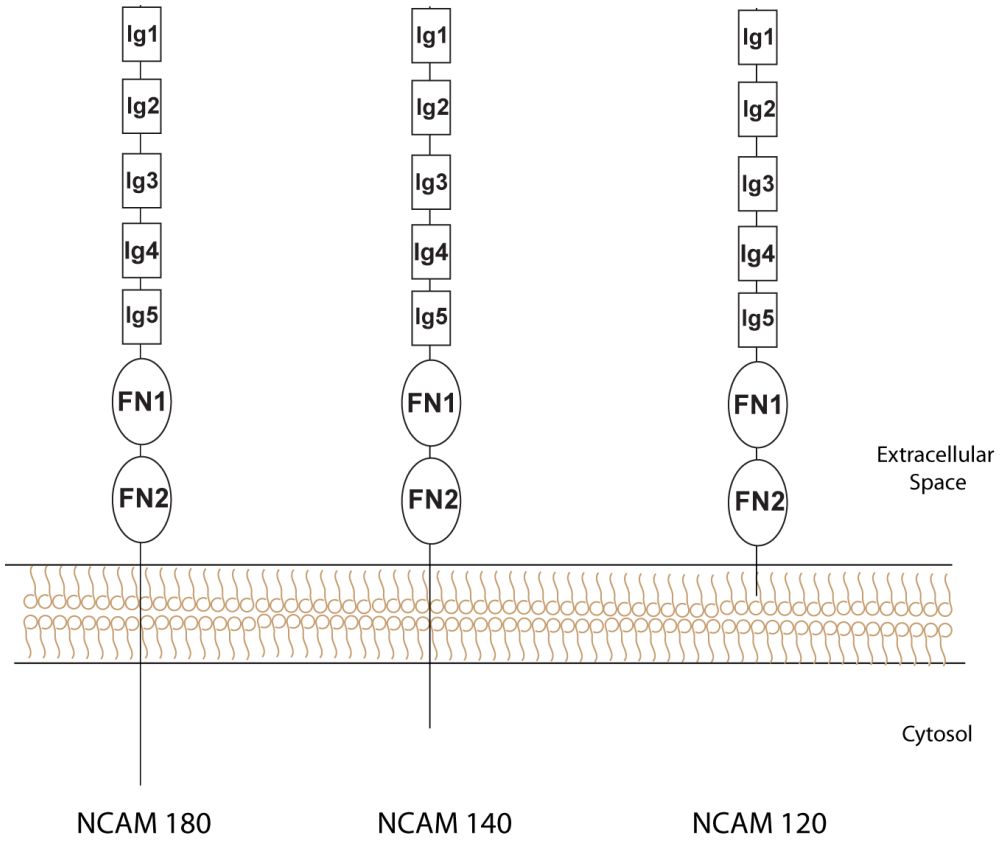


Figure 6. The three major NCAM isoforms. NCAM-180 and NCAM-140 both have a transmembrane region that transverses the lipid bilayer. In comparison to NCAM-140, NCAM-180 has 261 additional amino acids in the cytosolic tail. NCAM-120 is linked to the lipid bilayer by a GPI-anchor. All three NCAM isoforms are identical in their extracellular portions with five Ig-like domains (Ig1-5) and two fibronectin type III repeats (FN1 and FN2).

NCAM Mediated Cell Adhesion

NCAM mediates cell-cell adhesion through Ca^{2+} independent homophilic interactions between NCAM molecules on apposing cells. NCAM mediated adhesion can trigger signaling cascades that will be discussed below. The specifics of how NCAM forms homophilic interactions are debated, but it is generally believed that it involves an antiparallel interaction of the first 3 Ig domains of two NCAM proteins. Initial work on the mechanism of NCAM adhesion suggested that Ig3 was necessary for homophilic binding (78). Later work using soluble purified domains suggested that NCAM binds in an anti-parallel fashion with the Ig3 domain showing strong self-association (79). This agreed well with the initial studies, however surface plasmon resonance and nuclear magnetic resonance experiments failed to detect self-interaction between Ig3 domains (80, 81). These methods suggested an alternate binding scheme where only Ig1 and Ig2 interacted as an anti-parallel tandem. Later crystallographic studies supported the ability of Ig1-Ig2 to form trans-homophilic tandems (80, 82-84). Molecular force measurements also confirmed a trans-interaction between the Ig1-Ig2 tandem that was strengthened when all five Ig domains were present (85).

Bock and colleagues (86) found that Ig1 and Ig2 can form cis interactions with other NCAM Ig1 and Ig2 domains on the same cell membrane. They suggested that adjacent NCAM Ig1-Ig2 tandems could interact to form homophilic NCAM cis-dimers. These cis interactions caused a revision in the way NCAM trans interactions were envisioned. It was proposed that these NCAM cis-dimers interacted in trans with apposing NCAM cis-dimers in a zipper-like manner. This model was supported by experiments showing that peptides corresponding to

the proposed cis-interaction surfaces inhibited neurite outgrowth, suggesting that NCAM trans-homophilic interactions were blocked (86).

The role of NCAM in cell signaling

Although NCAM does not possess intrinsic signaling activity, the ability of NCAM to bind to a variety of partners can activate signaling cascades. The most well studied signaling event is the interaction of NCAM with the fibroblast growth factor receptor (FGFR) in neurons (87). FGFR binds to NCAM through NCAM FN1 and FN2 domains as shown by surface plasmon resonance (88). FGFR binding to NCAM leads to dimerization and phosphorylation of FGFR (89-91). Phosphorylated FGFR then is capable of recruiting PLCγ. Recruitment of PLCγ causes a conversion of diacylglycerol (DAG) to arachidonic acid and increased intercellular calcium (92). These secondary messengers as well as activation of the MAP kinase pathway eventually promotes neurite outgrowth (93, 91). Other reported NCAM binding partners that result in signal cascades include the receptor protein tyrosine phosphatase, the non-receptor tyrosine kinase Fyn and the glial cell line derived neurotrophic factor (96-97).

As described above the NCAM protein has effects on both cell adhesion and cell signaling. However the addition of polySia to NCAM's N-glycans can cause a switch in these NCAM properties.

Synthesis of Polysialylated NCAM

Characterization of the Polysialyltransferases

Addition of polySia to NCAM's N-glycans is catalyzed by two polysialyltransferases (polySTs), ST8SiaII/STX and ST8SIaIV/PST. These polyST's share 59% amino acid identity and show features typical of the sialyltransferase protein family (55, 57-58). Both proteins are type II membrane proteins that have a cytosolic tail, an uncleavable signal sequence that acts as a membrane spanning region (signal anchor domain), a membrane proximal stem region, and large catalytic domain that faces the lumen of the Golgi (98). Both sialyltransferases and polysialyltransferases have four motifs required for catalytic activity (Figure 7) (99-101).

The large sialyl motif (SML) is thought to bind the activated nucleotide-sugar CMP-sialic acid (102). The small sialyl motif (SMS) participates in binding the donor sugar while also binding the carbohydrate acceptor (103). The very small sialyl motif (SMVS) contains a histidine residue conserved in all sialyltransferases that is required for catalytic activity (104). A fourth motif comprised of just four amino acids, motif III, is also conserved in the sialyltransferases and has been implicated in acceptor recognition (105).

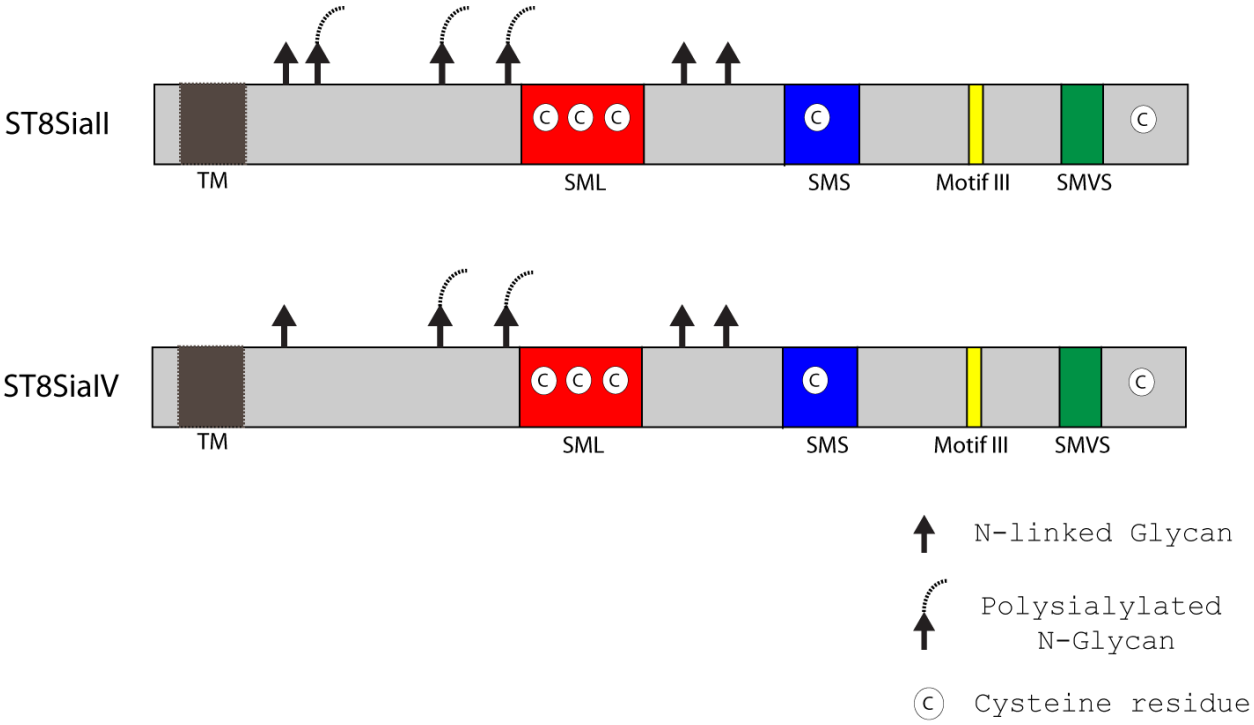


Figure 7. The two polysialyltransferses, ST8SiaII/STX and ST8SiaIV/PST. Both polysialyltransferases are type II membrane proteins localized in the Golgi. Sequences conserved in all sialyltransferases that are involved in CMP-sialic acid donor and glycan substrate recognition and catalysis are indicated: the large sialyl motif (SML) is shown in red, the small sialyl motif (SMS) in blue, the very small sialyl motif (SMVS) in green, and motif III in yellow. The trans-membrane domain (TM) is in black. Each enzyme is autopolysialylated on N-linked glycans with the major sites of polysialylation shown. Enzyme autopolysialylation is not required for substrate polysialylation.

Both of the polySTs have three conserved cysteine residues in the SML, one in the SMS and one in the C-terminus (Figure 7). Proper disulfide bonding between the three cysteines is crucial for catalytic activity (106). Proper bonding results in a disulfide bridge between the first SML cysteine and the SMS cysteine, and a disulfide bond between the second SML cysteine and the C-terminal cysteine (106). The third unpaired cysteine may be used as a reactive thiol during catalysis, as experiments in which it was mutated, showed weakened catalytic activity (106, 107).

The polyST's not only polysialylate NCAM but are themselves polysialylated on N-linked glycans (66, 108). This autopolysialylation however, is not a requirement for the initiation of enzymatic polysialylation of proteins but it does seem to enhance efficiency of the reaction (66, 109, and 110). The primary sites of N-linked polySia addition on the polySTs are the second and third N-glycans in ST8SiaIV/PST, and the second, fourth, and fifth N-glycans in ST8SIaII/STX (Figure 7) (109, 110).

Expression of the Polysialyltransferases

The genes encoding the two polySTs are located on separate chromosomes. The *ST8Sia2* gene is located at 15q26 and the *ST8Sia4* gene located at 5q21. The location of the two enzymes on separate chromosomes indicates that they diverged early in evolution (111). They do not share homology in their promoter regions implying that each gene is regulated independently of the other (112). The expression patterns of the two proteins differ as well. ST8SiaII/STX is expressed at high levels during development from embryonic day 9. After birth the levels of ST8SiaII/STX decline (113, 114). ST8SiaIV/PST is expressed at moderate levels during development, but after birth persists in specific areas of the brain that are involved in

synaptic plasticity and neuronal regeneration (114-116). In the adult animal each polyST exhibits distinct expression in specific tissues such as heart, lung and thymus (111, 113).

Synthesis of PolySia on NCAM

The major substrate for the polySTs is NCAM. PolySia is added to two N-linked glycans in the fifth Ig domain that have terminal α2, 3- or α2, 6-linked sialic acid residues (117, 118). The length of these polySia chains can exceed 50 units in mouse brain (119, 120) and extend to over 100 units in cancer cells (119). Addition of polySia does not require an initiator sialyltransferase to form the first α2, 8-sialic acid linkage (121). The polySTs do not polysialylate de-sialylated glycoproteins and will not act on glycans that do not terminate in either an α2, 3- or α2, 6-linked sialic acid residue (121, 122). Analysis of polysialylation of N-linked glycans reveals that core glycans can be di- tri- or tetra-antennary (123). The core sugars of polysialylated N-linked glycans are usually fucosylated on inner GlcNAc residues and can be modified by the HNK-1 carbohydrate structure (124-126).

Although the polySTs are expressed at different levels and in different locations, both are capable of independently polysialylating NCAM. ST8SiaII/STX shows a preference for polysialylating the N-linked glycan on the fifth glycosylation in Ig5, whereas ST8SiaIV/PST shows a slight preference for the N-glycan on the sixth N-linked glycosylation site in Ig5 (106). In *in vitro* studies ST8SiaIV/PST generated longer polySia chains than ST8SiaII/STX, however a synergistic effect was observed when both enzymes were present. Further cooperation between the polySTs was suggested when ST8SiaIV/PST was shown to be capable of elongating polySia chains initiated by ST8SiaII/STX (127).

Many of the differences between the polySTs observed in *in vitro* studies were not observed *in vivo*. Both enzymes generated polysialic chains of greater than 50 sialic acid residues. Galuska and colleagues (128, 129) showed that both polySTs efficiently added polySia to the fifth and sixth N-linked glycans in NCAM. Interestingly, *in vivo* studies showed that eliminating ST8SiaII/STX resulted in a greater loss of polySia than eliminating ST8SiaIV/PST (128, 129). The presence of a single allele of *ST8Sia2* in the absence of *ST8Sia4* resulted in 90% NCAM polysialylation. In contrast, the presence of a single allele of *ST8Sia4* in the absence of *ST8Sia2* resulted in just 30% NCAM polysialylation (128, 129). This suggests that while both enzymes are capable of polysialylating NCAM ST8SiaII/STX may be more efficient. In addition, these studies demonstrated that both polySTs generated the same length polySia chains (~55 units) in mouse brain (128, 129).

Effects of NCAM Polysialylation on Cell Adhesion

The major substrate for the two polySTs is NCAM. NCAM has six N-linked glycosylation sites (Figure 8). The primary sites of polysialylation are N-linked glycans 5 and 6 in the Ig5 domain (117). The presence of long, negatively charged, highly hydrated polySia chains on NCAM serves to block NCAM-NCAM interactions and non-NCAM interactions that promote cell adhesion (130-132). It has been demonstrated that NCAM linked polySia effectively blocks the adhesive ability of other cell surface proteins such as cadherins (131). In addition, Rutishauser and colleagues have shown that enzymatic removal of polySia from the cell surface decreases space between neighboring cells (130). PolySia acts as a global regulator of cell-cell interactions.

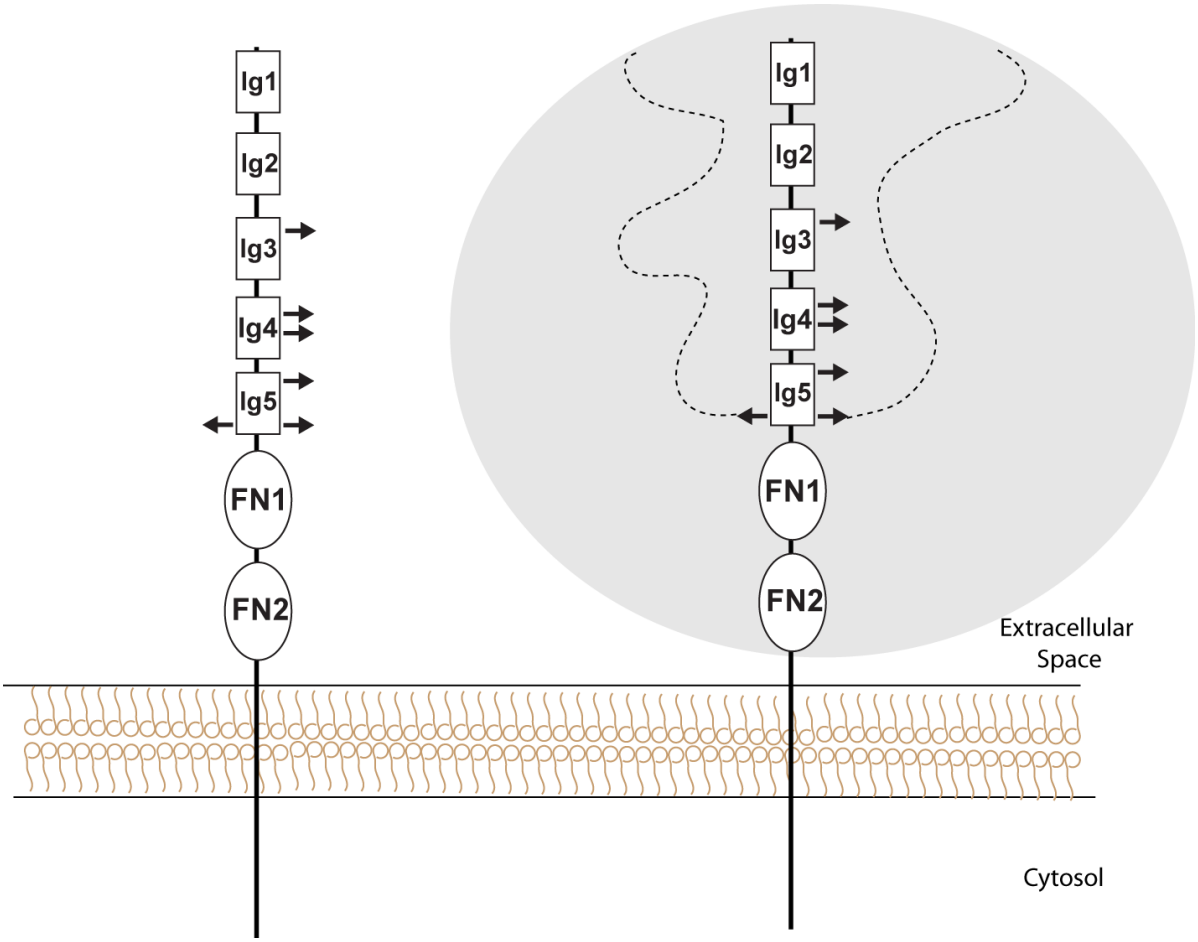


Figure 8. Polysialylated NCAM. NCAM contains six N-linked glycosylation sites marked by arrows. Sites 5 and 6 in the Ig5 domain are modified by the addition of long polysialic acid chains denoted as dashed lines. Polysialic acid is negatively charged and highly hydrated which increases the hydrodynamic radius of NCAM, denoted by the gray circle.

Effects of NCAM Polysialylation on Signaling

As discussed previously, NCAM can modulate signaling events in the cell by interaction with various binding partners. The polysialylation of NCAM that prevents cell-cell adhesion does not have the same effect on binding signaling partners. In fact removing polySia inhibits FGFR signaling and neurite outgrowth (133). A model has been suggested in which unpolysialylated NCAM forms cis homophilic interactions that exclude FGFR from binding NCAM and eliminate NCAM-stimulated FGFR signaling. Once NCAM is polysialylated, the NCAM-NCAM cis-interactions are disrupted and FGFR can now bind to NCAM in cis, which stimulates signaling and neurite outgrowth. (134). An alternative hypothesis is that the steric effects of polySia lock NCAM into positions that are favorable for FGFR binding (135). Recent results demonstrate that polySia is capable of binding a variety of neurotrophic factors. For example, the polySia on NCAM binds brain derived neurotrophic factor (BDNF) and stimulates signaling from BDNF receptors (136, 137).

Expression of Polysialylated NCAM

Polysialylated NCAM in Development

Polysialylated NCAM is most highly expressed during development coinciding with the high expression of the polySTs. Its expression is concentrated in the neural tissue, but it also shows transient expression in all three germ layers (138). The major role of polysialylated NCAM in the developing embryo is to allow for proper formation of the nervous system (139-141). Mice null for all three isoforms of NCAM were viable but had decreased brain weight and smaller olfactory bulbs (142). Further studies revealed that enzymatic removal of polySia from

NCAM on progenitor cells at the sub ventricular zone prevented these cells from migrating through the rostral stream into the olfactory bulb (143, 144), again leading to reduced olfactory bulb size. The progenitor cells lacking polySia did not migrate because they had adhered and differentiated before moving through the rostral stream (144). In normal animals once migrating cells reach their target destination polySia expression lost (141). This indicated that proper migration of cells was dependent on the expression of polySia and that it prevents contact dependent differentiation from occurring until the correct location is reached.

The presence of polysialylated NCAM on axons prevents these cells from making premature contacts and is critical in axon path finding and targeting (139-141). In the developing peripheral nervous system polysialylated NCAM expression is increased on migrating axons as these cells reach the plexus region. This reduces inappropriate axon-axon contacts mediated by the L1 cell adhesion molecule. These axons can then go on to form the correct muscle specific fascicles (145-147).

Polysialylated NCAM in the Adult Animal

Polysialylated NCAM in adult animals is mainly found in areas of the brain that retain on-going neurogenesis or require plasticity related responses. These include, the olfactory bulb, hippocampus, and hypothalamus (141, 142 and 148). NCAM knockout mice not only displayed smaller olfactory bulbs and decreased brain weight but also showed deficiencies in spatial learning and exploratory behavior (142). Synaptic plasticity is required for learning and memory in the hippocampus (149). Removing polySia from NCAM in hippocampal slice cultures

caused a decreased in long term potentiation (LTP) that impaired synaptic plasticity responses. This decrease in LTP could be restored by supplying exogenous BDNF (150).

Knockout of NCAM isoforms or enzymatic removal of polySia also affected mossy fiber axonal growth in the hippocampus (151, 152) and the ability of hippocampal progenitor cells to migrate properly (153). Showing that in adult animals, polysialylated NCAM is critical to axon path finding, cell migration and preventing premature differentiation in the hippocampus.

Control of Polysialylated NCAM Expression

The expression of the polySTs is the major factor controlling polysialylated NCAM expression, however polyST mRNA has been detected in tissues where NCAM is not polysialylated (115). In the chick ciliary ganglion, NCAM polysialylation is not controlled by changes in polyST expression, instead polySia synthesis is dependent on Ca^{2+} concentration (154). Thus, in some situations, Ca^{2+} regulated exocytosis may serve to regulate polysialylation of NCAM (155). For example, after enzymatic removal of polySia from cortical neurons and insulin secreting β cells in culture, activation of these cells resulted in re-expression of polysialylated NCAM in a Ca^{2+} dependent manner (155).

The serine/threonine kinase, protein kinase C delta (PKCδ) can induce phosphorylation of both polySTs and this phosphorylation is associated with reduced enzymatic activity (155, 156). In rats, expression of PKCδ in Golgi membrane fractions from the hippocampal dentate gyrus inversely correlates with NCAM polysialylation. Inhibition of PKCδ in these fractions stimulates polySia expression (157). In adult animals, it seems likely that polysialylated NCAM

is controlled by maintaining the polySTs in a non-functional state which can then be rapidly reactivated to allow NCAM polysialylation (157).

Phenotypes of polyST and NCAM null mice

Mice null for NCAM showed decreased brain weight and smaller olfactory bulbs but were otherwise normal (142). This came as a surprise given that NCAM is a major adhesion molecule and the carrier of polySia. Mice in which the polySTs have been deleted show why this phenotype was mild and help distinguish the role of NCAM from polysialylated NCAM.

Single Knockouts of ST8SiaII/STX or ST8SiaIV/PST

Mice null for ST8SiaIV/PST showed normal brain weight, olfactory bulb size, and hippocampal mossy fiber growth. After about six weeks of age, these mice displayed reduced polySia expression in several brain regions, with the exception of the rostral stream which showed expression of ST8SiaII/STX. Hippocampal slices of CA1 synapses showed decreased LTP. In the NCAM knockout mouse, both CA1 and CA3 synapses were impaired in synaptic plasticity, suggesting a role for both polysialylated and unpolysialylated NCAM in this process (158).

ST8SiaII/STX knockout mice also were viable. Despite being the primary polyST expressed during development, body and brain weight was relatively normal (159). Expression of polySia was reduced in the olfactory bulb, cerebral cortex, and dentate gyrus of the hippocampus. PolySia expression persisted in the rostral stream indicating an overlap of expression of both polySTs in this region. Mossy fibers in the hippocampus displayed altered axonal targeting in these knockout mice, resulting in a reduced fear response and increased exploratory drive (159).

Double Knockout of ST8SiaII/STX and ST8SiaIV/PST

Animals with both polySTs deleted were born at Mendalian ratios, but displayed growth retardation, failed to thrive, and few survived longer than four weeks (160). These mice possessed smaller olfactory bulbs and altered mossy fiber lamination seen in the NCAM knockout mice. In addition to these defects, the double knockout mice displayed hydrocephalus and brain wiring defects (160). Specific defects included abnormal forebrain organization, increased platelet derived growth factor (PDGF) induced glial cell differentiation, and decreased neuronal migration (161). This caused both neuronal and glial cells to be incorrectly positioned during formation of the cortex. These results confirmed the role of polySia in controlling migration and correct differentiation of neurons during development and its importance in nervous system development.

Triple Knockout of NCAM and the PolySTs

Deletion of the two polySTs and their substrate NCAM rescued the lethal phenotype seen in the double knockout animals (160). The triple knockout showed a phenotype similar to the NCAM knockout mouse. This indicated that smaller olfactory bulb and aberrant mossy fiber lamination are defects caused by the lack of polySia independent of NCAM, and highlight the role of polySia as a global regulator of cell adhesion and the importance of temporally regulating NCAM-mediated adhesion during development. The severe defects seen in the double polyST knockout animal is likely caused by an unregulated NCAM adhesive function that would not occur in the presence of polySia. It is conceivable that improper adhesion events

and changes in cell signaling could play a role in the defects seen in the polyST null mice. In sum, the analysis of polyST and NCAM null animals demonstrates that polySia regulates the interactions of NCAM and other cell adhesion molecules to insure that they take place in the appropriate place and time.

Polysialic Acid and Disease

In adult animals, ongoing expression of polySia is confined to select areas in the brain including the hippocampus and hypothalamus (140, 141). Disruption of polySia expression in these areas can lead to neuropsychiatric and neurodegenerative disorders. Expression of polySia is observed on cancer cells and promotes cancer cell growth and metastasis.

Polysialic acid and Tumor Metastasis

PolySia has been found to be re-expressed in small cell lung carcinomas (SCLC's) and could be used as a marker to distinguish SCLC's from other lung tumors (162). Tumors investigated in a study by Scheidigger et al. (163) demonstrated that high levels of polysialylated NCAM expression correlated with increased incidence of metastasis in nude mice *in vivo* and reduced cell aggregation *in vitro*. Additional work showed that ST8SiaII/STX was the polyST responsible for the NCAM polysialylation in SCLC (58). In mice, polysialylated NCAM was been shown to increase the metastasis on non-small cell lung carcinoma (NSCLC) (164), and ST8SiaII/STX expression was again correlated with tumor progression (165).

A variety of other cancers express high levels of polysialylated NCAM (166). Wilms' tumor is a malignant kidney tumor usually found in children, which displays features of

embryonic kidney cells. Developing kidneys express polysialylated acid and it is re-expressed in both the primary tumor and in metastasized cells (167, 168). Neuroblastomas are tumors of neuroectodermal origin that also display high levels of polySia. PolySia chains of over 100 residues were found attached to NCAM on human neuroblastoma cells (119). Both polySTs were found to be expressed in cultured tumor cells and from tumor biopsy cells (169). It was shown that polySia on neuroblastoma tumor cells inhibited MAPK activation by decreasing heterophilic NCAM signaling, and that this decreased MAPK activation promoted tumor progression (170).

Polysialic acid and Neuropsychiatric Disorders

Schizophrenia affects 1% of the world population and symptoms include social difficulties, cognitive dysfunction, delusions, and auditory hallucinations (171). The disease usually begins in adolescence or early adulthood. The neurodevelopmental hypothesis of schizophrenia proposes that normal maturation and connectivity of neurons is disrupted during development and that in turn leads to the activation of pathological neural circuits during early adulthood (172). Polysialylated NCAM expression was evaluated in the postmortem brains of schizophrenic individuals (173). The analysis revealed a reduction of polysialylated NCAM in the hilus region of the hippocampus in a majority of cases. Total NCAM levels were unaffected (173). The younger individuals showed a more dramatic decrease of polysialylated NCAM compared to normal individuals. Whether this reduced polysialylation of NCAM is important to the pathology of the disease or is a consequence of it is not yet known (173).

Hildebrandt et al. used different allelic combinations of *ST8Sia2 ST8Sia4* and *NCAM* in postnatal day one mice and demonstrated a correlation between increased levels of unpolysialylated NCAM and defects in brain connectivity (174). The defects observed in the mouse brain were reminiscent of those seen in schizophrenic patients. A single copy of *ST8Sia2* allele was able to prevent these defects in mice. These studies suggest improper expression of unpolysialylated NCAM may lead to a predisposition to schizophrenia (174).

Mechanism of Protein-Specific NCAM Polysialylation

Prior work in the Colley laboratory focused on determining what NCAM domains are critical for polysialylation. Removing the first four Ig domains from the extracellular portion of NCAM showed no effect on the polysialylation (Figure 9, NCAM3). Deletion of the second FN III repeat also had no effect on the polysialylation of NCAM (Figure 9, NCAM2) (175). The minimal deletion protein that exhibited polysialylation comparable to wild type NCAM, was NCAM4, a protein consisting of the cytosolic tail, transmembrane region, Ig5 and FN1 domain (Figure 9). NCAM5 consisting of the cytosolic tail, transmembrane region and Ig5 was not polysialylated (Figure 9) (175). Surprisingly, NCAM7 that consisted of the cytoplasmic tail, transmembrane region, FN1 and FN2 and lacking Ig5, was polysialylated at lower levels on O-glycans in the FN1 domain, while NCAM6 that lacked both FN1 and FN2 was polysialylated at very low levels (Figure 9) (175). These results suggested that the FN1 domain was required for the efficient polysialylation of NCAM Ig5 N-glycans.

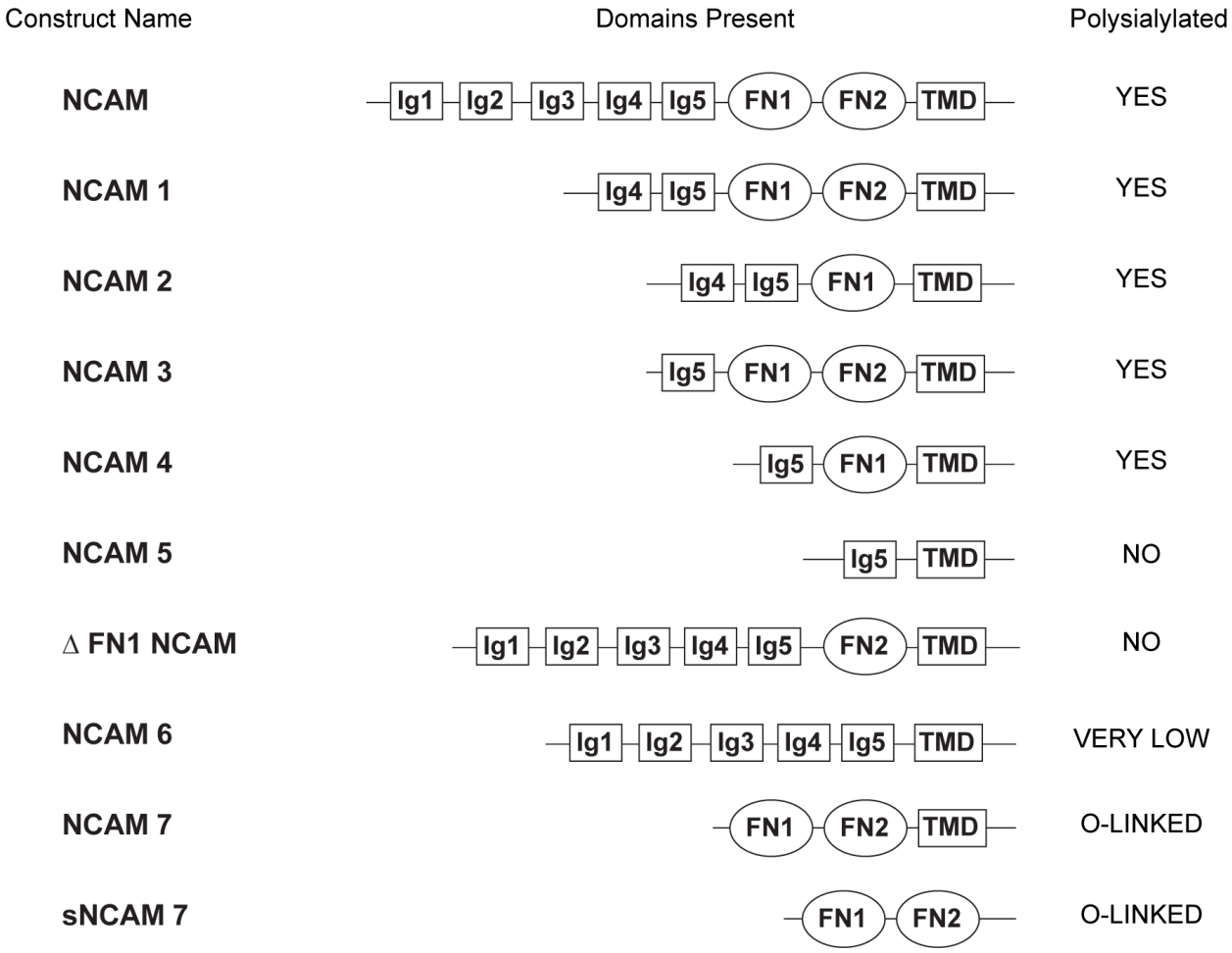


Figure 9. NCAM domain deletion mutants and their ability to be polysialylated. Removing the first four Ig domains showed no effect on the ability of NCAM to be polysialylated. The NCAM4 protein that possesses the Ig5 and FN1 domains was polysialylated, but the NCAM5 protein that possessed Ig5 but lacked FN1 was not. Deletion of the FN1 domain caused a loss of polysialylation (ΔFN1). The NCAM6 protein that lacked the FN1 and FN2 showed a very small amount of polysialylation, while the NCAM7 protein that lacked all the Ig domains and possessed on FN1 and FN2 was polysialylated on O-glycans in the FN1 domain. sNCAM 7 has the same domains as NCAM 7 but is missing the cytoplasmic tail and transmembrane region. The sNCAM7 protein is not membrane bound and is secreted into the media.

Shalu Shiv Mendiratta created an NCAM protein that lacked the FN1 domain (NCAM ΔFN1) (Figure 9). The NCAM ΔFN1 protein could not be polysialylated by either ST8Sia II/STX or ST8SiaIV/PST (176). This led to the hypothesis that the FN1 domain was necessary for the polysialylation of N-glycans in the Ig5 domain, perhaps by providing a docking site for the polyST's as illustrated in Figure 10. In collaboration with Arnon Lavie's laboratory, the Colley lab obtained the crystal structure of NCAM FN1 using a molecular replacement method that modeled this domain on the NMR structures of the Robo2 fibronectin type III repeat (176). This structure revealed two unique features of the FN1 domain, an acidic surface patch and a novel α–helix comprised of residues 551-558 connecting strands 4 and 5 of the β sandwich structure that was not found in any other fibronectin type III repeat (Figure 11) (176, 177).

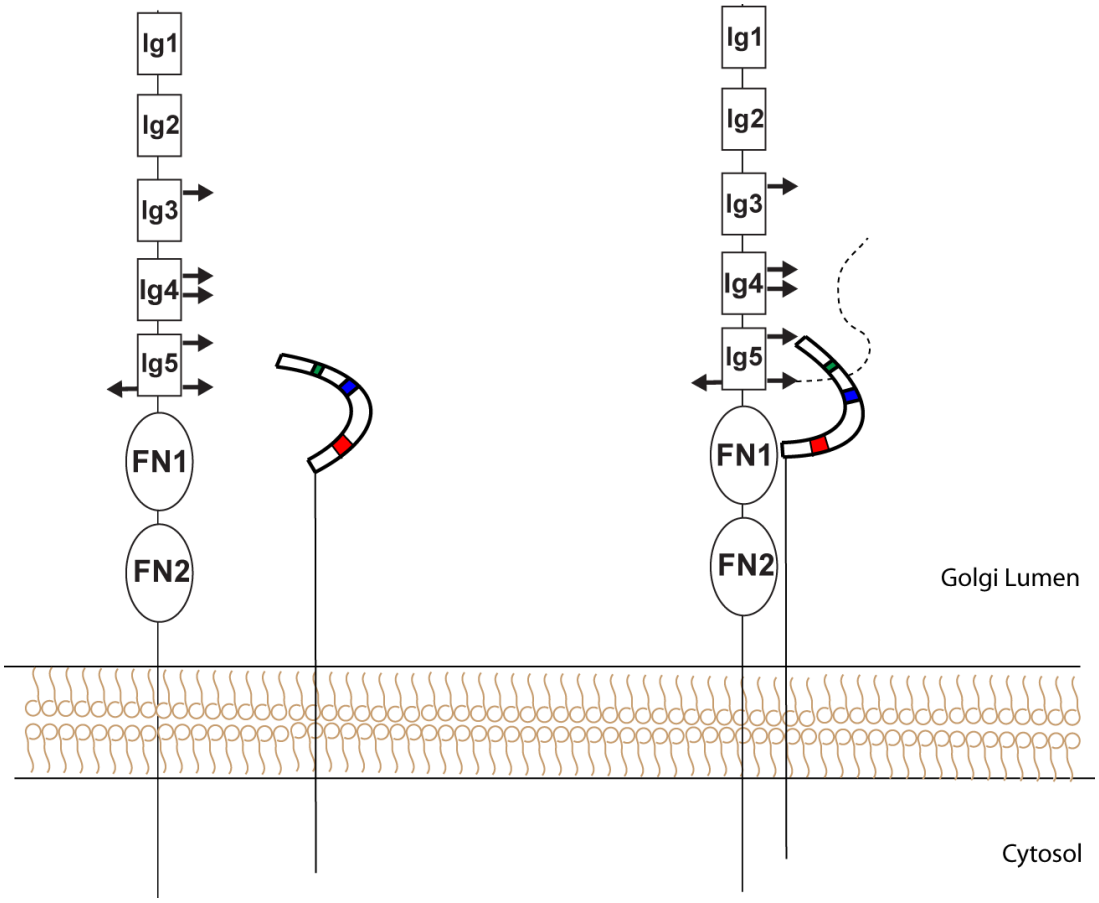


Figure 10. Model of NCAM-polyST interaction. In this early model, the polyST binds to sequences in NCAM FN1 and is positioned to polysialylate N-linked glycans in Ig5.

A later collaboration between Deirdre Foley and Arnon Lavie resulted in the crystal structure of the NCAM Ig5-FN1 tandem (178). The gap bridged by the α-helix was replaced by two threonine residues that bridge the same strands in Robo2. This Δhelix-TT mutation did not affect NCAM expression and still allowed NCAM polysialylation, however the polySia had been added to O-linked glycans (179). This O-linked polysialylation was found to be on threonine residues in FN1 (179). This shift in polysialylation may have been caused by a change in positioning of the polyST that places N-linked target glycans out of reach of the polyST, which then polysialylates O-linked glycans in FN1 that are within its range.

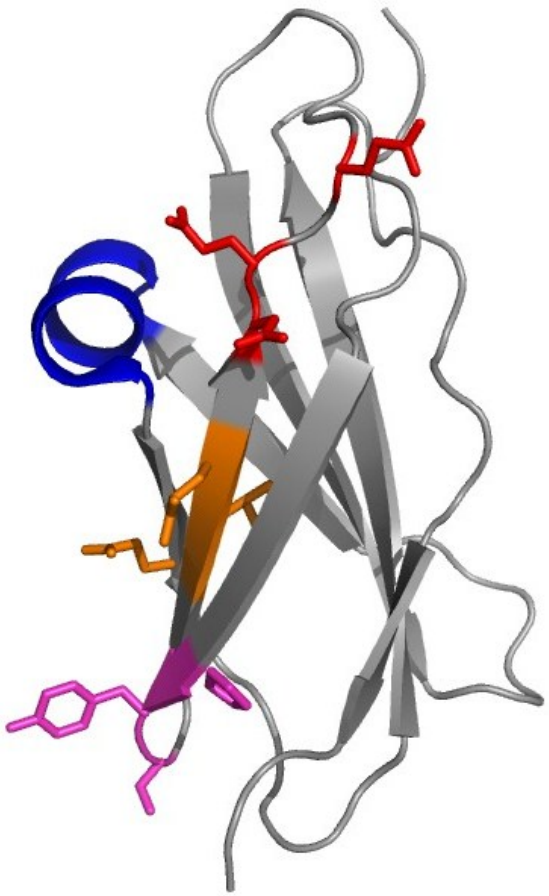


Figure 11. The structure of the NCAM FN1 domain. Sequences targeted for mutation are highlighted with side chains displayed. The surface acidic patch (Asp^{520}, Glu^{521}, Glu^{523}) is highlighted in red. The α helix is highlighted in blue. The QVQ (Gln^{516}, Val^{517}, Gln^{518}) sequence is in orange and the PYS (Pro^{510}, Tyr^{511}, Ser^{512}) sequence is in magenta.

The surface acidic patch in NCAM FN1 is comprised of four residues; Glu^{506}, Asp^{520}, Glu^{521} and Glu^{523}. Mutation of Asp^{520}, Glu^{521} and Glu^{523} individually to alanine had little effect on polysialylation, however simultaneous mutation of these residues to alanine led to a reduction in polysialylation (see Figure 11 for acidic patch location) (176). When the same three residues were mutated to arginine, a more drastic change, polysialylation was nearly eliminated (176). This indicated that this site may be working in conjunction with other residues to promote the polysialylation of NCAM and that the more drastic arginine replacement was required to disrupt contributions from other residues.

Two other, three amino acid sequences in NCAM FN1, Pro^{510}, Tyr^{511}, Ser^{512} (PYS) and Glu^{516}, Val^{517}, Glu^{518} (QVQ), when mutated, had distinct effects on NCAM polysialylation. Mutation of the QVQ sequence caused a switch of polysialylation from N-linked glycans to O-linked glycans, similar to replacement of the α-helix. Mutation of the PYS sequence caused a slight decrease in polysialylation of wild type NCAM (179).

Significance and Summary

Polysialylated NCAM has been shown to play crucial roles in development, neural regeneration and learning. It also has detrimental effects when incorrectly expressed in tumor cells and a decrease in NCAM polysialylation during development may lead to neuropsychiatric disorders later in life. Many of these effects require NCAM to display polySia in the correct place and time yet it is not entirely clear how the polySTs recognize NCAM to specifically add polySia. Prior work in our lab has indicated that NCAM FN1 is necessary for NCAM polysialylation,. Previous lab members found an acidic patch on the surface of FN1 is important

for polysialylation, but their work suggested that other sequences are also involved. In my thesis work, I created mutant and chimeric proteins and analyzed their polysialylation and polyST binding to determine the requirements for polyST recognition and binding to NCAM. I established that full length NCAM binds to the polyST and that the FN1 domain is necessary for binding and that the acidic patch plays a role in polyST-NCAM interaction (Chapter III). I demonstrated that sequences common to NCAM and OCAM FN1 domains that are found at the Ig5-FN1 interface are critical for NCAM polysialylation. Two of these sequences are likely required to maintain the Ig5-FN1 relationship, while the third is part of a surface that includes the acidic patch residues and plays a role in polyST binding (Chapter IV). In collaboration with Deirdre Foley, I found that specific residues in the NCAM Ig5 domain are required for NCAM polysialylation, and I demonstrated that these residues are involved in polyST-NCAM binding (Chapter V). This finding changed our model of polyST-NCAM interaction from one where the polyST interacts solely with the FN1 sequences to modify Ig5 N-glycans, to a new model where the polyST makes protein-protein contacts with both the Ig5 and the FN1 domains. The ultimate goal is to precisely define the sequences involved in polyST-NCAM interaction so that approaches can be devised to either enhance or eliminate this recognition event in order to contribute to therapies to mitigate tumor invasiveness (180) and re-activate NCAM polysialylation in nervous system repair (181, 182).

Chapter II

Materials and Methods

Acquisition of Reagents

The cDNA for human NCAM 140 was a gift from Dr. Nancy Kedersha (Brigham and Women's Hospital, Boston MA). The cDNAs for ST8SiaII and ST8SiaIV were gifts from Dr. John Lowe (Genentech, South San Francisco, CA) and Dr. Minoru Fukuda (Sanford Burnham Medical Research Institute, La Jolla, CA), respectively. Phage PK1E endo-N-acylneuraminadase (Endo N) cloned into the pEndo-N-expression plasmid was a gift from Dr. Eric Vimr (University of Illinois, Urbana, IL). The cDNA for mouse OCAM was obtained from Dr. Yoshihiro Yoshihara (RIKEN Brain Science Institute, Wako, Japan).

Reagents used in tissue culture, polymerase chain reaction experiments, and Western blotting experiments were purchased from Invitrogen (Carlsbad, CA). These reagents include: Dulbecco's modified Eagle's medium (DMEM), F-12 medium, Opti-MEM I, Lipofectin, lipofectamine, fetal bovine serum (FBS), oligonucleotides, PCR supermix, primers for mutagenesis and construct design, and anti-V5 antibody. The Quickchange™ site directed mutagenesis kit as well as *Pfu* polymerase were purchased from Stratagene (La Jolla CA). Kits for purifying DNA were purchased from Qiagen (Venlo, Netherlands). Protein-A Sepharose used in immunoprecipitation experiments was purchased from GE Healthcare (Waukesha, WI). Restriction enzymes, T4 DNA ligase, and peptide-N-glycosidase F (PNGase F) were purchased from New England Biolabs (Ipswich, MA). Polyclonal anti-myc epitope tag antibodies were purchased from Abcam Inc. (Cambridge, United Kingdom). Supplies for SDS PAGE, including

Precision Plus Protein™ standards and Mini Protean™ precast gels were purchased from Bio-Rad (Hercules, CA). Nitrocellulose membranes were purchased from Schleicher and Schuell (Keene, NH). Horseradish peroxidase (HRP)-conjugated- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies used in immunoblotting and immunofluorescence visualization were purchased from Jackson Immunoresearch (West Grove, PA). Super Signal West Pico chemiluminescence reagent was purchased from Pierce (Rockford, IL). ^{35}S-Express Protein Labeling mix was purchased from PerkinElmer Life Sciences (Boston, MA). Other chemicals and reagents used in experiments and to make buffers and reagents were purchased from Sigma Aldrich (St. Louis, MO) and Fisher Scientific (Hampton, NH).

Experimental Methods

Construction of Truncated NCAM Proteins

The construction of several truncated, soluble and membrane-associated NCAM proteins was described in (175). Several of these constructs were used in this work, including soluble NCAM (sNCAM) lacking the transmembrane region (TM) and cytosolic tail, NCAM3 that consists of Ig5-FN1-FN2-TM-tail, NCAM4 that consists of Ig5-FN1-TM-tail, and NCAM7 that consists of FN1-FN2-TM-tail. Soluble forms of NCAM4 and NCAM7 which lack the TM and cytosolic tail were also used.

Construction of Soluble ST8SiaIV/PST

Soluble ST8SiaIV-myc (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 (175) using primers 1 and 2 (Table 3). This

DNA was digested with HindIII and EcoRV and ligated into pcDNA3.1/Myc-HisB vector with a stop codon inserted before the His_6 tag to generate an expression vector including the signal peptide at the amino terminus and myc tag at the carboxyl terminus but no His_6 tag. The signal peptide sequence was introduced into the ST8SiaIV/PST Δtail and ΔTM template using primers 4 and 5 (Table 3). This fragment was digested with EcoRV and Xba1 and ligated into the modified signal peptide/myc tag vector.

Table III Primers used in construction of soluble NCAM and soluble ST8SiaIV/PST.

Primer	Primer name	Direction	Template	Primer sequence
1	Sp-NCAM signal peptide	5’-3’	NCAM 2	AAAAAAGATATCCAGGAGTCCTTCGAATTC
2	Sp-NCAM signal peptide	3’-5’	NCAM 2	AAAAAATCTAGACCGGTGCTCAGCCGTGAG
3	Sp-PST signal peptide	5’-3’	ΔTM PST	AAAACGTACCGCTAGCTTGCTTGTTC
4	Sp-PST signal peptide	3’-5’	ΔTM PST	AAAAGATATCGGATCCTCTAGAGTCAACG

Site Directed Mutagenesis

Site directed mutagenesis was performed PST using the Qiagen QuikChange™ mutagenesis kit, and was used to delete, add or change single or multiple amino acids in NCAM, OCAM and ST8SiaIV/PST. Primers for individual mutants are listed in Table 3. The basic protocol involves the addition of 5μl of 10X reaction buffer to 22μl of sterile water. Ten microliters of both the forward and reverse primer at a concentration of 1μg/ul are added to this mixture to yield a total reaction volume of 47μl. One microliter of a 10mM dNTP mix (2.5 mM concentration for each of the 4 nucleotides) is added, as well as 1μl of template DNA at a concentration of 30ng/ul. Finally 1μl (2.5 units) of *Pfu* polymerase is added to give a final reaction volume of 50μl. Reactions were carried out in a Geneamp™ thermocycler using the following conditions: Denaturation at 95^{o}C for 30 seconds, followed by 16 cycles consisting of, 30 seconds denaturation at 95^{o}C, annealing for 45 seconds at 56^{o}C, and elongation at 68^{o}C for 12 min. A final step at 68^{o}C for seven minutes is conducted to make sure all DNA is elongated properly. After the PCR reaction, 1μl (10 units) of Dpn I is added to each sample and the sample is incubated at 37^{o}C to allow enzyme to digest methylated and hemi-methylated template DNA to select for newly synthesized mutant DNA. PCR products are then transformed into competent *Escherichia coli* XL1-Blue cells as described below.

Transformation of Competent Cells

Escherichia coli XL1-Blue cells that are suspended in TSS buffer and previously frozen are thawed on ice. Once thawed, 10μl of the Dpn I digested PCR product is added to the cells and incubated on ice for 15 min. This is followed by 45 second incubation in a 42^{o}C water bath,

after which the cells are placed on ice for 5 min. Three hundred microliters of sterile LB media is added to the cells, and they are incubated at 37^{o}C for 1-2 h. Cells are pelleted at 5000 rpm for 5 min re-suspended in 50μl of LB media, and spread on LB agarose plates containing 100 ug/ml Ampicillin (LB-Amp plates) to select for cells that have incorporated the pcDNA 3.1 plasmid carrying the mutation of interest. The pcDNA3.1/Myc-HisB vector contains an Ampicillin resistance gene conveying Ampicillin resistance to cells that are transformed. Plates are incubated overnight at 37^{o}C to allow colonies to grow, selected colonies are chosen for small-scale plasmid DNA purification.

Small-Scale Plasmid DNA Preparation

Bacterial colonies that grew on the LB-Amp plates are transferred to 3ml of LB-Amp liquid media using sterilized wooden sticks. Cultures are grown at 37^{o}C with shaking (255 rpm) for 12-16 h. Prior to purification, 1 μl of each culture is spotted on an LB-Amp reference plate for future large-scale DNA purification. Two microliters of culture are transferred to a micro centrifuge tube and centrifuged at 5000 rpm for 5 min to pellet the cells. The supernatant is discarded and cells are then re-suspended and purified using the Qiagen Qiaprep Spin Miniprep Kit according to the manufacturer's instructions. Once purified, all mutant DNAs are sent to the University of Illinois at Chicago Research Resource Center for sequencing. Mutant replicates are selected from the reference plate and used to inoculate 400 ml of LB-Amp for large scale purification.

Large-Scale Plasmid DNA Preparation

Four hundred milliliters of LB-Amp media are inoculated with the appropriate colony from the reference plate, as described above. The 400 ml culture is grown overnight at 37°C with shaking (255 rpm). Plasmid DNA is purified using the Qiagen Qiaprep Spin Maxiprep Kit according to the manufacturer's instructions. The resulting DNA pellet is re-suspended in 300-600 μl sterile DNAse free water. The DNA concentration is determined by measuring absorbance at λ 260nm, and the DNA is stored at -20°C.

Transfection of Cells for Immunofluorescence Microscopy

Twelve millimeter glass coverslips are placed into a 24 well plate. COS-1 cells grown in DMEM supplemented with 10% FBS or Lec2 CHO cells grown in F-12 media supplemented with 10% FBS are plated on the coverslips. Cells are grown until 50-75% confluent, at which point 300μl of transfection mixture is added to each well. The transfection mixture is prepared as follows: 0.5μg plasmid DNA is diluted with 150μl of serum free Opti-MEM I. In a separate tube, 3μl of Lipofectin is diluted with 150 μl of serum free Opti-MEM I. After incubation for 30 min at room temperature, the two tubes are combined, gently mixed, and allowed to incubate for an additional 15 min. Growth media is removed from the cells, each coverslip is washed with 1 ml of serum free Opti-MEM I, and the transfection mixture is added. Cells are then placed back in the 37°C incubator at 5% CO_2 for 6 h, One milliliter of growth media (DMEM or F-12, 10% FBS) is added to each coverslip and expression is allowed to continue in the 37°C incubator at 5% CO_2 for 16-18 h.

Transfection of Cells for Biochemical Studies

COS-1 or Lec 2 CHO cells are grown on 100 mm plates in the appropriate media until 50-75% confluent for COS-1 cells or 90% confluent for Lec2 cells. Cells are then incubated with 3 ml of transfection mixture made as follows: 20 μg of plasmid DNA is incubated with 1.5 ml of Opti-MEM I, and in a separate tube 30 μl of Lipofectin (for COS-1 cells) or 20 μl of Lipofectamine (for Lec2 CHO cells) is incubated with 1.5 ml of Opti-MEM I. The two mixtures are allowed to incubate for 30 min at room temperature after which time they are combined, gently mixed, and incubated for another 15 min. Growth media is removed from cells growing on 100 mm tissue culture plates, and the cells washed with 10ml Opti-MEM I. Three milliliters of transfection mixture is then added to each plate, and cells are incubated at 37°C and 5% CO_2 for 6 h. Following this incubation, 7 ml of the appropriate growth media (DMEM or F-12, 10% FBS) is added and the cells were allowed to grow 16-18 h before harvesting.

Analysis of NCAM and OCAM Proteins by Indirect Immunofluorescence Microscopy

To insure that mutant proteins fold and traffic correctly, NCAM, OCAM, mutant and chimeric proteins tagged with the V5 epitope were expressed in COS-1 cells and these proteins localized by indirect immunofluorescence microscopy. To examine the effects of mutations on substrate polysialylation, the altered, V5-tagged protein coding sequence is co-transfected with a myc-tagged mutant form of ST8SiaIV/PST, PST Mut 2.3. This mutant is missing the second and third glycosylation sites and can no longer be autopolysialylated but is still capable of polysialylating substrates (66). Co-expression with this mutant enzyme allows substrate polysialylation to be examined by immunofluorescence microscopy with anti-polySia

antibodies, in the absence of enzyme autopolysialylation. Cells are plated in coverslips and transfected as described above. After removing the growth media and washing each coverslip twice with 1 ml PBS, cells are fixed and permeabilized using 1 ml of -20^{o}C methanol for 5 min. This allows the visualization of both internal structures and the cell surface. Cells are then incubated with 1 ml 5% goat serum in PBS for 1 h at room temperature to block nonspecific interactions. For protein localization, cells are next incubated for 1 h with anti-V5 epitope tag antibody diluted 1:250 in 5% goat serum, PBS. For analysis of substrate polysialylation, cells are then incubated for 1 h at room temperature in the OL.28 anti-polySia antibody diluted 1:100 in 5% goat serum, PBS. Coverslips are then washed 4 times with 1 ml PBS for 5 min. Cells are next incubated for 45 min at room temperature with secondary antibodies diluted 1:100 in 5% goat serum, PBS. FITC-conjugated goat anti-mouse IgG was used as a secondary antibody for detecting the mouse anti-V5 epitope tag antibody, and FITC-conjugated goat anti-mouse IgM was used as the secondary antibody to detect the OL.28 anti-polySia monoclonal antibody. Coverslips are then washed four times with PBS with 5 min incubations between washes. Coverslips are then rinsed in sterile deionized water and then blotted to remove excess water without damaging fixed cells. Coverslips are then mounted on glass slides using 20 μl of mounting media (15% w/v Vinol 205 polyvinyl alcohol, 33% w/v glycerol, 0.1% azide in PBS at pH 8.5). Mounting media is allowed to dry overnight, and cells are visualized using a Nikon Axiophot microscope equipped with epifluorescence illumination and a 60X oil immersion Plan Apochromatic objective. Pictures are taken using a SPOT RT color digital camera and processed using SPOT RT software version 3.5.1 (Diagnostic Instruments Inc., Sterling Heights, MI).

Analysis of NCAM and OCAM Protein Polysialylation

COS-1 cells plated on 100 mm tissue culture plates expressing NCAM, OCAM, their mutants and chimeras and ST8Sia IV/PST are washed with 10 ml PBS and lysed in 1 ml of immunoprecipitation buffer (50 mM Tris-HCL, pH 7.5, 150mM NaCl, 5mM EDTA, 0.5% Nonidet P-40, 0.1% SDS). Cell lysates are then frozen on dry ice and then thawed at room temperature or kept at -20^{o}C overnight. Thawed lysate is passed through a 25 gauge needle five times to shear large membrane fragments, and debris is pelleted by centrifugation at 13, 000 rpm for 5 min. Fifty microliters of the supernatant is reserved to evaluate protein expression, and the remaining supernatant is used for immunoprecipitation. In preparation for immunoprecipitation, the cell lysate supernatant is incubated with 50 μl of a protein-A Sepherose bead slurry (50% in PBS) for 1 h at 4^{o}C with end over end rotation. Beads and non-specifically binding proteins are pelleted and the remaining supernatant is transferred to a new tube and 1 μl of anti-V5 epitope tag antibody added and incubation continued with end-over-end rotation for 2h at 4^{o}C. Following this incubation, 35 μl of the protein-A Sepharose slurry is added and the incubation continued for 1 h at 4^{o}C. Beads and associated immune complexes are pelleted, supernatant removed and the beads washed four times with 250 μl of immunoprecipitation buffer. To remove immune complexes from the beads, beads are suspected in 25 μl of Laemmli sample buffer containing 5% β-mercaptoethanol, and heated to 65^{o}C for 8-10 min. Proteins are then separated on a 4-15% gradient SDS polyacrylamide gel. Twenty five microliters of Laemmli sample buffer are added to the 50 μl aliquot removed before immunoprecipitation, the sample is heated to 100^{o}C for 10 min (removes polySia) and

subjected to immunoblotting, as described below, to evaluate relative substrate protein expression.

Immunoblot Analysis of NCAM and OCAM Proteins

Following SDS PAGE, proteins are transferred to nitrocellulose membranes at 500 mA for 2.5 h in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes are then blocked for 1 h at room temperature in blocking buffer (5% nonfat powdered milk in Tris buffered saline, pH 8.0, 0.1% Tween 20). To visualize polySia, membranes are incubated overnight in a 1:200 dilution of OL.28 anti-polySia antibody in 2% nonfat dry milk in Tris buffered saline, pH 8.0, followed by two 15 min washes in TBS (Tris buffered saline, pH 8.0 with 0.1% Tween). Membranes are then incubated in a 1:4000 dilution of HRP-conjugated goat anti-mouse IgM in blocking buffer. To compare relative expression of substrate proteins, membranes are incubated overnight in blocking buffer with a 1:10,000 dilution of anti-V5 epitope antibody. After two 15 min washes in TBS, the membranes are incubated for 1 h in a 1:4000 dilution of HRP-conjugated goat anti-mouse IgG in blocking buffer. Finally, membranes are washed 4 times in Tris buffered saline, pH 8.0, 0.1% Tween 20, and visualized using the SuperSignal West Pico chemiluminescence kit and Bioexpress Blue Ultra Autorad film.

Evaluation of the N-glycosylation, Sialylation and Polysialylation of NCAM and OCAM Proteins

Endo- and exoglycosidase digestion is used to detect the presence of N-linked glycans and the type of glycan present on a protein. Peptide N-glycosidase F (PNGase F) selectively removes N-linked glycans (high mannose, hybrid and complex) from proteins (183). To evaluate the general N-glycosylation of a protein, one of a duplicate pair of immunoprecipitated samples is washed and resuspended in 77 μl sterile H_2O, 10 μl Nonidet P-40, 10 μl G7 buffer (0.5 M sodium phosphate, pH 7.5) with 3 μl (1500 units) of PNGase F and incubated with agitation at 37^{o}C overnight. Following glycosidase digestion, Laemmli sample buffer is added to treated and untreated samples, heated at either 65°C (to retain polySia) or 100°C for 8-10 min, and proteins are separated on SDS polyacrylamide gels, as described above.

Co-immunoprecipitation of NCAM and OCAM Proteins with ST8SiaIV/PST

To evaluate the interaction between NCAM or OCAM proteins with ST8SiaIV/PST, Lec2 CHO cells maintained in F-12, 10% FBS are co-transfected with a V5-tagged substrate construct and with either ST8SiaIV/PST-myc construct or an empty pcDNA3.1 Myc/HisB vector. Expressing cells are washed with 10 ml of PBS, scraped off plates in 1 ml lysis buffer (50 mm Hepes, 100 mm NaCl, 1% Triton X-100, pH 7.2), and incubated for 15 min on ice to allow lysis. Debris is removed by centrifugation and 500 μl of the supernatant is transferred to new microcentrifuge tubes. ST8SiaIV/PST is immunoprecipitated from cell lysates using 1.0 μl of anti-myc antibody. After 2 h of rotation at 4 °C, 35 μl of protein A-Sepharose (50% suspension in PBS) is added to each sample and rotated at 4 °C for 1 h. The protein A-Sepharose beads and

associated immune complexes are pelleted and washed 4 times with 250 μL of cold cell lysis buffer. Samples are resuspended in Laemmli sample buffer containing 5% β-mercaptoethanol and heated to 100 °C for 10 min. Precipitated proteins are separated on 4-15% gradient SDS-polyacrylamide gel and then subjected to immunoblotting as described above. To evaluate relative NCAM or OCAM protein expression levels, a 50 μl aliquot of cell lysate is removed before immunoprecipitation, and 25 μl of Laemmli sample buffer, 5% β-mercaptoethanol is added. Samples are then heated at 100 °C for 10 min, separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting, as described above. To quantify changes in polyST binding to NCAM mutants, OCAM or related chimeras, I used NIH ImageJ software and compared the ratio of precipitated to expression control for the mutants/chimeras *versus* wild type NCAM (set to 100%). In the event that nonspecific binding was detected (in samples without co-expressed ST8SiaIV/PST), this was subtracted from the final value.

Chapter III

The FN1 domain of NCAM is required for its recognition and polysialylation

Introduction

Addition of polySia chains to a protein is an uncommon modification occurring on only a small group of proteins. NCAM is the most abundant polysialylated protein of this group. We hypothesized that due to the rarity of this modification; the polyST's specifically recognize and form an initial protein-protein interaction with their substrates including NCAM. This is different from most glycosylation enzymes that show no preference for the glycoproteins they modify, and specifically recognize and modify the appropriate glycan substrates regardless of the protein carrier.

Previous work in the Colley laboratory evaluated which NCAM domains are required for NCAM recognition and polysialylation by the polySTs. They found that that a truncated protein consisting of Ig5-FN1-TM-tail (NCAM4) was polysialylated like wild type NCAM when co-expressed with the polySTs, and that deleting the FN1 domain from the full length NCAM protein (ΔFN1) eliminated its polysialylation (176). Interestingly, a protein consisting of just FN1-FN2-TM-tail (NCAM7) that possesses no N-glycans, was polysialylated on O-glycans. These results implied that the FN1 domain of NCAM was required for the polysialylation of two N-glycans on the adjacent Ig5 domain, and in the absence of Ig5, its own O-glycans (175, 176). Additional work showed that an acidic surface patch on FN1 comprised of Asp^{509}, Asp^{520}, Glu^{521},

Glu^{523} plays a role in NCAM polysialylation and that a unique α-helix in FN1 was required for the polysialylation of Ig5 N-glycans; in its absence, NCAM was polysialylated on FN1 O-glycans. Replacing two other sequences in NCAM FN1, Pro^{510}, Tyr^{511}, Ser^{512} (PYS) and Gln^{516}, Val^{517}, Gln^{518} (QVQ), also lead to changes in NCAM polysialylation. Replacing the QVQ sequence with alanine residues caused a switch of polysialylation from N-linked glycans to O-linked glycans, similar to replacement of the α-helix, while replacing the PYS sequence with alanines caused a slight decrease in polysialylation of wild type NCAM (177).

At this point, several sequences in NCAM FN1 had been shown to play roles in polysialylation, however it was not known whether the FN1 domain itself, and these FN1 sequences in particular, mediated direct polyST interaction or impacted polysialylation in another way. In addition, the polysialylation of NCAM persisted at lower levels even when all of these sequences were replaced, suggesting that other sequences might also be involved. In this chapter, I report my studies to determine how replacing these FN1 sequences impacts polyST-NCAM interactions.

Results

To explore whether the FN1 domain and specific sequences in this domain are critical for polyST interaction/recognition I took a co-immunoprecipitation approach. I used Lec 2 CHO cells to transiently co-express differentially tagged polySTs and NCAM proteins for these studies. Lec2 CHO cells have a mutation in the CMP-sialic acid transporter that prevents CMP-sialic acid from being imported to the Golgi where it is used as a donor by all sialyltransferases, including the polySTs (184). Consequently, Lec2 CHO cells possess no sialylated or

polysialylated glycans. I chose this cell line to control for the possibility that the presence of polySia chains on NCAM may attenuate the polyST-substrate interaction.

The first step was to use NCAM domain deletion constructs to determine whether polysialylation correlated with polyST-NCAM binding. I co-expressed ST8SiaIV/PST-myc with full length NCAM, NCAM3 (Ig5-FN1-FN2-TM-tail) and NCAM4 (Ig5-FN1-TM-tail) in Lec 2 CHO cells, immunoprecipitated myc-tagged ST8SiaIV/PST, and determined whether the V5-tagged NCAM proteins co-precipitated by immunoblotting. To insure that all NCAM mutants were equally expressed by the Lec2 CHO cells, one tenth of the cell lysate was subjected to immunoblotting with anti-V5 antibody. As seen in Figure 12, all three NCAM proteins co-precipitate with ST8SiaIV/PST, and no non-specific interaction with the anti-myc antibody or protein A-Sepharose beads is observed. This matches well with the observed ability of NCAM3 and NCAM4 to be recognized and polysialylated like wild type NCAM (175).

Table IV. Primer used in NCAM FN1 sequence replacements described in Chapter III.

Primer	Direction	Template DNA	Primer sequence
Acidic patch DEE/AAA	5’-3’	NCAM, NCAM 7	GCAGTTTGCTGCACCAGCGGC
Acidic patch DEE/AAA	3’-5’	NCAM, NCAM 7	CTGTGGCCGCTGGTGCAGCAA
Acidic patch DEE/RRR	5’-3’	NCAM, NCAM 7	GCAGTTTCGTCGACCACGGGCCACAGG
Acidic patch DEE/RRR	3’-5’	NCAM, NCAM 7	CCTGTGGCCCGTGGTCGACGAAACTGC
Δ Helix/TT	5’-3’	NCAM, NCAM7	CAAGTGGTATGATACTACTATCGTCACCATC
Δ Helix/TT	3’-5’	NCAM, NCAM7	GATGGTGACGATAGTAGTATCATACCACTTG
QVQ/AAA	5’-3’	NCAM, NCAM7	CAGCACAGCCGCGGCGGCGTTTGATGAAC
QVQ/AAA	3’-5’	NCAM, NCAM7	GTTCATCAAACGCCGCCGCGGCTGTGCTG
PYS/AAA	5’-3’	NCAM, NCAM7	CAGGTGGAGGCAGCCGCCAGCACAGCC
PYS/AAA	3’-5’	NCAM, NCAM7	GGCTGTGCTGGCGGCTGCCTCCACCTG

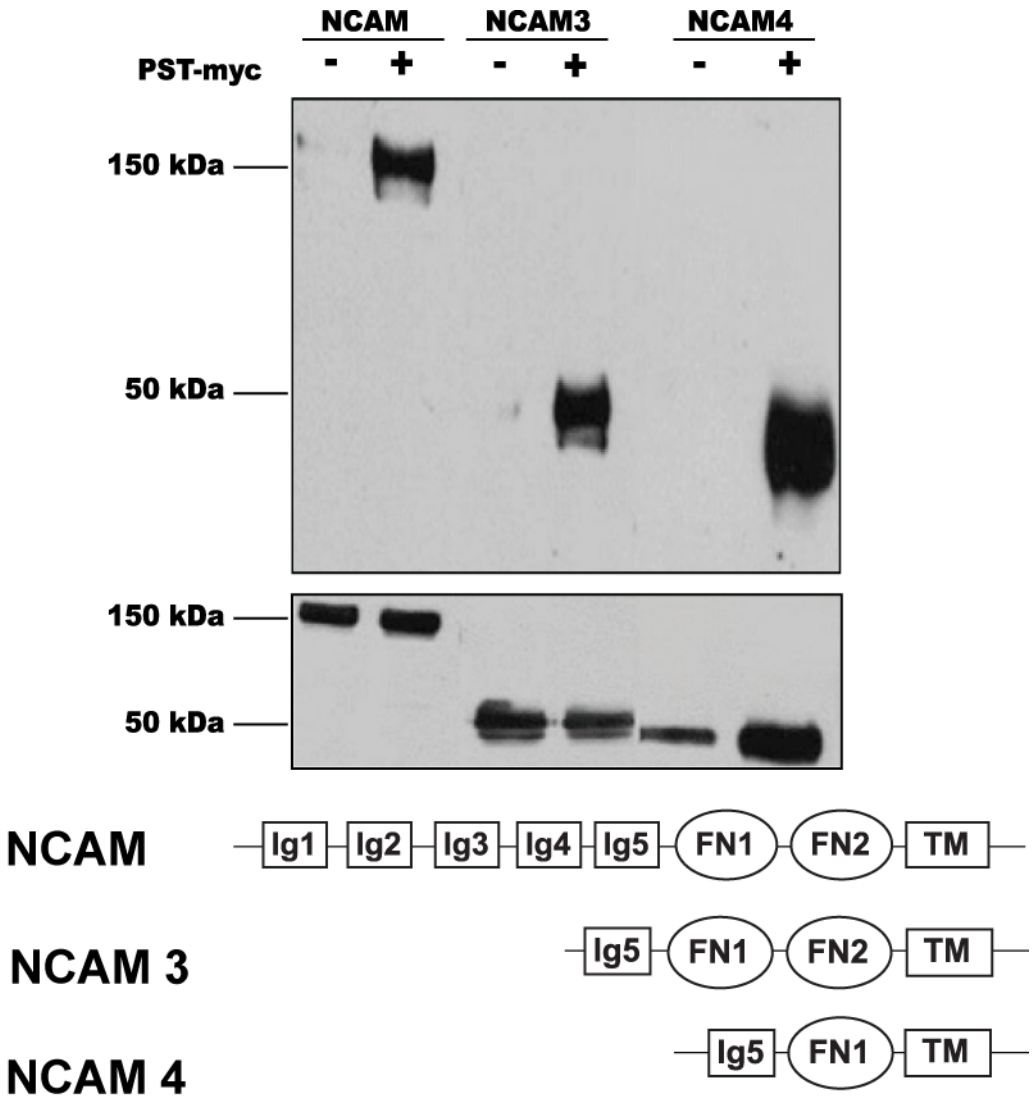


Figure 12. Full length NCAM and NCAM deletion constructs bind to the ST8SiaIV/PST polysialyltransferase. *Upper panel,* Lec2 CHO cells were co-transfected with V5-tagged NCAM proteins and myc-tagged ST8SiaIV/PST (+ lanes) or empty vector (- lanes). Cells were lysed and ST8SiaIV/PST was immunoprecipitated using an anti-myc epitope antibody. Immunoprecipitated proteins were subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody to visualize co-precipitated NCAM proteins. *Lower panel,* to evaluate the relative expression of NCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody. NCAM, NCAM3 and NCAM4 are schematically represented on the right side of the figure. Ig, immunoglobulin domain; FN1, first fibronectin type III repeat; FN2, second fibronectin type III repeat; TM, transmembrane region.

Next, I wanted to determine whether FN1 alone could specifically bind to ST8SiaIV/PST. However, previous work showed that the FN1-TM-tail protein or soluble FN1 were unstable when expressed in mammalian cells. I initially attempted to evaluate the binding of NCAM7 (FN1-FN2-TM-tail) to ST8SiaIV/PST. However, I found that this NCAM construct bound nonspecifically to the protein A-Sepharose beads (data not shown). Instead, I evaluated whether a soluble form of ST8SiaIV/PST (sPST) and a soluble form of NCAM7, both lacking the cytosolic tail and TM region of the proteins, could interact. These two soluble, secreted proteins were co-expressed in Lec 2 CHO cells and co-immunoprecipitated from the cell media. I observed that sNCAM7 bound specifically to sPST (Fig. 13). Neither sNCAM7 nor sPST are membrane bound, and thus they are free from alignment constraints. Consequently, these results show that the interaction of these two proteins did not require the use of TM regions or membrane clustering, a factor that impacts the interactions between some membrane-anchored proteins (185).

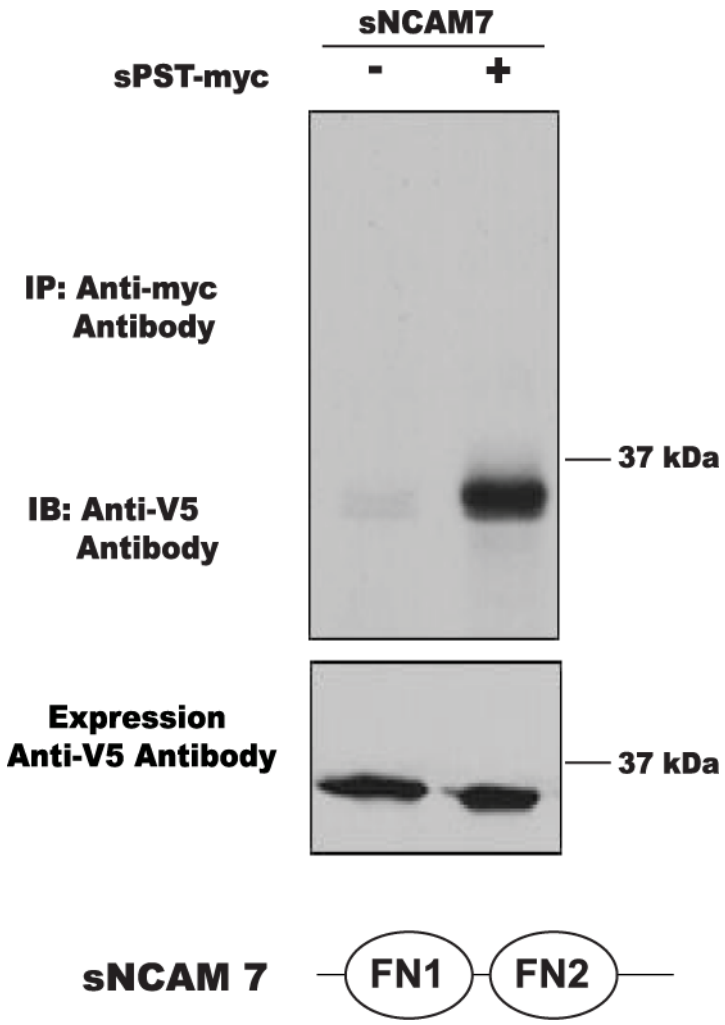


Figure 13. A soluble NCAM construct consisting of only FN1 and FN2 can bind to soluble ST8SiaIV/PST. *Upper panel, Lec 2* CHO cells were co-transfected with V5-tagged sNCAM7 and myc-tagged sPST (+ lanes) or empty vector (- lanes). Cell media was collected and sPST was immunoprecipitated with an anti-myc epitope antibody. Immunoprecipitated proteins were subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody to detect co-precipitated NCAM proteins. *Lower panel,* to evaluate the relative expression of NCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody. sNCAM7 is schematically represented on the right side of the figure. FN1, first fibronectin type III repeat; FN2, second fibronectin type III repeat. Soluble ST8SiaIV/PST (sPST) lacks the enzyme's N-terminal cytosolic tail and transmembrane region.

The experiments in Figures 12 and 13 showed that proteins containing the FN1 domain that are polysialylated also were able to bind to ST8SiaIV/PST. But what about NCAM mutants that are not polysialylated or polysialylated at lower levels? At the time I began on this project, the only unpolysialylated NCAM mutant was the ΔFN1 protein that lacks the FN1 domain (176). I found that this unpolysialylated mutant was unable to bind ST8SiaIV/PST (Figure 14). Taken together, these results are consistent with the conclusion that the FN1 domain is critical for polyST recognition and binding.

I next analyzed full length NCAM with mutations in the FN1 domain that altered the location or decreased overall polysialylation to determine whether decreases in polyST binding matched changes in polysialylation (Fig. 15). Previous work demonstrated that replacing the core residues of the acidic patch (Asp^{520}, Glu^{521}, Glu^{523}) with alanine (AP-AAA) decreased polysialylation of NCAM, while replacing these same residues with arginine (AP-RRR) eliminated NCAM polysialylation (176). Interestingly, both the AP-AAA and AP-RRR mutants exhibited equal decreases in ST8SiaIV/PST binding of from 30-40% (Fig. 15). The Δhelix-TT mutant that is polysialylated on FN1 O-glycans exhibited enhanced binding to ST8SiaIV/PST, although it exhibited substantial non-specific binding to the protein A-Sepharose beads (Fig. 15). Unlike, the Δhelix mutants, the QVQ-AAA mutant showed little change in ST8SiaIV/PST binding even though like the Δhelix mutant its polySia was found on O-glycans (Fig. 15). Not surprisingly, the PYS-AAA mutant that exhibited little change in overall polysialylation, also showed little change in enzyme binding (Fig. 15).

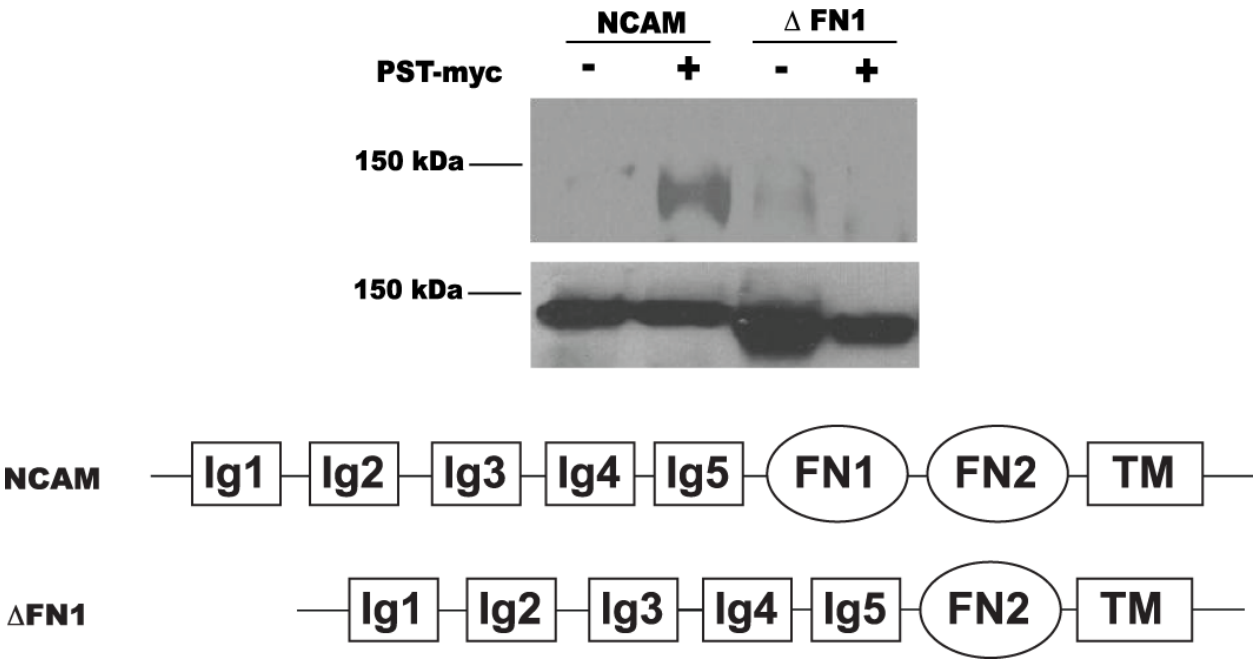


Figure 14. ΔFN1 NCAM does not interact with ST8SiaIV/PST. *Upper panel,* Lec2 CHO cells were co-transfected with V5-tagged NCAM or NCAM ΔFN1 and myc-tagged PST (+ lanes) or empty vector (- lanes). Cells were lysed and ST8SiaIV/PST was immunoprecipitated using an anti-myc epitope antibody. Immunoprecipitated proteins were subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody to visualize co-precipitated NCAM proteins. *Lower panel,* to evaluate the relative expression of NCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody. NCAM and NCAM ΔFN1 are schematically. Ig, immunoglobulin domain; FN1, first fibronectin type III repeat; FN2, second fibronectin type III repeat; TM, transmembrane region.

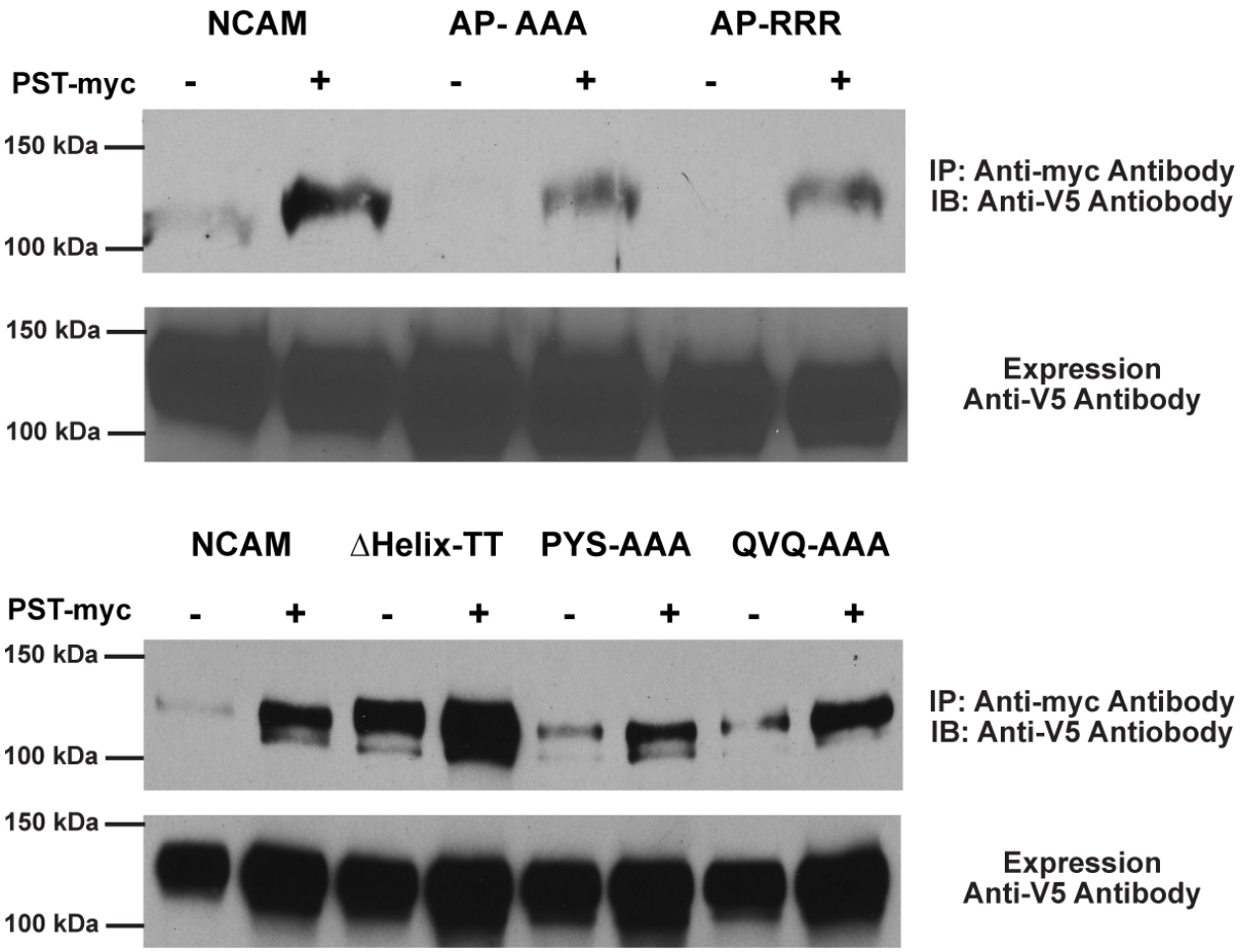


Figure 15. Replacing the core residues of the FN1 acidic patch partially disrupts the NCAM-ST8SiaIV/PST interaction. *Upper panel,* Lec2 CHO cells were co-transfected with V5-tagged NCAM or NCAM mutant and myc-tagged PST (+ lanes) or empty vector (- lanes). Cells were lysed and PST was immunoprecipitated by anti-myc epitope antibody. Immunoprecipitated proteins were subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody to visualize co-precipitated NCAM proteins. *Lower panel,* to evaluate the relative expression of NCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody.

These results suggested that other sequences in NCAM FN1, in addition to the acidic patch, are responsible for polyST interaction, and/or the polySTs may interact with other regions of NCAM, and in particular the Ig5 domain. With the latter possibility in mind, I decided to determine whether the same mutations in the FN1 domain of NCAM7 altered its polysialylation and binding to ST8SiaIV/PST equally. Since NCAM7 (FN1-FN2-TM-tail) lacks the Ig5 domain, I predicted that if the Ig5 domain or another Ig domain contributes to polyST binding, some of the FN1 mutants that showed partial effects in full length NCAM polysialylation might show larger decreases in NCAM7 polysialylation. First, I evaluated the impact of the acidic patch (AP-AAA), Δhelix-TT, PYS-AAA and QVQ-AAA mutants on NCAM7 polysialylation. Figure 16 illustrates that these FN1 mutations had similar, but in some cases more severe effects on polysialylation of NCAM7, then they did on the polysialylation of full length NCAM. As expected replacing QVQ with alanines and the α-helix with two threonines, that switched the polysialylation of full length NCAM to O-linked glycans in FN1 (176, 177), had little effect on NCAM7 polysialylation. However, the NCAM7 QVQ-AAA mutant does migrate at a lower molecular weight suggesting that less polySia may be added to its glycans (Fig. 16). In contrast, replacing the core acidic patch residues with alanines (AP-AAA) lead to a large decrease in NCAM7 polysialylation and replacing these residues with arginines (AP-RRR) almost completely eliminated polysialylation (Fig. 16). This correlates with the results seen with full length NCAM, but the severity of the disruption is much greater, especially for the AP-AAA mutant (176). These results suggest the possibility that the polySTs may interact with multiple domains in NCAM.

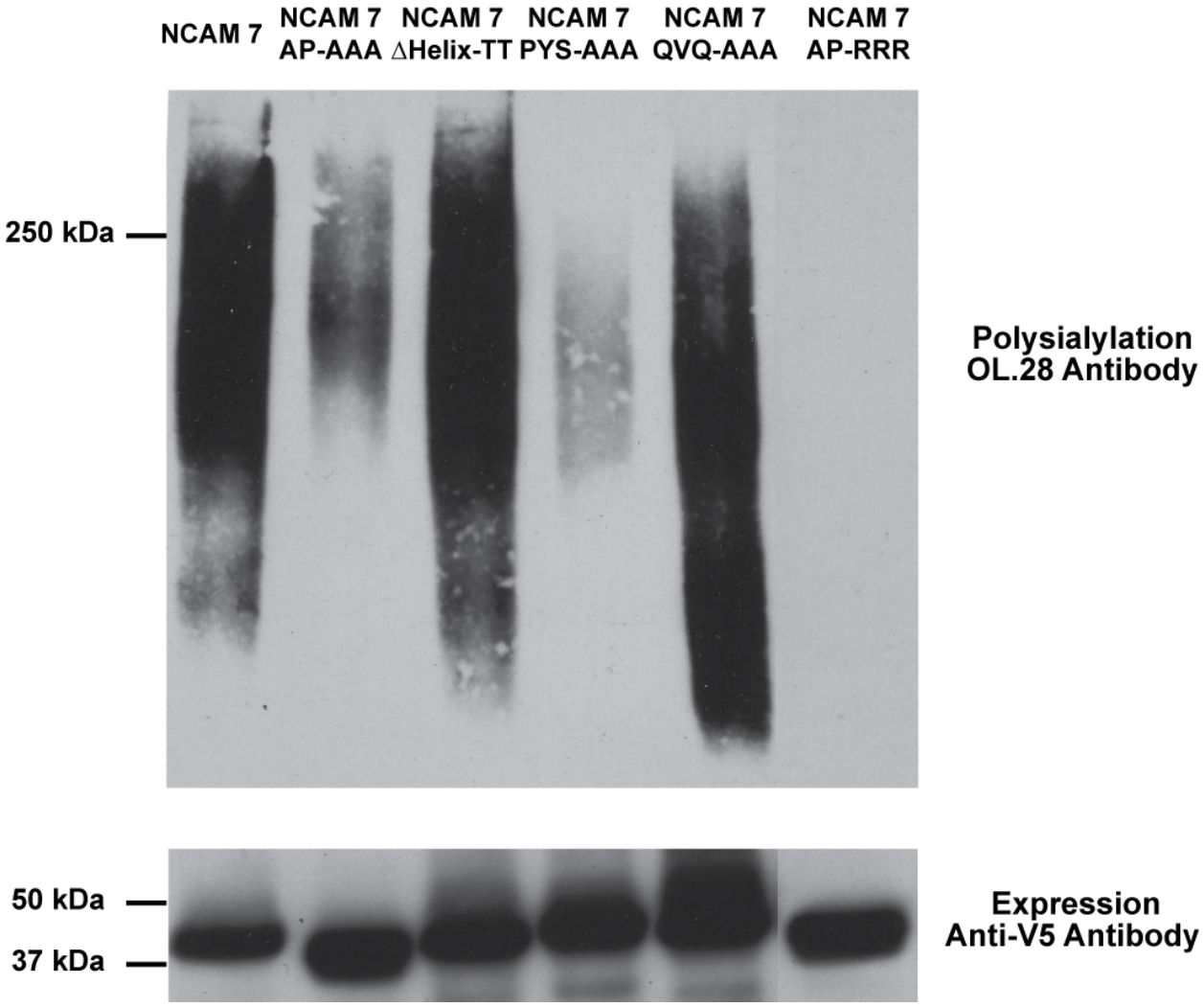


Figure 16. Mutations of FN1 sequences in NCAM 7 decrease or eliminate O-linked polysialylation. *Upper panel,* COS-1 cells were co-transfected with V5-tagged NCAM7 or NCAM7 mutants and myc-tagged PST. Cells were then lysed and the NCAM7 proteins immunoprecipitated using an anti-V5 antibody. Immunoprecipitated proteins were separated by SDS PAGE and subjected to immunoblotting with the OL.28 anti-polySia antibody. *Lower panel*, to evaluate the relative expression of NCAM and NCAM-OCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody.

The effects of mutating the PYS sequence to alanines in NCAM7 were surprisingly severe. In contrast to the small decrease in polysialylation of the full length NCAM PYS-AAA mutant, the polysialylation of the NCAM7 PYS-AAA mutant was nearly eliminated (179 and Fig.16). At first we thought that the serine in PYS was O-glycosylated and subsequently polysialylated and that changing it to alanine was eliminating a site of O-linked polysialylation in NCAM7 that is not used in full length NCAM. However, I replaced each residue in the PYS sequence individually and found that no single change replicated the replacement of the entire PYS sequence (data not shown). It may be that mutation of all three residues in PYS has propagated a structural change in NCAM7 (but not full length NCAM) that affects the NCAM7-ST8SiaIV/PST interaction or the access of the polyST to FN1 O-glycans.

The AP-AAA mutation had a more negative effect on NCAM7 polysialylation than it did on the polysialylation of full length NCAM. To evaluate whether I could connect the decrease in NCAM7 AP-AAA polysialylation to a decrease in ST8SiaIV/PST binding, I attempted to perform a co-immunoprecipitation experiment with sPST and sNCAM7-AP-AAA. Unfortunately, the soluble NCAM7 mutant exhibited substantial non-specific binding to the protein A-Sepharose beads and I was unable to draw any conclusions. I will return to this question concerning the role of Ig5 sequences in polyST recognition in Chapter 5.

Discussion

In this study I investigated if specific sequences in NCAM FN1 that affect the polysialylation of N-glycans in the Ig5 domain were also involved in the polyST-NCAM interaction. To explore these questions I made use of minimal NCAM constructs in which

extracellular domains of NCAM were selectively deleted. Removing the first four Ig domains (NCAM 3) did not impact the interaction of NCAM with ST8SiaIV/PST (Fig. 12). Further deletion of the FN2 domain (NCAM 4) also did not disturb the enzyme substrate interaction (Fig. 12). However, a NCAM protein lacking FN1 (ΔFN1) did show a loss of interaction with ST8SiaIV/PST indicating that NCAM FN1 is necessary for its interaction with the polyST, ST8SiaIV/PST (Fig. 14). Consistent with this, I found that a soluble construct consisting of only the FN1 and FN2 domains (sNCAM 7) interacted with a soluble version of ST8SiaIV/PST (Fig. 13). This result demonstrated that the cytosolic sequences and transmembrane region of NCAM and ST8SiaIV/PST are not required for their interaction.

Prior work had investigated how specific FN1 domain mutations impacted polySia addition to both full length and truncated NCAM proteins. Here, I evaluated whether these mutations had an effect on the ability of NCAM to interact with ST8SiaIV/PST. Replacing the QVQ and α-helix sequences in FN1 had been shown to cause a change in the location of polySia addition from N-linked glycans in Ig5 to O-linked glycans in FN1 (177). In co-immunoprecipitation studies I observed that the relative level of NCAM-ST8SiaIV/PST binding is similar for NCAM and the QVQ-AAA mutant (Fig. 15). This result might suggest that replacing the QVQ sequence altered the relationship between the Ig5 and FN1 domains, placing the Ig5 N-glycans out of reach of the polyST that is interacting with the FN1 domain. Alternatively, replacing these sequences may have altered the way the polyST interacts with the FN1 domain so that it no longer can access the Ig5 N-glycans. While replacing the FN1 α-helix also shifted polySia addition to FN1 O-glycans, this mutation had a different effect on polyST-NCAM binding. The Δhelix-TT mutant exhibited increased polyST binding relative to wild type NCAM (Fig. 15).

Much of this increase can be explained by an increase in nonspecific interaction with the protein-A Sepharose beads. It is likely that replacing the α-helix exposed a usually hidden hydrophobic surface that lead to this non-specific interaction. In sum, replacing the QVQ and α-helix sequences may have altered the FN1 domain structure and/or revealed cryptic binding site(s) for ST8SiaIV/PST, which then positioned the enzyme to polysialylate FN1 O-linked glycans.

Mutation of the FN1 PYS sequence showed little effect on full length NCAM polysialylation (179), and I found that the interaction of the PYS-AAA mutant with ST8SiaIV/PST was similar to that of wild type NCAM (Fig. 15). Interestingly, when this sequence was replaced in the NCAM7 protein a large decrease in polySia addition was observed (Fig. 16). I ruled out the possibility that replacing Ser^{512} in the PYS sequence eliminated an O-linked glycosylation site that was polysialylated (data not shown). Mutation of other potential O-glycosylation sites suggested that Ser^{489} may be a major site of O-glycan polysialylation in the NCAM7 protein (data not shown). It is likely that replacing the PYS sequence in the truncated NCAM7 protein propagated a structural change in the FN1 domain that in turn disrupted the NCAM7-ST8SiaIV/PST interaction. The presence of Ig5 and other Ig domains in full length NCAM may constrain the folding of FN1 and minimize the impact of replacing the PYS sequence in the full length protein, explaining the minimal effect of replacing PYS in full length NCAM.

The AP-AAA and AP-RRR FN1 acidic patch mutants were the only FN1 mutants examined that showed a decrease in polyST binding (Fig. 15). Replacing the acidic patch in full length NCAM with alanine residues (AP-AAA) reduced polysialylation by approximately 30%, and the

same mutant showed a 26% decrease in ST8SiaIV/PST binding. In contrast, replacing the acidic patch in full length NCAM with arginine residues (AP-RRR) nearly eliminated NCAM polysialylation (174), but again only decreased ST8SiaIV/PST binding by approximately 30% (Fig. 15). These results suggest that the presence of arginine residues is causing a further disruption that impacts polysialylation more strongly than polyST binding. These results and my observation that replacing acidic patch residues with alanine or arginine has a more substantial effect on NCAM 7 polysialylation than full length NCAM polysialylation, suggest that other residues in both FN1 and in other NCAM domains, such as Ig5, are playing a role in polyST recognition. In the next chapters I investigate if there are other residues in FN1 that together with the acidic patch comprises the binding site for ST8SiaIV/PST and if sequences in Ig5 contribute to the binding of ST8SiaIV/PST.

Chapter IV

Sequences in NCAM FN1 That Are Common to NCAM and OCAM Are Required for NCAM Polysialylation and May Maintain an Ig5-FN1 Relationship Necessary for NCAM Polysialylation.

Introduction

While I was searching for other NCAM FN1 residues that form part of the polyST binding region, another student in the laboratory, Deirdre Foley, discovered that the first fibronectin type III repeat of the Olfactory Cell Adhesion Molecule (OCAM) could replace NCAM FN1 and allow the polysialylation of an NCAM-OCAM chimera (179). OCAM is a cell adhesion protein that is expressed by neurons in the olfactory system and by other neural tissues such as the retina (186). OCAM has the same extracellular domain organization as NCAM with five Ig domains and two fibronectin type III repeats (Fig. 17). It contains eight potential N-glycosylation sites, two of which correspond precisely to the polysialylated N-glycosylation sites 5 and 6 in NCAM Ig5 (186). The overall level of identity between the two proteins is 44.5% with the Ig1 Ig2 and Ig5 domains having a higher percentage of identity (45-55%) than Ig3 Ig4 and the two FN domains (36-40%). The two FN1 domains exhibit 37.5% identity. OCAM is expressed in many of the same places as NCAM often at the same time, yet despite all of these similarities OCAM is not polysialylated (186).

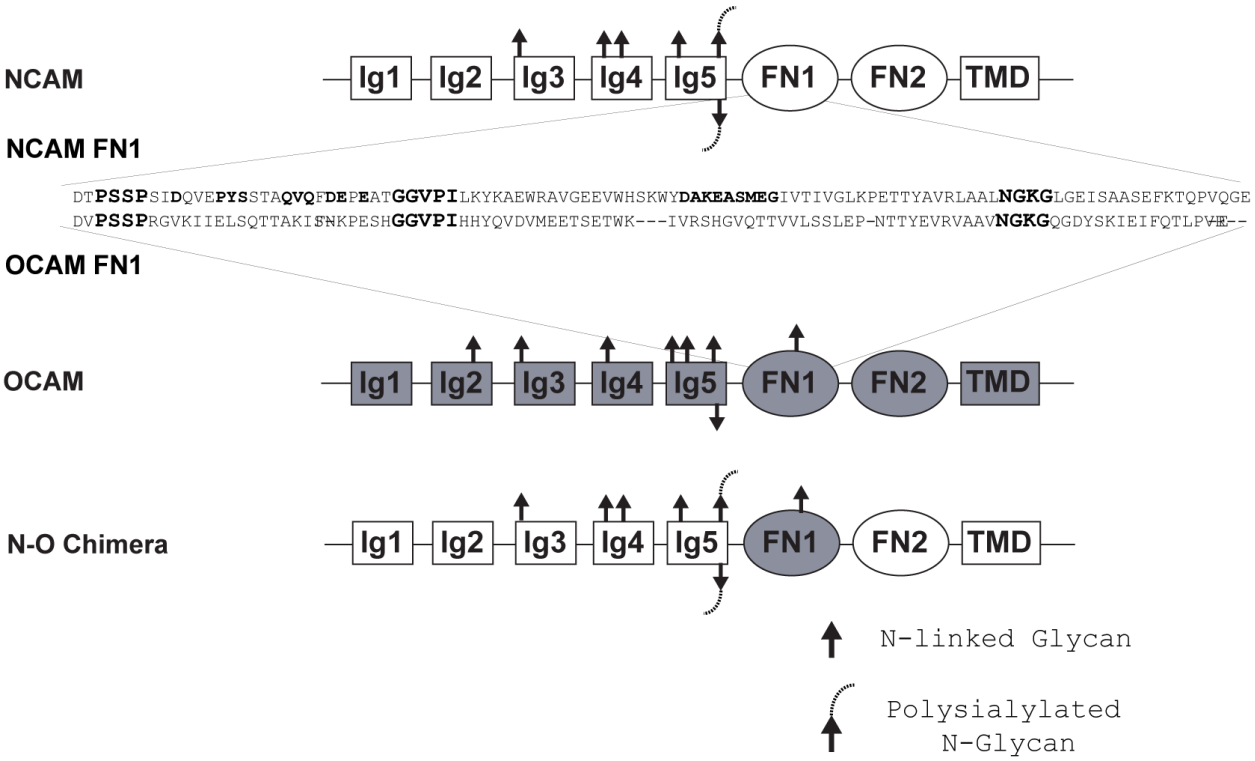


Figure 17. Comparison of NCAM, OCAM and the NCAM-OCAM chimera extracellular domains and NCAM and OCAM FN1 sequences. NCAM and OCAM show similarity in their extracellular domains both containing five Ig domains (Ig1-Ig5) and two fibronectin type III repeats (FN1, FN2). NCAM contains six consensus N-linked glycosylation sites with sites 5 and 6 modified by the addition of polySia. OCAM has eight consensus N-glycosylation sites and is not modified by polySia. The chimera protein (N-O Chimera) was created by replacing NCAM FN1 with OCAM FN1. The sequences of NCAM and OCAM FN1 domains are compared and three common sequences are highlighted and enlarged. Also shown are the NCAM FN1 acidic patch (D/DE_E), QVQ, PYS and α-helix (DAKEASMEG) in bold).

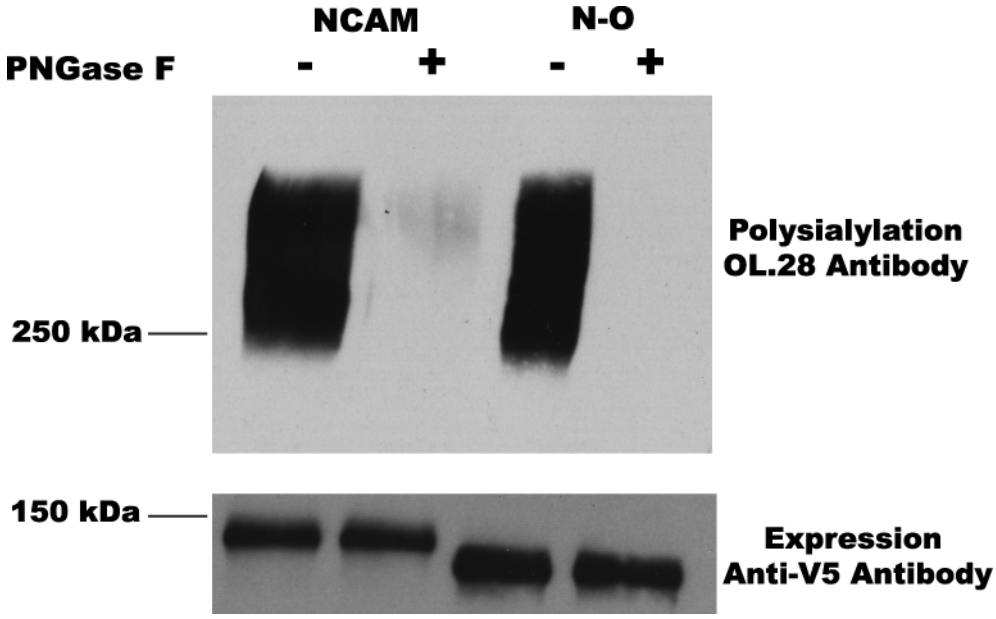


Figure 18. Replacing the NCAM FN1 domain with the FN1 domain from OCAM allows for polysialylation of an NCAM-OCAM chimera. *Upper panel,* COS-1 cells were transiently co-transfected with myc-tagged ST8SiaIV/PST and either V5-tagged NCAM or an NCAM-OCAM chimera. NCAM or the NCAM-OCAM chimera was immunoprecipitated from cell lysates using an anti-V5 epitope antibody. Half of the immunoprecipitated sample was treated with PNGase-F to cleave N-linked glycans, the other sample was left untreated. Samples were then separated by SDS PAGE and the polysialylation of immunoprecipitated proteins analyzed by immunoblotting with the OL.28 anti-polySia antibody. *Lower panel*, to evaluate the relative expression of NCAM and NCAM-OCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody.

The original purpose of making the NCAM-OCAM chimera, in which the first FN1 domain of NCAM was replaced with that of OCAM, was to provide an appropriately folded, non-polysialylated scaffold for reconstitution studies in which NCAM FN1 sequences would be added back to see if polysialylation could be restored. However, co-expression of ST8SiaIV/PST with the chimera revealed that it could be polysialylated on N-linked glycans just like wild type NCAM (Fig. 18). This suggested that OCAM FN1 contained sequences that could be recognized by the polySTs, and lead us to consider the possibility that sequences common to NCAM and OCAM FN1 domains may be critical for polyST recognition. To investigate this possibility, I compared the primary sequences for NCAM and OCAM FN1 to determine if any extended regions of identity exist. In this chapter I report my analysis of three common NCAM and OCAM FN1 sequences, and the role they play in maintaining the Ig5-FN1 relationship and NCAM recognition by the polySTs.

Results

Inspection of the NCAM and OCAM FN1 sequences revealed three common regions of 4–5 amino acids: $Pro^{500}Ser^{501}Ser^{502}Pro^{503}$ (*PSSP*), $Gly^{526}Gly^{527}Val^{528}Pro^{529}Ile^{530}$ (*GGVPI*), and $Asn^{580}Gly^{581}Lys^{582}Gly^{583}$ (*NGKG*). Interestingly, when these common sequences are mapped onto the structure of NCAM Ig5-FN1 (PDB ID 3MTR), I found that all three were located at or near the Ig-FN1 interface (Fig. 19). GGVPI and NGKG comprise the two loops connecting strands 2 and 3 and strands 6 and 7 of the FN1 β sandwich, respectively. These loops flank the Ig5-FN1 linker region (Fig.19, GGVPI in *green* and NGKG in *purple*). PSSP is two amino acids away from the Ig5-FN1 junction (Fig. 19, PSSP in *red*). I also noticed that the PSSP sequence lies next to the

core residues of the acidic patch (Asp^{520}, Glu^{521}, and Glu^{523}) (Fig. 19, compare the location of acidic patch in *orange* and PSSP in *red*).

To evaluate the role of these three common regions in NCAM polysialylation, I replaced each sequence in full length NCAM with alanines. I also replaced another sequence common to NCAM and OCAM FN1, $Lys^{532}Tyr^{533}Lys^{534}$ (KYK) with alanines. I co-expressed V5-tagged NCAM and the mutant NCAM proteins with myc-tagged ST8SiaIV/PST in COS-1 cells (Fig. 20, panel A). Cells were then lysed and the NCAM proteins were immunoprecipitated using an anti-V5 antibody to recover the NCAM proteins. Immunoprecipitated proteins were separated by SDS PAGE and immunoblotted with the OL.28 anti-polySia antibody (Fig. 20, panel A). As a comparison, the impact of replacing the acidic patch (AP-AAA, AP-RRR), α-helix (Δhelix-AA), and QVQ (QVQ-AAA) on NCAM polysialylation is shown (Fig. 20, panel B).

Table V. Primers used in NCAM/OCAM common sequence replacements described in Chapter IV.

Primer	Direction	Template DNA	Primer sequence
GGVPI-AAAAA	5’-3’	NCAM, NCAM 7	GAGGCCACAGGTGCGGCGGCCGCCCTCAAATAC
GGVPI-AAAAA	3’-5’	NCAM, NCAM 7	GTATTTGAGGGCGGCCGCCGCACCTGTGGCCTC
NGKG-AAAA	5’-3’	NCAM, NCAM 7	CTGGCGGCGCTCGCTGCCGCAGCGCTGGGTGAGATC
NGKG-AAAA	3’-5’	NCAM, NCAM 7	GATCTCACCCAGCGCTGCGGCAGCGAGCGCCGCCAG
PSSP-AAAA	5’-3’	NCAM, NCAM 7	CAAGCAGACACCGCCGCTGCAGCATCCATCGACCAG
PSSP-AAAA	3’-5’	NCAM, NCAM 7	CTGGTCGATGGATGCTGCAGCGGCGGTGTCTGCTTG
KYK-AAA	5’-3’	NCAM	GTGCCCATCCTCGCAGCCGCAGCTGAGTGGAG
KYK-AAA	3’-5’	NCAM	CTCCACTCAGCTGCGGCTGCGAGGATGGGCAC
PSSP-GSSG	5’-3’	NCAM	CAAGCAGACACCGGCTCTTCAGGATCCATCGACCAG
PSSP-GSSG	3’-5’	NCAM	CTGGTCGATGGATCCTGAAGAGCCGGTGTCTGCTTG
PSSP-PGGP	5’-3’	NCAM	CTTGTTCAAGCAGACACCCCCGGTGGTCCATCC ATCCATCGACGAGGT
PSSP-PGGP	3’-5’	NCAM	ACCTCGTCGATGGATGGATGGACCACCGGGGGTGT CTGCTTGAACAAG

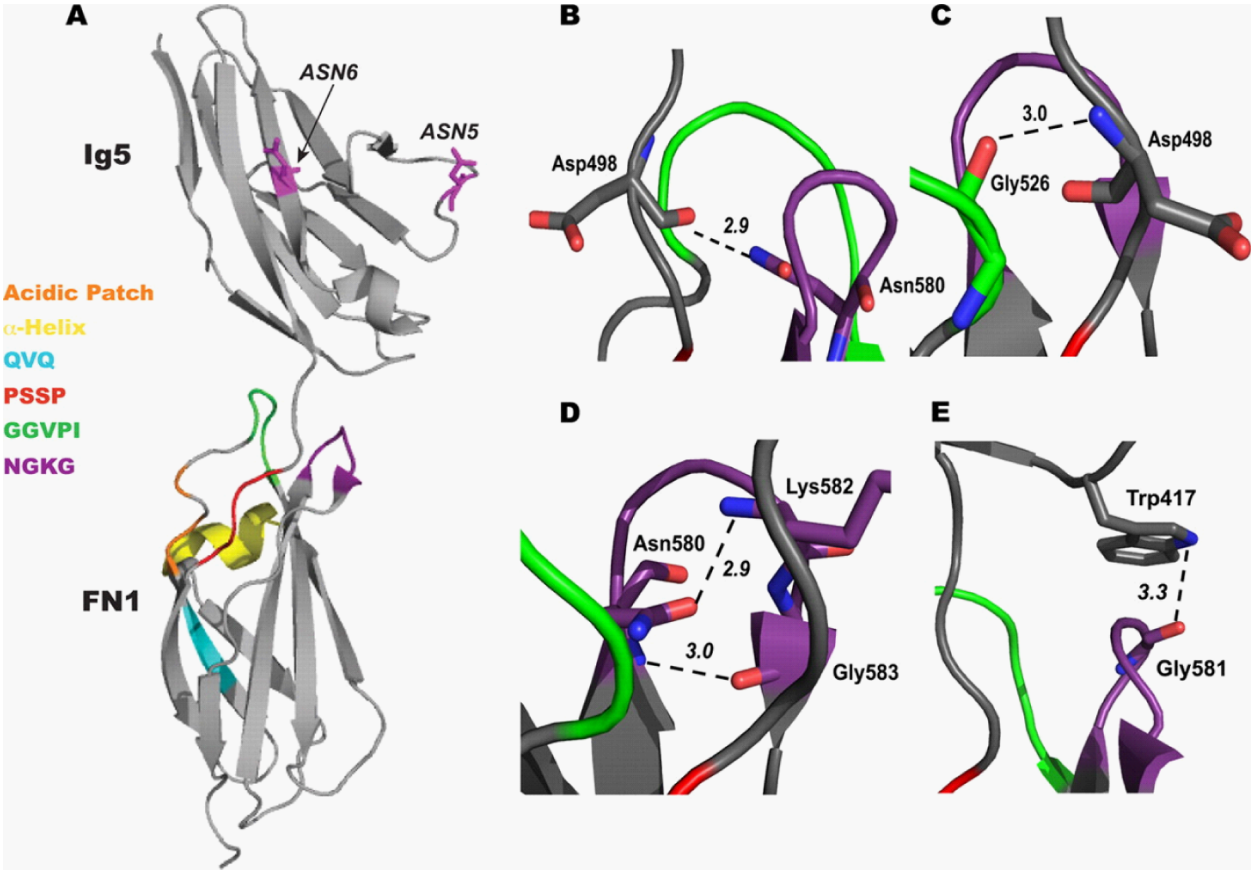


Figure 19. Crystal structure of the Ig5-FN1 tandem highlighting the location of NCAM and OCAM FN1 common sequences and showing interactions that may stabilize the Ig5-FN1 relationship. A) The crystal structure of Ig5-FN1 with key residues highlighted. Sequences unique to NCAM that have demonstrated roles in polysialylation are indicated in orange (acidic patch), yellow (α-helix), and blue (QVQ). The three regions of identity between NCAM and OCAM are shown in red (PSSP), green (GGVPI), and purple (NGKG). Note that these three sequences they cluster around the strand linking Ig5 and FN1. The location of Asn5 and Asn6, the N-glycan attachment sites that are polysialylated in Ig5, are shown in magenta. (B-D) Possible interactions that could stabilize the Ig5-FN1 interaction and affect relative orientation of the two domains are shown. Bond distance is reported in angstroms and is calculated using the measurement function in the Pymol™ software.

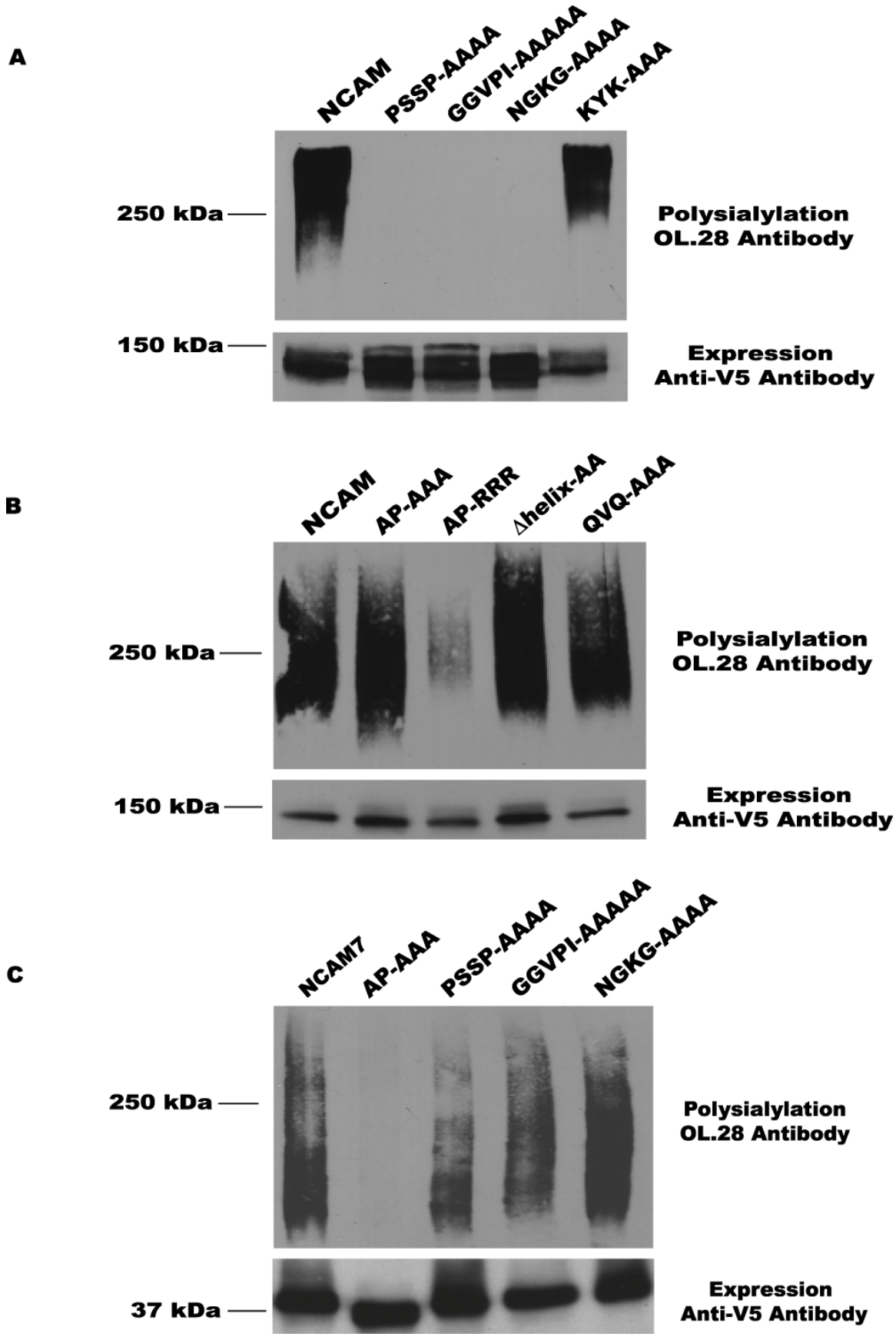

A
NCAM
PSSP-AAAA
GGVPI-AAAAA
NGKG-AAAA
KYK-AAA
250 kDa
Polysialylation
OL.28 Antibody
150 kDa
Expression
Anti-V5 Antibody
B
NCAM
AP-AAA
AP-RRR
Δhelix-AA
QVQ-AAA
250 kDa
Polysialylation
OL.28 Antibody
150 kDa
Expression
Anti-V5 Antibody
C
NCAM7
AP-AAA
PSSP-AAAA
GGVPI-AAAAA
NGKG-AAAA
250 kDa
Polysialylation
OL.28 Antibody
37 kDa
Expression
Anti-V5 Antibody

Figure 20. (A) Replacing sequences common to NCAM and OCAM FN1 near the Ig5-FN1 interface eliminates polysialylation. *Upper panel,* COS-1 cells were co-transfected with V5-tagged NCAM or NCAM mutants along with myc-tagged ST8SiaIV/PST. NCAM proteins were immunoprecipitated from cell lysates using an anti-V5 epitope antibody. Immunoprecipitated proteins were separated by SDS PAGE and protein polysialylation was analyzed by immunoblotting with the OL.28 anti-polySia antibody. *Lower panel*, to evaluate the relative expression of the NCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody. **(B) Only replacing the FN1 acidic patch with arginine residues substantially decreases NCAM polysialylation.** *Upper panel,* COS-1 cells were co-transfected with V5 tagged NCAM or NCAM mutants and myc-tagged ST8SiaIV/PST. The polysialylation of NCAM mutants with the acidic patch (AP-AAA, AP-RRR), α-helix (Δhelix-AA), and QVQ (QVQ-AAA) replaced was analyzed and compared to the polysialylation of wild type NCAM as described above. **(C) Replacing sequences common the NCAM and OCAM FN1 near the Ig5-FN1 interface does not result in the elimination of NCAM7 O-glycan polysialylation.** *Upper panel,* COS-1 cells were co-transfected with V5-tagged NCAM7 or NCAM7 PSSP, GGVIP or NGKG replacement mutants along with myc-tagged ST8SiaIVPST. The polysialylation of these mutant NCAM7 proteins was compared to that of the unaltered protein as described above.

As seen in Figure 20, replacing the NCAM/OCAM common sequences near the Ig5-FN1 interface with alanine caused an elimination of polySia addition. In contrast, replacing the KYK sequence that is not located near the Ig5-FN1 interface did not substantially affect NCAM polysialylation. We wanted to rule out the possibility of these replacement mutants causing misfolding and improper trafficking that would give a false positive for loss of polysialylation. To check for proper trafficking we used immunofluorescence microscopy to visualize NCAM and the mutant proteins traffic through the secretory pathway and get to the cell surface (Fig. 21).

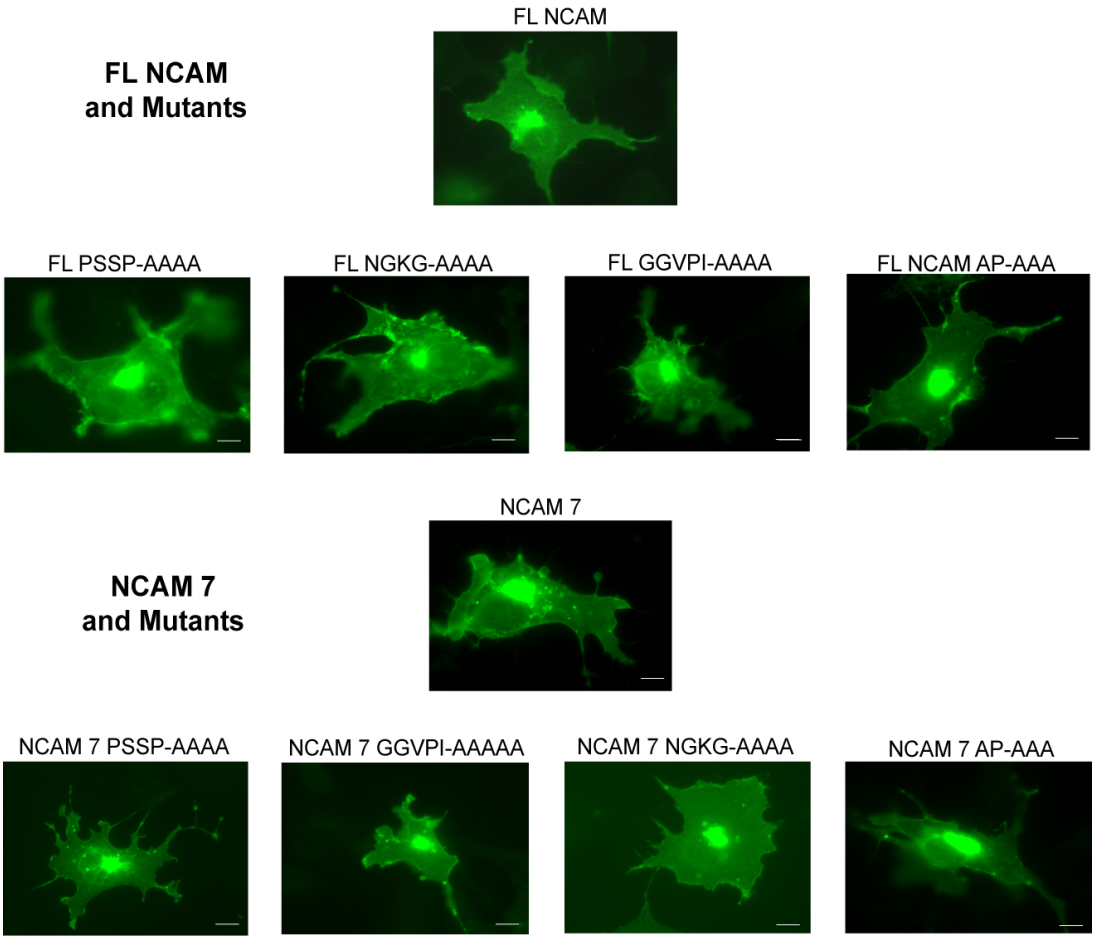


Figure 21. Localization of NCAM and NCAM7 mutants with changes in sequences common to NCAM and OCAM. COS-1 cells were grown on coverslips and co-transfected with V5-tagged NCAM and myc-tagged PST. Cells were allowed to grow overnight and then were fixed and permeabilized with methanol. Cells are incubated with anti-V5 antibody to bind to NCAM and a FITC-conjugated goat anti-mouse IgG to visualize the proteins. Coverslips are then mounted on slides and examined using a Nikon Axiophot microscope equipped with epifluorescence illumination and a 60X oil immersion Plan Apochromat objective.

Another possibility was that Long stretches of alanine residues, which have a propensity to form α-helicie, may distort the structure of FN1. In order to rule out the possibility that we had introduced unwanted secondary structure elements into NCAM FN1, I replaced the NCAM/OCAM common sequences with glycine residues, and found that these changes resulted in the same loss of polysialylation (data not shown). I also evaluated the effects of individually replacing residues in these sequences to determine if the loss of polysialylation I observed was due to removing "key" residues. The only individual amino acid replacements that impacted polysialylation were the proline residues Pro^{500} and Pro^{503} in the PSSP sequence, as seen in Fig. 22.

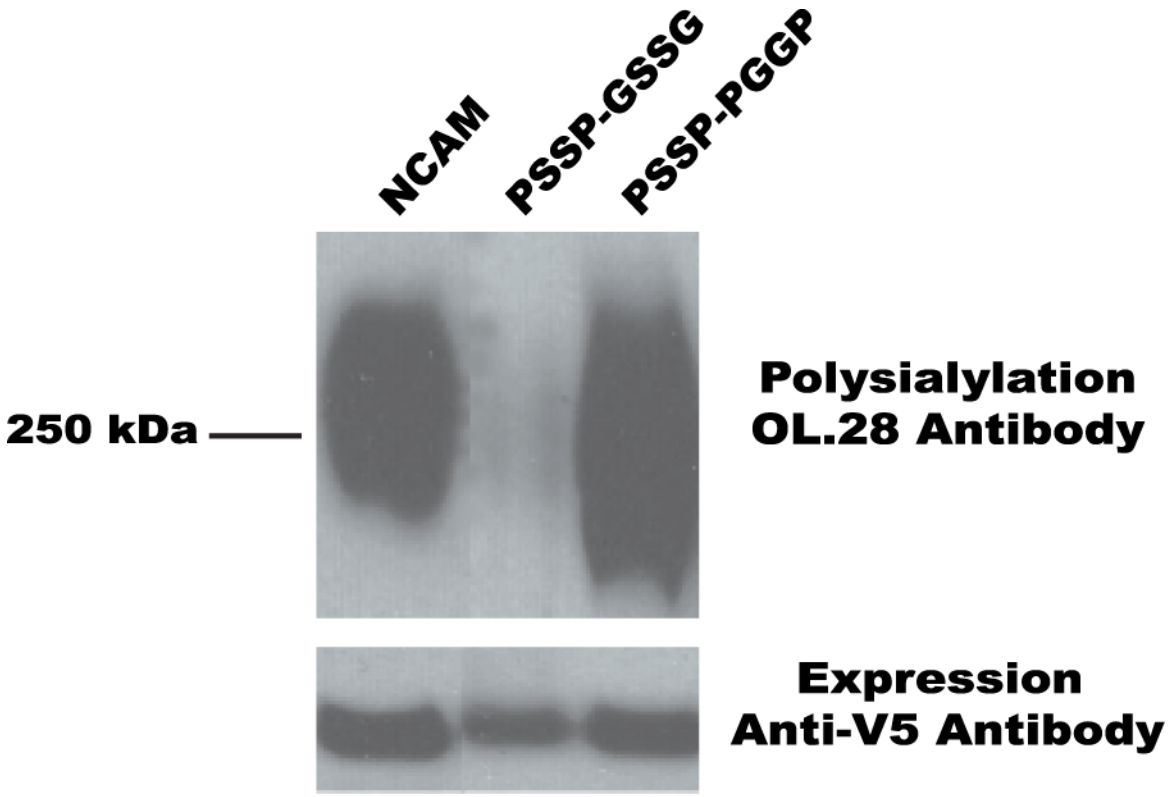


Figure 22. Replacing Pro^{500} and Pro^{503} in the PSSP sequence eliminates NCAM polysialylation, while replacing Ser^{501} and Ser^{502} has no effect. *Upper panel,* COS-1 cells were co-transfected with V5-tagged NCAM or NCAM mutants and myc-tagged ST8SiaIV/PST. NCAM proteins were immunoprecipitated from cell lysates using an anti-V5 epitope antibody. Immunoprecipitated proteins were separated by SDS PAGE and protein polysialylation was analyzed by immunoblotting with the OL.28 anti-polySia antibody. *Lower panel*, to evaluate the relative expression of the NCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody.

In comparison with the effects on NCAM polysialylation observed when the acidic patch, α-helix and QVQ sequences were replaced, replacement of the NCAM/OCAM common sequences had a much more drastic effect. Only replacing the FN1 acidic patch with arginine residues had a similar detrimental impact on NCAM polysialylation (Fig. 20, panel B, AP-RRR). These results suggested two possibilities. One possibility is that the NCAM/OCAM common sequence mutants disrupted the NCAM-ST8SiaIV/PST recognition and binding region. The second possibility was that because the common sequences clustered near the Ig5-FN1 interface, replacing these sequences disrupted the relative orientation of NCAM Ig5 and FN1 and this in turn prevented polysialylation of the Ig5 N-glycans.

To test the validity of either of these possibilities, we replaced the common sequences in the NCAM 7 protein that lacks Ig5 and is polysialylated on FN1. We reasoned that if the common sequences were involved in interaction with the polyST then NCAM7 would not be polysialylated when these sequences were replaced. However if the common sequence mutants acted to disrupt the Ig5-FN1 relationship, then NCAM7 would still be polysialylated. I found that replacing GGVPI or NGKG resulted in very little change in NCAM7 polysialylation, while replacing PSSP lead to a decrease in polysialylation compared to wild type NCAM 7, but not to the same degree as the AP-AAA acidic patch mutant. The results of this experiment indicated that the loss of polysialylation in the NCAM/OCAM common sequence mutants was largely due to a disruption of the Ig5-FN1 interaction, and only replacing PSSP may directly or indirectly impact polyST recognition (Fig. 20, panel C).

To determine whether the observed decrease in polysialylation in the PSSP, GGVPI and NGKG mutants had any impact on the NCAM-polyST interaction, I decided to perform a co-immunoprecipitation experiment to see if the NCAM/OCAM common sequence mutants were still capable of interacting with ST8SiaIV/PST. Lec2 CHO cells were co-transfected with V5-tagged NCAM or NCAM mutant as well as myc-tagged PST. ST8SiaIV/PST was immunoprecipitated using an anti-myc antibody, the immunoprecipitated proteins separated by SDS PAGE and subjected to immunoblotting with an anti-V5 antibody to detect co-precipitated NCAM proteins. I found that replacing NCAM FN1 GGVPI or NGKG had little effect on the NCAM-polyST interaction, while replacing PSSP lead to a more substantial decrease in NCAM-polyST interaction (Fig. 23, bottom). The decrease in NCAM-ST8SiaIV/PST binding observed in the PSSP mutant (~60% decrease) was similar to the decrease in binding observed when the acidic patch residues were replaced (~40% decrease) (Fig 23, top, AP-AAA or AP-RRR). The decrease in NCAM-ST8SiaIV/PST binding observed with the PSSP mutant correlated the decrease in polysialylation observed when this sequence was mutated in the NCAM7 protein (Fig. 20, panel C), and suggested again that the PSSP sequence directly or indirectly plays a role in NCAM-polyST binding.

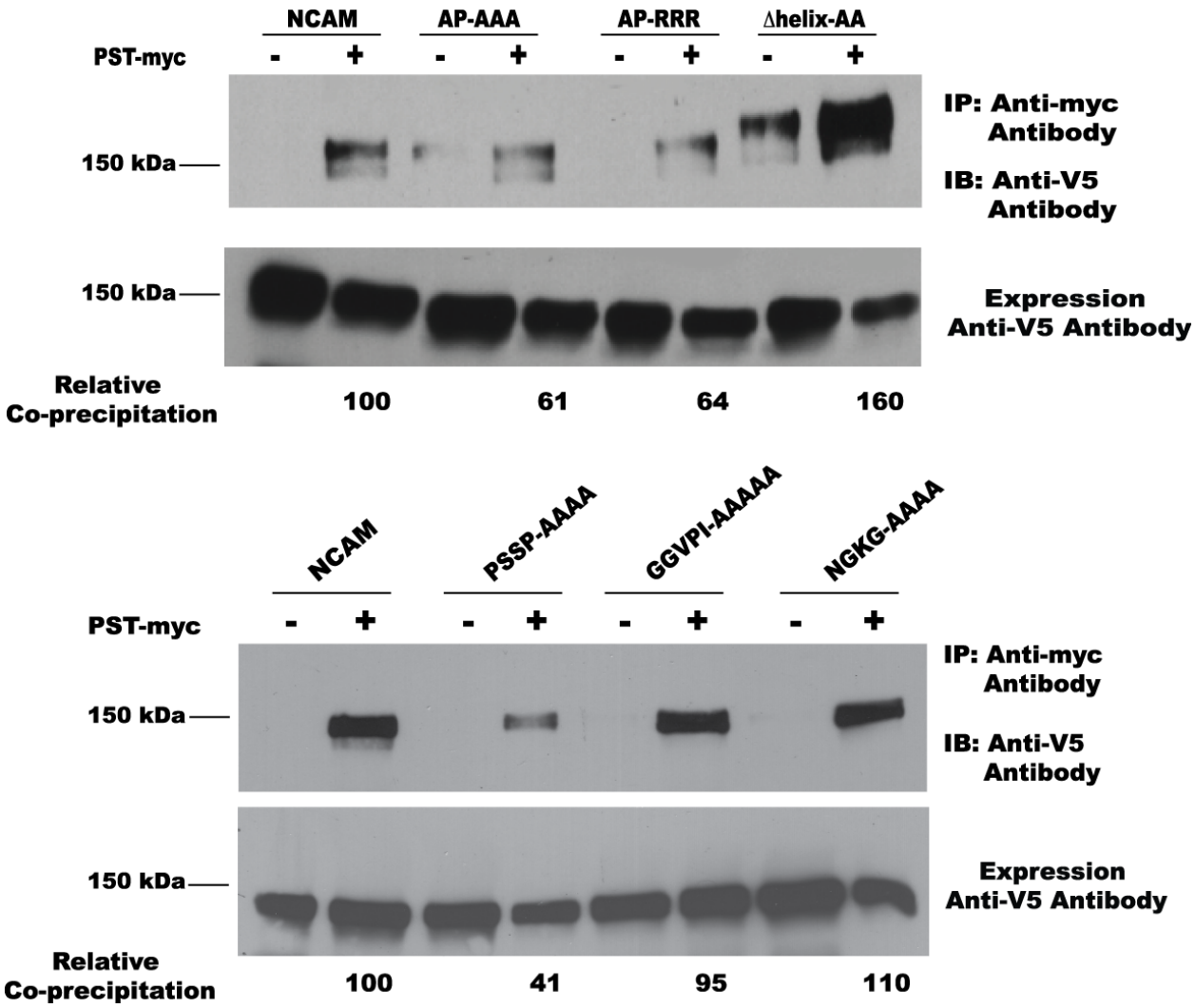

NCAM
AP-AAA
AP-RRR
Δhelix-AA
PST-myc
- + - + - + - +
150 kDa
IP: Anti-myc Antibody
IB: Anti-V5 Antibody
150 kDa
Expression Anti-V5 Antibody
Relative Co-precipitation
100 61 64 160
NCAM
PSSP-AAAA
GGVPI-AAAAA
NGKG-AAAA
PST-myc
- + - + - + - +
150 kDa
IP: Anti-myc Antibody
IB: Anti-V5 Antibody
150 kDa
Expression Anti-V5 Antibody
Relative Co-precipitation
100 41 95 110

Figure 23. (A) Mutations in the FN1 acidic patch partially disrupt the NCAM-ST8SiaIV/PST interaction. *Upper panel,* Lec2 CHO cells were co-transfected with V5-tagged NCAM or NCAM mutants and myc- tagged ST8SiaIV/PST (+lanes) or empty vector (-lanes). Cells were lysed and ST8SiaIV/PST was immunoprecipitated using an anti-myc epitope antibody. Immunoprecipitated proteins were subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody to visualize co-precipitated NCAM proteins. *Lower panel,* to evaluate the relative expression of NCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody. (**B) NCAM proteins with mutations in sequences at the Ig5-FN1 interface common to both NCAM and OCAM FN1 show variable disruption in NCAM-polyST interaction.** The ability of NCAM mutants with PSSP, GGVPI and NGKG sequences replaced with alanine residues to bind ST8SiaIV/PST was compared to that of wild type NCAM as described above. Relative levels of co-immunoprecipitation were calculated for each mutant relative to wild type NCAM and indicated below the immunoblots in (A) and (B). Only the PSSP mutant showed a reduction in NCAM-ST8SiaIV/PST binding and this was comparable to that observed with the acidic patch replacement mutants (AP-AAA and AP-RRR) in (A).

Discussion

In this study I evaluated the effects of replacing sequences common to both NCAM and OCAM FN1 domains in the polysialylation of NCAM. I 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. 20). However, I did observe a distinct decrease in NCAM7 polysialylation when the PSSP sequences were replaced, suggesting these may play a role in polyST recognition. Additional analysis of polyST-NCAM binding demonstrated that replacement of the PSSP sequence but not GGVPI or NGKG led to decrease in polyST-NCAM interaction. Taken together these results suggest that the GGVPI and NGKG sequences are likely to stabilize the Ig5-FN1 linker and the Ig5-FN1 relationship, whereas the PSSP sequence may play a dual role 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) 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, whereas NGKG is a classic β turn. Two hydrogen bonds are predicted to stabilize the NGKG β turn. These include a hydrogen bond between the side-chain carboxyl group of Asn^{580} and the main chain amide of Lys^{582} and a hydrogen bond

between the main chain amide group of Asn^{580} and the main chain carbonyl of Gly^{583} (Fig. 18D). Hydrogen bonds between residues in the GGVPI and NGKG loops and Ig5-FN1 linker region residue Asp^{498} 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 Asp^{498} and the side chain amide group of Asn^{580} (Fig. 19B) and a hydrogen bond between the main chain carbonyl of Gly^{526} in *G*GVPI and the main chain amide of Asp^{498} (Fig. 19C). Finally, a hydrogen bond may form between the amine group in the indole ring of Trp^{417} in Ig5 and the main chain carbonyl of Gly^{581} in the N*G*KG turn that may further stabilize and orient Ig5 and FN1 (Fig. 19E).

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, Val or Pro or Ile in GGVPI) showed little or only partial decreases in polysialylation (data not shown). 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 chain 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 Pro^{500} and Pro^{503}. Although glycine and alanine residues make a region more flexible, proline residues reduce the allowed torsional angles and make a region more rigid (186). 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 (^{496}Q(A/D)TPSSP^{503}) 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 (AAA or GGG) were inserted at the Ig5-FN1 junction (179). Together, these results led 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 polySia attachment sites (188, 189). Work and models by various groups supported the idea that this flexible hinge might be found either between Ig4 and Ig5 (190) or Ig5 and FN1 (188, 191). However, recent crystal structures of the NCAM FN1-FN2 tandem suggest that the flexible hinge may exist between these two domains (192). Our own crystal structure of the NCAM Ig5-FN1 tandem exhibited an extended relationship between these two domains, but other analysis suggested that there may be flexibility in the interdomain relationship (178).

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 (Asn^{449} (Asn^{5}) and Asn^{478} (Asn^{6})) that carry polySia (178). Subsequent analysis of the ability of N-glycans on novel engineered glycosylation sites to be acceptors for polySia demonstrated that glycans further away from the FN1 domain or closer to the unpolysialylated Asn^{423} (Asn^{4}) glycosylation site found on the opposite side of the Ig5 domain could be polysialylated (178). 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.

The data presented in this chapter suggest that sequences at or near the Ig5-FN1 junction and interface are critical for NCAM Ig5 *N*-glycan polysialylation, and suggest that the Ig5-FN1 relationship is relatively rigid. Interestingly, recent crystal structures that allowed the reconstruction of the entire OCAM extracellular portion strongly suggest that in OCAM there is considerable flexibility between the Ig5 and FN1 domains (192). This flexibility may be what is preventing OCAM polysialylation. In sum, these results indicate a model of NCAM polysialylation that requires that the polyST can recognize and bind NCAM FN1, and also requires the correct relative orientation of NCAM Ig5 and FN1 domains to allow for *the* polysialylation of Ig5 N-glycans.

Chapter V

NCAM Ig5 Sequences Play a Role in the NCAM-PolyST Interaction

Introduction

Previous work demonstrated that the FN1 domain was critical to the polysialylation of NCAM and deletion of this domain caused a disruption of the NCAM-polyST interaction (176, 177). Investigation of specific sequences in NCAM FN1 in chapter III revealed that the acidic patch was part of a larger binding region (176), and studies in chapter IV suggested that disruption of the Ig5-FN1 domain relationship could disrupt NCAM polysialylation (179 and Chapter IV). This evidence indicated that the Ig5-FN1 tandem is critical for polysialylation. What was not clear was whether the Ig5 domain contributed to forming a binding region for ST8SiaIV/PST.

Earlier studies using the NCAM-OCAM chimera demonstrated that OCAM FN1 is able to support polyST recognition and polysialylation when used to replace NCAM FN1 (179 and Chapter IV). This not only led me to look for sequences common to NCAM and OCAM FN1 that might mediate polyST recognition and polysialylation (Chapter IV), but also made us wonder why OCAM wasn't polysialylated. Could it be that the FN1 domain of OCAM did not support polysialylation? Or that the Ig5-FN1 relationship in OCAM was not optimal for polysialylation? Or both? In addition, I wondered whether other sequences in the FN1 domain are required for polyST recognition. To test the idea that sequences in the NCAM Ig5 domain contributes to

polyST recognition, I decided to take a reconstitution approach using chimeric proteins containing analogous domains from NCAM and OCAM.

Results

In order to evaluate whether the Ig5 domain is involved in the ST8SiaIV/PST interaction with NCAM, I used a series of OCAM-NCAM chimeras made by Deirdre Foley (Fig. 24). We reasoned that if wild type OCAM is not polysialylated, then we could use it as a background to determine which NCAM Ig5 and FN1 sequences are sufficient for polyST recognition and polysialylation. This approach would also allow us to test the role of each domain in recognition and polysialylation individually and together.

The first step was to see if replacing the OCAM Ig5 domain with that of NCAM would allow polysialylation. Deirdre created three OCAM-NCAM (O-N) chimeras: O-N FN1 with NCAM FN1 replacing OCAM FN1, O-N Ig5 with NCAM Ig5 replacing OCAM Ig5, and O-N Ig5-FN1 with NCAM Ig5-FN1 replacing OCAM Ig5-FN1 (Fig.24). I found that all of the chimeras were efficiently localized at the cell surface like wild type OCAM and NCAM, suggesting than none of these proteins was grossly misfolded (Fig. 25). I analyzed the polysialylation of OCAM and the three chimeras by co-expressing each protein with ST8SiaIV/PST in COS-1 cells, immunoprecipitating the OCAM or OCAM chimeras and evaluating their polysialylation by immunoblotting with the OL.28 anti-polySia antibody (Fig. 26). I found that while OCAM and O-N FN1 proteins were not polysialylated, OCAM could be polysialylated when NCAM Ig5 or Ig5-FN1 were inserted. The sizeable increase in polysialylation observed when both the Ig5 and FN1 domains from NCAM replaced OCAM sequences over the polysialylation of the O-N Ig5 chimera suggested that both the Ig5 and FN1 domains likely contribute to polysialylation (Fig. 26).

Table VI. NCAM Ig5 residue replacements and OCAM Ig5-FN1 sequence replacements described in Chapter V.

Primer	Direction	Template DNA	Primer sequence
NCAM S448K	5’-3’	NCAM	CTGCTGCCAAGCAAGAATTACAGCAATATC
NCAM S448K	3’-5’	NCAM	GATATTGCTGTAATTCTTGCTTGGCAGCAG
NCAM N476R	5’-3’	NCAM, NCAM S448K	GAGAATGATTTTGGGCGCTACAACTGTAC
NCAM N476R	3’-5’	NCAM, NCAM S448K	GTACAGTTGTAGCGCCCAAAATCATTCTC
NCAM S448A	5’-3’	NCAM	CTGCTGCCAAGCGCCAATTACAGCAATATC
NCAM S448A	3’-5’	NCAM	GATATTGCTGTAATTGGCGCTTGGCAGCAG
NCAM N476A	5’-3’	NCAM, S448A	GAGAATGATTTTGGGGCATACAACTGTAC
NCAM N476A	3’-5’	NCAM ,S448A	GTACAGTTGTATGCCCCAAAATCATTCTC
NCAM S448E	5’-3’	NCAM	CTGCTGCCAAGCGAGAATTACAGCAATATC
NCAM S448E	3’-5’	NCAM	GATATTGCTGTAATTCTCGCTTGGCAGCAG
NCAM N476E	5’-3’	NCAM, S448E	GAGAATGATTTTGGGGAGTACAACTGTAC
NCAM N476E	3’-5’	NCAM ,S448E	GTACAGTTGTACTCCCCAAAATCATTCTC
OCAM NQA	5’-3’	OCAM K444S R472N OCAM K444S R472N +Strand	CCCAAGAGTAATACATG GCCTGATTTGAAACAAAC
OCAM NQA	3’-5’	OCAM K444S R472N OCAM K444S R472N +Strand	GTTTGTTTCAAATCAGGC CATGTATTACTCTTGGG
OCAM N520D K521E	5’-3’	OCAM NQA K444S R472N +Strand	GGCAAGATCTCTTTCGAT GAACCCGAATCCCAT
OCAM N520D K521E	3’-5’	OCAM NQA K444S R472N +Strand	ATGGGATTCGGGTTCATT CGAAAGAGATCTTGGC

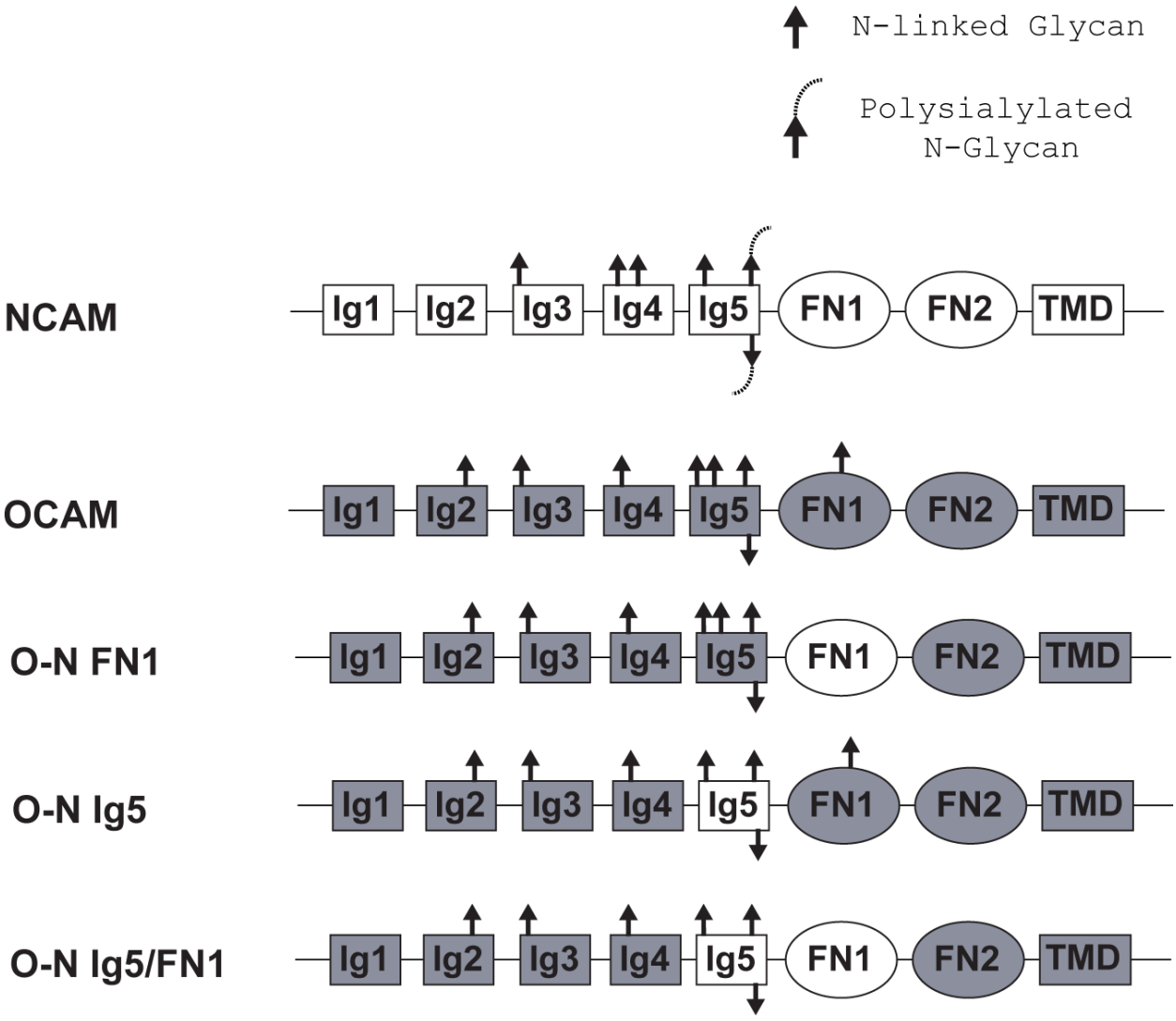


Figure 24. Organization of NCAM-OCAM chimeras. Chimeras were constructed by replacing either the Ig5, FN1 or both domains of OCAM with the analogous NCAM domains. Ig, immunoglobulin domain; FN, fibronectin type III repeat; TMD, transmembrane domain. Arrows indicate sites of N-linked glycosylation. Arrows with extension indicate sites of N-glycan polysialylation.

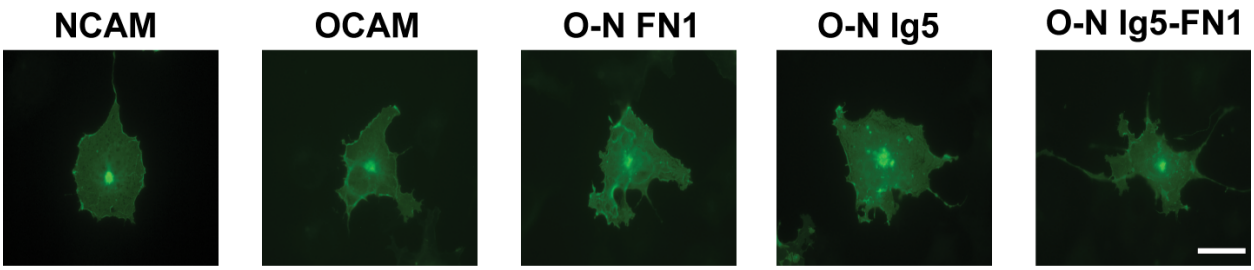


Figure 25. OCAM-NCAM chimeric proteins are localized to the cell surface like wild type OCAM and NCAM. COS-1 cells were grown on coverslips and co-transfected with V5-tagged NCAM or chimera DNA and myc-tagged PST DNA. Cells were allowed to grow overnight and then were fixed and permeabilized with methanol. Cells are incubated with anti-V5 antibody to bind to NCAM/chimera proteins and a FITC-conjugated goat anti-mouse IgG to visualize the proteins. Coverslips are then mounted on slides and examined using a Nikon Axiophot microscope equipped with epifluorescence illumination and a 60X oil immersion Plan Apochromat objective. Bar = 10 µm.

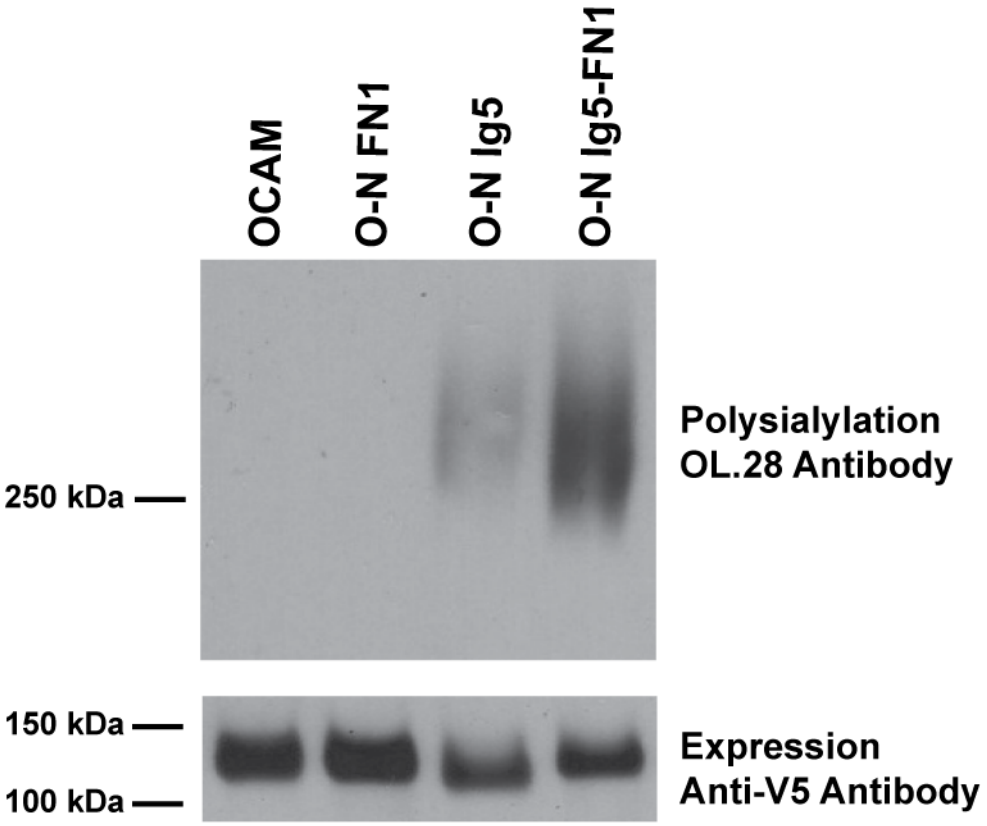


Figure 26. OCAM Ig5 prohibits OCAM polysialylation. *Upper panel,* COS-1 cells were co-transfected with V5-tagged NCAM or an OCAM-NCAM chimera along with myc-tagged ST8SiaIV/PST. NCAM or the OCAM-NCAM chimeras were immunoprecipitated from cell lysates using an anti-V5 epitope antibody. Imunoprecipitated proteins were then separated by SDS PAGE and their polysialylation analyzed by immunoblotting with the OL.28 anti-polySia antibody. *Lower panel*, to evaluate the relative expression of NCAM and the OCAM-NCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody. These data show that inserting NCAM Ig5 into OCAM allows its

polysialylation and that the greatest polysialylation is observed when both Ig5 and FN1 from NCAM are inserted into OCAM.

To determine whether the presence of both domains was critical for polyST recognition, I next evaluated the ability of the OCAM chimeras to bind to the ST8SiaIV/PST. Based on our analysis of chimera polysialylation, I expected to see the greatest interaction between ST8SiaIV/PST and the O-N Ig5-FN1 protein. The analysis of enzyme-chimera binding revealed some interesting results about the ability of the Ig5 and FN1 domains to mediate ST8SiaIV/PST interaction (Fig. 27). I found that wild type OCAM is unable to interact with ST8SiaIV/PST. As expected the OCAM chimera with both NCAM Ig5-FN1 was able to interact with PST better than the other chimeric proteins. This matched with the result showing it to be the most polysialylated of all the O-N chimeras. The OCAM chimera with NCAM FN1 (O-N FN1), which was not polysialylated, interacted slightly with the ST8SiaIV/PST. The OCAM chimera with NCAM Ig5 that was polysialylated showed no interaction with the polyST in this blot however longer exposure does show a minimal interaction with the polyST (data not shown). Taken together these results suggested that the combination of NCAM Ig5-FN1 allowed much greater polyST interaction than either NCAM Ig5 or FN1 alone, and suggested that the polyST may make a direct contact with sequences in Ig5.

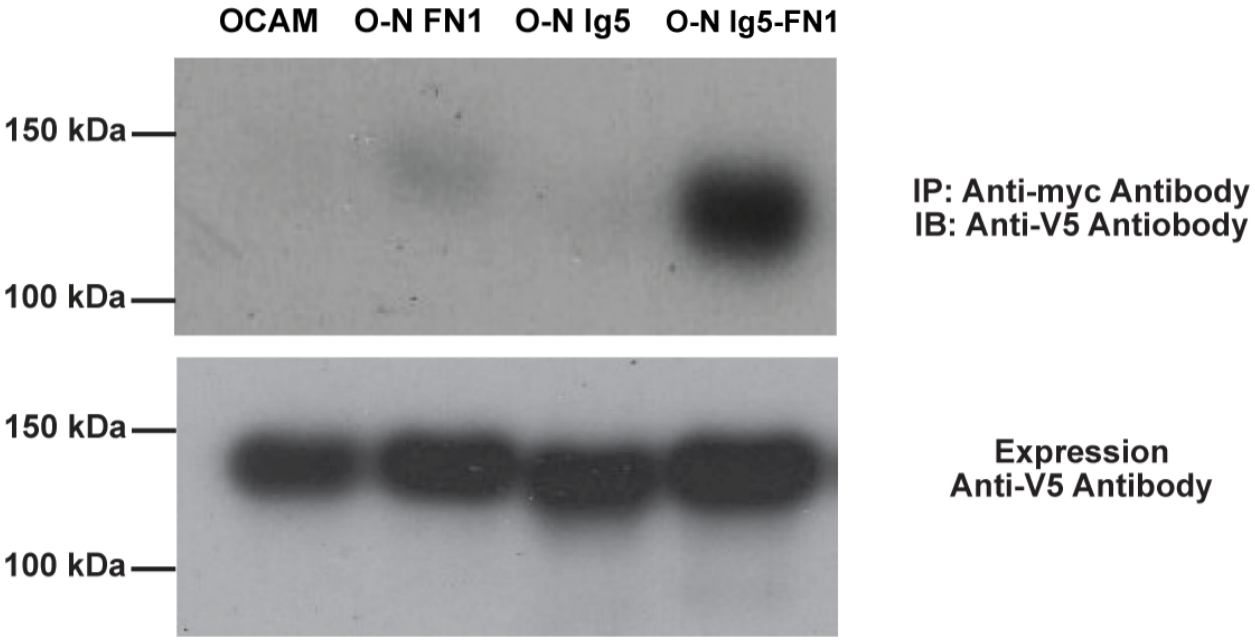


Figure 27. An OCAM-NCAM chimera containing NCAM Ig5 can bind ST8SiaIV/PST, but an OCAM-NCAM chimera containing NCAM Ig5 and FN1 binds the enzyme most strongly. *Upper panel,* Lec2 CHO cells were co-transfected with V5-tagged NCAM, or OCAM-NCAM chimeras and myc-tagged PST. Cells were lysed and ST8SiaIV/PST was immunoprecipitated using an anti-myc epitope antibody. Immunoprecipitated proteins were subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody to visualize co-precipitated NCAM or OCAM-NCAM proteins. *Lower panel,* to evaluate the relative expression of NCAM and OCAM-NCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody.

We next directed our attention towards answering the question of why OCAM Ig5 was blocking polysialylation. Initially, work by Deirdre Foley showed that an extra N-glycan in OCAM Ig5 when inserted into NCAM Ig5 eliminated polysialylation (see Fig. 17 in Chapter IV). However, removal of this extra glycan from OCAM did not allow for OCAM polysialylation (data not shown). Since the FN1 domain of OCAM was shown to allow polysialylation of an NCAM-OCAM chimera (see Fig. 18 in Chapter IV), it was likely that there were key differences in the OCAM Ig5 domain, in addition to the extra N-glycan, that were preventing polysialylation of OCAM. Deirdre began this analysis by comparing the sequences of the NCAM and OCAM Ig5 domains and replacing large segments of NCAM Ig5 with non-homologous segments of OCAM Ig5, however she saw no substantial decreases in NCAM polysialylation (data not shown). Looking more closely at individual residues that differed in the NCAM and OCAM Ig5 domains, we found two residues adjacent to or nearby the two asparagine residues that carry the polysialylated glycans that differed substantially in the two proteins. In NCAM Ig5, Ser^{448} is adjacent to Asn ^{449} (Asn5) (Fig. 28). It is replaced by a lysine residue (Lys^{444} adjacent to Asn^{445}) in OCAM. Also in NCAM Ig5, Asn^{476} is a residue removed from Asn^{478} (Asn6) (Fig. 28). In OCAM Ig5 it is replaced by an arginine residue (Arg^{472} nearby Asn^{474}) in OCAM. Deirdre's preliminary work suggested that replacing Lys^{444} with serine and Arg^{472} with asparagine allowed some polysialylation of OCAM but not as much as if the entire Ig5 domain was replaced by that of NCAM (data not shown).

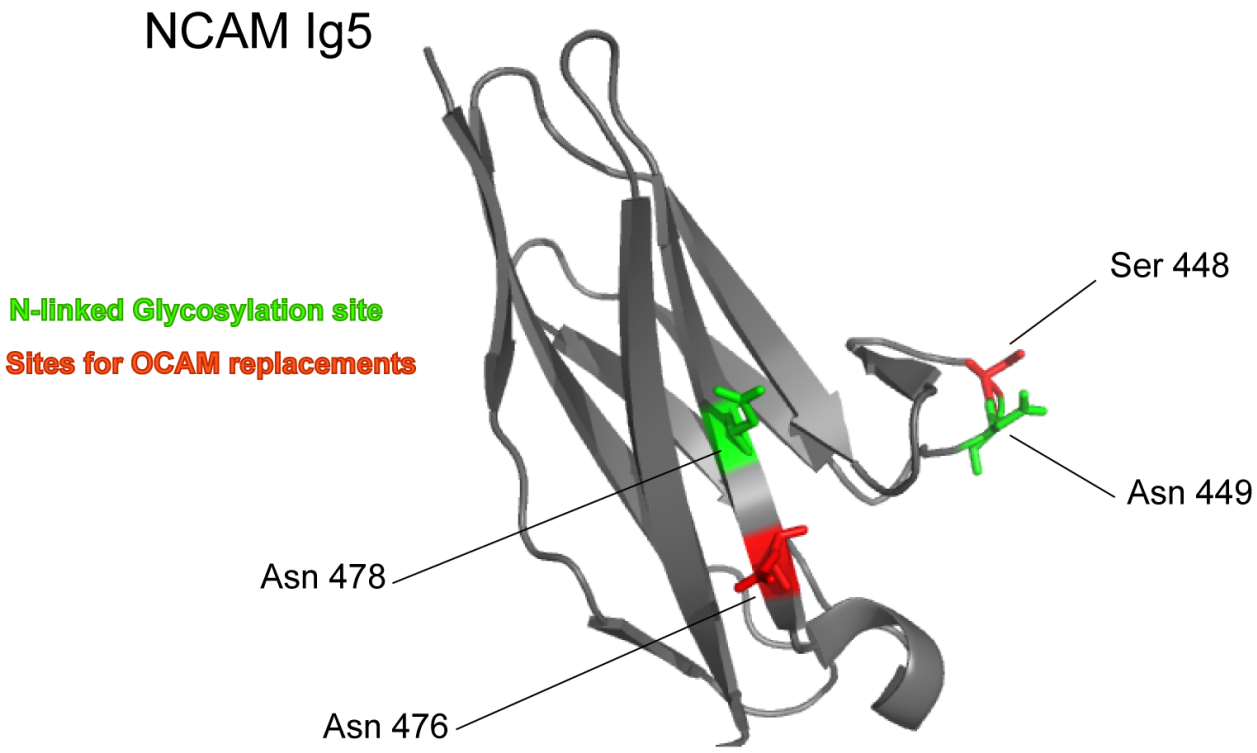


Figure 28. Relative locations of polysialylated N-glycans, Asn 5 (Asn^{449}) and Asn6 (Asn^{478}), in NCAM, and nearby residues that differ in NCAM and OCAM. Ser^{448} adjacent to the Asn5 glycosylation site and Asn^{476} adjacent to the Asn6 glycosylation site in NCAM are replaced by lysine and arginine residues in OCAM Ig5, respectively.

I began my analysis by determining whether replacing NCAM Ig5 Ser^{448} and Asn^{476} with lysine and arginine, respectively, would decrease NCAM polysialylation. In order to determine whether the actual amino acid side chain or the charge of the amino acid was critical, Ser^{448} and Asn^{476} in NCAM Ig5 were also replaced with alanine or glutamic acid residues. These NCAM Ig5 mutants were then tested for both polysialylation by, and binding to, ST8SiaIV/PST (Fig. 29 A and B). Prior to this analysis, I checked the localization of these mutants following expression in COS-1 cells to ensure they were efficiently transported to the cell surface, and found that all of the mutants were localized on the cell surface like wild type NCAM (Fig. 30). I found that replacing of Ser^{448} with Lys^{444} substantially decreased both NCAM polysialylation (Fig. 29A, S448K) and ST8SiaIV/PST binding (Fig. 29 B, S448K). Replacing Asn^{476} with Arg^{472} had an even greater effect on NCAM polysialylation, nearly eliminating it (Fig. 29A, N476R). However, polyST binding was decreased to the same extent as the S488K mutant (by ~50%) (Fig. 29 B). Simultaneously replacing both residues with the OCAM equivalents (S448K N476R) effectively eliminated NCAM polysialylation and reduced polyST binding to approximately 25% of the wild type protein, but did not eliminate it (Fig. 29 B, S448K N476R). Interestingly, replacing both residues with either alanines or glutamic acids (S448A N476A or S448E N476E) did not have a substantial effect on ST8SiaIV/PST binding and did not eliminate NCAM polysialylation (Fig. 29 A and B). However, both the S448A N476A and S448E N276E polysialylated proteins did migrate with a lower molecular mass than polysialylated NCAM, suggesting that these proteins likely have fewer or shorter polySia chains on their N-glycans.

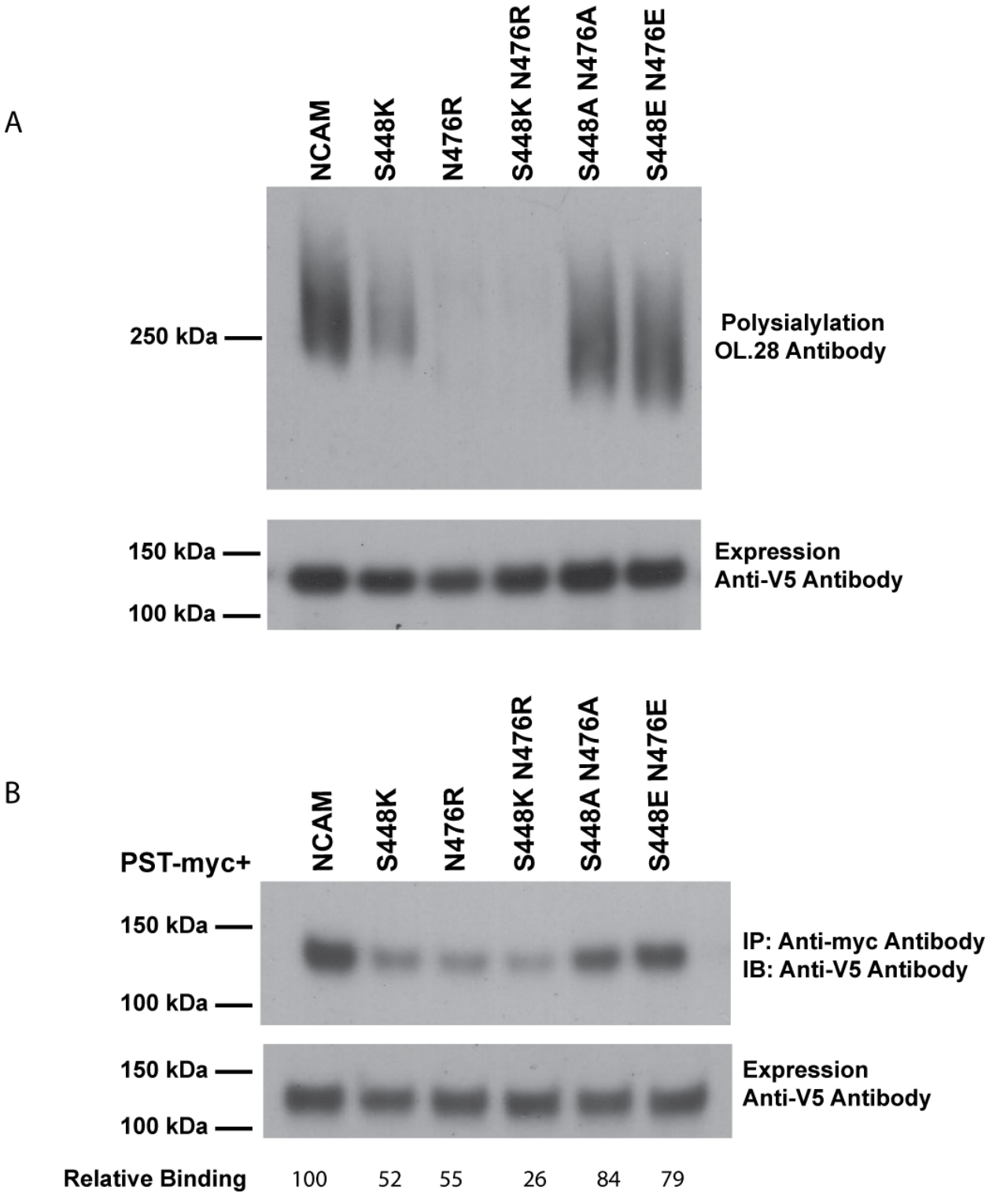

A
NCAM
S448K
N476R
S448K N476R
S448A N476A
S448E N476E
250 kDa
Polysialylation
OL.28 Antibody
150 kDa
100 kDa
Expression
Anti-V5 Antibody
B
PST-myc+
NCAM
S448K
N476R
S448K N476R
S448A N476A
S448E N476E
150 kDa
100 kDa
IP: Anti-myc Antibody
IB: Anti-V5 Antibody
150 kDa
100 kDa
Expression
Anti-V5 Antibody
Relative Binding
100
52
55
26
84
79

Figure 29. (A) Replacing NCAM Ig5 Ser^{448} and Asn^{476} with lysine and arginine residues found in analogous positions in OCAM Ig5 severely decreases polysialylation. Ser^{448} and Asn^{476} in NCAM Ig5 were replaced individually or simultaneously with lysine and arginine, respectively (S448K, N476R, S448K N476R). These residues were also replaced simultaneously with either alanine or glutamic acid residues (S448A N476A and S448E N476E). *Upper panel,* COS-1 cells were co-transfected with V5-tagged NCAM or NCAM mutants and myc-tagged ST8SiaIV/PST. NCAM or NCAM mutants were immunoprecipitated from cell lysates using an anti-V5 epitope antibody. Imunoprecipitated proteins were then separated by SDS PAGE and their polysialylation analyzed by immunoblotting with the OL.28 anti-polySia antibody. *Lower panel*, to evaluate the relative expression of NCAM and NCAM mutant proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody. **B) Co-immunoprecipitation analysis shows that replacing NCAM Ig5 Ser^{448} with lysine and Asn^{476} with arginine disrupts the NCAM-polyST interaction.** *Upper panel,* Lec2 CHO cells were co-transfected with V5-tagged NCAM or NCAM mutants and myc-tagged ST8SiaIV/PST. Cells were lysed and ST8SiaIV/PST was immunoprecipitated using an anti-myc epitope antibody. Immunoprecipitated proteins were subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody to visualize co-precipitated NCAM proteins. *Lower panel,* to evaluate the relative expression of NCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody.

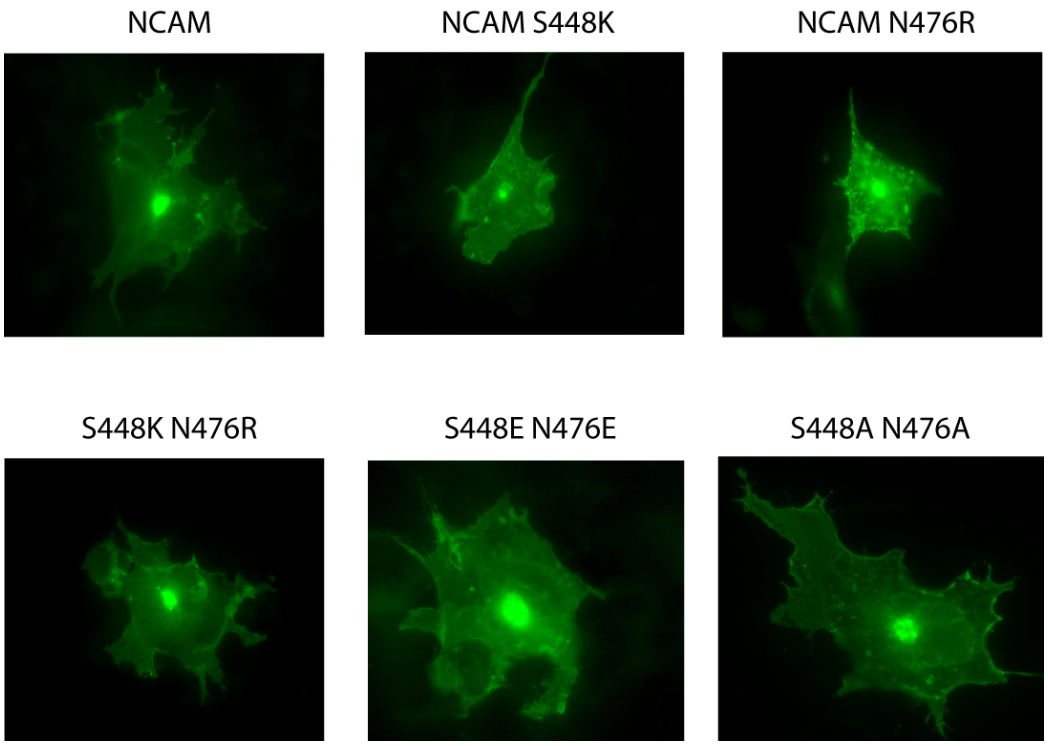


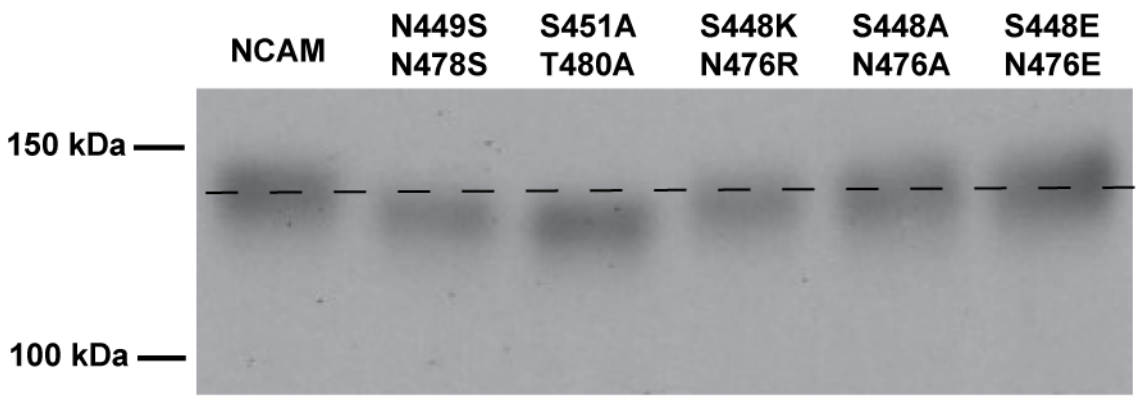
Figure 30. Localization of NCAM and NCAM mutants. COS-1 cells were grown on coverslips and co-transfected with V5-tagged NCAM and myc-tagged PST. Cells were allowed to grow overnight and then were fixed and permeabilized with methanol. Cells are incubated with anti-V5 antibody to bind to NCAM and a FITC-conjugated goat anti-mouse IgG to visualize the proteins. Coverslips are then mounted on slides and examined using a Nikon Axiophot microscope equipped with epifluorescence illumination and a 60X oil immersion Plan Apochromat objective.

These results strongly suggest that the OCAM equivalents of NCAM Ser^{448} and Asn^{476} have a negative effect on OCAM binding and recognition by ST8SiaIV/PST and this compromises subsequent polysialylation. How was this occurring? One possibility was that introduction of a positively charged lysine and arginine residues close to Asn5 and Asn6 glycosylation sites, respectively, may have disrupted glycan attachment to these sites. As a result, the "foundation" for polySia addition was no longer present. In addition, if the glycans on these sites are critical for polyST binding, then if the glycans are no longer present in the S448K and N476R mutants, then polyST binding would be decreased. Essentially, if the glycosylation of Asn5 and Asn6 in NCAM Ig5 is blocked, both polyST interaction and polysialylation could be blocked independently.

To test if the Ser^{448} and Asn^{476} NCAM mutants were being properly glycosylated, we made two types of mutants in each of the two glycosylation sites. In one set of mutants we replaced the asparagine residues with serines (N449S N478S). In the second set of mutants we replaced the third position in each consensus glycosylation site (Asn-X-Ser/Thr) with alanine residues (S451A T480A). We then expressed these V5-tagged mutants in COS-1 cells, immunoprecipitated using an anti-V5 antibody, and subjected them to SDS PAGE (4-15% gradient gel) and immunoblotting to determine whether the Ser^{448} and Asn^{476} mutants exhibited changes in molecular weight suggestive of the absence of these two N-glycans (Fig. 31 A). The Ser^{448} and Asn^{476} mutants as well as mutants in N-linked glycosylation were also tested for their ability to interact with ST8SiaIV/PST (Fig. 31 B). These mutants were transfected into Lec2 CHO cells with myc-tagged PST and were co-immunoprecipitated as described above. I found, as expected, that the glycosylation site mutants led to a caused a decrease in molecular

weight due to elimination of the glycans at site 5 and 6 (Fig. 31 A, N449S N478S and S451A T480A). Mutation of Ser^{448} and Asn^{476} to lysine and asparagine as found in OCAM Ig5 did not lead to a downshift in NCAM molecular weight (Fig. 31 A, S448K N476R). Neither did replacing these residues with alanines or glutamic acids (Fig. 30A, S448A N476A and S448E N476E). From this we concluded that the OCAM replacements that caused both a decrease in polysialylation and polyST interaction did not do so by disrupting the glycosylation of Asn5 and Asn6 in the Ig5 domain.

A


NCAM
N449S
N478S
S451A
T480A
S448K
N476R
S448A
N476A
S448E
N476E
150 kDa
100 kDa

B

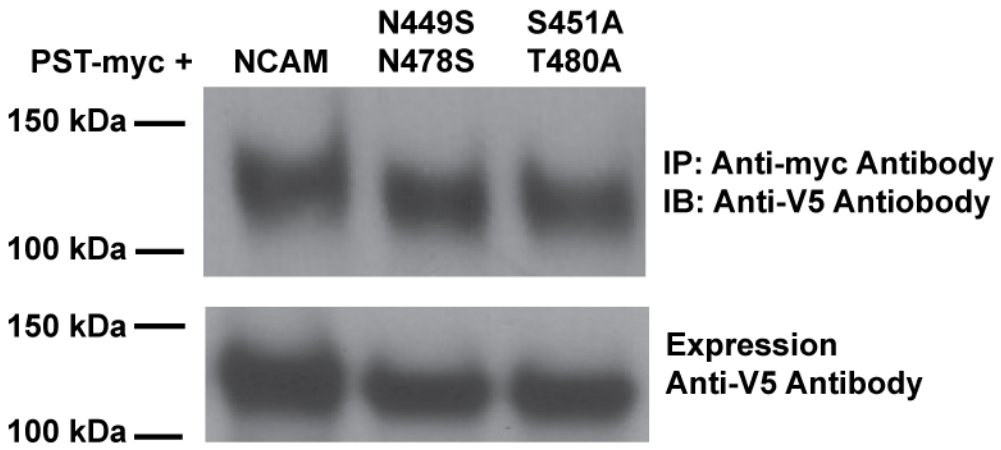

PST-myc +
NCAM
N449S
N478S
S451A
T480A
150 kDa
100 kDa
IP: Anti-myc Antibody
IB: Anti-V5 Antiobody
150 kDa
100 kDa
Expression
Anti-V5 Antibody

Figure 31. (A) Replacing NCAM Ig5 Ser^{448} and Asn^{476} with lysine and arginine residues found in analogous positions in OCAM Ig5 does not compromise glycosylation of Asn^{449} (Asn5) and Asn^{478} (Asn6). The molecular weight of NCAM mutants lacking the Asn-X-Thr/Ser consensus glycosylation sites for Asn^{449} (Asn5) and Asn^{478} (Asn6), N449S N478S and S451A T480A (third position mutant), was compared to Ser^{448} and Asn^{476} replacement mutants to determine whether altering these residues compromised Asn5 and Asn6 glycosylation. COS-1 cells were transfected with V5-tagged NCAM or NCAM mutants. Cells were then lysed and immunoprecipitated with an anti-V5 antibody. Samples were then separated on 4-15% SDS gels and transferred to nitrocellulose membranes. Nitrocellulose membranes were blotted with anti-V5 antibody to detect the NCAM proteins. A size shift due to loss of N-glycans is not seen in any of the NCAM mutants where Ser^{448} and Asn^{476} are replaced. **B) NCAM N-glycans on Asn5 and Asn6 are not necessary for NCAM-polyST interaction.** *Upper panel,* Lec2 CHO cells were co-transfected with V5-tagged NCAM or NCAM glycosylation site mutants and myc-tagged ST8SiaIV/PST. Cells were lysed and ST8SiaIV/PST was immunoprecipitated using an anti-myc epitope antibody. Immunoprecipitated proteins were subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody to visualize co-precipitated NCAM proteins. *Lower panel,* to evaluate the relative expression of NCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody.

Next I determined whether the absence of the Ig5 N-glycans on Asn5 and Asn6 altered NCAM-ST8SiaIV/PST binding taking the co-immunoprecipitation approach described above. I found that direct replacement of Asn5 and Asn6 with serine residues or mutating the third site in the Asn-X-Thr/Ser consensus sequence that lead to a loss of N-glycans at these sites did not impact the ability of NCAM to interact with ST8SiaIV/PST as shown in Figure 30B. This result shows that the polyST does not require these N-glycans for binding to NCAM. Taken together, the results shown in this chapter suggest that the OCAM Ig5 lysine and arginines residues that replace NCAM Ig5 Ser^{448} and Asn^{476} are blocking polyST-OCAM interaction and subsequent polysialylation.

The NCAM Ig5 residues Ser448 and Asn476 appear to be necessary for polyST recognition and NCAM polysialylation, but could the absence of these residues and the presence of basic, positively charged residues in addition to the "extra" N-glycan in OCAM Ig5 be the only factors blocking OCAM polysialylation? Recently, Kulahin et al. (193) obtained a series of crystal structures of OCAM fragments that allowed them to assemble a composite structure of an entire OCAM extracellular domain. Their structures suggested that there is likely considerable flexibility in the linker between Ig5 and FN1 in OCAM. In addition, two of the three core acidic patch residues are missing from OCAM FN1. I wanted to determine whether removing the extra Ig5 N-glycan and then adding back some or all of these sequences would lead to OCAM polysialylation (Fig. 32).

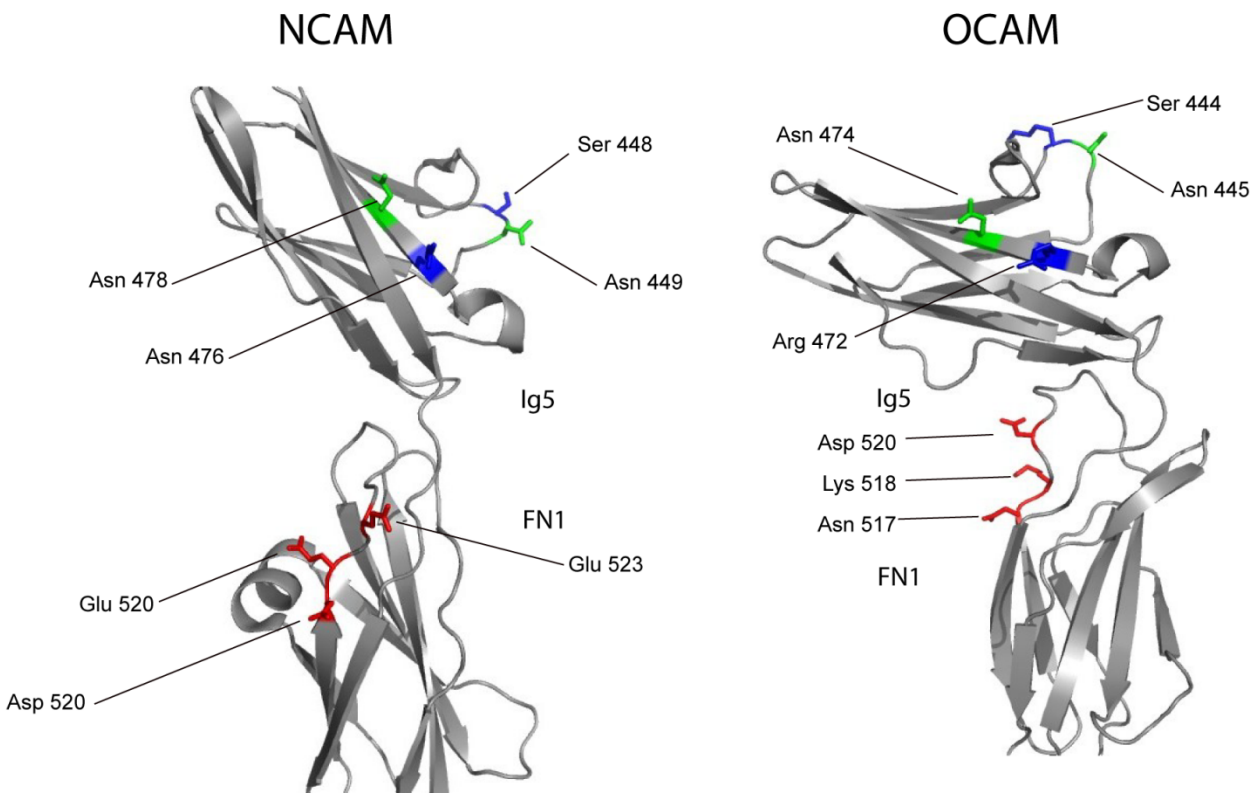


Figure 32. Location of Ig5 and FN1 sequences replaced in OCAM to reconstitute polysialylation. Sites in NCAM and the corresponding residues in OCAM are shown in the above figure. Ser^{448} and Asn^{476} in NCAM Ig5 correspond to Ser^{444} and Arg^{472} in OCAM Ig5. Acidic residues in NCAM FN1 correspond to the residues in OCAM highlighted in red. In OCAM only Asp^{520} is acidic. NCAM structure (170, PDB ID:3MTR). OCAM structure (184, PDB ID: 2XYC).

I began by replacing residues Lys^{444} with serine and Arg^{472} with asparagine in OCAM Ig5. These changes and all the mutations I make in this section were made in an OCAM construct in which the extra N-glycan consensus sequence of NQT was changed to NQA, because this N-glycan, when its attachment site was inserted into NCAM, blocked NCAM polysialylation (178). Changing these Ig5 residues in the NQA mutant led to a very small amount of OCAM polysialylation that was only apparent after a long exposure of the immunoblot (Fig. 33) Considering the possibility that the NCAM Ig5-FN1 relationship may be more rigid than that of OCAM, I replaced the strand connecting OCAM Ig5 and FN1 with the equivalent NCAM strand in the context of the NQA/K444S/A472N mutant to determine how this impacted the polysialylation of OCAM (Fig. 33). This seemed to improve the polysialylation of OCAM over that observed in the NQA/K444S/R472N mutant (Fig. 33, NQA/K444S/R472N/Strand), Finally, converting OCAM FN1 NKPE to the NCAM core acidic patch (DEPE) in the context of the other changes led to a further increase in polysialylation of the OCAM mutant (Fig. 33, long exposure, NQA/K444S/R472N/Strand/NKPE-DEPE). However, none of these changes led to the level of polysialylation observed when NCAM Ig5-FN1 replaces the equivalent domains in OCAM (Fig. 33, short and long exposure, O-N Ig5-FN1). These results demonstrate that all these sequences play a role in polysialylation, however in aggregate they still cannot recapitulate the polysialylation of intact NCAM Ig5-FN1 in the context of the OCAM protein.

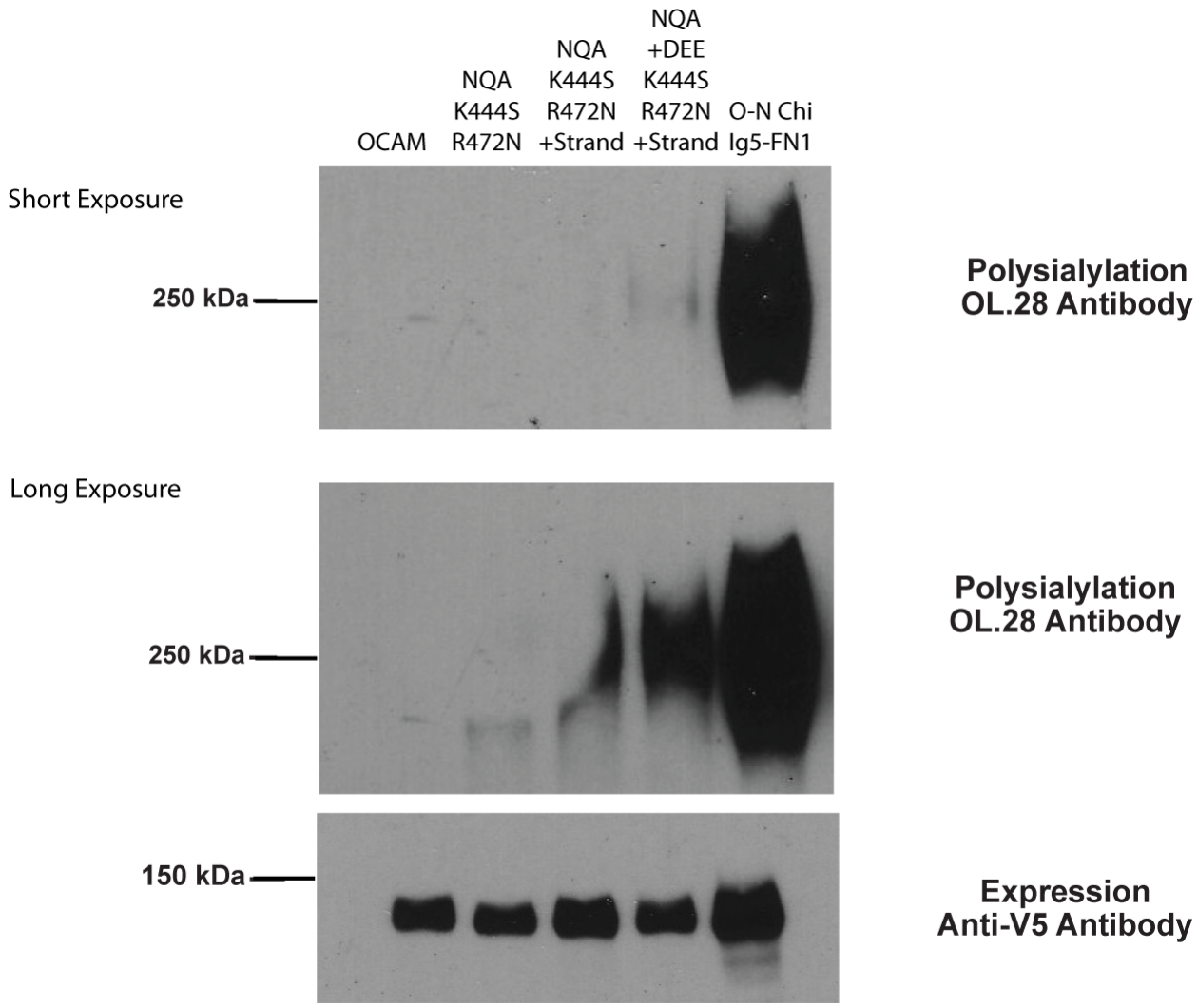

OCAM
NQA K444S R472N
NQA K444S R472N +Strand
NQA +DEE K444S R472N +Strand
O-N Chi Ig5-FN1
Short Exposure
250 kDa
Polysialylation OL.28 Antibody
Long Exposure
250 kDa
Polysialylation OL.28 Antibody
150 kDa
Expression Anti-V5 Antibody

Figure 33. Sequential addition of NCAM residues back into OCAM increases the polysialylation of OCAM but not to the same extent as replacing OCAM Ig5-FN1 with NCAM Ig5-FN1. *Upper panels,* COS-1 cells were co-transfected with V5-tagged OCAM or OCAM mutants and myc-tagged ST8SiaIV/PST. Cells were lysed and ST8SiaIV/PST was immunoprecipitated using an anti-myc epitope antibody. Immunoprecipitated proteins were subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody to visualize co-precipitated NCAM proteins. The first blot was exposed for 30 seconds (Short exposure). The second blot shows a 2 min exposure (Long exposure). *Lower panel,* to evaluate the relative expression of NCAM proteins, one tenth of the cell lysate was subjected to SDS PAGE and immunoblotting with an anti-V5 epitope antibody. Note all the OCAM mutants have the extra Ig5 N-glycan removed.

Discussion

In this study, I evaluated the effects of replacing OCAM domains with equivalent NCAM domains to investigate the Ig5-FN1 relationships effects on polysialylation. OCAM was chosen as the background for these reconstitution studies because it shares a fair amount of identity (44%) with NCAM but is not polysialylated endogenously (193). In NCAM-OCAM chimeras replacing the NCAM FN1 domain with the OCAM FN1 domain allowed for N-linked polysialylation (Fig. 18). This indicated that something in OCAM was actively prohibiting polysialylation of N-linked glycans in the protein.

Deirdre Foley had previously shown that an extra N-linked glycan found in the Ig5 domain of OCAM on Asn^{432}, disrupted NCAM disrupted polysialylation when its attachment site was inserted into the NCAM sequence. However removing this N-glycan site from OCAM did not restore polysialylation. This suggested that in addition to the non-permissive N-glycan, other factors in OCAM Ig5 block polysialylation. We discovered that two OCAM Ig5 residues, Lys^{444} and Arg^{472} located close to the 7th and 8^{th} glycosylation site in OCAM (equivalent to the 5th and 6^{th} glycosylation sites in NCAM) are replaced with neutral Ser and Asn residues in NCAM. When these basic residues were used to replace the neutral residues in NCAM (Ser^{448} and Asn^{476}) NCAM polysialylation was substantially reduced (Fig. 29). However, replacing OCAM Lys444 and Arg472 with serine and asparagine residues, respectively, in the absence of the non-permissive extra Ig5 N-glycan, did not allow polysialylation of OCAM (Fig. 33).

A possible clue as to why OCAM is not polysialylated comes from the recent structures of OCAM suggest that the linker between Ig5 and FN1 may be very flexible (195), This flexibility of the OCAM Ig5-FN1 tandem may weaken or prohibit simultaneous interaction of the polyST

with both the FN1 and Ig5 domains thereby blocking OCAM polysialylation. Removing the extra Ig5 N-glycan, inserting the two neutral Ig5 residues, and replacing the last β strand in Ig5 that links to FN1 allowed for only a small amount of OCAM polysialylation (Fig. 33). However, recreating the full acidic patch in the OCAM FN1 domain enhanced polysialylation O-N Ig5-FN1 chimera., but this was not comparable to that observed for the O-N Ig5-FN1 chimera.

The work presented in this chapter also revealed critical details of the NCAM Ig5-FN1 relationship that allow for polysialylation. From chimera studies, I found that the NCAM Ig5-FN1 tandem interacts more readily with the polyST and is better polysialylated than the O-N Ig5 chimera that has the OCAM FN1 domain and NCAM Ig5 domain. Work represented in this chapter shows that the Ig5-FN1 tandem is critical for polysialyltransferase recognition and NCAM polysialylation and for the first time shows that the polySTs make a protein-protein contact with both the Ig5 and FN1 domains. Additionally, my results highlight differences in both OCAM Ig5 and FN1 domains that compromise OCAM polysialylation.

Chapter VI

Concluding Remarks

NCAM is a cell adhesion protein that is the major carrier of polySia in mammals. The addition of polySia to NCAM in the correct spatio-temporal manner is critical in nervous system development, and in learning and memory, neuronal regeneration and synaptic plasticity in adult animals. Incorrect expression of polysialylated NCAM has been implicated in neuropsychiatric disorders and tumor growth and invasiveness. Although polysialylated NCAM is involved in many important functions, the specificity of the NCAM-polysialyltransferase interaction that generates polysialylated NCAM is still being explored.

Prior work in our lab had implicated the first fibronectin type III repeat (FN1) to be critical to the NCAM-polyST interaction. Molecular modeling and structural analysis of NCAM FN1 revealed features not seen in other FN1 structures. These included an α-helix and an acidic surface patch. Mutational analysis of these regions showed that the loss of the acidic patch correlated with a decrease in NCAM polysialylation, where replacement of the α helix caused a switch of polysialic acid addition from N-linked glycans in the Ig5 domain to O-linked glycans in the FN1 domain.

The goal of my thesis work was to further examine the interaction of NCAM with the polySTs and to determine regions of NCAM critical for proper polysialylation. Using mutational analysis and chimeric proteins, I investigated the role of FN1 and Ig5 sequences in the polysialylation of NCAM.

The NCAM FN1 domain and acidic sequences in this domain are required for polyST recognition/binding and NCAM polysialylation. In Chapter III, I used co-immunoprecipitation experiments to show that a minimal NCAM construct consisting of just FN1 and FN2 can in interact with the polyST. This implied that the interaction site for the polyST resides in FN1 and that the transmembrane region is not needed for this interaction. Further co-immunoprecipitation experiments using a ΔFN1 NCAM construct showed no interaction with the polyST. Previous work in the lab had shown that NCAM FN1 had several unique sequences including; the α-helix, an acidic patch comprised of residues Asp^{509}, Asp^{520}, Glu^{521}, Glu^{523}, and two sequences Pro^{510}, Tyr^{511}, Ser^{512} (PYS), Gln^{516}, Val^{517}, Gln^{518} (QVQ) that when mutated affected polySia addition to NCAM.

I evaluated the ability of mutants, in which these identified FN1 sequences were replaced, to bind to the ST8SiaIV/PST polyST using co-immunoprecipitation experiments as above. I found that replacing the residues of the acidic patch with alanine or a charge switch mutation to arginine led to a decrease but did not eliminate the NCAM-polyST interaction. Replacing either the α-helix or QVQ, which had shifted polySia to O-linked glycans, did not disrupt the NCAM-polyST interaction. Mutation of PYS also had no effect on the interaction.

These results suggest that the acidic patch in NCAM FN1 is part of the binding region for the polySTs, but it is not the sole determinant. To investigate if residues outside of the FN1 domain are involved in the polyST interaction, I evaluate the impact of mutating the acidic patch residues in the truncated NCAM7 construct that lacked the Ig domains (FN1-FN2-TM-tail). I found that replacing the acidic patch in NCAM7 more severely compromised its polysialylation

than it did for full length NCAM. The AP-AAA mutation greatly decreases NCAM7 O-glycan polysialylation, while the AP-RRR mutation eliminates its polysialylation. In full length NCAM these mutants decreased but did not eliminate polySia addition. This was a first indication that sequences outside the FN1 domain may play a role in the recognition of full length NCAM by the polySTs.

In sum results from this study confirmed that the FN1 domain is necessary for the NCAM-polyST interaction, but also indicated that this domain may not represent the whole polyST binding region. My results also demonstrated that while the acidic patch is part of the binding region, the other sequences (α-helix, QVQ, PYS) played other roles. For example, the α–helix and QVQ may alter the binding of the polyST placing the Ig5 N-glycans out of its range. Alternatively, replacing these sequences may have altered the Ig5-FN1 relationship leading to the same change in the location of polySia. The results of mutating the PYS sequence are not as clear, indicating that this sequence has a role in O-linked polysialylation or causes a disruption of the FN1 structure. Our initial model of the NCAM-polyST interaction seen in Figure 10 simply predicted that interaction of the polyST with FN1 led to polysialylation of Ig5 N-glycans. I found that other sequences in the FN1 domain besides the acidic patch that is likely to play a role in polyST binding, also impact the recognition and polysialylation of NCAM. The possible roles of all these FN1 residues are summarized in Figure 34.

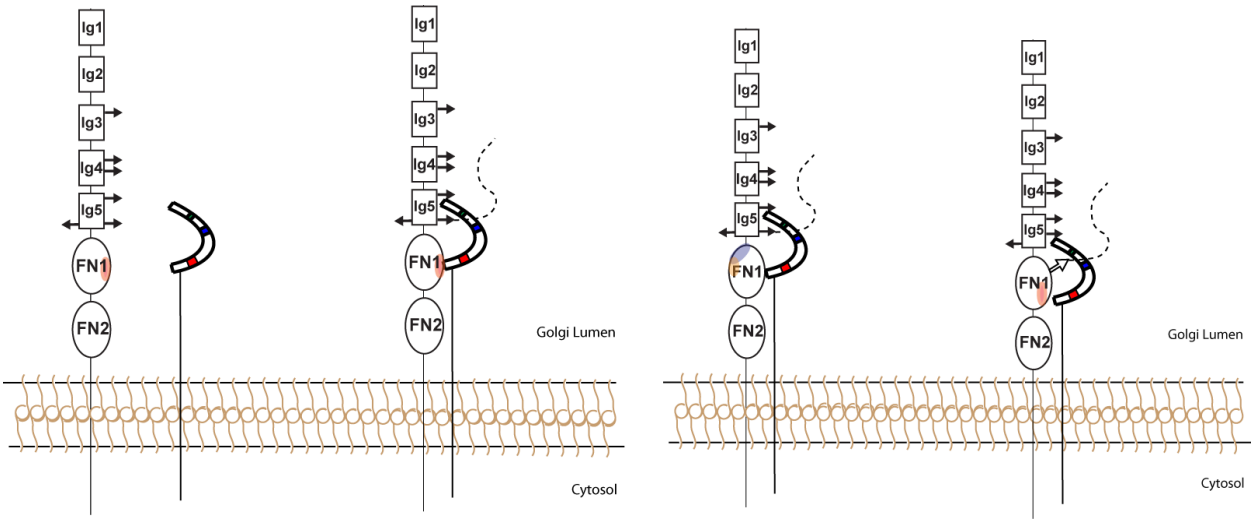


Figure 34. Effects of unique FN1 sequences on NCAM polysialylation.

A) NCAM FN1 contains an acidic patch (red oval) that acts as part of the binding region for the polyST. Additional sequences in Ig5 may also play a role in this interaction.

B) Left side, NCAM FN1 α-helix (blue oval) and QVQ (orange oval) may affect the relationship of the Ig5 and FN1 domain to allow for proper N-linked polysialylation of N-linked glycans (solid arrows) in NCAM Ig5. *Right side,* Replacement of either of these sequences leads to the addition of polySia on available O-linked glycans in FN1 (unfilled arrow). One possibility is that these sequences are required for restraining the polyST interaction with the acidic patch and in their absence the polyST binds in a different way and is not able to access the Ig5 N-glycans and polysialylates on available O-linked glycans in FN1. An alternative possibility is that replacement of these sequences causes a mis-alignment of Ig5-FN1 but does not disrupt the polyST interaction with FN1. This misalignment makes N-linked glycans in Ig5 inaccessible.

Sequences common between NCAM and OCAM FN1 are critical to maintaining the Ig5-FN1 relationship.

Results from Chapter III suggested that other sequences in addition to the acidic patch were involved in the interaction of NCAM with the polyST. I followed up on this possibility in Chapters IV and V. Work by Deirdre Foley in the lab found that the first fibronectin type III domain of unpolysialylated OCAM was able to replace the FN1 domain of NCAM to allow proper N-linked polysialylation of Ig5 glycans. Although OCAM is not polysialylated endogenously, this new finding indicated that sequences common between the two domains may be involved in interaction with the polyST. OCAM does not have sequences equivalent to the NCAM α-helix and did not have a complete acidic patch, but it did have three regions comprised of 4 or more amino acids that were identical to those in NCAM. These sequences were: $Pro^{500}Ser^{501}Ser^{502}Pro^{503}$ (*PSSP*), $Gly^{526}Gly^{527}Val^{528}Pro^{529}Ile^{530}$ (*GGVPI*), and $Asn^{580}Gly^{581}Lys^{582}Gly^{583}$ (*NGKG*).

When we mapped these sequences onto the crystal structure of NCAM Ig5-FN1 we found that they were located near the interface of the two domains. In addition, the PSSP sequence was adjacent to the acidic patch on the FN1 surface. I mutated the residues in all three sequences to alanine or glycine and examined the effects on polysialylation. Mutation of any common sequence caused an elimination of NCAM polysialylation. Mutation of homologous NCAM/OCAM sequences away from the ig5-FN1 interface did not show this loss of polysialylation.

Mutation of single residues in these regions could not replicate the loss of polySia seen when all residues were replaced. The exception to this was that replacement of the two proline residues in the PSSP sequence caused a decrease in polysialylation comparable to replacement

of the entire sequence. This would indicate that loss of polySia in this mutant is due to a change in conformation upon replacing necessary proline residues.

Since all three sequences were near the interface of Ig5 and FN1 it seemed likely that the effects seen on polysialylation were due to a change in the relationship of the Ig5 and FN1 domains when these sequences were replaced. To test this, I used the NCAM7 construct that does not possess an Ig5 domain. We reasoned that since this construct is polysialylated on O-linked glycans in FN1, the mutation of common sequences would not have an effect on polysialylation of this construct. As seen in Figure 19 C, mutation of the common sequences that eliminated full length NCAM polysialylation had little to no effect on O-linked polysialylation of FN1 in the NCAM7 protein. Mutation of the PSSP sequence did cause a slight decrease of NCAM7 polysialylation. Due to its location near the acidic patch mutation of this sequence may have somewhat disrupted the NCAM-polyST interaction. I examined the binding of NCAM with these common sequences replaced to ST8SiaIV/PST polysialyltransferase and found that only mutation of the PSSP sequence showed a decrease in binding. This correlated with the effects seen in the NCAM7 mutants and indicates that PSSP is either directly part of the binding region or impacts the binding region due to its close proximity to the FN1 surface acidic patch.

The likely cause for the loss of polysialylation in the NCAM/OCAM common sequence mutants is a disruption of the Ig5-FN1 tandem relative orientation. Residues in GGVPI and NGKG form loops that likely stabilize the Ig5-FN1 interaction through main chain hydrogen bonding. The PSSP sequence is part of the linking strand connecting Ig5 to FN1. I have shown

that the two proline residues in this sequence are critical for maintaining NCAM polysialylation. Disruption of these prolines likely increased the flexibility between the two domains and compromising the polyST's access to the Ig5 N-glycans.

These results are incorporated into my model of NCAM-polyST interaction in Figure 35. Replacing QVQ or the α-helix may have allowed the polyST to bind in such a way that only FN1 O-glycans can be polysialylated. In contrast, replacing the NCAM/OCAM common sequences likely destabilized the Ig5-FN1 linker region and altered the orientation of Ig5 relative to FN1. In this case, the polySTs still bind FN1, but the Ig5 N-linked glycans are no longer accessible to the bound polyST and no polysialylation occurs.

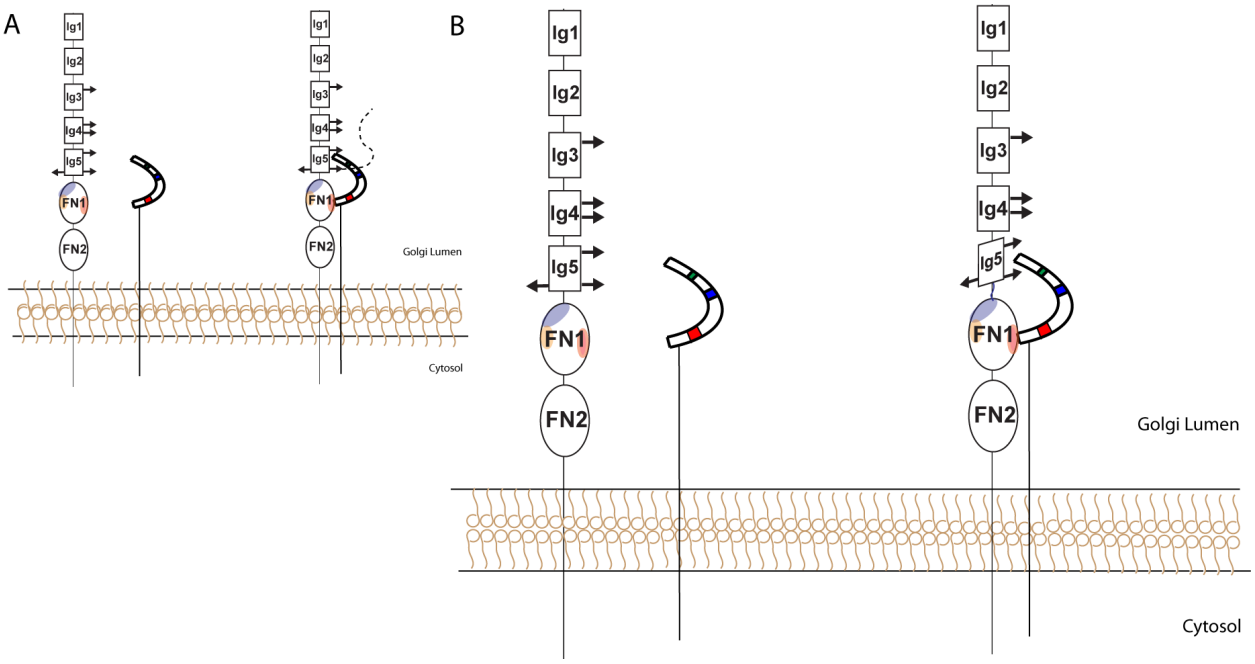


Figure 35. Sequences common between NCAM and OCAM FN1 hold Ig5-FN1 in the correct orientation for polysialylation of N-linked Ig5 glycans.

A) Model of NCAM-polyST interaction with acidic patch region (red oval) and the QVQ (orange oval) and α-helix (blue oval) allowing for correct Ig5-FN1 alignment.

B) Replacing the NCAM/OCAM FN1 common regions disrupts the Ig5-FN1 orientation without altering the polyST interaction with NCAM FN1. This alters the location of Ig5 N-glycans relative to the FN1-bound polyST and eliminates polysialylation.

The role of Ig5 sequences in the NCAM-polyST interaction.

In Chapter IV, I investigated the effects of replacing OCAM Ig5 or FN1 with the analogous domains from NCAM. This allowed us to examine the relative contributions of each domain to polyST interaction and polysialylation. These O-N chimeric proteins also allowed for the investigation of specific residues in Ig5 that may affect NCAM polysialylation.

An O-N chimeric protein in which OCAM Ig5 was replaced with NCAM Ig5 showed increased polysialylation over wild type OCAM. However, replacing OCAM Ig5-FN1 with the equivalent NCAM domains (O-N Ig5-FN1) showed even greater polysialylation. Interestingly, replacing OCAM FN1 with NCAM FN1 did not enhance chimera polysialylation, but did increase the interaction with the polyST. This result was intriguing, because the N-O FN1 chimera, where OCAM FN1 was placed into an NCAM background, interacted with polyST like wild type NCAM. This suggested that that OCAM FN1 can interact with the polyST in the NCAM context, but in wild type OCAM it does not.

This result, along with previous data showing that an extra N-glycan in OCAM Ig5 when inserted into NCAM Ig5 inhibited polysialylation, caused us to look into what in OCAM, besides the non-permissive "extra" Ig5 N-glycan may be preventing polysialylation. We found that removing the extra N-glycan from OCAM Ig5 was not enough induce polysialylation of OCAM, and this implied that other aspects of the OCAM Ig5 domain were blocking polysialylation.

We identified two large basic residues (Lys^{444} and Arg^{472}) in OCAM Ig5 nearby the asparagines that are equivalent to those that carry the polysialylated N-glycans in NCAM. In NCAM, the equivalent residues are small and neutral (Ser^{448} and Asn^{476}). I replaced NCAM Ser^{448}

and Asn^{476} with the OCAM equivalents (S448K and N476R) and examined the effect on NCAM polysialylation and interaction with the polyST. Replacing either Ig5 residue decreased polysialylation and binding, with N476R mutant showing the greatest effect on both. Replacing both residues showed a severe decrease in polysialylation and binding (25% of wild type). When Ser^{448} and Asn^{476} were replaced with alanines or glutamic acid residues these effects were not observed, but the polysialylated NCAM proteins migrated with slightly lower molecular weights suggesting that fewer or shorter polySia chains may have been added to these proteins. Interestingly, these mutants bound at an intermediate level between the S448K N476R mutants and wild type NCAM. Notably, the effects on binding and polysialylation I observed were not due to a loss of glycosylation at the asparagines near to the basic residues, nor did a loss of binding reflect a change in the N-glycan orientation because I found that the Asn5 and Asn6 N-glycans are not required for polyST binding to NCAM. Taken together, these results indicate that large, basic residues in these positions in the Ig5 domain are particularly detrimental to the NCAM-polyST interaction and strongly suggest that the polyST not only interacts with the NCAM FN1 domain, but also sequences in the Ig5 domain.

Based on these results, I attempted to reconstitute the polysialylation of OCAM by replacing both Lys^{444} and Arg^{472} with the NCAM equivalents (Ser and Asn). I found that this allowed barely detectable levels of polysialylation. However, more polysialylation was observed if I also replaced the final Ig5 β strand linking Ig5 to FN1 with that from NCAM, and recreated the entire acidic patch in the OCAM FN1 domain. However, I was never able to achieve the same level of polysialylation as that observed for the O-N Ig5-FN1 chimera. Taking a step back, I attempted to add critical residues to the O-N chimeric proteins to see if we could enhance

polysialylation or polyST interaction. I recreated the FN1 acidic patch in the O-N Ig5 chimera and observed a slight increase in polysialylation and polyST binding over O-N Ig5. In the O-N FN1 chimera replacement of Lys^{444} and Arg^{472} with NCAM equivalents did not increase polysialylation but did increase binding to levels seen in the O-N Ig5-FN1 chimera. These results were further indication that the acidic patch is part of the binding region for the polyST and that residues Lys^{444} and Arg^{472} specifically inhibit interaction with the polyST.

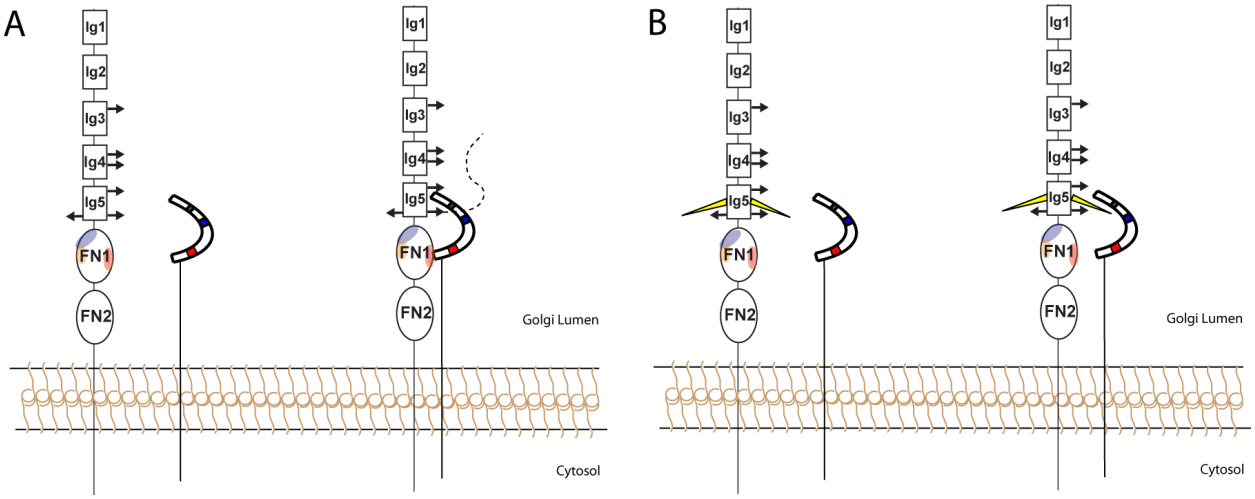


Figure 36. Replacing Ser^{448} and Asn^{476} in NCAM Ig5 with Lys and Arg inhibits polysialylation and NCAM-polyST interaction.

A) The productive NCAM-polyST interaction that leads to NCAM polysialylation involves polyST contact with both the Ig5 and FN1 domains.

B) Inserting lysine and arginine residues (yellow triangles) in place of Ser^{448} and Asn^{476} in NCAM Ig5 in prevents interaction with the polyST and polysialylation.

We could not reconstitute polysialylation in OCAM to the same level as seen in NCAM without replacing the entire Ig5-FN1 tandem with NCAM sequences. Although several of the regions we targeted showed large effects in NCAM they were insufficient to reconstitute polysialylation in OCAM alone or in aggregate. Part of the reason for this may be due to the likely flexibility in the linker between OCAM Ig5 and FN1 domains that is suggested by a series of OCAM structures reported by Kulahin and colleagues. We attempted to correct this by inserting the Ig5 linker strand into OCAM along with other key residues, but clearly this was not sufficient to completely reconstitute polysialylation. This interdomain flexibility in the OCAM Ig5-FN1 may have prevented the proper orientation of Ig5 and FN1 even when key residues in the Ig5 and FN1 domains were inserted.

Final Conclusions

In this work the interaction of NCAM with the polySTs was investigated. The critical role of FN1 for polyST interaction and NCAM polysialylation was established and the effects of specific sequences in this domain were documented. The FN1 acidic surface patch plays a role in polyST interaction. Other sequences both unique to NCAM FN1 and in common with OCAM FN1 seem to affect the Ig5-FN1 relationship which is critical for proper polysialylation. PolyST interaction and polysialylation of a chimeric protein was most successful when both Ig5 and FN1 were inserted into OCAM. Two residues in OCAM Ig5, Lys^{444} and Arg^{472}, were shown to be detrimental to interaction with the polyST in both OCAM and when placed in NCAM. Figure 37 models our current understanding of the NCAM-polyST interaction.

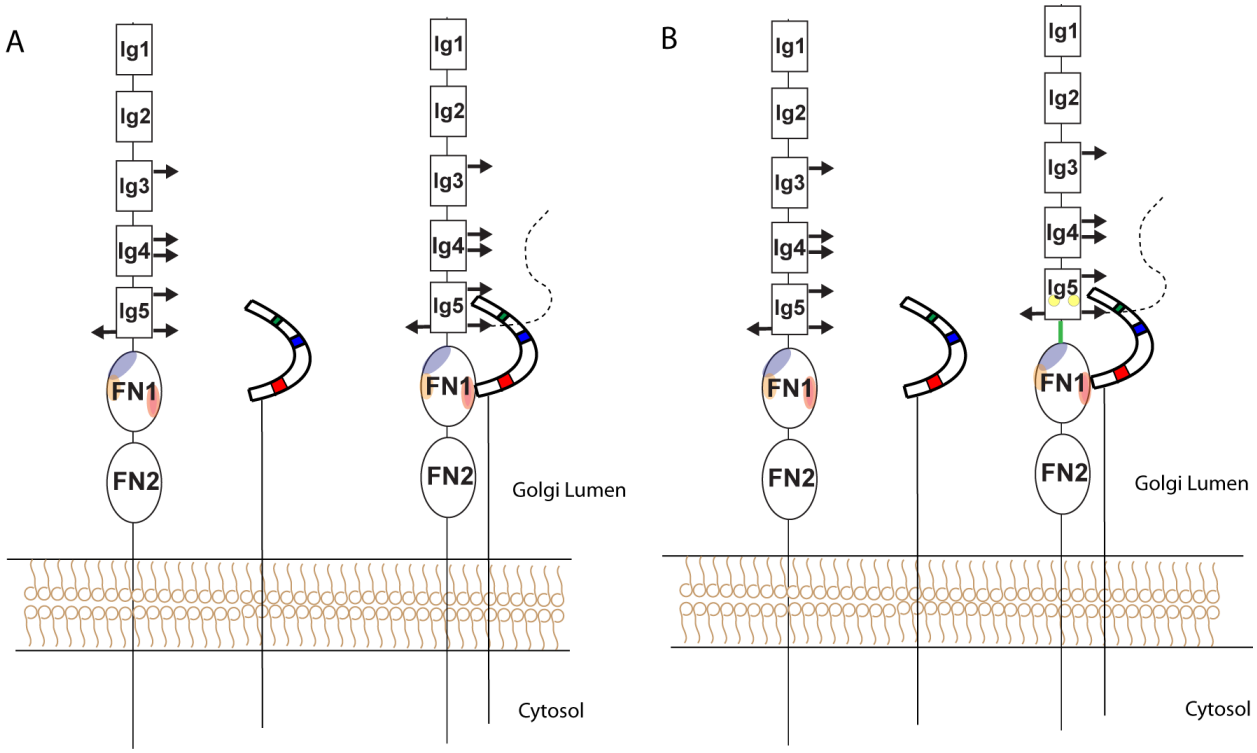

A
Ig1
Ig2
Ig3
Ig4
Ig5
FN1
FN2
Golgi Lumen
Cytosol
B
Ig1
Ig2
Ig3
Ig4
Ig5
FN1
FN2
Golgi Lumen
Cytosol

Figure 37. Summary of effects on NCAM polysialylation and polyST-NCAM interaction.

A) The initial model of the NCAM-polyST interaction involved the binding of NCAM FN1 by the polyST to the surface acidic patch (red 0val)and then polysialylating N-linked glycans (dashed line) in Ig5. While the α helix (blue oval) and the QVQ (orange oval) sequence caused a switch to O-linked polysialylation in FN (not shown).

B) The expanded model of the NCAM-polyST interaction. The polyST binds to the acidic patch in NCAM FN1 and to other residues in the NCAM Ig5 domain. Residues at location 448 and 472 (yellow circles) must be permissive for this interaction to occur. Sequences in the Ig5-FN1 interface (PSSP, GGVPI, NGKG) (green line) hold Ig5 and FN1 in the correct orientation to allow polysialylation of N-linked glycans by the bound polyST. Finally The α helix and the QVQ sequence restrain the interaction of the polyST to the correct location to insure polysialylation on Ig5 N-glycans.

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Publications- Journals

Thompson, M.G., Foley, D. A., Colley, K. J., (2012) The polysialyltransferases recognize sequences in two domains of the neural cell adhesion molecule to allow its polysialylation. *In preparation.*

Thompson, M. G., Foley, D. A., Swartzentruber, K.G., Colley**,** K. J., (2011) 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. J. Biol. Chem. 286, 4525-4534.

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Publications –Abstracts

Colley, K. J., Foley, D. A., Swartzentruber, K., and **Thompson, M.** (2009) The polysialylation of the neural cell adhesion molecule. Glycobiology 19, abstract 35.

Presentations

Thompson, M. G., and Colley, K. J., (2010) (Poster Presentation) Sequences in NCAM FN1 critical to interaction with the polysialyltransferase. University of Illinois College of Medicine Research Forum, Chicago Il.

Thompson, M. G., and Colley, K. J., (2009) (Poster Presentation and Oral Presentation) Sequences in NCAM FN1 critical to NCAM polysialylation. Annual Conference for the Society for Glycobiology, San Diego, CA.

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