Structural Basis for the Polysialylation of the Neural Cell Adhesion Molecule

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Introduction

Polysialic Acid: An Important Regulator of NCAM-dependent and NCAM-independent Adhesion

NCAM is an immunoglobulin super family protein that consists of five immunoglobulin (Ig) domains, two fibronectin type III (FN_{III}) repeats, and a transmembrane (TM) region plus cytoplasmic tail (NCAM140 and NCAM180) or a glycosyl-phosphatidylinositol (GPI) anchor (Fig. 1) [3]. Studies by Nelson et al. [4] have demonstrated that of NCAM's six N-glycosylation sites, sites 5 and 6 on the fifth Ig domain (Ig5) carry the bulk of NCAM's polysialic acid (polySia) (Fig. 1). Other work has demonstrated that the presence of long chains of polySia on the N-glycans of NCAM negatively modulates NCAM-dependent and NCAM-independent cell interactions [5,6]. More recently, elegant biophysical studies by Leckband, Rutishauser and colleagues [1] have quantitatively demonstrated the negative effects polySia has on both NCAM-mediated and cadherin-mediated adhesion processes.

The expression of polySia is developmentally regulated [2]. Polysialylated NCAM is highly expressed in the embryo and neonate, and exhibits a decreased and more restricted expression in the adult animal, where it is found in areas of the adult brain that require morphofunctional plasticity [7–9]. During development, polySia promotes axon guidance and targeting, and the separation and migration of cells (reviewed in [7–10]). The presence of surface polySia also allows cells to exhibit reversible interactions needed for cell movement (for example [11]). In adult animals, continued polySia expression is observed in specific areas of the brain, such as the hippocampus, the suprachiasmatic nucleus, and on olfactory bulb precursors that persist in their ability to generate neurons and show physiological

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V. Berezin (ed.), *Structure and Function of the Neural Cell Adhesion Molecule NCAM*, *Advances in Experimental Medicine and Biology* 663, DOI 10.1007/978-1-4419-1170-4_7,

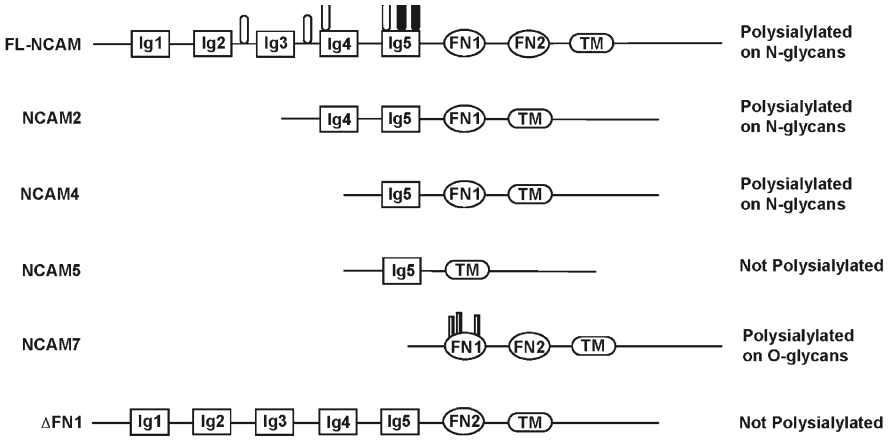

Fig. 1 NCAM and Domain Deletion Mutants. Shown are full length NCAM (FL-NCAM), NCAM2 (Ig4-Ig5-FN1-TM-Tail), NCAM4 (Ig5-FN1-TM-Tail), NCAM5 (Ig5-TM-Tail), NCAM7 (FN1-FN2-TM-Tail) and ΔFN1 (Ig1-Ig2-Ig3-Ig4-Ig5-FN2-TM-Tail). Ig, immunoglobulin domain; FN, fibronectin type three repeat; TM, transmembrane region. White elongated ovals represent N-glycans and black elongated ovals represent polysialylated N-glycans [1,2]. Sites of O-polysialylation in the NCAM7 protein (Thr 505, Thr 516 and Thr 553) are indicated by gray rectangles. The ability of each protein to be polysialylated by coexpressed polySTs, and the predominant type of glycan polysialylated are indicated

or morphological plasticity [12–15]. PolySia is also reexpressed during axon regeneration, and in various muscle diseases involving dystrophy and denervation [9,16,17]. High levels of polySia are also observed on the surface of some cancer cells, such as neuroblastomas, medulloblastomas, gliomas, small cell lung carcinomas, Wilms' tumor, and in other pediatric tumors, where its presence is frequently correlated with the cancer's highly metastatic behavior [9,16–25].

Recent studies using polysialyltransferase (polyST) and NCAM knockout mice have revealed the critical role of polySia in brain development. Mice lacking NCAM exhibit no obvious developmental defects, but do show a reduction in the size of the olfactory bulb, as well as deficits in spatial learning [26]. Mice lacking either ST8Sia IV/PST or ST8Sia II/STX exhibit a partial loss of polySia that results in compromised long term potentiation, long term depression, axon guidance, and synapse formation in the hippocampus [27,28]. However, mice lacking both polySTs and, therefore, all polySia, exhibit severe defects in brain development, fail to thrive, and die within 1 month after birth [29,30]. They also have smaller olfactory bulbs, as was observed in NCAM knockout mice, due to a decrease in neuroblast migration to the olfactory bulb in the absence of polySia. Strikingly, elimination of NCAM in the double polyST knockout mice reversed the developmental defects observed when polySia alone was eliminated, suggesting that the regulated polysialylation of NCAM glycans and the resulting decrease in cell adhesion is critical for brain development and animal survival [30].

Polysialylation of NCAM: A Protein Specific Modification

In mammals, polySia is found as homopolymers of α2, 8-linked Neu5Ac capping terminal α2, 3- or α2, 6-linked sialic acid on the N-linked and O-linked glycans of a small group of proteins. The polySTs, ST8Sia IV/PST and ST8Sia II/STX, are responsible for the polysialylation of mammalian proteins [31]. The proteins specifically modified by polySia include NCAM [2,32], the α subunit of the voltage dependent sodium channel [2,32–34], the two polySTs [35], a form of the scavenger receptor, CD36, found in milk [36], and neuropilin-2 found on dendritic cells [37]. Interestingly, the polySia on CD36 and neuropilin-2 is found exclusively on O-glycans [36,37]. The limited number of polysialylated proteins in mammalian cells suggests that the process of polysialylation is protein-specific. In other words, it requires that the polySTs recognize specific features of their glycoprotein substrates, and this allows the polymerization of polySia chains on the glycans of these substrates.

In most cases, the types of glycosyltransferases expressed by a cell and their levels of expression will determine structures of glycans found on proteins and lipids [38]. General variations in glycan structures may be related to a protein's folding pattern or subunit interactions, which may enhance or limit access to particular glycosylation enzymes [39]. However, in some instances, a protein's sequence and/or structure may be recognized by a particular enzyme, and this interaction will increase the efficiency of a glycosylation event. Examples of such protein-specific glycosylation events include the biosynthesis of the mannose 6-phosphate recognition marker on lysosomal enzymes (mediates their lysosomal targeting) [40], the biosynthesis of the GalNAc-4-SO_4 structure on N-glycans of pituitary glycoprotein hormones (mediates their clearance from the circulation) [41], the biosynthesis of $Glc_1Man_9GlcNAc_2$ glycans on misfolded glycoproteins in the endoplasmic reticulum (allows chaperone interactions) [42,43], and the addition of GlcNAc to O-fucose on epidermal growth factor repeats by Fringe (modifies Notch ligand interactions and signaling) [44,45].

In the case of mannose 6-phosphate and GalNAc-4-SO_4 biosynthesis, enzyme recognition is mediated by specific protein features. GalNAc-4-SO_4 is found at the termini of the N-glycans modifying pituitary glycoprotein hormones, and regulates the circulatory half-lives of these proteins by mediating their internalization by a hepatic receptor and their subsequent degradation in lysosomes [41]. Recognition of the glycoprotein hormones by a specific N-acetyl-galactosaminyltransferase requires a cluster of basic residues in an α-helix positioned 6-9 amino acids amino terminal to the hormone's N-glycan [46,47]. Mannose-6-phosphate residues on the N-linked glycans of lysosomal enzymes are required for their recognition by specific receptors in the *trans* Golgi network and subsequent trafficking to lysosomes [48,49]. The N-acetylglucosamine-1-phosphotransferase that catalyzes the first step in the two-step biosynthesis of mannose 6-phosphate requires maintenance of lysosomal enzyme tertiary structure and a signal patch that includes specific lysine residues [40,50–53].

Early studies first suggested the possibility that the polySTs recognize specific features of the NCAM protein. Kojima et al. [54] found that while ST8Sia II/STX could polysialylate a variety of glycoprotein substrates under in vitro conditions, NCAM served as a 1500-fold better substrate than the next best substrate, fetuin. Angata et al. [55] demonstrated that both polySTs could add polySia to glycan substrates, but that the polysialylation of glycans attached to the NCAM protein was much more efficient. In addition, using catalytically active, but unpolysialylated forms of polySTs (sites of autopolysialylation mutated), we demonstrated that no endogenous proteins in COS-1 cells are polysialylated when these enzymes are expressed, and that polySia is only detected when NCAM is coexpressed with the mutant enzymes [56,57]. Taken together, these results suggest that the polySTs must recognize some protein features of NCAM and their other substrates for efficient polysialylation of substrate glycans. Our work, described below, confirms that NCAM polysialylation, like the examples above, exhibits specific protein sequence and structural requirements and, thus, is a protein-specific glycosylation event.

NCAM Domains Required for Polysialylation

Early studies by Nelson et al. [4] demonstrated that a truncated NCAM protein consisting of the Ig4, Ig5, and the first FN_{III} repeat (FN1) fused to the TM region, and cytoplasmic tail was polysialylated by the endogenous polyST activity of F11 rat/mouse hybrid cells. In this truncated NCAM protein, Ig4 could be partially replaced by another Ig domain from the L1 cell adhesion molecule, but FN1 was absolutely required and could not be deleted or replaced by the second FN repeat (FN2) of L1. They proposed that the polySTs recognize and bind all three NCAM domains (Ig4, Ig5, FN1) simultaneously. We reevaluated the NCAM domain requirements for polysialylation using the two polySTs that were not available to these researchers and a series of domain deletion mutants [58] (see Fig. 1). We found that a protein possessing Ig4, Ig5 and FN1 (NCAM2) was efficiently polysialylated by coexpressed polySTs, in agreement with the earlier work of Nelson et al. [4]. However, we also observed that a shorter protein possessing just Ig5 and FN1 (NCAM4) was efficiently polysialylated, suggesting that Ig4 was not necessary for recognition and polysialylation [58]. Other work by Fujimoto et al. [59] agreed with our findings. The most important observation in our study was that, while NCAM4 possessing both the Ig5 and FN1 domains was polysialylated by co-expressed polySTs, a protein containing only the Ig5 domain (NCAM5) was not polysialylated [58]. This comparison suggested that NCAM FN1 was necessary for the polysialylation of the N-glycans on the adjacent Ig5 domain. Additional experiments with soluble NCAM proteins showed that membrane association was not necessary for their polysialylation, but did in some cases enhance the level of polysialylation [58]. This enhancement is likely the result of optimum positioning and concentration of the membrane-associated polyST and NCAM proteins on the lumenal face of the Golgi membrane. Taken together, these results suggest a model

in which the polySTs recognize features of NCAM FN1, and this "docking" positions them for polysialylation of the N-glycans on the adjacent Ig5 domain.

Fibronectin type I, II and III repeats were first identified in fibronectin [60] and were subsequently found in many other proteins [61]. The FN_{III} repeat is the largest of the three (~90 amino acids) and the most common. It is estimated to be present in approximately 2% of all mammalian proteins and has been identified in neural cell adhesion molecules, receptor protein kinases and phosphatases, adhesive matrix proteins, cytokine receptors, cytoplasmic muscle proteins, and prokaryotic carbohydrate cleavage enzymes [61]. The sequence homology for different FN_{III} repeats is relatively low (<20%), while the sequence homology for the same (similarly positioned) FN_{III} repeat in the same protein across different species is high (80–90%) [62]. In contrast to their low sequence homology, FN_{III} repeats exhibit high structural homology and fold into a β sandwich structure with one three-strand and one four-strand anti-parallel β sheet (Fig. 2) [63].

To address the question of whether the structure and/or sequence of NCAM FN1 was important for its role in polysialylation, we asked whether any other FN repeat could fulfill the requirement for NCAM FN1 in polysialylation. We deleted FN1 from the full-length NCAM (FL-NCAM) sequence, effectively placing FN2 in its position (Fig. 1). A test of the expression and trafficking of the ΔFN1 protein showed that it was well expressed, and efficiently left the endoplasmic reticulum and trafficked to the cell surface, indicating that it was not grossly misfolded. We observed no polysialylation of ΔFN1 by ST8Sia IV/PST or ST8Sia II/STX following expression in COS-1 cells. These results suggested that specific features of NCAM FN1 are critical for polyST recognition and NCAM polysialylation [64].

The Unique β Sandwich Structure of NCAM FN1: The Role of an Acidic Surface Patch and Novel α-Helix in NCAM Polysialylation

Our next challenge was to determine the features of NCAM FN1 required for polyST recognition and NCAM polysialylation. A comparison of NCAM FN1 and FN2 amino acid sequences demonstrated that they are only ~25% identical. Nevertheless, because they were FN_{III} repeats, we predicted that they are likely to have similar structures. We modeled the structure of human NCAM FN1 based on the known structure of rat NCAM FN2 (see Fig. 2 for rat NCAM FN2 structure). What was immediately apparent was the presence of an acidic surface patch found on FN1, but missing from FN2 [64]. In collaboration with Drs. Arnon Lavie and Nikolina Sekulic, we obtained the X-ray crystal structure of human NCAM FN1 (Fig. 2 and 3) [65]. We found that NCAM FN1 did indeed fold into a β sandwich structure, but that it had two unique features. We confirmed the presence of the acidic surface patch formed from Asp 497, Asp 511, Glu 512 and Glu 514 (Fig. 3) on one face of the β sandwich. We also found a novel, eight amino acid α-helix

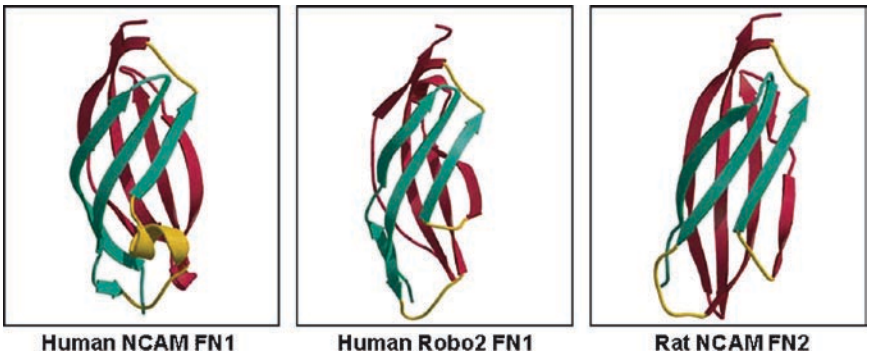

Fig. 2 Comparison of human NCAM FN1 structure to other fibronectin type III repeats. Structural representations of human NCAM FN1 (Protein Data Bank ID 2HAZ), human Robo2 FN1 (Protein Data Bank ID 1UEM), and rat NCAM FN2 (Protein Data Bank ID 1LWR). Note the absence of the α-helix from the latter two structures. In the Δhelix-TT proteins, the eight amino acids of the helix are replaced by two threonines as found in the corresponding region of Robo2 FN1

linking the fourth and fifth strands of the NCAM FN1 β sandwich, which has not been observed in any other FN_{III} repeat structure solved to date (Fig. 3) [65].

To evaluate the role of the acidic patch in NCAM polysialylation, we replaced the three core amino acids of this patch, Asp 511, Glu 512 and Glu 514, with both arginine and alanine residues in FL-NCAM, and in the truncated mutant NCAM4, which possesses the Ig5 and FN1 domains [64] (Fig. 3). Replacing the acidic residues with arginine eliminated the polysialylation of FL-NCAM and NCAM4 by the polySTs without detectably compromising their expression, folding, or trafficking [64]. Interestingly, replacing the acidic residues with alanine dramatically decreased NCAM4 polysialylation, but did not substantially impact FL-NCAM polysialylation [64]. From these and other results, we predicted that the acidic patch was likely to be a small part of a larger polyST interaction domain. We hypothesized that NCAM4 is not optimally positioned vis à vis the membrane-associated polySTs because it is missing the FN2 domain, and, as such, may not make as many contacts with the polySTs as FL-NCAM. If this is the case, then even replacing the NCAM4 acidic patch residues with neutral alanine residues may disrupt an already compromised interaction between enzyme and substrate [64].

Naturally, we considered the possibility that the FN1 α-helix may be part of an extended polyST recognition region on FN1, and we sought to replace the amino acids of the α-helix to test this idea. The Robo2 FN1 NMR structures were used for the molecular replacement solution of the NCAM FN1 structure (see Fig. 2 for Robo2 FN1 structure) [65]. In Robo2 FN1, two threonine residues link the fourth and fifth strands of the β sandwich. Overlay of the structures of human NCAM FN1 and Robo2 FN1 demonstrated that the eight amino acid α-helix in NCAM FN1 spanned the same distance as these two threonine residues [65]. Therefore, we rationalized that replacing the α-helix with two threonine residues would not

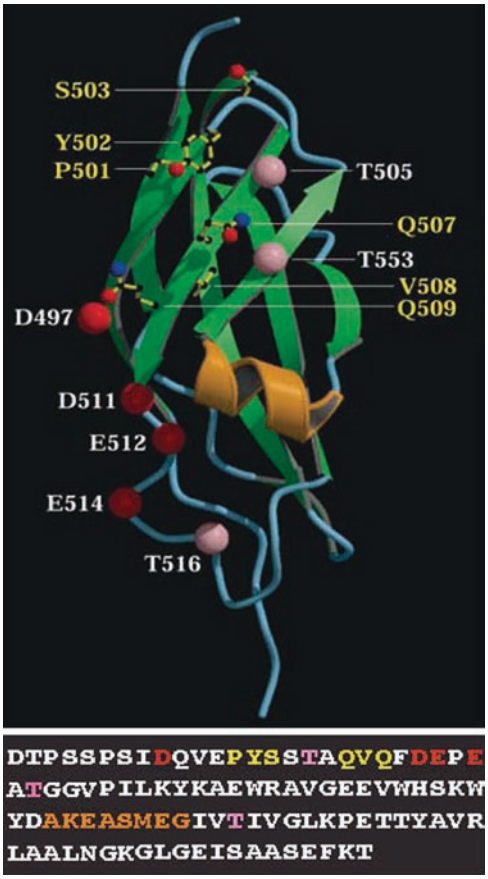

Fig. 3 Location of NCAM FN1 acidic patch, α-helix, Pro_{501}-Tyr_{502}-Ser_{503}, Gln_{507}-Val_{508}-Gln_{509} and sites of O-glycan polysialylation. Acidic patch residues Asp497, Asp511, Glu512 and Glu514 are indicated in red in the sequence and by large red balls in the structural representation (D497, D511, E512 and E514). The eight amino acids of the α-helix are indicated in orange in both the structural representation and the sequence. The side chains of $Pro_{501}Tyr_{502}Ser_{503}$ and $Gln_{507}Val_{508}Gln_{509}$ (P501, Y502, S503 and Q507, V508, Q509) are shown in yellow on strands β1 and β2 in the structural representation, with the small red balls representing the oxygens of the side chains of Tyr_{502}, Ser_{503}, Gln_{507} and Gln_{509}. In the sequence, these residues are indicated in yellow. The three threonine residues that are modified with O-linked polysialylated glycans in the Δhelix proteins are represented by large pink balls (T505, T516, and T553) in the structural representation and shown in pink in the sequence

substantially alter the strand arrangement and overall structure of NCAM FN1. We generated a Δhelix-TT protein and found that it was expressed well and trafficked to the cell surface just like FL-NCAM [65]. We were initially disappointed to find that the Δhelix-TT protein was as efficiently polysialylated as FL-NCAM when coexpressed with ST8Sia IV/PST, suggesting that the α-helix did not play a role in polyST recognition. However, additional analyzes with the enzyme peptide

N-glycosidase F (PNGase F) that specifically cleaves N-linked, but not O-linked glycans, led to a surprising finding-that the polySia on the Δhelix-TT protein was modifying O-linked glycans and not N-linked glycans [65]. One possibility was that the two, newly added threonine residues were serving as sites for O-linked glycosylation, and that these O-glycans were being polysialylated. However, a replacement of the helix with two alanine residues revealed that this was unlikely. The Δhelix-AA protein, like the Δhelix-TT protein, was polysialylated on O-linked glycans [65]. These results suggested that while the α-helix is not necessary for polyST recognition, it is important for the positioning of Ig5 N-glycans for polysialylation.

Are Interdomain Interactions Critical for Polysialylation of the Ig5 N-glycans?

The presence of polySia on O-glycans has now been observed on a muscle specific domain of an NCAM splice variant [66,67], the CD36 scavenger receptor [36], and most recently on neuropilin-2 [37]. In addition, early work in our laboratory had also detected polySia on O-glycans on unidentified cancer cell proteins [68], on FL-NCAM (small proportion of total polySia) [58], and on a domain deletion mutant, NCAM7 (all polySia), after coexpression with ST8SiaIV/PST in COS-1 cells [58]. NCAM7 consists of FN1, FN2, the TM region and cytoplasmic tail of NCAM140, and was originally intended to be a negative control (Fig. 1) [58]. Treatment of immunoprecipitated NCAM7 with PNGase F, followed by immunoblotting with the OL28 anti-polySia antibody, demonstrated that this protein is polysialylated on O-glycans. Considering these previous results along with the O-polysialylation of the Δhelix proteins, we wondered whether the FN1 α-helix is necessary for an interaction between Ig5 and FN1 that allows the correct positioning of Ig5 N-glycans for polysialylation by the polyST docked on FN1. If this is the case, then one would predict that this interaction would not occur in the absence of the α-helix, that the Ig5 N-glycans would not be polysialylated, and instead O-glycans in the FN1-FN2 region might be polysialylated (see models in Fig. 4). Initial experiments supported this premise. First, we found that O-glycans in the FN1 domain are polysialylated in the Δhelix proteins [65]. Using a prediction program designed by Igor Almedia and Rafel Torres, Jr. (University of Texas, El Paso), we identified three threonine residues that were likely sites of O-glycosylation (Fig. 3). Replacement of these threonines in the Δhelix-AA protein abolished polysialylation, thus demonstrating that the polysialylated O-glycans of the Δhelix proteins are found in FN1 [65]. Second, we found that replacement of the acidic patch residues in either Δhelix-AA or NCAM7 proteins substantially decreases or eliminates polysialylation of the O-glycans in these proteins, suggesting that the polySTs interact with the same site or overlapping sites on FN1 for both N- and O-glycan polysialylation [65]. In sum, our results indicate that the FN1 α-helix is not necessary for polyST recognition, but does play an important role in positioning

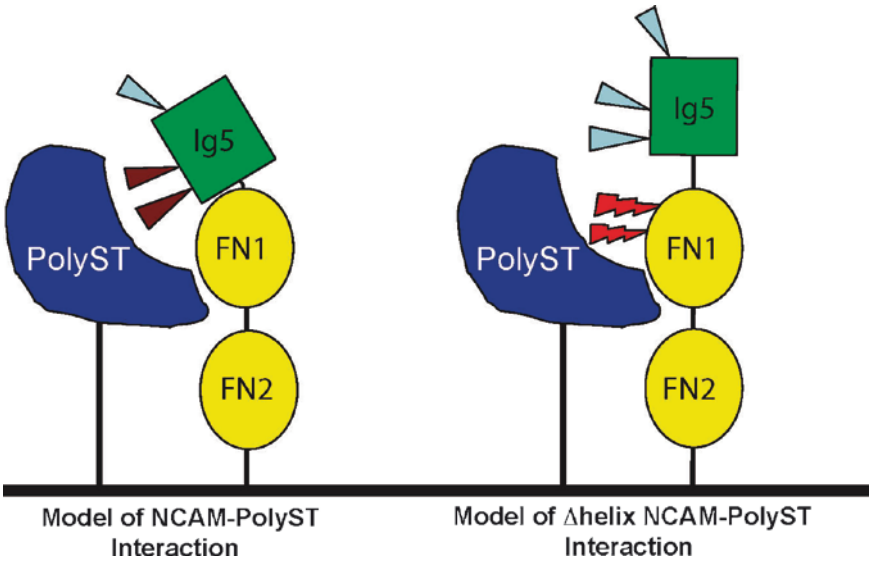

Fig. 4 Model of possible Ig5-FN1 interactions and changes upon elimination of the α-helix. We predict that the polyST recognizes and docks on the FN1 repeat of NCAM, and an interaction between the Ig5 and FN1 domains requiring the α-helix in FN1 positions the Ig5 N-glycans for polysialylation by the "docked" polyST (model on left). If the α-helix is replaced, the Ig5-FN1 interaction no longer occurs and the Ig5 N-glycans (dark red triangles) are moved away to reveal O-glycans in the FN1 repeat (red lightning bolts) that are then polysialylated by the "docked" polyST

the Ig5 N-glycans for polysialylation. Solid evidence for an interaction between Ig5 and FN1 requires additional experiments, however, it is intriguing that rotary shadowing electron microscopy detects a bend in the NCAM molecule that is predicted to be in the vicinity of the Ig5-FN1 region [69,70].

Reconstitution of PolyST Recognition and Polysialylation of an Unpolysialylated NCAM-OCAM Chimeric Protein

Up until this point, we had identified amino acids and structural features of NCAM FN1 necessary for NCAM polysialylation. Recently, we decided to determine whether these sequences were also sufficient for NCAM polysialylation. To do this, we generated a chimeric protein in which NCAM FN1 was replaced by the analogous FN1 repeat of the olfactory cell adhesion molecule, OCAM. OCAM is an NCAM family member that is expressed postnatally in the brain, olfactory epithelium, and retina [71–73]. OCAM is 45% identical to NCAM, has the same domain structure, and possesses the consensus glycosylation sites corresponding to the fourth, fifth and sixth N-glycans found in NCAM's Ig5 domain [73].

Despite these similarities, OCAM is not polysialylated [73]. The FN1 repeats of NCAM and OCAM are 37% identical, making the OCAM FN1 domain an excellent background for inserting NCAM FN1 sequences. Notably, comparison of the known structure of human NCAM FN1 with a modeled structure of OCAM FN1 reveals that OCAM FN1 lacks the acidic surface patch and α-helix observed in the NCAM FN1 structure. Initially, we inserted the four acidic patch residues and the β4-α-helix-β5-region from NCAM FN1 into the OCAM FN1 of the NCAM-OCAM chimera, and found that these sequences were sufficient for recognition and low levels of chimera polysialylation (data not shown). We were able to increase the level of polysialylation by adding six additional amino acids, $Pro_{501}Tyr_{502}Ser_{503}$ and $Gln_{507}Val_{508}Gln_{509}$, from NCAM FN1 (Fig. 3 and Fig. 5). These residues were

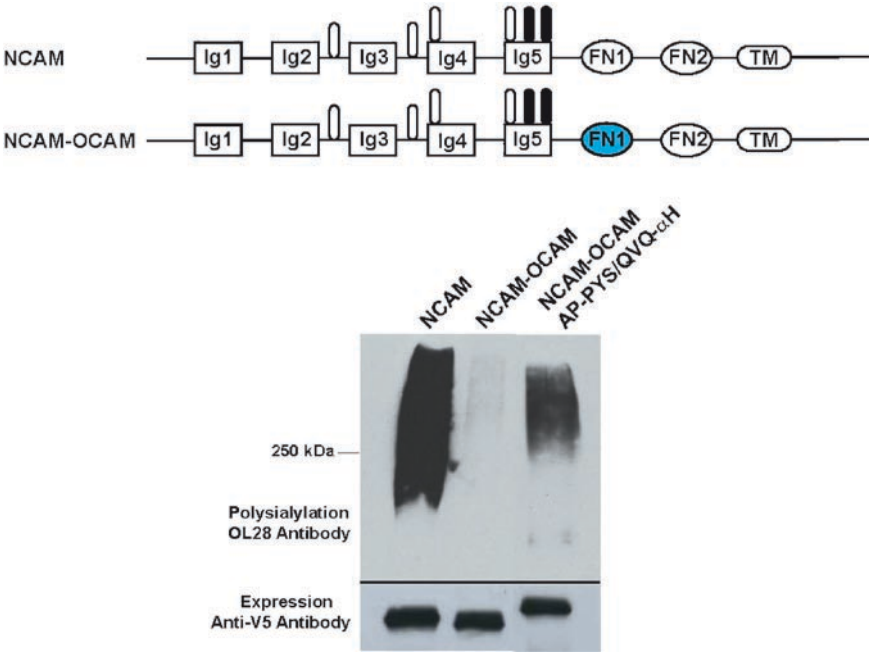

Fig. 5 Reconstitution of polysialylation of an NCAM-OCAM chimera by insertion of NCAM FN1 sequences. Top, schematic representation of FL-NCAM and the NCAM-OCAM chimera. The OCAM FN1 is indicated in turquoise. Bottom, a comparison of the polysialylation of FL-NCAM, NCAM-OCAM and NCAM-OCAM including specific NCAM FN1 sequences. FL-NCAM, the NCAM-OCAM chimera and the NCAM-OCAM chimera containing the NCAM FN1 acidic patch residues, $Pro_{501}Tyr_{502}Ser_{503}$ and $Gln_{507}Val_{508}Gln_{509}$, and the β4 strand-α-helix-β5 strand sequences (NCAM-OCAM AP-PYS/QVQ-αH) were coexpressed with ST8Sia IV/PST in COS-1 cells. NCAM proteins were immunoprecipitated using an antibody against the V5 epitope tag and subjected to immunoblotting using the anti-polySia antibody, OL28 (Polysialylation, OL28 Antibody). NCAM protein expression was determined by immunoblotting sample aliquots with anti-V5 antibody (Expression, Anti-V5 Antibody) (see Methods in references [62,63])

chosen because they were near the acidic patch region on strands 1 and 2 of the β sandwich and were some of the most divergent between the NCAM and OCAM FN1 domain sequences. Currently, we are determining which FN1 sequences are absolutely required for polyST recognition and which glycans are polysialylated in the modified NCAM-OCAM chimeras.

A Model of PolyST-NCAM Interaction

Our results suggest a model of polyST-NCAM interaction that depends upon two protein-protein interactions. One that involves the "docking" of the polyST on the NCAM FN1 domain, and a second between the Ig5 and FN1 domains that positions the Ig5 N-glycans for polysialylation. We predict that the polyST-FN1 interaction is the basis for the protein specificity of polysialylation, but also may be required for the polymerization of the polySia chains on the NCAM glycans. In fact, the affinity of this interaction could either be envisioned to dictate the length of the polySia chains polymerized or the number of NCAM proteins in a population polysialylated. Interestingly, preliminary co-immunoprecipitation experiments in our laboratory suggest that the polySTs bind directly to NCAM and that ST8Sia II/STX has a greater affinity for NCAM than does ST8Sia IV/PST (B. E. Close and K. J. Colley, unpublished observations). Recent in vivo work by Galuska et al. [74,75] demonstrates that the two polySTs synthesize polySia chains of the same average length, but that they differentially modify the two major attachment sites in NCAM, and that ST8Sia II/STX more efficiently polysialylates an entire population of NCAM molecules. This work suggests that potential differences in the affinities of the two polySTs for NCAM have a greater impact on the glycans modified and the efficiency of the polysialylation process, and less impact on polySia chain length per se.

Future Directions

Several additional questions must be addressed in order to understand the mechanism of protein polysialylation. We have reconstituted polyST recognition and polysialylation in the NCAM-OCAM chimera by inserting several different sequences from NCAM FN1; however, the contribution of each is not understood, nor is the identity of the glycans polysialylated. Our model predicts that the polySTs directly bind NCAM and our preliminary data suggest this is so. However, it is not known which of the FN1 residues required for NCAM polysialylation are also required for polyST-NCAM binding. In addition, our data suggest that the Ig5 and FN1 domains may interact to position the Ig5 N-glycans for polysialylation. Additional structural analysis and binding studies using these two domains should provide the answer to this question and reveal whether polyST binding is necessary

to induce an Ig5-FN1 interaction. An unexplored area concerns the contribution of protein-glycan interactions to the initial engagement of the polyST and NCAM, and/or to the chain elongation process. It is also not known how other polyST substrates are recognized by the polySTs and whether similar amino acids and structural features mediate these interactions. Notably, none of these other proteins contain FN_{III} repeats. Finally, once the requirements for NCAM recognition by the polySTs are defined, it would be useful to design inhibitors of the polyST-NCAM interaction and NCAM polysialylation.

Acknowledgments I would like to thank Deirdre Foley for providing Figure 5. I am also very grateful to Dr. Nikolina Sekulic and Saugata Hazra for providing the structural representations in Figures 2 and 3. I would also like to extend my thanks to members of my laboratory (K. Swartzentruber, D. Foley, W. Zhao, and M. Thompson) for critiquing this manuscript and helping with its assembly. This work was supported by NIH RO1 GM63843 to K. J. C.

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