Autopolysialylation of polysialyltransferases is required for polysialylation and polysialic acid chain elongation on select glycoprotein substrates

Polysialic acid (polySia) is a large glycan polymer that is added to some glycoproteins by two polysialyltransferases (polySTs), ST8Sia-II and ST8Sia-IV. As polySia modulates cell adhesion and signaling, immune cell function, and tumor metastasis, it is of interest to determine how the polySTs recognize their select substrates. We have recently identified residues within the ST8Sia-IV polybasic region (PBR) that are required for neural cell adhesion molecule (NCAM) recognition and subsequent polysialylation. Here, we compared the PBR sequence requirements for NCAM, neuropilin-2 (NRP-2), and synaptic cell adhesion molecule 1 (SynCAM 1) for polysialylation by their respective polySTs. We found that the polySTs use unique but overlapping sets of PBR residues for substrate recognition, that the NCAM-recognizing PBR sites in ST8Sia-II and ST8Sia-IV include homologous residues, but that the ST8Sia-II site is larger, and that fewer PBR residues are involved in NRP-2 and SynCAM 1 recognition than in NCAM recognition. Noting that the two sites for ST8Sia-IV autopolysialylation flank the PBR, we evaluated the role of PBR residues in autopolysialylation and found that the requirements for polyST autopolysialylation and substrate polysialylation overlap. These data together with the evaluation of the polyST autopolysialylation mechanism enabled us to further identify PBR residues potentially playing dual roles in substrate recognition and in polySia chain polymerization. Finally, we found that ST8Sia-IV autopolysialylation is required for NRP-2 polysialylation and that ST8Sia-II autopolysialylation promotes the polymerization of longer polySia chains on SynCAM 1, suggesting a critical role for polyST autopolysialylation in substrate selection and polySia chain elongation.

Polysialic acid (polySia) is a large glycan polymer composed of large linear chains of 8–400 α2,8-linked sialic acids, which in mammalian cells caps the N-linked or O-linked glycans of a small set of glycoproteins (1–3). polySia serves as both a “global regulator of cell adhesion” (4) and as a modulator of select signaling pathways (1, 5). The latter function is likely due to its ability to modulate protein–protein interactions and could also be a result of its ability to serve as a reservoir for biologically active molecules like neurotrophins, neurotransmitters, and growth factors (1, 2, 5). polySia is independently synthesized by two polysialyltransferases (polySTs), ST8Sia-II and ST8Sia-IV, whose expression is transcriptionally regulated (6, 7).

The most abundant and well-studied polysialylated protein is the neural cell adhesion molecule, NCAM. polyST and NCAM knock-out mice demonstrated that temporal control of NCAM adhesion and associated signaling is not only critical for proper brain formation but also for the maintenance and function of other parts of the nervous system (8–11). Other studies demonstrate its role in developmental and regenerative processes in extraneural tissues like immune cells, lung, placenta, testis, and liver (1, 12) and suggest its contribution to cancer cell invasion and metastasis (13).

In addition to NCAM, the voltage-dependent sodium channel α subunit (14), a small proportion of the CD36 scavenger receptor found in human milk (15), synaptic cell adhesion molecule 1 (SynCAM 1) (16), NRP-2 (17), C-C chemokine receptor 7 (18), and E-selectin ligand-1 (19) are also polysialylated. The polySTs are also capable of autopolysialylation; however, the presence of polySia on their glycans is not required for the polysialylation of NCAM (20–23). Interestingly, although NCAM can be polysialylated by either polyST, it has been established that NRP-2 is exclusively polysialylated by ST8Sia-IV in vivo (17, 24), and SynCAM 1 is exclusively polysialylated by ST8Sia-II in vivo (16, 25).

The small number of polyST substrates, and the inefficient polysialylation of free glycans relative to those on proteins (26), suggested to us that polysialylation is a protein-specific modification that requires an initial protein–protein interaction between enzyme and substrate. Work in our labora-
Results for polysialyltransferase substrate recognition

tory has abundantly supported this notion (1, 3, 27). We have shown that an acidic patch on the first fibronectin type III repeat of NCAM (FN1) is required for the recognition, binding, and polysialylation of two N-glycans in the adjacent Ig5 domain (28–30). The same pattern holds for NRP-2, where a pair of acidic residues on the surface of the meprin-A5 antigen–μ-tyrosine phosphatase (MAM) domain are essential for polysialylation of O-glycans in the adjacent linker region (31).

With these data in mind, we identified a conserved amino acid stretch in the polySTs that is enriched in basic residues that might act as a complementary binding region for the acidic patches in the NCAM FN1 domain and NRP-2 MAM domain. This polybasic region (PBR) is composed of residues 86–120 in ST8Sia-II and residues 71–105 in ST8Sia-IV (32). We found that the ST8Sia-IV PBR was required for NCAM binding, and replacing PBR residues Arg82 and Arg93 substantially decreased NCAM polysialylation and blocked the ability of an inactive ST8Sia-IV enzyme (H331K) to serve as a competitive inhibitor of NCAM polysialylation by wild-type ST8Sia-IV (33). These results suggested that these basic residues are required for ST8Sia-IV recognition of NCAM (33). Recently, we confirmed this notion using biophysical techniques to demonstrate a direct interaction between the isolated PBR region and NCAM FN1 that depended upon PBR residues Arg82 and Arg93 and FN1 residues Asp520, Glu521, and Glu523 (34).

In this work, to understand how the two polySTs differentially recognize their substrates, we have compared the polyST PBR residues required for polysialylation of NCAM, SynCAM 1, and NRP-2, as well as enzyme autopolsialylation. A competition assay is then used to determine whether loss of substrate polysialylation reflects the loss of polyST recognition. We find that distinct sets of overlapping PBR residues in each polyST are required for the recognition and polysialylation of NCAM, NRP-2, or SynCAM 1. We also provide evidence for the mechanism of polyST autopolsialylation and make a surprising observation that polyST autopolsialylation is required for NRP-2 polysialylation and promotes SynCAM 1 polySia chain elongation.

Results and discussion

Previous work established the role of the ST8Sia-IV PBR residues Arg82 and Arg93 in NCAM recognition and polysialylation (32–34). In addition, we found that Arg82 plays a role in NRP-2 polysialylation but Arg93 does not (33). In this work, our overall goal was to determine whether each polyST-substrate pair requires a unique combination of ST8Sia-IV or ST8Sia-II PBR basic residues and how those residues contribute to the polysialylation process.

To do this, each arginine or lysine residue within the ST8Sia-II and ST8Sia-IV PBR regions was individually mutated to alanine (Fig. 1, underlined). Additionally, all ST8Sia-II and ST8Sia-IV PBR alanine mutants, as well as these PBR mutants of ST8Sia-II H346K and ST8Sia-IV H331K, inactive enzymes that are used in competition assays (see below), were examined for proper cellular localization by individually expressing each Myc-tagged protein in COS-1 cells and staining the cells with anti-Myc antibody. All of the polyST mutants co-localize with the GM130 Golgi marker, indicating that these proteins are correctly folded, exit the endoplasmic reticulum, and are transported to the Golgi where they can fulfill their function (data not shown).

Previous attempts to use a co-immunoprecipitation approach to evaluate the role of specific PBR residues in substrate binding provided inconclusive results. This is most likely due to multiple interactions between the polySTs and their substrates and the inability to distinguish productive and non-productive interactions (35). To isolate those interactions that lead to protein-specific polysialylation, we have taken a competition approach in which PBR residues are replaced in a catalytically inactive polyST, ST8Sia-II H346K or ST8Sia-IV H331K, and those inactive mutants that exhibit a reduced ability to compete with the active enzyme to block substrate polysialylation identify PBR residues that are likely to play a role in substrate recognition (33). In other words, if competition is decreased or lost for an inactive PBR mutant, and polysialylation of the substrate is recovered, then that residue is likely to be part of the enzyme recognition site for that substrate or alternatively influence the structure of the enzyme recognition site. Here, we present the comparisons of the impact of replacing basic PBR residues in the wild-type and inactive polySTs for each substrate–polyST pair to define residues involved in substrate recognition and polysialylation.

Overlapping sets of PBR basic residues impact NCAM and NRP-2 recognition and polysialylation by ST8Sia-IV

To compare the ST8Sia-IV PBR residues required for NCAM and NRP-2 recognition and polysialylation, Myc-tagged wild-
type and PBR mutant ST8Sia-IV proteins were co-expressed with V5-tagged substrates in COS-1 cells. Substrates were immunoprecipitated from cell lysates, and the level of substrate polysialylation was assessed by immunoblotting with an anti-polySia antibody (Figs. 2A and 3A, left top panels). Relative expression levels of NCAM, NRP-2, and the polyST were determined as described under “Experimental procedures” (middle and bottom panels). The relative amount of substrate polysialylation observed in the presence of each polyST PBR mutant was normalized to substrate expression levels and quantified using ImageJ software (National Institutes of Health) and indicated as a percentage of NCAM or NRP-2 polysialylation by unaltered ST8Sia-IV. Results from three or more repeats were averaged, and standard deviation (S.D.) and significance (p value) were assessed using a one-way ANOVA test with Dunnett’s post hoc test (Figs. 2A and 3A, right top scatterplots).

As observed previously by Foley et al. (32), replacing ST8Sia-IV Arg82 and Arg93 with alanines greatly reduced the polysialylation of NCAM to 49 ± 16% (S.D.) and 17 ± 14% (S.D.) of that seen with the wild-type enzyme, respectively (Fig. 2A and Table 1). Although smaller reductions in NCAM polysialylation are seen with other ST8Sia-IV PBR mutants, such as K83A (73 ± 4% (S.D.)), R87A (67 ± 11% (S.D.)), and K103A (70 ± 4% (S.D.)), Arg82 and Arg93 are the most critical ST8Sia-IV PBR basic residues for NCAM polysialylation. When NRP-2 polysialylation by ST8Sia-IV PBR mutants was evaluated, we found that replacing either Arg82 or Lys99 significantly reduced NRP-2 polysialylation to 9 ± 0.6% (S.D.) and 12 ± 12% (S.D.) of that seen with the wild-type enzyme, respectively (Fig. 3A and Table 1). In addition, replacing Arg87 also decreases polysialylation to a lesser extent (72 ± 14% (S.D.)).

Next, to evaluate whether the ST8Sia-IV PBR residues that contributed to NCAM and NRP-2 polysialylation also play roles

Figure 2. NCAM recognition and polysialylation requires Arg82 and Arg93 in the ST8Sia-IV PBR.
in substrate recognition, V5-tagged NCAM or NRP-2 were expressed with the untagged active polyST and Myc-tagged inactive ST8Sia-IV H331K and its PBR mutants in COS-1 cells at a ratio of 1:1:6 (substrate/wild-type ST8Sia-IV/ST8Sia-IV competitor) (Figs. 2B and 3B). V5-tagged substrates were immunoprecipitated from cell lysates, and their polysialylation was assessed by immunoblotting with an anti-polySia antibody (Figs. 2B and 3B, upper panels). Relative expression levels of NCAM, NRP-2, as well as wild-type and competitor polyST expression were determined as described under "Experimental procedures" (Figs. 2B and 3B, middle and bottom panels). Relative substrate polysialylation observed in the presence of ST8Sia-IV H331K and its PBR mutants was quantified using ImageJ software and reported as polysialylation recovery (fold recovery) observed with each PBR mutant as compared with the substrate polysialylation observed in the presence of the competitive inhibitor, ST8Sia-IV H331K.

In the case of NCAM, R82A and R93A mutations that significantly reduce its polysialylation also reduce the ability of the inactive H331K mutant to compete with the active enzyme, leading to an increase in NCAM polysialylation (fold recovery, R82A/H11005 2.94 ± 0.45 (S.D.); R93A/H11005 0.3 (S.D.)) (Fig. 2B).

Figure 3. NRP-2 polysialylation requires Arg82 and Lys99 in the ST8Sia-IV PBR, but only Arg82 is required for recognition. A, left panels, V5-tagged NRP-2 was co-expressed with Myc-tagged ST8Sia-IV or its mutants in COS-1 cells. After 24 h, NRP-2 was recovered from cell lysates by immunoprecipitation using an anti-V5 antibody and subjected to SDS-PAGE and immunoblotting with the 12F8 anti-polySia antibody to analyze the level of NRP-2 polysialylation (upper panel). Relative NRP-2 and ST8Sia-IV expression levels were determined as described under "Experimental procedures" (middle and bottom panels). Data are reported as % of wild-type enzyme polysialylation.

B, left panels, V5-tagged NRP-2 was co-expressed in COS-1 cells with untagged wild-type ST8Sia-IV and Myc-tagged inactive ST8Sia-IV H331K or its PBR mutants in a ratio of 1:1:6. NRP-2 polysialylation was determined as described above (upper panel). The relative expression levels of NRP-2 and ST8Sia-IV H331K or its mutants were determined as described under "Experimental procedures" (middle and bottom panels). Data are reported as fold recovery from competition with ST8Sia-IV H331K. A and B, right scatterplots, results from four and three repeats, respectively, were averaged, and standard deviation and significance were assessed using a one-way ANOVA test with a Dunnett’s post hoc test, where *, 0.01 < p < 0.05; **, 0.001 < p < 0.01; ***, 0.0001 < p < 0.001; ns, p > 0.05.

Table 1

|       | R72A | R82A | K83A | R87A | R93A | K99A | K103A |
|-------|------|------|------|------|------|------|-------|
| NCAM polysialylation | 89% ± 3% | 49 ± 16% | 73 ± 4% | 67 ± 11% | 17 ± 14% | 97 ± 13% | 70 ± 4% |
| NRP-2 polysialylation | 78 ± 12% | 9 ± 6% | 89 ± 9% | 72 ± 14% | 95 ± 10% | 12 ± 12% | 107 ± 12% |
| Autopolysialylation | 88 ± 9% | 47 ± 13% | 88 ± 10% | 109 ± 6% | 98 ± 4% | 24 ± 11% | 98 ± 14% |

* Values represent % of wild type ST8Sia-IV polysialylation.
and Table 2). This observation is in accordance with our recent data demonstrating that these residues are essential for the direct interaction between an isolated PBR peptide and a recombinant NCAM FN1 domain (34). Of the other PBR mutants that show a lesser impact on NCAM polysialylation (replacement of Lys83, Arg87, or Lys103), only the K83A mutation in the ST8Sia-IV H331K protein led to a reduction in competition and a significant recovery of NCAM polysialylation (fold recovery, K83A = 2.58 ± 0.57 (S.D.)) (Fig. 2B and Table 2).

Competition experiments for NRP-2 polysialylation revealed that replacing Arg82 and Arg87 in the ST8Sia-IV H331K protein led to a reduction in competition and a recovery of NRP-2 polysialylation (fold recovery, R82A = 4.75 ± 0.41 (S.D.); R87A = 3.35 ± 0.52 (S.D.)) (Fig. 3B and Table 2). As in the NCAM competition experiments, the K83A mutant, which did not significantly alter NRP-2 polysialylation, did lead to some recovery of NRP-2 polysialylation (fold recovery, K83A = 2.73 ± 0.58 (S.D.)). Finally, the most surprising result came for the K99A mutant, which substantially reduced NRP-2 polysialylation but exhibited a lower level of recovery of NRP-2 polysialylation in the competition experiment (fold recovery, K99A = 1.96 ± 0.82 (S.D.)) relative to the other mutants (Fig. 3B and Table 2).

These results demonstrate the common role of Arg82 in the recognition and polissialylation of NCAM and NRP-2. However, recognition and polissialylation requirements then deviate. NCAM requires Arg93 for recognition and polissialylation, whereas Lys99 plays a key role in NRP-2 polissialylation but may only weakly contribute to recognition. The mismatched contribution of Lys93 (NCAM) or Lys93 and Arg87 (NRP-2) to recognition and polissialylation of these substrates is difficult to explain, but it could reflect a change in local structure that impacts the availability of key interacting residues that is more readily detected in the competition assay. For example, any change in interaction surface of the inactive competitor, even a local structural change that slightly compromises access to key binding residues, would make it easier for the wild-type enzyme to replace the competitor and polissialylate the substrate. In contrast, the same mutant in the wild-type enzyme may have a much reduced impact on polissialylation if the enzyme could “hold on” to the substrate using a partial recognition site.

Table 2

| Impact of replacing ST8Sia-IV PBR residues on ST8Sia-IV H331K competition |
|---------------------------|-----------------|----------------|----------------|----------------|----------------|
|                           | R72A            | R82A           | K83A           | R87A           | R93A           | K99A           | K103A          |
| NCAM competition          | 1.23 ± 0.48 (1.18) | 2.94 ± 0.45 (10) | 2.58 ± 0.57 (8.78) | 1.46 ± 0.16 (4.97) | 2.51 ± 0.3 (8.54) | 1.63 ± 0.16 (5.54) | 0.76 ± 0.23 (2.59) |
| NRP-2 competition          | 1.64 ± 0.82 (3.45) | 4.75 ± 0.41 (10) | 2.73 ± 0.58 (5.75) | 3.35 ± 0.52 (7.05) | 1.24 ± 0.12 (2.61) | 1.96 ± 0.82 (4.13) | 1.81 ± 0.34 (3.81) |

Values represent fold recovery of polissialylation relative to the competition with the ST8Sia-IV H331K with an unaltered PBR. Numbers in parentheses were generated by setting the highest fold recovery to 10 and adjusting other values accordingly.

Polissialylation of NCAM and SynCAM 1 by ST8Sia-II requires the contribution of a larger set of PBR basic residues

We took the same approaches described above to evaluate the recognition and polissialylation of NCAM and SynCAM 1 by ST8Sia-II. At first, we used membrane-anchored substrates but realized that ST8Sia-II was co-precipitating with NCAM and SynCAM 1, something we had not observed with ST8Sia-IV. Consequently, we were concerned that the inconsistent results we initially obtained related to how much autopolissialylated ST8Sia-II was co-precipitating with the autopolissialylated substrate. To address this challenge, we used Fc-tagged soluble forms of substrates, denoted henceforth as NCAM-Fc and SynCAM-Fc. We reasoned that because these soluble forms are polissialylated in the Golgi by the Golgi-localized ST8Sia-II and then secreted into the cell culture medium, we could recover them without lysing the cells and thereby avoid co-precipitation with membrane-anchored enzyme.

The Fc-tagged substrates were precipitated from the cell culture media using protein A-Sepharose beads, and their polissialylation was assessed by immunoblotting with an anti-polySia antibody (Figs. 4A and 5A, left upper panels). The relative expression levels of the soluble substrates and the membrane-associated polySTs were determined as described under “Experimental procedures” (Figs. 4A and 5A, left middle and bottom panels). We confirmed the absence of any co-precipitated ST8Sia-II following protein A-Sepharose precipitation of Fc-tagged substrates from the cell culture medium by performing an immunoblot using the anti-V5 antibody (data not shown).

When NCAM-Fc was co-expressed with wild-type ST8Sia-II and PBR mutants in COS-1 cells, replacing Arg97 and Lys103, residues homologous to ST8Sia-IV Arg82 and Arg87, significantly reduced NCAM-Fc polissialylation to 33 ± 13% (S.D.) and 31 ± 12% (S.D.) of that seen with the wild-type enzyme, respectively (Figs. 4A and 3). Interestingly, replacing Lys114 and Lys118 in the ST8Sia-II PBR also significantly reduced NCAM-Fc polissialylation to 42 ± 18% (S.D.) and 31 ± 11% (S.D.) of that seen with the wild-type enzyme, respectively (Fig. 4A and 3). In contrast, replacing the analogous residues in the ST8Sia-IV PBR, Lys99 and Lys103, had no impact or a much reduced impact on NCAM polissialylation (97 ± 13% (S.D.) and 70 ± 4% (S.D.)) (Fig. 2A and 1). We also noted that replacing Lys102 led to a moderate reduction in NCAM polissialylation (66 ± 18% (S.D.)).

SynCAM 1 polissialylation by ST8Sia-II requires a somewhat different set of PBR residues. We observed that replacing Arg87 had a smaller impact on SynCAM-Fc polissialylation (51 ± 16% (S.D.)) than NCAM polissialylation (33 ± 13% (S.D.)) (Fig. 5A and 3, compare with Fig. 4A and 1). However, replacing Lys114 and Lys118 shows a greater reduction in SynCAM 1 polissialylation (3 ± 5% (S.D.) and 2 ± 3% (S.D.)) than in NCAM polissialylation (42 ± 18% (S.D.) and 31 ± 11% (S.D.)) (Fig. 5A and 3, compare with Fig. 4A and 1). Notably, replacing Lys102 also more greatly impacted SynCAM 1 polissialylation (21 ± 20% (S.D.) than NCAM polissialylation (66 ± 18% (S.D.)) (Fig. 5A and 1, compare with Fig. 4A and 3).
To evaluate the role of ST8Sia-II PBR residues in the recognition of NCAM-Fc and SynCAM-Fc, we created the PBR mutants in the ST8Sia-II H346K catalytically inactive enzyme and co-expressed these and the wild-type enzyme with NCAM-Fc or SynCAM-Fc in COS-1 cells. The impact of these enzyme competitor mutants on competition and recovery of NCAM-Fc and SynCAM-Fc polysialylation was determined as described above. In the case of NCAM-Fc, replacing several PBR residues led to a loss of competition by the ST8Sia-II H346K mutant. Replacing ST8Sia-II Arg97 and Lys108, which are homologous to the ST8Sia-IV Arg82 and Arg93 that are key for NCAM recognition and polysialylation by this polyST, led to the most loss of competition in the H346K background and maximum polysialylation recovery (fold recovery, 11.48 ± 2.54 (S.D.) and 9.54 ± 1.95 (S.D.)) (Fig. 4B and Table 4). Replacing other ST8Sia-II PBR basic residues that also reduced NCAM polysialylation, such as Lys102, Lys114, and Lys118, also allowed polysialylation recovery in competition assays (fold recovery, K102A = 5.83 ± 1.53 (S.D.), K114A = 6.79 ± 1.40 (S.D.), and K118A = 5.91 ± 1.32 (S.D.) (Fig. 4B and Table 4).

Table 3
Impact of replacing ST8Sia-II PBR residues on substrate polysialylation and enzyme autopolsialylation

|          | R87A | R95A | R97A | K98A | K102A | K108A | K114A | K118A |
|----------|------|------|------|------|-------|-------|-------|-------|
| NCAM polysialylation | 83 ± 10% | 87 ± 12% | 33 ± 13% | 87 ± 11% | 66 ± 18% | 23 ± 12% | 42 ± 18% | 31 ± 11% |
| SynCAM polysialylation | 77 ± 99% | 79 ± 19% | 51 ± 16% | 97 ± 12% | 21 ± 20% | 59 ± 20% | 3 ± 5% | 2 ± 3% |
| Autopolysialylation | 100 ± 13% | 83 ± 12% | 75 ± 9% | 102 ± 17% | 95 ± 21% | 102 ± 6% | 32 ± 9% | 15 ± 7% |

* Values represent % of wild type ST8Sia-II polysialylation.
For SynCAM-Fc, the greatest recovery of polysialylation in the competition assays was observed for the H346K K102A mutant (fold recovery, K102A/H11005 3.64/H11006 0.44 (S.D.) (Fig. 5B and Table 4)). Surprisingly, for the ST8Sia-II K114A and K118A mutants that nearly eliminated SynCAM-Fc polysialylation, only the H346K K114A mutant exhibited a statistically significant loss of competition and recovery of SynCAM-Fc polysialylation (fold recovery, 2.19/H11006 0.21 (S.D.) (Fig. 5B and Table 4)).

In sum, ST8Sia-II recognition and polysialylation of NCAM-Fc require Arg97 and Lys108 that are homologous to Arg82 and Arg93, the key residues for ST8Sia-IV substrate recognition and polysialylation, but other residues, Lys102, Lys114, and Lys118, may also play a role in recognition. SynCAM-Fc polysialylation appears to be quite different with modest contributions by Arg97 and Lys108, and stronger contributions by Lys102, Lys114, and Lys118. Strikingly, for SynCAM-Fc, only the H346K K102A and K114A mutants exhibit any significant loss of competition and recovery of polysialylation, suggesting these residues are involved in SynCAM 1 recognition. Based on their impact on NCAM-Fc and SynCAM-Fc polysialylation, we might have expected a larger recovery of polysialylation in the competition assays for the K114A and K118A mutants, and this

Table 4

| R87A | R95A | R97A | K98A | K102A | K108A | K114A | K118A |
|------|------|------|------|-------|-------|-------|-------|
| NCAM competition | 3.25 ± 0.84 (2.83) | 1.15 ± 0.32 (1) | 11.48 ± 2.54 (10) | 3.97 ± 2.43 (3.46) | 5.83 ± 1.53 (5.07) | 9.54 ± 1.95 (8.31) | 6.79 ± 1.40 (5.91) | 5.91 ± 1.32 (5.15) |
| SynCAM competition | 2.1 ± 0.27 (5.77) | 0.5 ± 0.44 (1.37) | 1.76 ± 0.29 (4.84) | 0.84 ± 0.36 (2.31) | 3.64 ± 0.44 (10) | 0.95 ± 0.74 (2.61) | 2.19 ± 0.21 (6.02) | 1.54 ± 0.80 (4.23) |

*Values represent fold recovery of polysialylation relative to the competition with the ST8Sia-II H346K with an unaltered PBR. Numbers in parentheses were generated by setting the highest fold recovery to 10 and adjusting other values accordingly.

For SynCAM-Fc, the greatest recovery of polysialylation in the competition assays was observed for the H346K K102A mutant (fold recovery, K102A = 3.64 ± 0.44 (S.D.)) (Fig. 5B and Table 4). Surprisingly, for the ST8Sia-II K114A and K118A mutants that nearly eliminated SynCAM-Fc polysialylation, only the H346K K114A mutant exhibited a statistically significant loss of competition and recovery of SynCAM-Fc polysialylation (fold recovery, 2.19 ± 0.21 (S.D.)) (Fig. 5B and Table 4).

In sum, ST8Sia-II recognition and polysialylation of NCAM-Fc require Arg97 and Lys102 that are homologous to Arg82 and Arg93, the key residues for ST8Sia-IV substrate recognition and polysialylation, but other residues, Lys102, Lys114, and Lys118, may also play a role in recognition. SynCAM-Fc polysialylation appears to be quite different with modest contributions by Arg97 and Lys102, and stronger contributions by Lys102, Lys114, and Lys118. Strikingly, for SynCAM-Fc, only the H346K K102A and K114A mutants exhibit any significant loss of competition and recovery of polysialylation, suggesting these residues are involved in SynCAM 1 recognition. Based on their impact on NCAM-Fc and SynCAM-Fc polysialylation, we might have expected a larger recovery of polysialylation in the competition assays for the K114A and K118A mutants, and this

Figure 5. SynCAM 1 polysialylation primarily requires Lys102, Lys114, and Lys118 in the ST8Sia-II PBR with Lys102 and Lys114 making the most significant contributions to recognition. A, left panels, SynCAM-Fc was co-expressed with V5-tagged ST8Sia-II or its PBR mutants in COS-1 cells. After 24 h, SynCAM-Fc was recovered from cell medium using protein A-Sepharose beads, and its polysialylation was assessed by SDS-PAGE and immunoblotting with the 12F8 anti-polySia antibody (upper panel). Relative expression levels of SynCAM-Fc, ST8Sia-II, or its mutants were determined as described under “Experimental procedures” (middle and bottom panels). Data are reported as % of wild-type enzyme polysialylation. B, left panels, SynCAM-Fc was co-expressed in COS-1 cells with V5-tagged wild-type ST8Sia-II and Myc-tagged inactive ST8Sia-II H346K or its PBR mutants in a ratio of 1:1:6. SynCAM-Fc polysialylation was determined as described above (upper panel). The relative expression levels of SynCAM-Fc and ST8Sia-II H346K or its mutants were determined as described under “Experimental procedures” (middle and bottom panels). Data are reported as fold recovery from competition with ST8Sia-II H346K. A and B, right scatterplots, results from three repeats were averaged, and standard deviation and significance were assessed using a one-way ANOVA test with a Dunnett’s post hoc test, where *, 0.01 < p < 0.05; **, 0.001 < p < 0.01; ***, 0.0001 < p < 0.001; ns, p > 0.05.
mismatch might reflect additional roles for these residues in the polysialylation process.

Potential roles of polyST PBR residues in substrate polysialylation

The loss of polysialylation versus loss of competition/gain of polysialylation numbers for some PBR mutants do not precisely match. In an effort compare the impact of these mutations for different polyST-substrate pairs, we set the highest fold recovery to 10 for each enzyme/substrate pair and adjusted the other numbers to that scale. We then grouped residues impacting polysialylation and/or competition into three groups. Residues colored green are those that when replaced led to substrate polysialylation of between 60 and 0% that seen with the wild-type enzyme and rated between 5 and 0 on the loss of competition/recovery of polysialylation scale. For these residues, loss of polysialylation matches the loss of substrate recognition. Residues colored red are those that when replaced led to substrate polysialylation of between 51 and 100% of that seen with the wild-type enzyme and rated between 5 and 10 on the loss of competition/recovery of polysialylation scale. For these residues, loss of recognition/competition was greater than observed loss in polysialylation. Residues colored blue are those that when replaced led to substrate polysialylation of between 0 and 50% that seen with the wild-type enzyme and rated between 0 and 4.99 on the loss of competition/recovery of polysialylation scale. For these residues loss of polysialylation was greater than the observed loss of recognition/competition.

Those residues that impact autopolysialylation when replaced with alanines are indicated by a *.

Specific PBR mutants have an effect on ST8Sia-IV and ST8Sia-II autopolysialylation

To evaluate the possibility that changes to the polyST PBRs may alter autopolysialylation, we individually expressed Myc-tagged ST8Sia-II and ST8Sia-IV PBR mutants in COS-1 cells, immunoprecipitated them using an anti-Myc antibody, and assessed their polysialylation by immunoblotting with an anti-polySia antibody (Fig. 8, A and B). For ST8Sia-IV, replacing PBR could serve as part of an extended basic surface that engages the growing polySia chain and promotes its polymerization and even the continued engagement of the substrate and enzyme once the protein-protein interaction no longer is possible (36–38), or they could play structural roles and/or be essential for catalytic activity. We created a model structure of ST8Sia-IV using the SWISS-MODEL server based on the crystal structure of another α2,8-sialyltransferase (ST8Sia-III) solved by Volkers et al. (38–42). This structure reveals the disposition of the PBR residues, their proximity to the two sites of ST8Sia-IV autopolysialylation, and to other basic residues of the polysialyltransferase domain (PSTD), identified by Nakata et al. (43), which together with the PBR could form a basic surface to promote polySia chain elongation (Fig. 7) (38). This model depicts the ST8Sia-IV PBR as a broken helix that has three parts. The first section is composed of Ser39–Ser42-Leu43 (NCAM), Arg42 (NRP-2), and ST8Sia-II Arg77, Lys108, Lys114, and Lys118 (NCAM), and Lys4 and Lys14 (SynCAM 1). Category 2 (Fig. 6, red) includes those residues that when replaced reduce competition but have a lesser impact on substrate polysialylation, suggesting that their replacement may impact local structure and the presentation of key recognition residues, an effect that may be more apparent in a competition assay. These residues include ST8Sia-IV Lys43 (NCAM) and ST8Sia-II Arg87 (NRP-2). Finally, category 3 (Fig. 6, blue) includes those residues that when replaced led to a loss of substrate polysialylation that was not associated with an accompanying loss of competition, suggesting that they are involved in something besides substrate recognition. These include ST8Sia-IV Lys43 (NRP-2) and Lys118 (SynCAM 1). Other residues such as ST8Sia-II Lys102 (NCAM) and Arg97 (SynCAM 1), which might be included in the green and blue groups, respectively, have intermediate impact on polysialylation and competition, making it difficult to categorize them.

What function could the residues in category 3 play? In addition to making contacts with substrates, basic residues in the
Arg82 and Lys99 led to reduced autopolysialylation (47 ± 13% (S.D.) and 24 ± 11% (S.D.) of that of the wild-type enzyme, respectively) (Fig. 8A and Table 1). Interestingly, although both mutants reduced overall levels of autopolysialylation, the K99A mutant was notable in that it maintained the ability to generate very high molecular mass forms of the enzyme (Fig. 8A).

For ST8Sia-II, replacing Lys114 (homologous to ST8Sia-IV Lys99) and Lys118 led to reduced autopolysialylation (32 ± 9% (S.D.) and 15 ± 7% (S.D.) of that of the wild-type enzyme, respectively), although replacing Arg97 (analogous to ST8Sia-IV Arg82) led to a smaller decrease in autopolysialylation (75 ± 9% (S.D.) of that of the wild-type enzyme) (Fig. 8B and Table 3). How could these mutations be impacting polyST autopolysialylation? For Arg82 and Lys99 in ST8Sia-IV, their proximity to the two sites of autopolysialylation suggests that replacing these residues might cause a local structural change that hinders the enzyme N-glycan access to the active site, and thus its autopolysialylation. This is unlikely to be the case for Lys114 and Lys118 because ST8Sia-II autopolysialylation occurs on three N-glycans that are not close to these two residues (23).

Because the polyST PBR residues that are essential for enzyme autopolysialylation are also involved in substrate recognition and polysialylation, we considered what processes could be common to both types of polysialylation. Both autopolysialylation and substrate polysialylation would certainly require a catalytically active enzyme. This possibility could be definitively ruled out for the ST8Sia-IV K99A mutant that shows a dramatic decrease in NRP-2 polysialylation (12 ± 12% (S.D.)), a smaller decrease in autopolysialylation (24 ± 11% (S.D.)), and very efficient NCAM polysialylation (97 ± 13% (S.D.)) (Table 1). The ST8Sia-IV R82A mutant appears to equally impact autopolysialylation and NCAM polysialylation reducing these to 47 ± 13% (S.D.) and 49 ± 16% (S.D.) of that seen with the wild-type enzyme. However, this mutation has a much greater impact on NRP-2 polysialylation reducing it to 9 ± 6% (S.D.) of that seen with the wild-type enzyme (Table 1).
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If there is a reduction in activity for the R82A mutant, it would only be ~50%. In contrast, the ST8Sia-II K114A and K118A mutants dramatically reduce SynCAM-Fc polysialylation to 3 ± 5% (S.D.) and 2 ± 3% (S.D.) of that seen with the wild-type enzyme, but have a smaller impact on NCAM-Fc polysialylation (42 ± 18% (S.D.) and 31 ± 18% (S.D.), respectively) (Table 3). Their impact on autopolysialylation is mixed with the K114A mutant reducing ST8Sia-II autopolsialylation to 32 ± 9% (S.D.) of that of the wild-type enzyme and the K118A mutant reducing ST8Sia-II autopolsialylation to 15 ± 7% (S.D.) of that of the wild-type enzyme (Table 3). Again, these mutants are not completely inactive and at least retain 30–40% of wild-type enzyme activity.

Two other processes might be required for both autopolsialylation and substrate polysialylation. First, autopolsialylation might require a protein–protein interaction like the one observed to initiate substrate polysialylation. For the autopolsialylation process, this would be an interaction between two enzyme monomers that would then polysialylate their partner’s glycans. Second, both autopolsialylation and substrate polysialylation may require the same or overlapping basic surfaces to optimally elongate growing polySia chains to lengths recognized by most anti-polySia antibodies (usually ≥8 units) (1, 2). It is important to note here that it is difficult to separate reduced catalytic activity, which would be involved in both chain initiation and elongation, from disruption of the elongation process due to lack of an adequate basic surface to stabilize the growing chain. To get insight into the roles of these PBR residues in substrate polysialylation and enzyme autopolsialylation, below we evaluate the mechanism of autopolsialylation and ask whether polyST autopolsialylation is required for NRP-2 and SynCAM 1 polysialylation.

**Autopolsialylation of ST8Sia-IV appears to be self- and not cross-polsialylation**

To evaluate whether ST8Sia-IV autopolsialylation requires one enzyme monomer to polysialylate a partner’s N-glycans (cross-polsialylation), we used two non-autopolysialylated but active mutant enzymes, ST8Sia-IV mut2.3, in which the second and third glycosylation sites, Asn219 and Asn234, that carry the polysialylated glycans, are mutated to serine and glutamine, respectively (22), and ST8Sia-IV L151A that localizes to the Golgi but is also not autopolysialylated.4 Both of these mutant enzymes are non-autopolysialylated but able to polysialylate NCAM (Fig. 9, A and C). We co-expressed V5-tagged mut2.3 and L151A with Myc-tagged inactive ST8Sia-IV H331K, immunoprecipitated the Myc-tagged protein from the cell lysates, and immunoblotted using an anti-polySia antibody to assess the ability of the active but non-autopolysialylated mutants to polysialylate the inactive polyST. We found that the non-autopolysialylated ST8Sia-IV mutants were not capable of polysialylating co-expressed ST8Sia-IV H331K (Fig. 9B). These results strongly suggest that autopolsialylation is a self- and not a cross-modification event.

So, despite the fact that both ST8Sia-IV Arg82 and ST8Sia-IV Lys114 are involved in substrate recognition, these results suggest that the decreased autopolsialylation of the ST8Sia-IV R82A and ST8Sia-IV K114A mutants is not due to reduction in the interaction of two enzyme monomers and may be more likely due to nearly equivalent reductions in catalytic activity/chain elongation. The decreased autopolsialylation of the K118A mutant that decreases NCAM recognition but not SynCAM 1 recognition likely reflects an ~30% decrease in catalytic activity plus an additional ~15% decrease possibly due to a disruption of the basic surface required for polySia chain elongation. Finally, the reduction seen in K99A autopolsialylation may be explained by a local structural alteration that hampers polyST N-glycan polysialylation. Another possibility is that this residue is an essential part of the basic surface required for chain elongation. We think that this possibility is unlikely because the small proportion of the K99A mutant that is polysialylated migrates as very high molecular mass forms suggesting that chain elongation *per se* is not compromised (Fig. 8A).

What was particularly striking in our comparisons of the impact of ST8Sia-IV Arg82 and Lys99 and ST8Sia-II Lys114 and Lys118 on enzyme autopolsialylation and substrate polysialylation was how replacing these residues had a much more dramatic impact on NRP-2 and SynCAM 1 polysialylation than it did on NCAM polysialylation (Tables 1 and 3). We have previously demonstrated that polyST autopolsialylation was not required for NCAM polysialylation, but these current data suggest that changes in the autopolsialylation of the PBR mutants may be able to explain, at least in part, the decreases observed in NRP-2 and SynCAM 1 polysialylation by the same mutants.

**ST8Sia-IV autopolsialylation is required for NRP-2 polysialylation and ST8Sia-II autopolsialylation promotes SynCAM 1 polySia chain elongation**

To determine whether enzyme autopolsialylation is required for NRP-2 polysialylation, we employed two ST8Sia-IV mutants used above that are catalytically active but not autopolsialylated. We found that although ST8Sia-IV mut2.3 and L151A can polysialylate NCAM, they cannot polysialylate NRP-2 (Fig. 9C). Consequently, the R82A and K99A mutations that compromise ST8Sia-IV autopolsialylation would in turn be expected to reduce NRP-2 polysialylation. Does the presence of polySia chains on ST8Sia-IV in some way promote polySia chain elongation on NRP-2 to lengths recognized by the anti-polySia antibodies (1, 2)? In support of this potential role for polyST polySia, we found that a non-autopolysialylated mutant of ST8Sia-II, mut2.4.5, could not generate a high molecular mass population of polysialylated SynCAM-Fc that was synthesized by the wild-type enzyme (Fig. 9D). As the fourth and fifth glycosylation/polysialylation sites (Asn219 and Asn234) are not near ST8Sia-II Lys114 and Lys118 in the linear sequence or in the predicted structure, it seems very unlikely that alterations in these PBR residues would structurally impact glycosylation of these sites or the availability of glycans for autopolsialylation (and in fact we see no evidence of the molecular mass change reflective of a loss of glycosylation at one or more sites). Instead, we favor the idea that Lys114 and Lys118 serve as part of a basic surface that is used for both substrate recognition as well as polySia chain elongation.
How would polyST autopolysialylation promote substrate polysialylation? One possibility is that the polySia chains on the polySTs stabilize or direct substrate interactions by blocking interactions with inappropriate basic surfaces on the enzyme and in this way direct acidic surfaces of substrate’s recognition domain to bind to the polyST’s PBR sequences. This would necessitate the enzyme’s polySia chains to bind back to the surface of the enzyme without binding to the PBR and blocking substrate access.

Another possibility is that the polySia chains on the polyST could bind to a basic surface on the substrate to prevent this surface from binding the growing polySia chain and hindering its elongation. For example, the O-glycans on NRP-2 that are polysialylated reside in a linker region between the second Factor V/Factor VIII homology domain and the MAM domain that serves as the NRP-2 recognition domain. The Factor V/Factor VIII domain is highly basic with a predicted pI of 8.31 (45), and it could interact with the growing, negatively charged polySia chain on the NRP-2 O-glycans. Could the ST8Sia-IV polySia chains block the interaction of the growing polySia chains with this basic domain, thereby promoting chain polymerization? This clearly would not be the case for SynCAM 1 that is polysialylated on one N-terminal Ig domain (25). Notably, for NCAM polysialylation that is not

Figure 9. polyST autopolysialylation is self- and not cross-polysialylated and is required for NRP-2 polysialylation and optimal SynCAM 1 polysialylation. A, V5-tagged ST8Sia-IV or non-autopolysialylated ST8Sia-IV mutants (mut2.3 and L151A) were expressed in COS-1 cells. B, Myc-tagged, inactive ST8Sia-IV H331K was co-expressed with V5-tagged mut2.3 or L151A catalytically active but non-autopolysialylated enzyme mutants in COS-1 cells. Myc-tagged ST8Sia-IV was expressed alone as an autopolysialylation control. C, V5-tagged ST8Sia-IV and its non-autopolysialylated mutants mut2.3 and L151A were co-expressed in COS-1 cells with Myc-tagged NCAM or NRP-2. D, Fc-tagged NCAM and SynCAM 1 were co-expressed separately with V5-tagged ST8Sia-II or its non-autopolysialylated mutant (ST8Sia-II mut2.4.5) in COS-1 cells. For all panels, protein polysialylation and relative protein expression levels were assessed by immunoblotting with either the anti-polySia 12F8 antibody (A–C) or the anti-polySia 735 antibody (D) and appropriate anti-tag antibodies as described under “Experimental procedures.” All experiments were performed at least three times, but quantitation was not performed because under the conditions tested there was no polysialylation (A–C) or the absence of a specific polysialylated form (D).
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impacted by polyST autopolylysialylation, the domain adjacent and N-terminal to the polylysialated Ig5 domain is Ig4 and it has a more acidic pI of 5.2 (45) than the analogous NRP-2 domain.

Another attractive possibility is that the polySia chains on the polySTs may stabilize or promote the elongation of polySia chains on the NRP-2 or SynCAM 1 substrates via direct carbohydrate–carbohydrate interactions. Although it might seem counterintuitive for two highly negatively charged chains to interact, using atomic force microscopy, Finne and co-workers (46) demonstrated that oligomers of polySia with 12 or more sialic acid units can assemble into filament networks. It is interesting to note that both NRP-2 and SynCAM 1 appear to engage fewer ST8Sia-IV and ST8Sia-II PBR residues than NCAM, and this observation begs the question of whether for these substrates an interaction with the polyST polySia chains provides that additional and, in the case of NRP-2, necessary stability required to maintain contact and achieve optimal polysialylation.

In summary, we have identified basic amino acids in the PBR sequences of ST8Sia-II and ST8Sia-IV that are required for substrate polysialylation. We note that these requirements vary for each substrate but do include common residues. Of the PBR residues required for substrate polysialylation, some are involved exclusively in substrate recognition. These include ST8Sia-IV Arg91 (NCAM), ST8Sia-II Arg97, Lys102, and Lys108 (NCAM), and Lys102 (SynCAM 1). Other PBR residues play roles in both substrate recognition and autopolylysialylation, a process that our data suggest is a true self-polysialylation and does not involve interaction between two enzyme monomers. These residues may serve two functions. First as contact points in the protein–protein interaction between substrate and polyST, and second as part of a basic surface on the polyST that engages growing polySia chains to stabilize them and promote elongation. This latter function would be shared by both substrate polysialylation and polyST autopolylysialylation. Residues in this category include ST8Sia-IV Arg82 (NCAM and NRP-2), ST8Sia-II Lys114 and Lys118 (NCAM), and ST8Sia-II Lys114 (SynCAM 1). For SynCAM 1 polysialylation, Lys118 may be solely involved in polySia chain elongation. Finally, we found for the first time that NRP-2 polysialylation by ST8Sia-IV requires that this polyST be autopolylysialylated and that the autopolylysialylation of ST8Sia-II promotes the polymerization of longer chains on SynCAM 1. From this, we can conclude that the impact of replacing PBR residues that are key for enzyme autopolylysialylation (ST8Sia-IV Arg82 and Lys99; ST8Sia-IV Lys114 and Lys118) on NRP-2 or SynCAM 1 polysialylation may at least in part reflect a loss of enzyme autopolylysialylation that in turn compromises these substrates’ polysialylation. The one case where a loss of polyST autopolylysialylation may be the predominant factor in a loss of substrate polysialylation is the ST8Sia-IV K99A mutant, which dramatically decreases enzyme autopolylysialylation to 24% that of the wild-type enzyme, and NRP-2 polysialylation to 12% that of wild-type enzyme, without substantially impacting NCAM polysialylation.

Our data and that of others (36–38, 43) allow us to propose a general model for substrate polysialylation. We predict that polysialylation is a two-step process. The first step consists of an initial protein–protein interaction between polyST and substrate that involves specific residues in the PBR of the polyST and a complementary acidic region of the enzyme. The second step occurs when the polySia chain is long enough to prohibit the protein–protein interaction and may shift the engagement to a protein–glycan interaction or a glycan–glycan interaction between polyST and substrate. For example, in the case of NCAM polysialylation by ST8Sia-II, Arg97, Lys108, Lys114, and Lys118 may all play roles in the initial protein–protein interaction, whereas Lys114 and Lys118 also take part in the second step to engage the growing polySia chain on NCAM. This step is also predicted to include basic residues in the PSTD region of the enzyme (38, 43). In the case of NRP-2 polysialylation by ST8Sia-IV, Arg82 is essential for the polyST–substrate protein–protein interaction, and although Arg82 and Lys99 may be involved in promoting polySia chain elongation on this substrate as part of a basic surface on the polyST, we propose that the polySia chains on the enzyme itself may directly impact continued substrate engagement and polySia chain polymerization. Future work will be directed at testing these models.

Experimental procedures

The cDNA for full-length human NCAM140 was a gift from Dr. Nancy Kedersha (Brigham and Women’s Hospital, Boston). The cDNAs for full-length human SynCAM 1 and NRP-2 were obtained from Dr. Thomas Biederer (Tufts University, Boston) and Dr. Nicholas Stamatos (University of Maryland School of Medicine, Baltimore, MD), respectively. The cDNAs for full-length human ST8Sia-II and ST8Sia-IV were obtained from Dr. John Lowe (Genentech, South San Francisco, CA) and Dr. Minoru Fukuda (Sanford Burnham Medical Research Institute, La Jolla, CA), respectively. pcDNA4-NCAM-Fc was a kind gift from Dr. Ken Kitajima (Nagoya University, Nagoya, Japan), and pcAG-ST8Sia-II-V5 was gifted by Dr. Stephen Dalton (University of Georgia, Athens, GA). Tissue culture media and reagents, including Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Opti-MEM I, Lipofectin, and Lipofectamine-2000 transfection reagents, as well as anti-V5 epitope tag antibody (catalogue no R960-25), and 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) were purchased from Thermo Fisher Scientific. Oligonucleotides were obtained from Thermo Fisher Scientific and Integrated DNA Technologies (Coralville, IA). QuikChange™ site-directed mutagenesis kit and Pfu DNA polymerase were purchased from Agilent (La Jolla, CA). Small- and large-scale plasmid DNA purification kits were obtained from Qiagen (Valencia, CA). In-Fusion HD kit was from Clontech. Protein A-Sepharose beads were purchased from GE Healthcare. Precision Plus™ protein standards, Clarity™ ECL Western blotting substrate, and 4–15% Mini-PROTEAN TGX precast gels were purchased from Bio-Rad. Nitrocellulose membranes were purchased from Schleicher & Schuell. Mouse monoclonal anti-Myc epitope tag antibody (9B11) (catalogue no. 2276), used for immunoprecipitation and immunoblotting, and rabbit polyclonal anti-Myc epitope (catalogue no. 2272), used for immunofluorescence, were obtained from Cell Signaling Technologies (Danvers, MA). Rat monoclonal anti-mouse CD56 (12F8) (catalogue no. 712 J. Biol. Chem. (2018) 293(2) 701–716
and mouse monoclonal anti-GM130 antibody (catalogue no. 610822) were obtained from BD Biosciences. Anti-polysialic acid 735 antibody (catalogue no. AB00240-2.0) was purchased from Absolute Antibody Ltd. (Oxford, UK). Horse-radish peroxidase (HRP)-conjugated anti-human IgG (H+L) (catalogue no. W403B) was obtained from Promega (Madison, WI). HRP-conjugated anti-mouse (catalogue no. 115-035-020) secondary antibodies for immunofluorescence were obtained from Jackson ImmunoResearch (West Grove, PA). HyBlot CL autoradiography films were purchased from Denville Scientific (Holliston, MA). All other chemicals and reagents were purchased from Sigma, VWR Scientific, and Fisher.

**Construction of ST8Sia-II-Myc and ST8Sia-IV-Myc PBR mutants**

The ST8Sia-II and ST8Sia-IV cDNAs were cloned into the EcoRV and XbaI sites of a previously digested pcDNA3.1/Myc-HisB mammalian expression vector, containing a C-terminal Myc epitope tag and a stop codon prior to the His6 tag. The resulting plasmids containing the DNA sequences for ST8Sia-II-Myc or ST8Sia-IV-Myc served as the templates for the creation of all PBR mutants, in which each arginine or lysine residue within the PBR was individually replaced with alanine. All ST8Sia-II PBR mutants were created as described previously by Foley et al. (32). All ST8Sia-II PBR mutants were created using the QuickChange™ site-directed mutagenesis kit and Pfu DNA polymerase according to manufacturer’s instructions using the oligonucleotide primers listed in Table 5. To confirm the presence of the desired ST8Sia-II PBR mutations, the resultant DNAs were sequenced using the DNA Sequencing Facility at the Research Resources Center at the University of Illinois at Chicago.

**Table 5**

| Primer set | Mutant | Template | Primer sequence |
|------------|--------|----------|-----------------|
| 1          | R87A   | ST8Sia-II| 5’-GACTTTTAAATCTGGCCATTCGCAATCTGATGTTAC-3’ |
| 2          | R95A   | ST8Sia-II| 5’-GACAGAGACCATGAAGGAGAGATG-3’ |
| 3          | R97A   | ST8Sia-II| 5’-AACTTTTTTAAATCTGGCCATTCGCAATCTGATGTTAC-3’ |
| 4          | K98A   | ST8Sia-II| 5’-GACTTTTAAATCTGGCCATTCGCAATCTGATGTTAC-3’ |
| 5          | K102A  | ST8Sia-II| 5’-AGGAAGGAGAGATG-3’ |
| 6          | K108A  | ST8Sia-II| 5’-AATCTTTTTTAAATCTGGCCATTCGCAATCTGATGTTAC-3’ |
| 7          | K114A  | ST8Sia-II| 5’-GACTTTTAAATCTGGCCATTCGCAATCTGATGTTAC-3’ |
| 8          | K118A  | ST8Sia-II| 5’-GACTTTTAAATCTGGCCATTCGCAATCTGATGTTAC-3’ |
| 9          | H331K  | ST8Sia-IV| 5’-AGGAAGGAGAGATG-3’ |
| 10         | H346K  | ST8Sia-IV| 5’-AATCTTTTTTAAATCTGGCCATTCGCAATCTGATGTTAC-3’ |
| 11         | Amplification of pcDNA4-Fc vector for in-fusion | pcDNA4-NRP-2-Fc | 5’-ACAGAGAGGAGAGATG-3’ |
| 12         | Amplification of ectodomains to make SynCAM-Fc construct | SynCAM 1 | 5’-CCATCTATTAGCTCGAGATG-3’ |

**Construction of ST8Sia-II-Myc H331K and ST8Sia-IV-Myc H346K PBR mutants**

Catalytically inactive, full-length ST8Sia-IV-Myc H331K, ST8Sia-II-Myc H346K, and associated PBR mutants were created using the oligonucleotide primers listed in Table 5. Wild-type ST8Sia-II and ST8Sia-IV, as well as the catalytically active ST8Sia-II and ST8Sia-IV PBR mutants above, served as the templates for the introduction of either the H331K (ST8Sia-IV and ST8Sia-IV PBR mutants) or H346K (ST8Sia-II and ST8Sia-III PBR mutants) point mutations. All H331K and H346K mutations were created and confirmed as described above.

**Construction of the pcDNA4-SynCAM 1-Fc construct**

Extracellular portion of SynCAM 1 was amplified with primers that included a 15-bp overhang on each side with sequence corresponding to the pcDNA4 vector on the N terminus and Fc fragment on the C terminus (Table 5). pcDNA4-Fc vector was amplified separately using primers indicated in Table 5. The PCR-amplified products were mixed, and homologous recombination was carried out to create the pcDNA4-SynCAM 1-Fc construct using Clontech In-Fusion HD cloning kit, according to the manufacturer’s protocol.

**Immunofluorescence analysis of the intracellular localization of polyST mutants in COS-1 cells**

COS-1 cells maintained in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum (FBS) were plated onto 12-mm glass coverslips and grown in a 37 °C, 5% CO2 incubator until 50–70% confluent. Cells on each coverslip were transfected with 0.5 µg of Myc-tagged ST8Sia-II, ST8Sia-IV, ST8Sia-IV H331K, and ST8Sia-II H346K or associated PBR mutant cDNA in 300 µl of Opti-MEM I supplemented with 3 µl of Lipofectin transfection reagent. Cells were incubated with the transfection mixture for 6 h at 37 °C, followed by the addition of 1 ml of DMEM, 10% FBS, and allowed to grow in the
Recovery of NCAM, NRP-2, and the polySTs from cell lysates or A-Sepharose beads (50% suspension in PBS) for 1 h at 4°C with 
250 M inverted confocal microscope using a azide, pH 8.5), and the cells were visualized using a Zeiss Axio-

Transfection of COS-1 cells for polysialylation, 

Transfection of COS-1 cells for polysialylation, autopolysialylation, and competition experiments 

COS-1 cells maintained in DMEM, 10% FBS, were plated onto 60-mm tissue culture plates and grown in a 37°C, 5% CO2 incubator until 70–80% confluent. For polysialylation experiments, cells were co-transfected with 3 μg of substrate cDNA (NCAM, NRP-2, or SynCAM 1) and 3 μg of wild-type or mutant polyST cDNA in 1 ml of Opti-MEM I containing 12 μl of Lipofectamine 2000 transfection reagent per tube. For autopolysialylation experiments, 6 μg of Myc-tagged ST8Sia-II or ST8Sia-IV were transfected, as described above. For competition experiments, cells were transfected with 750 ng of V5-tagged substrate cDNA (NCAM, SynCAM 1, or NRP-2), 750 ng of untagged ST8Sia-II or ST8Sia-IV cDNA, and 4.5 μg of a Myc-tagged polyST mutant cDNA (ST8Sia-IV H331K, ST8Sia-II H346K, or their PBR mutants) in 1 ml of Opti-MEM I containing 12 μl of Lipofectamine 2000 transfection reagent. For all experiments, cells were incubated with the transfection mixture plus 3 ml of DMEM, 10% FBS for 24 h in a 37°C, 5% CO2 incubator.

Recovery of NCAM, NRP-2, and the polySTs from cell lysates or cell medium 

Proteins were immunoprecipitated as described previously (31). For the NCAM, NRP-2, and autopolysialylation experiments, 24 h post-transfection, cells were washed twice with PBS and lysed in 200 μl of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS). Cell lysates were pre-cleared with 25 μl of protein A-Sepharose beads (50% suspension in PBS) for 1 h at 4°C with rotation. V5-tagged NCAM and NRP-2 and Myc-tagged polySTs were immunoprecipitated by incubating cell lysates with 1 μl of anti-V5 tag antibody or 1 μl of anti-Myc antibody overnight at 4°C with rotation. Samples were then rotated with 25 μl of protein A-Sepharose beads for 1 h at 4°C. The beads were washed four times with immunoprecipitation buffer and once with immunoprecipitation buffer containing 1% SDS. Samples were then resuspended in 25 μl of Laemmli sample buffer containing 10% β-mercaptoethanol, heated for 8 min at 65°C. To determine the relative NCAM, NRP-2, and polyST protein expression levels, 20 μl of cell lysate was removed prior to immunoprecipitation, mixed with 20 μl of Laemmli sample buffer containing 10% β-mercaptoethanol, and heated for 8 min at 100°C to remove polySia. Fc-tagged NCAM or SynCAM 1 were precipitated from the cell medium using protein A-Sepharose beads, as described previously (31). To determine relative protein expression levels, 25% of these beads were resuspended in Laemmli buffer and heated to 100°C to release proteins and remove polySia, as described previously (31). All protein samples were separated on 4–15% Mini-PROTEAN TGX gels.

Immunoblot analysis of protein expression and polysialylation 

Following gel electrophoresis, the proteins were transferred to nitrocellulose membranes at 100 V for 2 h. Membranes were blocked for 1 h at 4°C in blocking buffer (5% nonfat dry milk in Tris-buffered saline, pH 8.0, 0.1% Tween 20). To detect NCAM, SynCAM 1, or NRP-2 polysialylation, nitrocellulose membranes were incubated overnight at 4°C with 12F8 anti-polySia antibody diluted 1:1000 in 2% nonfat dry milk in Tris-buffered saline, pH 8.0 (1:500 dilution of 12F8 anti-polySia antibody was used to detect SynCAM-Fc polysialylation). Anti-polySia 735 antibody was diluted to 1:2000 in 5% nonfat dry milk in Tris-buffered saline, pH 8.0, to detect NCAM and NRP-2 polysialylation and 1:1000 for SynCAM-Fc polysialylation. Expression of V5-tagged proteins was detected using a 1:10,000 dilution of anti-V5 antibody, and Myc-tagged proteins were detected using a 1:2500 dilution of anti-Myc antibody. Following overnight incubation with primary antibodies, membranes were then incubated for 1 h at 4°C with HRP-conjugated goat anti-rat IgM (12F8-treated membranes) or HRP-conjugated goat anti-mouse IgG (anti-V5 or anti-Myc or anti-polySia 735 antibody-treated membranes) secondary antibodies diluted 1:5000 in blocking buffer. Fc-tagged proteins were detected with HRP-conjugated anti-human IgG (H+L) diluted 1:2500 in blocking buffer containing high salt (500 mM NaCl, 150 mM Tris-HCl, pH 8.0, and 0.1% Tween 20) for 45 min. Membranes were washed four times before and four times after secondary antibody incubation with Tris-buffered saline, pH 8.0, 0.1% Tween 20 (TBST) for 10 min per wash with agitation. Membranes blotted with anti-human IgG were washed with TBST containing high salt as described previously (31). Immunoblots were developed using the Clarity ECL Western blotting substrate and HyBlot CL autoradiography films. Please note the reactivity of the anti-polySia antibodies 12F8 and 735 has previously been established by ourselves and others by demonstrating that the epitope recognized by these antibodies is destroyed by the polySia-specific bacteriophage enzyme endoneuraminidase N (47).

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