A Novel $\alpha$-Helix in the First Fibronectin Type III Repeat of the Neural Cell Adhesion Molecule Is Critical for N-Glycan Polysialylation*

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Shalu Shiv Mendiratta§, Nikolina Sekulic†, Francisco G. Hernandez-Guzman‡, Brett E. Close§, Arnon Lavie†, and Karen J. Colley∗†§

From the ∗†Department of Biochemistry and Molecular Genetics, College of Medicine, University of Illinois at Chicago, Chicago, Illinois 60607 and §Accelrys, Inc., San Diego, California 92121

Polysialic acid is a developmentally regulated, anti-adhesive glycan that is added to the neural cell adhesion molecule, NCAM. Polysialylated NCAM is critical for brain development and plays roles in synaptic plasticity, axon guidance, and cell migration. The first fibronectin type III repeat of NCAM, FN1, is necessary for the polysialylation of N-glycans on the adjacent immunoglobulin domain. This repeat cannot be replaced by other fibronectin type III repeats. We solved the crystal structure of human NCAM FN1 and found that, in addition to a unique acidic surface patch, it possesses a novel $\alpha$-helix that links strands 4 and 5 of its $\beta$-sandwich structure. Replacement of the $\alpha$-helix did not eliminate polysialyltransferase recognition, but shifted the addition of polysialic acid from the N-glycans modifying the adjacent immunoglobulin domain to O-glycans modifying FN1. Other experiments demonstrated that replacement of residues in the acidic surface patch alter the polysialylation of both N- and O-glycans in the same way, while the $\alpha$-helix is only required for the polysialylation of N-glycans.

Our data are consistent with a model in which the FN1 $\alpha$-helix is involved in an Ig5-FN1 interaction that is critical for the correct positioning of Ig5 N-glycans for polysialylation.
fibronectin type III (Fn III) repeats in its extracellular domain (Fig. 1, FL-NCAM) (32). Deletion analysis demonstrated that the first Fn III repeat of NCAM (FN1) is necessary for the polysialylation of the N-glycans on the adjacent immunoglobulin (Ig) domain (this is the fifth Ig domain-Ig5) (31). We found that the second Fn III repeat (FN2) cannot replace the first, suggesting that the polysialyltransferases recognize unique features of FN1 (30). Modeling the structure of FN1 using the NMR structure of rat NCAM FN2 revealed a putative acidic patch on the surface of FN1 (Asp511, Glu512, Glu514) that is not present on FN2 (30). Replacement of these negatively charged residues with positively charged arginine residues eliminated polysialylation of a truncated NCAM protein consisting of Ig5, FN1, the transmembrane region, and cytoplasmic tail (NCAM4) and full-length NCAM (FL-NCAM) (see Fig. 1 for a description of these proteins) (30). Interestingly, replacement of the acidic residues with alanine residues eliminated the polysialylation of NCAM4, but not of FL-NCAM. Additional experiments suggested that the absence of the FN2 domain in NCAM4, and the resulting change in positioning of the NCAM4 Ig5-FN1 unit relative to the membrane-associated polysialyltransferases, lead to a weaker and/or less extensive polysialylation-NCAM4 interaction (30). As a result, even alanine replacement mutants were able to impair polysialylation of NCAM4. These studies suggest that the FN1 acidic patch may be part of a larger interaction region that is critical for the polysialyltransferase-NCAM interaction and subsequent NCAM polysialylation.

In this work we sought to determine the structure of the FN1 repeat from human NCAM in an effort to confirm the existence of the acidic patch and to determine whether other unique features of this domain contribute to recognition and polysialylation of NCAM. We have solved the x-ray crystal structure of human NCAM FN1 and found that in addition to an acidic surface patch consisting of Asp97, Asp511, Glu512, and Glu514, it possesses a unique α-helix that links strands 4 and 5 of its β-sandwich structure. Replacement of this eight-amino acid linker with an analogous linker from another Fn III repeat does not eliminate polysialylation, but changes the sites of polysialylation from N-glycans modifying Ig5 to O-glycans modifying FN1. Our data are consistent with a model in which an Ig5-FN1 interaction involving the FN1 α-helix is necessary for the correct positioning of N-glycans on Ig5 for polysialylation.

**MATERIALS AND METHODS**

Tissue culture media and reagents, including Dulbecco’s modified Eagle’s medium, Opti-MEM I, Lipofectin and fetal bovine serum were purchased from Invitrogen Corp. (Carlsbad, CA). Nitrocellulose membranes were purchased from Schleicher and Schuell (Keene, NH). SuperSignal West Pico chemiluminescence reagent was obtained from Pierce. Protein molecular mass standards were purchased from Bio-Rad. Oligonucleotides and anti-V5 epitope tag antibodies were purchased from Invitrogen. The QuickChange™ site-directed DNA mutagenesis kit and Pfu DNA polymerase were obtained from Stratagene (La Jolla, CA). Peptide N-glycosidase F (PNGase F) was purchased from New England Biolabs (Beverly, MA). The cDNA for human ST8Sia IV/PST was obtained from Dr. Minoru Fukuda (Burnham Institute, La Jolla, CA) and the cDNA for ST8SiaII/STX was obtained from Dr. John Lowe (Case Western Reserve University). DNA purification kits were obtained from Qiagen (Valencia, CA). Protein A-Sepharose was obtained from Amersham Biosciences. Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Jackson Laboratories (West Grove, PA). Other chemicals and reagents were purchased from Sigma and Fisher Scientific (Hanover Park, IL).

**Construction of FN1 Bacterial Expression Construct—**A PCR fragment of FN1 cDNA encoding amino acids 487–590 was cloned into the pET14b bacterial expression vector (Novagen) in the NdeI and BamHI sites. This expression vector inserts a His6 tag and thrombin cleavage site at the N terminus of the FN1 domain. After cleavage of the His6 tag, our constructs contained 4 additional N-terminal residues (Gly-Ser-His-Met).

**Expression, Purification, and Crystallization of Human NCAM FN1—**BL21 (DE3) *Escherichia coli* were transformed with the above vector, grown to an optical density of 0.6–0.8 at which time the culture was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside and allowed to grow overnight at 22 °C. Cells were harvested by centrifugation and lysed by sonication. Ultracentrifugation yielded the supernatant that was loaded on a 1 ml HiTrap™ Chelating HP column (GE Healthcare) charged with nickel ions. The protein was cleaved from the His6 tag on the column with thrombin, collected, and concentrated to 4 mg/ml. Crystallization experiments conducted with several Hampton Research screens provided multiple hits. One of those hits was optimized to produce small cubic-shaped crystals (50 μm to an edge) at 0.3 M CaCl2, 17% PEG 3350, 10% PEG 400 (the latter only present in the drop).

**X-ray Data Collection, Structure Solution, and Modeling of the ΔHelix-TT Protein—**Diffraction data to 1.7 Å resolution were collected from a single crystal frozen in liquid nitrogen using beam line A1 at CHESS. Processing was accomplished with XDS (33). Initial molecular replacement using several Fn III repeat crystal structures as search models was unsuccessful. A second attempt for a search model was done with a BLAST experiment as implemented in the Protein Similarity Search module of the DS Modeling 1.1 program (Accelrys, San Diego, CA). This search indicated that the first Fn III repeat of human Roundabout homolog 2 (KIAA1568 protein, Protein Data Bank ID 1UEM) had the lowest Expectancy value at 3.2e-003 and hence the best sequence alignment hit. A molecular replacement search was done with all 20 NMR solution structures after truncation of the first 13 and last 4 residues for all structures. Molecular replacement was done automatically using the AMoRe software (34) as implemented in the HT-XMR module of DS Modeling 1.1. From the molecular replacement search of all 20 solution structures, 7 NMR structures gave the correct orientation, but the molecular replacement search using structure number 13 gave the highest quality solution. Full model building and refinement of the FN1 protein was done using the XBUILD (35) and CNX (Accelrys) modules, respectively, as implemented in DS Modeling 1.1 (Table 1). A final round of refinement done using REFMAC5 (36) yielded a model with good statistics. We could model all residues present in our FN1 construct, plus three additional N-terminal residues that remain after cleaving of the His6 tag with thrombin. In addition,
we modeled 97 water molecules and a single sodium ion observed to bridge two crystallographically related FN1 monomers. The accuracy of the model was verified by inspecting simulated annealing omit maps as implemented in CNS (37). The refined model and structure factors are deposited under Protein Data Bank ID 2HAZ.

To create the FN1 variant lacking the helical segment connecting strands 4 and 5 (Δhelix-TT), we exploited the model of the first Fn III repeat of Roundabout homolog 2, which lacks this helix, to design the appropriate loop. This loop substitutes the residues that form the helix (residues Asp543-Gly550) and the flexible segment preceding this helix, to design the appropriate loop. This loop substituting the residues that form the helix (residues Asp543-Gly550) and the flexible segment preceding this helix, to design the appropriate loop.

**Construction of FL-NCAM Mutant cDNAs—NCAM(AAA) and NCAM7(AAA) mutants were generated by simultaneously replacing Asp543, Glu544, and Glu545 with alanine using the primers 5’-CTGGTCCGGTGTGCAGCATGCTCC-3’ and 5’-CTGCACTTGGATCATAGTACCCGTCC-3’. NCAM(RRR) and NCAM7(RRR) mutants were generated by using the primers 5’-CTGGTCCGGTGTGCAGCATGCTCC-3’ and 5’-CTGCACTTGGATCATAGTACCCGTCC-3’. The Δhelix-TT change was generated in FL-NCAM and NCAM(AAA) by replacing Asp543, Glu544, and Glu545 with alanine using the primers 5’-CTGGTCCGGTGTGCAGCATGCTCC-3’ and 5’-CTGCACTTGGATCATAGTACCCGTCC-3’.

**TABLE 1**

| Data collection | Value |
|-----------------|-------|
| X-ray source    | CHESS |
| Wavelength (Å)  | 0.9766|
| Unit cell (Å)   | 42.76 |
| b               | 52.88 |
| c               | 74.07 |
| Space group     | C222  |
| No. molecules/a.u. | 1 |
| Resolution limit (Å) | 20–1.59 |
| Measured reflections | 68,622 |
| Unique reflections | 9,851 |
| Completeness (% overall/last shell)* | 99/94 |
| I/σ (overall/last shell)** | 12.88/3.02 |
| R yes (overall/last shell)*** | 9.2/48.7 |

**Refinement**

| Resolution limit (Å) | 20–1.70 |
| No. reflections (working/free) | 8,753/941 (9.7%) |
| R yes (overall/last shell)** | 0.212/0.298 |
| R free (overall/last shell)** | 0.286/0.431 |
| No. protein/ion/water non-H atoms | 103/1/97 |

**r.m.s.d. from ideal geometry (Å)**

| Bond angle | 0.010 |
| Angle distances | 1.286 |
| Estimated coordinate error (Å) | 0.155 |

**Ramachandran plot statistics**

| Residues in most favored regions | 93.1% |
| Residues in generously allowed regions | 6.9% |
| Residues in allowed regions | 0.0% |
| Residues in disallowed regions | 0.0% |

*Last shell = 1.69–1.79 Å.
**R yes = Σ|Fo|–|Fc|)/Σ|Fo|, 9.7% randomly omitted reflections were used for R free.
***R free = Σ|Fo|–|Fc|)/Σ|Fo|, 9.7% randomly omitted reflections were used for R free.

The refined model and structure factors are deposited under Protein Data Bank ID 2HAZ.

**Transfection of COS-1 Cells with NCAM and PST or STX cDNAs—COS-1 cells maintained in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum were plated on 100-mm tissue culture plates and grown in a 37 °C, 5% CO2 incubator until 50–70% confluent. Lipofectin transfections were performed according to the protocols provided by Invitrogen. Transfections were done in 3 ml of Opti-MEM 1 using 30 µl of Lipofectin and 10 µg each of FL-NCAM or NCAM mutant cDNAs cloned in the pcDNA 3.1 (V5-His B) expression vector that places the His6 and V5 epitope tags at the C termini of the protein, and PST or STX cDNA that were cloned in the pcDNA 3.1 (no tag) expression vector. Cells were harvested 18–24 h post-transfection for analysis.

**Immunoprecipitation and Immunoblot Analysis of NCAM Proteins—COS-1 cells were co-transfected with untagged polysialyltransferase cDNA and V5-tagged NCAM cDNA at a 1:1 ratio, as described above.** Following transfection, 7 ml of Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum was added to the cells. After incubation for 18 h at 37 °C in a 5% CO2 incubator, cells were washed with 10 ml of phosphate-buffered saline and lysed in 1 ml of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS). NCAM proteins were immunoprecipitated from cell lysates using 3 µl of anti-V5 epitope tag antibody and 50 µl of a 50% slurry of protein A-Sepharose, as previously described (30, 31). PNGase F treatment was performed following immunoprecipitation and while the immune complexes were still bound to the protein A-Sepharose beads. Briefly, two identical samples were resuspended in 77 µl of D2O, 10 µl of 10% Nonidet P-40, and 10 µl of 10× reaction buffer (0.5 M sodium phosphate, pH 7.5). PNGase F (3 µl, 1500 units) was added to one sample of the pair and both were incubated for 18 h with shaking at 37 °C. The samples (including the protein A-Sepharose beads) were then resuspended in 50 µl of Laemmli sample buffer containing 5% β-mercaptoethanol, heated for 10 min at 65 °C (to preserve polysialic acid), and directly loaded into the gel wells. Immunoprecipitated proteins were separated on a SDS-polyacrylamide gel (5% separating gel, 3% stacking gel). To evaluate protein expression, a 30-µl aliquot of each cell lysate was removed before immunoprecipitation. Following the addition of Laemmli sample buffer containing 5% β-mercaptoethanol, the sample was incubated at 110 °C for 10 min to remove polysialic acid and electrophoresed on a SDS-polyacrylamide gel (7.5% separating gel and 3% stacking gel). Following electrophoresis, proteins were electrophoretically transferred to nitrocellulose membranes for 16 h at 500 mA. To detect polysialylated proteins, membranes were incubated with proteins were generated by replacing Thr505, Thr516, and Thr553 sequentially with alanine in the Δhelix-AA protein using primers 5’-CTGCACTTGGATCATAGTACCCGTCC-3’ and 5’-CTGGTCCGGTGTGCAGCATGCTCC-3’. NCAM(AAA) and NCAM(RRR) were generated by using the primers 5’-CTGCACTTGGATCATAGTACCCGTCC-3’ and 5’-CTGGTCCGGTGTGCAGCATGCTCC-3’.
the OL28 anti-polysialic acid antibody (IgM) in blocking buffer 1 (2% nonfat dry milk in Tris-buffered saline, pH 8.0) and HRP-conjugated goat anti-mouse IgG diluted 1:8000 in blocking buffer 2 (5% nonfat dry milk in Tris-buffered saline, pH 8.0, 0.1% Tween 20). For immunoblotting of the cell lysate aliquots, the anti-V5 epitope tag antibody was diluted 1:5000 and HRP-conjugated goat anti-mouse IgG was diluted 1:8000 in blocking buffer 2. Immunoblots were developed using the SuperSignal West Pico chemiluminescence kit (Pierce) and exposed to Kodak Bio-Max MR film at room temperature.

RESULTS AND DISCUSSION

NCAM FN1 Possesses a Unique α-Helix That Links Strands 4 and 5 of Its β-Sandwich Structure—Fn III repeats contain ~100 amino acids and adopt the Ig-like β-sandwich fold (38). This domain is present in proteins with diverse functions such as cell adhesion proteins, extracellular matrix proteins, cytokine receptors, and muscle proteins. The Fn III repeat superfamily has 9037 members in the current Pfam database (39), and members share as low as 5% sequence identity. Our interest in the FN1 of NCAM originates from its critical role in mediating the specificity of NCAM polysialylation. We propose that interactions between the polysialyltransferase and the NCAM FN1 domain lead to the correct anchoring of the enzyme and subsequent polysialylation of N-glycans modifying the adjacent immunoglobulin domain (Ig5). Previously, we built a homology model of FN1 that revealed an acidic patch composed of three carboxylic acid residues on its surface (Asp<sup>511</sup>, Glu<sup>512</sup>, Glu<sup>514</sup>) (Fig. 1b, FN1) (30). Replacement of these three residues with alanine or arginine slightly decreased or eliminated the polysialylation of FL-NCAM, respectively (Fig. 1b, Acidic Patch Mutants) (30). To verify the existence of this structural feature, we solved the crystal structure of this domain to 1.7-Å resolution. Our experimentally derived structure confirmed the presence of the acidic patch, and showed that it includes a fourth carboxylic acid residue (Asp<sup>997</sup>) (Fig. 2). In an additional experiment, we found that replacing all four acidic patch residues with alanine did not further decrease the recognition and polysialylation of FL-NCAM (data not shown). This suggested that sequences beyond the acidic patch play a role in enzyme recognition and polysialylation.

The structure of NCAM FN1 also revealed an α-helix that links strands 4 and 5 of the β-sandwich fold (Fig. 2). Analysis of our model using the DALI server (40) indicates that this helical segment is unique among known Fn III repeats. Being in close proximity to the acidic patch, we surmised that the α-helix could be a structural element important for recognition of NCAM by the polysialyltransferases. To test this, we designed a mutant where we replaced the α-helix, the Acidic Patch Mutants that have Asp<sup>511</sup>, Glu<sup>512</sup>, and Glu<sup>514</sup> replaced with either alanine residues (AAA) or arginine residues (RRR), and the combination of both changes (Δhelix Acidic Patch Mutants). Several features of the FN1 domain are depicted including the seven strands of the β-sandwich fold (β1-β7, white ovals), the α-helix (black oval), and the sites of O-glycan polysialylation (Thr<sup>505</sup>, Thr<sup>516</sup>, and Thr<sup>553</sup>) are shown in the FN1 structure as stars.
cells, immunoprecipitated with anti-V5 epitope tag antibody and subjected to immunoblotting with the OL28 anti-polysialic acid antibody. We found that replacement of the H9251-helix did not eliminate recognition and polysialylation of either FL-NCAM or NCAM(AAA) (Fig. 3). These results suggested that the H9251-helix in FN1 is not necessary for recognition or polysialylation by PST.

**Replacement of the FN1 α-Helix Changes the Sites of NCAM Polysialylation**—In previous studies, we found that PST was capable of polysialylating both N- and O-glycans in different NCAM domains (31). NCAM7, a truncated form of NCAM lacking all the Ig domains and consisting only of the two Fn III repeats, transmembrane region and cytoplasmic tail (Fig. 1a), was polysialylated on O-glycans when co-expressed with PST in COS-1 cells ((31) and Fig. 5b). We considered the possibility that by replacing the H9251-helix, we had changed the sites of polysialylation from the N-glycans on Ig5 to O-glycans in the Fn III repeat region. To determine whether the Δhelix-TT protein was polysialylated on O-glycans, we treated the immunoprecipitated proteins with peptide N-glycosidase F (PNGase F) prior to OL28 immunoblot analysis. This enzyme cleaves all N-glycans from proteins without affecting O-glycans (41). As expected, FL-NCAM was polysialylated primarily on N-glycans that were sensitive to PNGase F treatment, with only a small amount of polysialic acid found on PNGase F-insensitive O-glycans (Fig. 4). In contrast, the vast majority of the polysialic acid on the Δhelix-TT protein was found on PNGase F-insensitive O-glycans (Fig. 4, left panel). Because threonine residues are sites for O-glycan addition, we considered the possibility that we had inadvertently introduced two new sites of O-glycosylation in the Δhelix-TT protein, and those glycans were being polysialylated. To evaluate this, we made a Δhelix-AAA protein in which two alanine residues were used to replace the Δhelix (Fig. 1b, FN1 Mutants, Δhelix-AAA). Like the Δhelix-TT protein, the Δhelix-AAA was polysialylated predominantly on O-glycans ruling out the possibility that the added threonine residues were acting as new sites for O-glycosylation and subsequent polysialylation (Fig. 4, right panel).

Why did replacing the α-helix alter the NCAM glycans that are polysialylated? One possibility is that replacing the α-helix eliminated an interaction between Ig5 and FN1 in FL-NCAM, and in the absence of this interaction, N-glycans on Ig5 are

**FIGURE 2.** The first FnIII repeat of NCAM contains a novel α-helix close to an acidic patch. a, ribbon diagram showing the β-sandwich fold of FN1, where one sheet is colored in purple and the second in turquoise. Linkers connecting the two sheets are yellow: the α-helix unique to the NCAM1 FN1 domain links strands β4 and β5. The side chains of the four carboxylic acid residues that form the acidic patch are displayed in red. b, view after a 90 degree rotation relative to that in a. c, electrostatic surface potential of FN1 in the same orientation as in b. Negatively charged surface is displayed in red, positively charged surface in blue, uncharged in white. Figures were prepared using Molscript (42) and Grasp (43). d, overlay of FN1 (red) and the first FnIII repeat of Roundabout homolog 2 protein (green) used to solve the structure by molecular replacement (r.m.s.d. 1.53 Å over 92 compared atoms). Note the α-helix that is unique to FN1. We designed a variant lacking this α-helix (close-up, in blue) by replacing these residues with a tandem of threonine residues (cyan) that link the analogous strands in the Roundabout homolog 2 protein.
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moved out of range of the polysialyltransferase, while O-glycans in the Fn III repeat region become accessible to the polysialyltransferase and are polysialylated. This would essentially mimic the situation with NCAM7. Alternatively, the α-helix may be a critical component of the primary polysialyltransferase interaction site on FN1, and in its absence, a secondary site of recognition is used that repositions the enzyme and changes the glycans polysialylated.

Polysialylation of O-Glycans on the ΔHelix-AA and NCAM7 Proteins Is Reduced or Eliminated When the Residues in the Acidic Patch Are Altered—To investigate the possibility that a secondary site of polysialyltransferase recognition and interaction is involved in O-glycan polysialylation, we evaluated the impact of altering the acidic patch residues in Δhelix-AA and NCAM7 O-glycan polysialylation and the role of the FN1 α-helix in NCAM7 O-glycan polysialylation. We previously found that replacing the core amino acids of the acidic patch with arginine residues dramatically reduced the polysialylation of FL-NCAM without drastically altering its folding ((30) and Fig. 5a). Making the same changes in the Δhelix-AA protein also substantially decreased its N- and O-glycan polysialylation (Fig. 5a). Consistent with this observation, polysialylation of NCAM7 O-glycans was also very sensitive to the replacement of acidic patch residues, and NCAM7(AAA) and NCAM7(RRR) acidic patch mutants exhibited significantly reduced or no polysialylation (Fig. 1b, Acidic Patch Mutants, and Fig. 5c). In contrast, we found that replacing the FN1 α-helix in the NCAM7 protein did not significantly impact the polysialylation of its O-glycans (Fig. 1b, FN1 Mutants, Δhelix-AA and Fig. 5c). These results suggest that the polysialyltransferases may be contacting the FN1 domain in the same or a similar fashion for the polysialylation of O-glycans (Δhelix-AA and NCAM7) that they do for the polysialylation of N-glycans (FL-NCAM and NCAM4). These results also demonstrate that while the FN1 α-helix is critical for the correct polysialylation of the N-glycans on Ig5, it is not required for the polysialylation of O-glycans in the Fn III repeat region (NCAM7).

The Polysialylated O-Glycans of the FL-NCAM Δhelix-AA Protein Are Found on O-Glycans Modifying Threonine Residues in the FN1 Domain—To determine where the polysialylated O-glycans are located in the FL-NCAM protein we used the NetOGlyc3.1 Server and O-Glycosylation Prediction Electronic Tool (OGPET v1.0) created by Rafael Torres Jr. and Igor C. Almeida. Using the FL-NCAM sequence the OGPET program predicted four potential sites of O-glycosylation (Ser504, Thr516, Thr553, and Thr561), while the NetOGlyc3.1 program predicted many more including Thr505 and several threonines in the cytoplasmic tail of the protein that cannot be glycosylated. We combined the results of the two predictions and started by generating two Δhelix-AA proteins in which two (Thr505/516) or three (Thr505/516/553) threonine residues were replaced by alanine (Fig. 6). We found that the T505A/T516A mutant showed a decrease in polysialylation; however some PNGase F-insensitive O-glycan polysialylation still persisted (Fig. 6). A complete elimination of PNGase F-insensitive O-glycan polysialylation was observed for the T505A/T516A/T553A mutant. These results support our hypothesis by demonstrating that the vast majority of polysialic acid on the Δhelix-AA protein is found on O-glycans modifying threonine residues 505, 516, and 553 in the FN1 domain.
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The FN1 Acidic Patch and α-Helix Play the Same Roles in PST and STX Polysialylation—Our previous work on the requirements for polysialyltransferase recognition and polysialylation of NCAM focused on one of the two polysialyltransferases, PST. To determine whether the acidic patch and α-helix are playing similar roles in STX polysialylation, we co-expressed this enzyme with FL-NCAM, NCAM(Δhelix-AA), and NCAM(Δhelix-A(AAA)) mutants, in which the α-helix or acidic patch core residues were replaced (Fig. 7). As with PST, the polysialic acid added by STX to FL-NCAM was found largely on PNGase F-sensitive N-glycans (Fig. 7). Replacing the FN1 α-helix (Fig. 1b, FN1 mutants, Δhelix-AA) shifted polysialylation to PNGase F-insensitive O-glycans, but did not appreciably decrease polysialylation (Fig. 7). In contrast, replacing the core residues of the acidic patch with alanine residues or arginine residues (Fig. 1b, Δhelix-AAA, Δhelix-ARRR) decreased or nearly eliminated polysialylation by STX (Fig. 7). These results show that polysialylation by either PST or STX is altered identically when the FN1 acidic patch or α-helix are replaced. This suggests that the mechanism of enzyme-NCAM interaction and the require-
ments for polysialylation of the N-glycans on Ig5 are the same or similar for the two polysialyltransferases.

In sum, we have found that the FN1 domain of NCAM has a unique α-helix linking strands 4 and 5 of its β-sandwich structure that is critical for the polysialylation of N-glycans on the adjacent Ig5 domain. Previous work demonstrated that the FN1 domain of NCAM is required for the polysialylation of Ig5 N-glycans and suggested that an acidic patch on the surface of FN1 is part of a larger polysialyltransferase recognition region (30, 31). We tested the possibility that the FN1 α-helix is part of the polysialyltransferase recognition region and found that replacing this helix did not eliminate polysialylation in the presence or absence of the acidic patch residues, but that its replacement changed the glycans that were polysialylated from O-glycans on FN1 to O-glycans on FN1. Our data suggest two levels of specificity for the process of polysialylation and a model where the FN1 α-helix is required for an interaction between Ig5 and FN1 that is necessary to position the N-glycans on Ig5 for polysialylation. Current work is focused on determining whether Ig5 and FN1 interact and whether the FN1 α-helix is involved in this potential interdomain interaction.

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