

The polysialyltransferase ST8Sia II/STX: posttranslational processing and role of autopolysialylation in the polysialylation of neural cell adhesion molecule

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The presence of α 2,8-linked polysialic acid on the neural cell adhesion molecule (NCAM) is known to modulate cell interactions during development and oncogenesis. Two enzymes, the α 2,8-polysialyltransferases ST8Sia IV/PST and ST8Sia II/STX are responsible for the polysialylation of NCAM. We previously reported that both ST8Sia IV/PST and ST8Sia II/STX enzymes are themselves modified by α 2,8-linked polysialic acid chains, a process called autopolysialylation. In the case of ST8Sia IV/PST, autopolysialylation is not required for enzymatic activity. However, whether the autopolysialylation of ST8Sia II/STX is required for its ability to polysialylate NCAM is unknown. To understand how autopolysialylation impacts ST8Sia II/STX enzymatic activity, we employed a mutagenesis approach. We found that ST8Sia II/STX is modified by six Asn-linked oligosaccharides and that polysialic acid is distributed among the oligosaccharides modifying Asn 89, 219, and 234. Coexpression of a nonautopolysialylated ST8Sia II/STX mutant with NCAM demonstrated that autopolysialylation is not required for ST8Sia II/STX polysialyltransferase activity. In addition, catalytically active, nonautopolysialylated ST8Sia II/STX does not polysialylate any endogenous COS-1 cell proteins, highlighting the protein specificity of polysialylation. Furthermore, immunoblot analysis of NCAM polysialylation by autopolysialylated and nonautopolysialylated ST8Sia II/STX suggests that the NCAM is polysialylated to a higher degree by autopolysialylated ST8Sia II/STX. Therefore, we conclude that autopolysialylation of ST8Sia II/STX, like that of ST8Sia IV/PST, is not required for, but does enhance, NCAM polysialylation.

Key words: autopolysialylation/glycosylation/NCAM/polysialyltransferase/ST8Sia II

Introduction

The addition of sialic acid polymers (polysialic acid) to the termini of glycoprotein oligosaccharides is a posttranslational

and developmentally regulated event (Kiss and Rougon, 1997; Muhlenhoff *et al.*, 1998). Though this unusual carbohydrate structure is found in neuroinvasive bacteria (Robbins *et al.*, 1974; Vimr *et al.*, 1995) and in fish and sea urchin eggs (Inoue and Iwasaki, 1978; Kitazume *et al.*, 1994), the expression of polysialic acid in mammalian cells is of particular interest because of its established role as an antiadhesive agent during development and in metastasis. Though many forms of sialic acid have been identified, the predominant form of polysialic acid found in mammals is a linear homopolymer of 5-N-acetylneuraminic acid (Neu5Ac) linked by α 2,8-glycosidic bonds (Troy, 1992). Mammalian polysialic acid is unique in that it is found on a small group of proteins including the α -subunit of the rat brain voltage-sensitive sodium channel (Zuber *et al.*, 1992) and the polysialyltransferase enzymes themselves (Close and Colley, 1998). However, the most abundant carrier of polysialic acid in mammals is the neural cell adhesion molecule (NCAM) (Edelman and Crossin, 1991; Troy, 1995).

During embryogenesis, NCAM is present in a highly polysialylated form, with sialic acid comprising ~30% of its molecular mass (Rothbard *et al.*, 1982; Goridis and Brunet, 1992). High levels of polysialylated NCAM are observed in developing tissues, such as heart and muscle (Finne *et al.*, 1987; Watanabe *et al.*, 1992; Dubois *et al.*, 1994), kidney (Roth *et al.*, 1987, 1988b; Troy, 1995), and brain (Finne *et al.*, 1983; Berardi *et al.*, 1995). However, the majority of adult tissues express very low levels or no polysialic acid (Edelman, 1985; Rutishauser *et al.*, 1988; Troy, 1995). Many lines of evidence indicate that the presence of highly polysialylated NCAM at the cell surface disrupts the homophilic binding properties of NCAM, as well as general cell adhesion (Sadoul *et al.*, 1983; Rutishauser *et al.*, 1985; Rutishauser, 1996). This in turn facilitates cell migration during development (Rutishauser *et al.*, 1988; Acheson *et al.*, 1991; Rutishauser and Landmesser, 1996) and neurite outgrowth (Kiss and Rougon, 1997; Franceschini *et al.*, 2001). Polysialylated NCAM is also reexpressed on some metastatic cancers, such as neuroblastoma (Livingston *et al.*, 1988; Moolenaar *et al.*, 1990; Seidenfaden and Hildebrandt, 2001), small cell lung carcinoma (Kibbelaar *et al.*, 1989; Nilsson, 1992; Miyahara *et al.*, 2001), and the highly metastatic kidney tumor, Wilms' tumor (Roth *et al.*, 1988a; Gluer *et al.*, 1998). Akin to its role during development, this reexpression of polysialic acid is thought to enhance the metastatic potential of tumor cells by decreasing their adhesion and facilitating their migration (Michalides *et al.*, 1994; Scheidegger *et al.*, 1994; Fukuda, 1996).

Biosynthesis of polysialic acid in mammalian cells is carried out by two closely related enzymes, ST8Sia IV/PST (Nakayama *et al.*, 1995; Yoshida *et al.*, 1995; Eckhardt *et al.*, 1995; Phillips *et al.*, 1997) and ST8Sia II/STX (Livingston and

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Paulson, 1993; Kojima *et al.*, 1995; Scheidegger *et al.*, 1995). Whereas ST8Sia IV/PST and ST8Sia II/STX both catalyze the same polysialylation reaction, their expression patterns and polysialylation site preferences on NCAM differ. ST8Sia IV/PST and ST8Sia II/STX mRNA are both highly expressed in fetal brain (Livingston and Paulson, 1993; Nakayama *et al.*, 1995; Yoshida *et al.*, 1995; Kurosawa *et al.*, 1997). However, though ST8Sia II/STX mRNA is absent in the adult brain (Livingston and Paulson, 1993), ST8Sia IV/PST mRNA expression persists in a number of somatic tissues, as well as in areas of the adult brain that require continuing neurogenesis and neural plasticity (Nakayama *et al.*, 1995; Yoshida *et al.*, 1995; Phillips *et al.*, 1997). The distinct temporal and tissue-specific expression patterns of the polysialyltransferases suggest independent regulation at the transcriptional level (Seidenfaden *et al.*, 2000), and imply that ST8Sia II/STX is responsible for the embryonic polysialylation of NCAM (Angata *et al.*, 1997), whereas ST8Sia IV/PST is the dominant polysialyltransferase in the adult brain (Hildebrandt *et al.*, 1998).

In addition to the differences in their expression patterns, ST8Sia IV/PST and ST8Sia II/STX also exhibit distinct preferences with regard to the NCAM oligosaccharides they polysialylate. Members of the immunoglobulin superfamily of proteins, the NCAM 140 and 180 isoforms are composed of five Ig-like domains, two fibronectin type III repeats, and a transmembrane spanning segment followed by a cytoplasmic tail (Cunningham *et al.*, 1987; Goridis and Brunet, 1992). NCAM is modified by six Asn-linked oligosaccharides, three of which reside within the fifth Ig-like domain (numbered 4 through 6) and serve as the acceptors for polysialic acid addition (Nelson *et al.*, 1995; Franceschini *et al.*, 2001). Work by Angata *et al.* (1998) demonstrated that ST8Sia IV/PST preferentially adds polysialic acid to the oligosaccharide on Asn 6, with a small amount added to the oligosaccharide on Asn 5. In contrast, ST8Sia II/STX evenly distributes polysialic acid between the oligosaccharides on Asn 5 and Asn 6 (Angata *et al.*, 1998). Furthermore, ST8Sia IV/PST synthesizes a larger amount of polysialic acid on NCAM than ST8Sia II/STX (Angata *et al.*, 1998). It is interesting to note that we have also observed that ST8Sia IV/PST is autopolsialylated to a greater degree than ST8Sia II/STX (Close and Colley, 1998).

We previously reported that the ST8Sia IV/PST and ST8Sia II/STX polysialyltransferases are modified by α 2,8-linked polysialic acid chains when expressed in COS-1 cells (Close and Colley, 1998), a process termed autopolsialylation (Muhlenhoff *et al.*, 1996). These autopolsialylated enzymes localize to the Golgi, the cell surface, and are found soluble in the extracellular space. In addition, following their expression in COS-1 cells, we found that these polysialyltransferase proteins are the only polysialylated proteins expressed, suggesting that polysialylation is a protein-specific modification (Close and Colley, 1998; Close *et al.*, 2000). Subsequent analysis revealed that ST8Sia IV/PST is modified by five Asn-linked oligosaccharides, and that the oligosaccharide modifying Asn 74 possesses the majority of the polysialic acid (Close *et al.*, 2000). Furthermore, when nonautopolsialylated mutants of ST8Sia IV/PST were coexpressed with full-length and soluble NCAM, we found that ST8Sia IV/PST autopolsialylation is not required for NCAM polysialylation, but that it did seem to increase the amount of polysialic acid added to NCAM (Close *et al.*, 2000). Both ST8Sia IV/PST and ST8Sia

II/STX share 57% amino acid identity and catalyze the same polysialylation reaction on NCAM. This high degree of similarity suggests that the glycosylation pattern and activity requirements of ST8Sia II/STX may parallel that of ST8Sia IV/PST. Here we report on the carbohydrate modifications of ST8Sia II/STX and identify the requirements for ST8Sia II/STX autopolsialylation, as well as the requirements for polysialylation of NCAM by ST8Sia II/STX.

Results

ST8Sia II/STX is modified by six Asn-linked oligosaccharides

In order to determine whether autopolsialylation of ST8Sia II/STX is a prerequisite for its enzymatic activity, we first had to determine which N-glycosylation consensus sites of ST8Sia II/STX are utilized. Human ST8Sia II/STX (Scheidegger *et al.*, 1995) has six Asn residues positioned in potential Asn-X-Ser/Thr glycosylation sites (Figure 1A), and a seventh Asn residue (Asn 206) found in a disallowed Asn-Pro-Ser/Thr sequence (Kornfeld and Kornfeld, 1985). Asn residues in each consensus glycosylation site were mutated to Ser, as described in *Materials and methods*. COS-1 cells expressing wild-type and mutant ST8Sia II/STX proteins were metabolically labeled with 35 S-Express protein labeling mix for only 1 h to detect N-glycosylated but nonautopolsialylated species (Close and Colley, 1998). V5 epitope-tagged enzymes were immunoprecipitated from cell lysates using the anti-V5 epitope tag antibody (V5 Ab) and separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Empirically, a single nonautopolsialylated Asn-linked oligosaccharide contributes approximately 4 kDa to the molecular mass of a protein. Thus, mutagenesis of the Asn residue in a glycosylated consensus sequence will result in a protein that migrates ~4 kDa smaller than the wild-type protein. Analysis of wild-type glycosylated but nonautopolsialylated ST8Sia II/STX by SDS-polyacrylamide gel electrophoresis (PAGE) revealed that the largest form of the enzyme migrated with an apparent molecular mass of 67 kDa. This corresponds to the expected molecular mass of human ST8Sia II/STX modified by the V5 epitope tag and six Asn-linked oligosaccharides (Figure 1B, WT ST8Sia II/STX). Interestingly, six additional lower molecular mass forms decreasing in size by ~4 kDa increments were also observed in the lane containing the immunoprecipitated wild-type protein. Based on their molecular masses (43–63 kDa), it is likely that these bands represent differentially glycosylated forms of the enzyme with zero to six Asn-linked oligosaccharides added. Some or all of these lower-molecular-mass forms were also observed in lanes containing the mutant ST8Sia II/STX proteins described below, and these observations indicate some inefficiency in ST8Sia II/STX glycosylation in the COS-1 cell system. Individual mutations of Asn 60, 72, 89, 134, 219, and 234 to Ser resulted in proteins that migrated with reduced molecular mass (63 kDa) in comparison to wild-type ST8Sia II/STX (Figure 1B, compare the largest forms of WT ST8Sia II/STX to those of N60S, N72S, N89S, N134S, N219S, and N234S). This demonstrated each of these Asn residues is modified by an Asn-linked oligosaccharide in the wild-type ST8Sia II/STX protein. In contrast, no change in molecular mass occurred when Asn 206 was converted to Ser, indicating that this residue is not glycosylated (data not shown). These

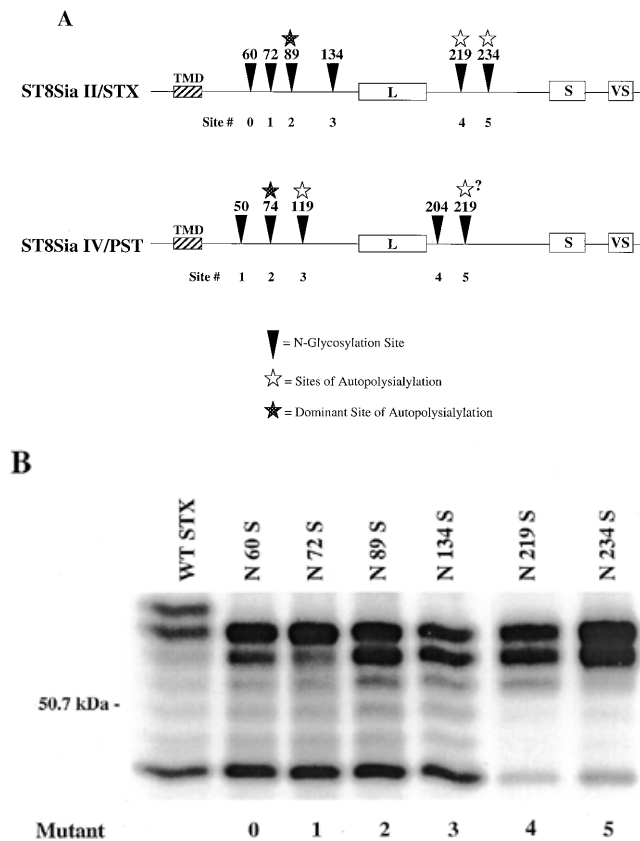


Fig. 1. ST8Sia II/STX is modified by five Asn-linked oligosaccharides. **(A)** Schematic representation of the ST8Sia II/STX and ST8Sia IV/PST enzymes. The numeric position of each Asn residue in the consensus N-glycosylation sites is given. Also shown are the transmembrane domain (TMD) and the sialyl motifs (L, S, and VS). The question mark (?) denotes a possible site of autopolysialylation of ST8Sia IV/PST. **(B)** Wild-type (WT) and mutant V5-tagged ST8Sia II/STX proteins were expressed in COS-1 cells, metabolically labeled for 1 h with ^{35}S -Express protein labeling mix and immunoprecipitated from cell lysates with V5 Ab. The samples were separated on 7.5% SDS–polyacrylamide gels, and radiolabeled protein bands visualized by fluorography. The molecular mass marker shown is 50.7 kDa, ovalbumin.

data demonstrate that human ST8Sia II/STX is modified by six Asn-linked oligosaccharides. For the purpose of directly comparing analogous glycosylation sites of ST8Sia II/STX and ST8Sia IV/PST (see Close *et al.*, 2000), we will refer to Asn residues 60, 72, 89, 134, 219, and 234 as Asn 0, 1, 2, 3, 4, and 5, respectively (Figure 1B, bottom).

Glycosylation of Asn 89 (site 2), Asn 219 (site 4), and Asn 234 (site 5) is necessary for ST8Sia II/STX autopolysialylation

To establish whether the ST8Sia II/STX glycosylation mutants were autopolysialylated like wild-type enzyme, we expressed these mutant proteins in COS-1 cells that lack endogenous polysialyltransferases and their known glycoprotein substrates (Close and Colley, 1998). The expressing cells were metabolically labeled with ^{35}S -Express protein labeling mix for 1 h and chased with unlabeled medium for 6 h; the mutant ST8Sia II/STX proteins were immunoprecipitated from the cell medium and analyzed by SDS–PAGE and fluorography. Wild-type ST8Sia II/STX appeared as two forms: a 60-kDa glycosylated but

nonautopolysialylated form and a polydisperse form with an apparent molecular mass ranging from ~68 kDa to ~160 kDa (Figure 2A, WT). Previous work established that the 60-kDa form represents a population of ST8Sia II/STX secreted from the cell modified only by high mannose oligosaccharides (Figure 2A, see arrow), whereas the larger form of ST8Sia II/STX represents the autopolysialylated enzyme (Close and Colley, 1998). Mutation of Asn 0, 1, or 3 to Ser resulted in proteins that appeared to be autopolysialylated to the same extent as the wild-type ST8Sia II/STX (Figure 2A, mutants 0, 1, and 3). In

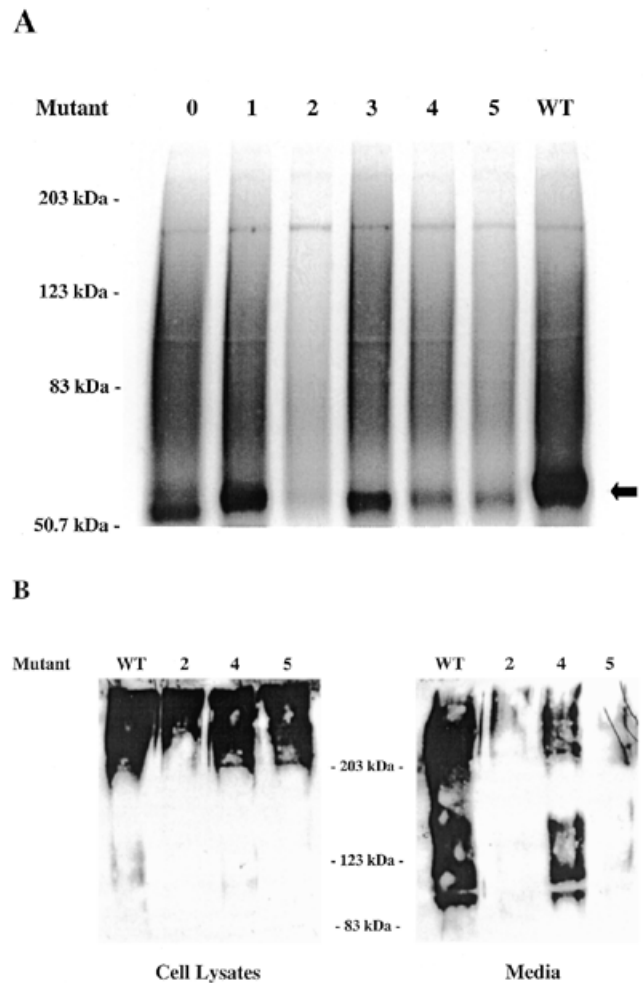


Fig. 2. Elimination of glycosylation site 2 (Asn 89), site 4 (Asn 219), or site 5 (Asn 234) decreases the autopolysialylation of ST8Sia II/STX. **(A)** Wild-type (WT) and single Ser mutant V5-tagged ST8Sia II/STX proteins were transiently expressed in COS-1 cells, metabolically labeled with ^{35}S -Express protein labeling mix for 1 h and immunoprecipitated from 6 h chase medium with the V5 Ab. The immunoprecipitated samples were electrophoresed on 7.5% SDS–polyacrylamide gels, and radiolabeled protein bands visualized by fluorography. The arrow indicates the position of the form of ST8Sia II/STX possessing only high-mannose oligosaccharides. **(B)** A parallel experiment was conducted without metabolic labeling of the polysialyltransferases. Wild-type (WT) and mutant V5-tagged ST8Sia II/STX proteins were immunoprecipitated from COS-1 cell lysates and medium 18 h posttransfection, separated on 7.5% SDS–polyacrylamide gels, and electrophoretically transferred to nitrocellulose membranes. The membranes were subjected to immunoblot analysis using OL28 Ab. Molecular mass markers are as follows: 203 kDa, myosin; 123 kDa, β -galactosidase; 83 kDa, BSA; 50.7 kDa, ovalbumin.

contrast, mutation of Asn 2, Asn 4, or Asn 5 to Ser resulted in a substantial reduction in the high-molecular-mass autopolysialylated form of the enzyme, suggesting that the oligosaccharides attached to these Asn residues were modified by polysialic acid (Figure 2A, mutants 2, 4, and 5). To confirm that the polydisperse, high-molecular-mass forms of wild-type and mutant ST8Sia II/STX proteins are modified by α 2,8-linked polysialic acid and that autopolysialylation of the mutant 2, 4, and 5 proteins is reduced, we performed a parallel immunoblot with the OL.28 antibody (Figure 2B). Proteins were expressed in COS-1 cells, immunoprecipitated from unlabeled cell lysates and medium, and analyzed by immunoblotting with the OL.28 anti-polysialic acid antibody (OL.28 Ab) that is specific for α 2,8-linked polyNeu5Ac of 5 units or longer (Close *et al.*, 2000; Sato *et al.*, 2000). We found that wild-type ST8Sia II/STX and the mutant proteins lacking the oligosaccharides on sites 0, 1, and 3 migrated with similar polysialylated molecular masses and exhibited equal intensities upon immunoblotting with OL.28 Ab (data not shown), suggesting that none of the oligosaccharides attached to these sites are polysialylated. Interestingly, although the autopolysialylation of the cell-associated mutant 2, 4, and 5 proteins was only marginally reduced compared to that of the wild-type ST8Sia II/STX, the autopolysialylation of the secreted mutant 2, 4, and 5 proteins was dramatically reduced relative to that of wild-type ST8Sia II/STX (Figure 2B, mutants 2, 4, and 5, Cell Lysates and Media).

From these data, we could not be sure whether the decrease in autopolysialylation of these three mutant proteins (mutants 2, 4, and 5) reflected the absence of polysialic acid attachment sites or problems with their intracellular trafficking. We considered two possibilities. First, were these proteins folding in such a way that they were slow to leave the endoplasmic reticulum (ER) or slow to leave the Golgi, resulting in their reduced appearance in the cell medium? If this was the case, could this be due to the absence of an oligosaccharide during the folding process or the presence of the foreign Ser residue? Second, was the polysialic acid divided evenly between the oligosaccharides attached to these three sites? If it were, would we observe a more substantial decrease in autopolysialylation if we mutated two or three glycosylation sites at one time?

To address these issues we replaced the Asn 2, 4, and 5 residues with Gln rather than Ser to test the possibility that the nature of the amino acid at this site was having an impact on folding, and that this was leading to differences in trafficking and/or enzymatic activity. To confirm that the oligosaccharides on Asn 2, 4, and 5 possess all the polysialic acid-modifying ST8Sia II/STX, we also made double glycosylation site mutants (mutants 2.4, 2.5, and 4.5) and triple glycosylation site mutants (mutant 2.4.5) using both Ser and Gln to replace Asn. We first analyzed these single, double, and triple glycosylation site mutants by immunofluorescence microscopy (Figures 3 [Ser mutants] and 4 [Gln mutants]). We used both the V5 Ab to localize the proteins within the cell, and the OL.28 Ab to evaluate the level of autopolysialylation of the mutant proteins, as well as where these autopolysialylated proteins were localized. Previous work by Close *et al.* (2000) demonstrated that no endogenous COS-1 cell proteins are substrates for ST8Sia IV/PST, and we expected the same situation for ST8Sia II/STX. Staining with the V5 Ab revealed that, like the wild-type ST8Sia II/STX protein, all the single, double, and triple mutants (either Ser or Gln replacements) were localized in the

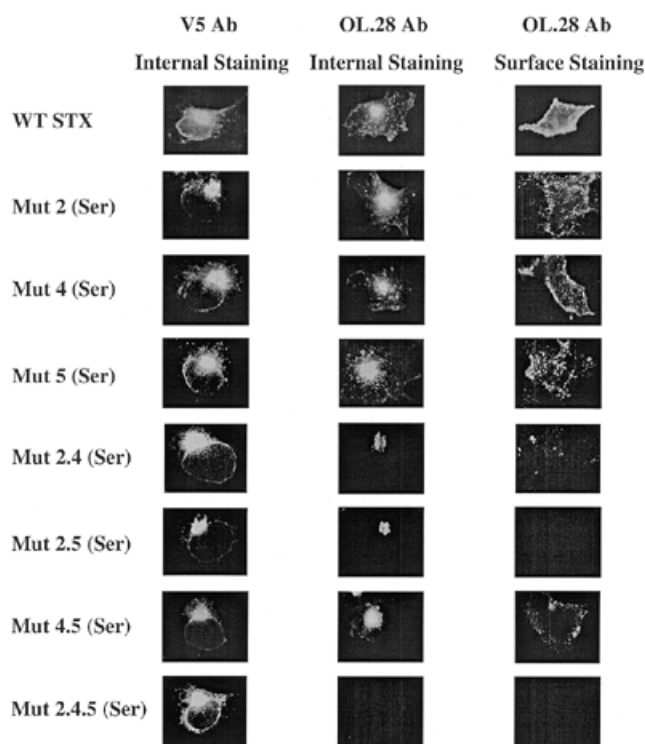


Fig. 3. Glycosylation sites 2, 4, and 5 are necessary for ST8Sia II/STX autopolysialylation: Localization and autopolysialylation of wild-type ST8Sia II/STX and Ser mutants. COS-1 cells transiently expressing wild-type ST8Sia II/STX (WT) or single, double, or triple Asn to Ser mutant ST8Sia II/STX proteins were analyzed by indirect immunofluorescence microscopy using either V5 Ab (internal staining) or OL.28 Ab (internal and surface staining), and the appropriate FITC-conjugated secondary antibodies. Prior to staining, cells were fixed and permeabilized with methanol to visualize internal structures or fixed with 3% paraformaldehyde to visualize only cell surface. Immunofluorescence was visualized using a Nikon Axiophot fluorescence microscope and a 60 \times oil immersion Plan Apochromat objective. Magnification, 750 \times .

Golgi. As more glycosylation sites were mutated, however, we did observe more ER staining in addition to distinct Golgi staining. This suggested that the presence of oligosaccharides at specific positions in the protein might aid the folding process. Notably, no differences were seen when we compared the localization of the Ser versus the Gln mutants.

Comparison of the autopolysialylated forms of these two sets of mutant proteins revealed only subtle differences in the level of autopolysialylation and localization. As was observed on the immunoblots in Figure 2B, the level of autopolysialylation of the intracellular single glycosylation mutants (Ser or Gln replacements) did not appear substantially decreased relative to wild-type ST8Sia II/STX (Figures 3 and 4, compare ST8Sia II/STX, Mut 2, Mut 4, and Mut 5, OL.28 Ab internal staining). Notably, the presence of these autopolysialylated mutant ST8Sia II/STX proteins was decreased on the cell surface relative to wild-type ST8Sia II/STX (Figures 3 and 4, compare ST8Sia II/STX, Mut 2, Mut 4, and Mut 5, OL.28 Ab surface staining). This again suggested that these mutants are slow to leave the Golgi, after which we would expect a smaller amount to move to the cell surface and the majority to enter a post-Golgi compartment where cleavage would occur leading to secretion

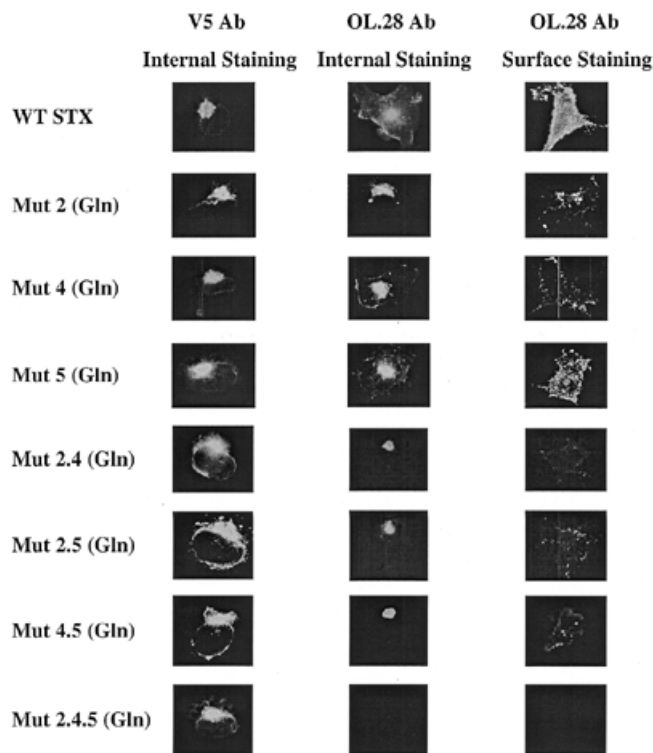


Fig. 4. Glycosylation sites 2, 4, and 5 are necessary for ST8Sia II/STX autopolysialylation: Localization and autopolysialylation of wild-type ST8Sia II/STX and Gln mutants. COS-1 cells transiently expressing wild-type ST8Sia II/STX (WT) or single, double, or triple Asn to Gln mutant ST8Sia II/STX proteins were analyzed by indirect immunofluorescence microscopy using either V5 Ab (internal staining) or OL28 Ab (internal and surface staining), and the appropriate FITC-conjugated secondary antibodies. Prior to staining, cells were fixed and permeabilized with methanol to visualize internal structures or fixed with 3% paraformaldehyde to visualize only cell surface. Immunofluorescence was visualized using a Nikon Axiophot fluorescence microscope and a 60 \times oil immersion Plan Apochromat objective. Magnification, 750 \times .

(see Ma *et al.*, 1997). Interestingly, we observed more cell surface autopolysialylated protein when Asn 2 or Asn 4 was replaced with Ser than when these amino acids were replaced by Gln, suggesting that the Gln mutants may be either more stably retained in the Golgi or have lower catalytic activity than the analogous Ser mutants (compare Mut 2 and Mut 4 OL28 Ab surface staining in Figure 3 to the analogous panels in Figure 4).

Evaluation of the double and triple glycosylation site mutants verified that the polysialic acid on ST8Sia II/STX is modifying the oligosaccharides on Asn 89 (site 2), Asn 219 (site 4), and Asn 234 (site 5). For both the Ser and Gln double glycosylation mutants, we observed a significant decrease in both intracellular and cell surface polysialic acid (Figures 3 and 4, Mut 2.4, Mut 2.5, and Mut 4.5, OL28 Ab internal and surface staining). Replacing all three glycosylation sites with either Ser or Gln, led to proteins that were primarily Golgi localized but that exhibited no autopolysialylation (Figures 3 and 4, Mutant 2.4.5). These results were also supported by OL28 immunoblot experiments shown in Figure 5 for the double and triple Ser glycosylation mutants. Notably, Mut 4.5

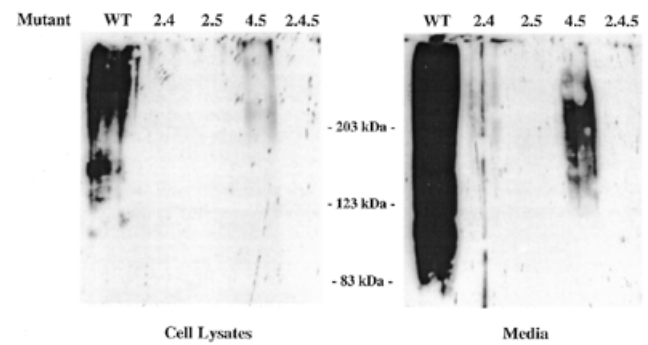


Fig. 5. Autopolysialylation of Asn to Ser ST8Sia II/STX mutant proteins. Wild-type ST8Sia II/STX (WT), double, or triple Asn to Ser mutant ST8Sia II/STX proteins were transiently expressed in COS-1 cells without metabolic labeling and immunoprecipitated from COS-1 cell lysates and medium 18 h posttransfection. Immunoprecipitated proteins were separated on 7.5% SDS–polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes, and subjected to immunoblot analysis using OL28 Ab. Molecular mass markers are as follows: 203 kDa, myosin; 123 kDa, β -galactosidase; 83 kDa, BSA.

Ser, which still possesses an oligosaccharide on Asn 89 (site 2) exhibits the most autopolysialylation of the three double glycosylation site mutants in both the immunoblot and immunofluorescence experiments (Figure 3 and Figure 5, Mut 4.5). This suggested that the oligosaccharide attached to Asn 89 (site 2) may, like the analogous oligosaccharide attached to ST8Sia IV/PST, possess the majority of the polysialic acid.

Retention of at least two Asn-linked oligosaccharides is sufficient for ST8Sia II/STX autopolysialylation in COS-1 cells

To evaluate the relative levels of polysialylation of the oligosaccharides attached to the oligosaccharides modifying Asn 89 (site 2), Asn 219 (site 4), and Asn 234 (site 5), we generated a series of mutants retaining one, two, or all three of these Asn-linked glycosylation sites (the “alone” mutants). These mutant proteins were analyzed by immunofluorescence microscopy to determine their intracellular localization and level of autopolysialylation, as done with the glycosylation site mutants in Figures 3 and 4. All the mutants retaining one, two, or three Asn-linked oligosaccharides were transported to the Golgi apparatus (Figure 6, V5 Ab internal). However, immunofluorescence microscopy experiments using the OL28 Ab revealed that none of mutants retaining only one of the three Asn-linked oligosaccharides was detectably autopolysialylated (Figure 6, 2 Alone, 4 Alone, and 5 alone, OL28 Ab internal and surface). In contrast, mutants retaining two of the three Asn-linked oligosaccharides, those modifying Asn 2 and 4 (2.4 Alone), Asn 2 and 5 (2.5 Alone), or Asn 4 and 5 (4.5 Alone), were found in their autopolysialylated forms in the Golgi and at the cell surface (Figure 6, OL28 Ab internal and surface). Likewise, the ST8Sia II/STX protein retaining Asn residues 2, 4, and 5 (2.4.5 Alone) was autopolysialylated to a level comparable to wild-type ST8Sia II/STX (Figure 6, OL28 Ab internal and surface, compare WT to 2.4.5 Alone). This experiment suggested that there were no substantial differences in the autopolysialylation of the three ST8Sia II/STX Asn-linked oligosaccharides in these “alone” mutant proteins. In addition, the results did support the previous conclusion that the

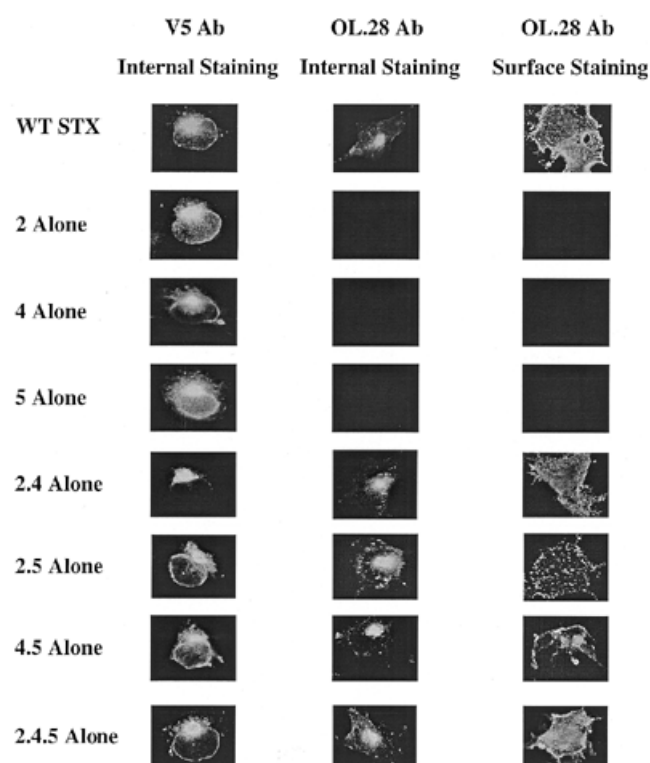


Fig. 6. Retention of two or more of the identified glycosylation sites (Asn 89, and/or Asn 219, and/or Asn 234) is sufficient for ST8Sia II/STX autopolysialylation. A series of mutants was generated that retain only one, two, or all three of the Asn 89, Asn 219, and/or Asn 234 glycosylation sites. The localization and autopolysialylation of these mutants was analyzed by indirect immunofluorescence microscopy using V5 Ab (internal staining), OL28 Ab (internal and surface staining), and the appropriate FITC-conjugated secondary antibodies. Immunofluorescence was visualized using a Nikon Axiophot fluorescence microscope and a 60 \times oil immersion Plan Apochromat objective. Magnification, 750 \times .

oligosaccharides on Asn 89, Asn 219, and Asn 234 possess the polysialic acid-modifying ST8Sia II/STX.

Why was the presence of at least two of the three glycosylation sites required for ST8Sia II/STX autopolysialylation? One obvious possibility is that the mutant proteins modified by only one glycosylation site were folding into forms that were able to exit the ER and be transported to the Golgi but lacked enzymatic activity. In this type of *in vivo* assay, a lack of enzymatic activity could either reflect the protein folding into an inactive state or the protein folding into a form that localized too early in the Golgi to be functional, such as in an early Golgi compartment that does not contain the CMP-NeuAc sugar nucleotide donor. These same issues were concerns for the triple glycosylation mutants, Mut 2.4.5 Ser and Mut 2.4.5 Gln, that were also nonautopolysialylated when expressed in COS-1 cells (Figures 3, 4, and 5).

ST8Sia II/STX autopolysialylation is not a requirement for polysialylation of NCAM

To evaluate the polysialyltransferase activity of both the ST8Sia II/STX triple glycosylation site mutants (Mut 2.4.5 Ser and Mut 2.4.5 Gln), as well as the mutant proteins possessing only one Asn-linked oligosaccharide (2 Alone, 4 Alone, and

5 Alone), we evaluated their ability to polysialylate NCAM when coexpressed with this substrate in COS-1 cells. First, to determine whether the 2 Alone, 4 Alone, and 5 Alone mutant ST8Sia II/STX proteins were active, we coexpressed these proteins with full-length NCAM and evaluated NCAM polysialylation by immunofluorescence microscopy with the OL28 Ab. We found that all three of these mutant proteins were capable of NCAM polysialylation, even though they were not autopolysialylated at a detectable level (Figure 7A). This indicated that the inability of these mutant ST8Sia II/STX proteins to autopolysialylate did not reflect a loss of catalytic activity or mislocalization to an early Golgi compartment.

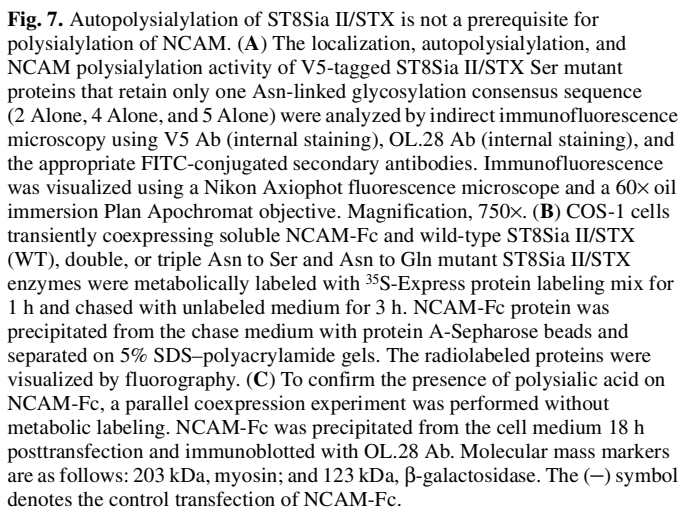
Next we evaluated the ability of both the double and triple glycosylation site mutants to polysialylate NCAM in a similar coexpression assay. Because the double glycosylation site mutants were autopolysialylated to some degree, we could not use immunofluorescence microscopy to evaluate NCAM polysialylation. Instead, we coexpressed soluble NCAM-Fc with wild-type ST8Sia II/STX and the double and triple mutant ST8Sia II/STX proteins (Ser and Gln replacements) in COS-1 cells. Expressing cells were radiolabeled for 1 h, chased with unlabeled medium for 3 h, and soluble NCAM-Fc was precipitated from the medium with protein A-Sepharose as described in *Materials and methods*. The NCAM-Fc monomer migrates on SDS-polyacrylamide gels with an apparent molecular mass of 180 kDa, and polysialylated NCAM-Fc appears as a polydisperse band with a molecular mass ranging from 180 kDa to above 200 kDa (Figure 7B). We confirmed that the polydisperse appearance of NCAM-Fc reflected polysialylation by immunoblotting NCAM-Fc with the OL28 Ab (Figure 7C). As expected, coexpression of wild-type ST8Sia II/STX with NCAM-Fc resulted in the polysialylation of the NCAM-Fc (Figures 7B and 7C, WT). Likewise, coexpression of the Ser and Gln double glycosylation mutants with NCAM-Fc also resulted in the polysialylation of NCAM-Fc (Figures 7B and 7C, Ser and Gln mutants 2.4, 2.5, and 4.5). Strikingly, we observed that the Mut 2.4 Gln and Mut 4.5 Gln proteins add less polysialic acid to NCAM than the comparable Ser double mutants (Figure 7C, compare especially the Gln and Ser mutants 2.4 and 4.5). Most significant was the finding Mut 2.4.5 Ser, which was shown to be nonautopolysialylated (Figures 3, 4, and 5) was still able to polysialylate NCAM-Fc in our coexpression assay, whereas the Mut 2.4.5 Gln appeared to be completely inactive (Figures 7B and 7C, Ser and Gln mutants 2.4.5).

Whether inability of the Mut 2.4.5 Gln to polysialylate NCAM reflects the protein's inactivity or its inability to compartmentalize correctly is not clear. More important, the ability of the nonautopolysialylated Mut 2.4.5 Ser protein to polysialylate NCAM nicely parallels our previous report that nonautopolysialylated ST8Sia IV/PST is capable of NCAM polysialylation (Close *et al.*, 2000) by demonstrating that autopolysialylation is not a requirement for ST8Sia II/STX enzymatic activity as well. In sum, our data in this work establish that autopolysialylation of ST8Sia II/STX, like that of ST8Sia IV/PST, is not a prerequisite for its ability to polysialylate NCAM. In addition, the mutant ST8Sia II/STX proteins analyzed in these experiments showed us that the level of NCAM polysialylation appears to depend on not only their autopolysialylation (as seen previously with the ST8Sia IV/PST glycosylation/autopolysialylation mutants [Close *et al.*, 2000])

Previously, we reported that both ST8Sia IV/PST and ST8Sia II/STX are autopolysialylated on complex type Asn-linked oligosaccharides when expressed in mammalian cells (Close and Colley, 1998). This was notable because polysialylation appears to be an extremely protein-specific event, with polysialic acid previously found only on the oligosaccharides attached to NCAM and the α subunit of the voltage sensitive sodium channel (Zuber *et al.*, 1992). The presence of polysialic acid on ST8Sia IV/PST and ST8Sia II/STX led us to question what role this modification plays in their enzymatic activity. We found that abrogation of ST8Sia IV/PST autopolysialylation by elimination of critical glycosylation sites or by a nonasparagine point mutation did not abolish its ability to polysialylate NCAM (Close *et al.*, 2000; Close and Colley, unpublished data). However, autopolysialylated ST8Sia IV/PST did seem to add more polysialic acid to NCAM, suggesting that polysialic acid may somehow stabilize the interaction of ST8Sia IV/PST with NCAM.

In this work we have demonstrated that ST8Sia II/STX has polysialic acid modifying the oligosaccharides attached to Asn 89, Asn 219, and Asn 234. These ST8Sia II/STX glycosylation sites are analogous to glycosylation sites 2, 4, and 5 in ST8Sia IV/PST (Close *et al.*, 2000). Elimination of combinations of two of these glycosylation sites (Mut 2.4, Mut 2.5, and Mut 4.5) led to significant decreases in the autopolysialylation of the mutant ST8Sia II/STX proteins, and elimination of all three sites completely abolished ST8Sia II/STX autopolysialylation (see Figures 3, 4, and 5). The observation that the Ser triple Mut 2.4.5 protein was not autopolysialylated yet still was able to polysialylate NCAM demonstrated that this mutant protein was still catalytically active and that autopolysialylation was not required for NCAM polysialylation (Figure 7).

ST8Sia IV/PST and ST8Sia II/STX share 57% amino acid identity and catalyze the same polysialylation of NCAM oligosaccharides, with some slight differences in site preference and length of polysialic acid chains added (Angata *et al.*, 1998; Kitazume-Kawaguchi *et al.*, 2001). Therefore, it was unexpected that the glycosylation and autopolysialylation of ST8Sia II/STX would be significantly different from that of ST8Sia IV/PST. Initially we were surprised that three sites in ST8Sia II/STX (rather than two sites as originally found for ST8Sia IV/PST) were autopolysialylated and that only one of these sites (site 2, Asn 74 in ST8Sia IV/PST and Asn 89 in ST8Sia II/STX) was the same for the two enzymes. In our original analysis of ST8Sia IV/PST polysialylation, we showed that the bulk of the polysialic acid (~80%) was found on the oligosaccharide attached to Asn 74 (site 2) (Close *et al.*, 2000). However, as clearly shown and discussed in that study, our data indicated that there is a residual amount of polysialic acid present on ST8Sia IV/PST after mutation of Asn 74. Experiments described in that work and from results of later unpublished work suggested that there was also polysialic acid modifying the oligosaccharide attached to Asn 119 (site 3). More recent experiments show that a myc-tagged version of the ST8Sia IV/PST



Mut 2.3 protein, in contrast to the previously analyzed V5-tagged protein, exhibits weak autopolysialylation. This suggests that a third site, possibly Asn 219 (site 5), possesses a small amount of polysialic acid and that autopolysialylation is very sensitive to subtle folding differences, even those generated by the addition of a short peptide epitope to the enzyme's carboxy-terminus (Close and Colley, unpublished data). Considered together, these data suggest that the autopolysialylation of the two polysialyltransferases may be more similar than we originally suspected.

Work with other sialyltransferases, such as ST8Sia IV/PST and the STTyr isoform of the $\alpha 2$, 6-sialyltransferase (ST6Gal I), has shown us that either the absence of an oligosaccharide or the presence of a foreign amino acid (Ser or Gln) at a consensus N-glycosylation site can have profound effects on protein trafficking and/or catalytic activity. ST8Sia IV/PST, ST8Sia II/STX, and the STTyr isoform of ST6Gal I are all transiently localized in the Golgi. By analogy with the STTyr isoform of the ST6Gal I, we believe that the majority of ST8Sia IV/PST and ST8Sia II/STX ultimately migrate to a post-Golgi compartment where it undergoes cleavage in the stem region and is then secreted. A smaller portion bypasses the cleavage event and is transported to the cell surface (see Ma *et al.*, 1997; Close and Colley, 1998). We found that replacement of Asn 158 (one of two glycosylation sites) of the ST6Gal I STTyr isoform with a Gln residue generated a misfolded protein that was retained in the ER and rapidly degraded (Chen and Colley, 2000). However, replacement of the same Asn residue with Ser generated an active protein that was stably localized in the Golgi and never cleaved and secreted into the cell medium (Chen and Colley, 2000). In stark contrast, replacing Asn 119 (glycosylation site 3) of ST8Sia IV/PST with a Ser rendered it completely inactive without slowing its ER to Golgi trafficking, whereas replacing the same Asn residue with Gln generated a completely active polysialyltransferase (Close *et al.*, 2000; Bharaty *et al.*, unpublished data).

Recently, Gerardy-Schahn and colleagues (Muhlenhoff *et al.*, 2001) reported that autopolysialylation of murine ST8Sia II/STX was dependent on the presence of specific N-glycans attached to Asn 89 and Asn 219. This correlates well with the data presented here that demonstrates the oligosaccharides modifying Asn 89, Asn 219, and Asn 234 are necessary for human ST8Sia II/STX autopolysialylation (see Figures 2, 3, and 5). Muhlenhoff *et al.* (2001) also reported that hamster ST8Sia IV/PST is modified by five Asn-linked oligosaccharides and that the bulk of the polysialic acid is present on the oligosaccharide attached to Asn 74. This nicely parallels our previously published report that established that human ST8Sia IV/PST is modified by five Asn-linked oligosaccharides and that the oligosaccharide modifying Asn 74 is the major acceptor of polysialic acid (Close *et al.*, 2000). Based on the inability of their Gln replacement mutant, ST8SiaII Δ 3,5,6 (analogous to sites 2, 4, and 5 in our numbering system), to polysialylate NCAM, Muhlenhoff *et al.* (2001) again suggested that elimination of ST8Sia II/STX autopolysialylation leads to an abrogation of polysialyltransferase activity. In contrast, we found that a nonautopolysialylated mutant of ST8Sia II/STX, triple Ser mutant 2.4.5, is still active to polysialylate NCAM (see Figure 7A, B, and C). Moreover, we found that substitution of Gln residues for Asn residues in

ST8Sia II/STX resulted in a catalytically inactive polysialyltransferase (see triple Gln mutant 2.4.5, Figure 7A, B, and C). Thus, it is likely that the inability of Muhlenhoff *et al.* (2001) to detect any NCAM polysialylation activity of their nonautopolysialylated ST8Sia II/STX mutants is the result of replacement of Asn residues with Gln residues and the concomitant subtle perturbation of protein structure that leads to an inactive enzyme, rather than being suggestive of a functional link between autopolysialylation and catalytic activity.

From the work presented herein, it is clear that the folding of ST8Sia II/STX is much more sensitive to replacement of amino acids and the elimination of glycosylation sites than that of ST8Sia IV/PST. Even the alteration of one Asn residue and the resultant elimination of one glycosylation site significantly slowed the cleavage and secretion of the ST8Sia II/STX protein and decreased the amount observed at the cell surface (Figures 2–5). These effects were even more severe when two or more glycosylation sites were eliminated (Figures 3–5). In addition, ST8Sia II/STX catalytic activity was clearly compromised when Gln residues were used to replace Asn 89, 219, and 234. This was surprising because Gln is structurally more similar to Asn than is Ser. The double Asn to Gln mutants (Mut 2.4 Gln and Mut 4.5 Gln) exhibited a decreased ability to add polysialic acid to NCAM relative to identical Ser double mutants, and NCAM proteins polysialylated by these mutant ST8Sia II/STX proteins migrated with a reduced molecular mass compared to NCAM proteins polysialylated by wild-type ST8Sia II/STX (Figure 7C). This result suggested that the presence of a Gln residue at amino acid 219 was particularly damaging to the ability of ST8Sia II/STX to fully polysialylate NCAM. Similarly, while a triple Asn to Ser replacement mutant (Mut 2.4.5 Ser) was able to polysialylate NCAM, although to a lesser extent than wild-type ST8Sia II/STX, an identical triple Asn to Gln replacement mutant (Mut 2.4.5 Gln) was completely inactive in NCAM polysialylation.

The “alone” mutant series was originally generated to confirm the polysialylation of the oligosaccharides attached to Asn 89, Asn 219, and Asn 234 (sites 2, 4, and 5, respectively), and to determine which Asn-linked oligosaccharides were sufficient for ST8Sia II/STX autopolysialylation. We found that mutant proteins retaining the single glycosylation sites Asn 89, Asn 219, or Asn 234 exhibited no detectable autopolysialylation (Figure 6). This was unexpected because previously we found a mutant ST8Sia IV/PST protein retaining a single glycosylation site at Asn 74 (2 Alone ST8Sia IV/PST) was polysialylated to nearly wild-type levels (Close *et al.*, 2000). Localization studies demonstrated the presence of these ST8Sia II/STX “alone” mutant proteins in the Golgi (Figure 6), and coexpression with full-length NCAM demonstrated that they were able to polysialylate NCAM despite their lack of detectable autopolysialylation (Figure 7A). These results suggest that the process of ST8Sia II/STX autopolysialylation may be cooperative, requiring more than one oligosaccharide to interact with the enzyme, or extremely sensitive to alterations in enzyme structure generated by the lack of oligosaccharides during the folding process.

In sum, we have definitively shown that autopolysialylation of both polysialyltransferases, ST8Sia IV/PST and ST8Sia II/STX, is not required for their ability to polysialylate NCAM. Gerardy-Schahn and colleagues (Muhlenhoff *et al.*, 1996; Windfuhr *et al.*, 2000) have suggested a functional link between

autopolysialylation and NCAM polysialylation. However, our previous (Close *et al.*, 2000) and present conclusions have been further substantiated by a recent report by Fukuda and colleagues (Angata *et al.*, 2001), in which they show that exogenous ST8Sia IV/PST expressed in insect cells is not autopolysialylated, yet is an active polysialyltransferase. Presently, the mechanism of enzyme autopolysialylation is not well understood. It is clear that polysialic acid chains are not preassembled on the oligosaccharides of the polysialyltransferase and then transferred to the oligosaccharides of NCAM (Muhlenhoff *et al.*, 1996). However, it is not known if autopolysialylation is a *cis* event, with one molecule of enzyme polysialylating its own oligosaccharides, or a *trans* event, with one molecule of enzyme polysialylating a neighbor's oligosaccharides. In addition, why the presence of polysialic acid on ST8Sia IV/PST and ST8Sia II/STX enhances the amount of polysialic acid added to NCAM, and whether this reflects increased stability of the enzyme–substrate interaction is not clear. More work is clearly required to answer these questions and elucidate the mechanisms of autopolysialylation and NCAM polysialylation.

Materials and methods

Tissue culture media and reagents, including Dulbecco's modified Eagle's medium (DMEM), Opti-MEM I, Lipofectin, and oligonucleotides were purchased from Life Technologies, a division of Invitrogen Corporation (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Nitrocellulose membranes were purchased from Schleicher and Schuell (Keene, NH). SuperSignal West Pico chemiluminescence reagent was obtained from Pierce Chemical (Rockford, IL). Protein molecular mass standards (myosin, 203 kDa; β -galactosidase, 123 kDa; bovine serum albumin [BSA], 83 kDa; ovalbumin, 50.7 kDa) were purchased from Bio-Rad Laboratories (Hercules, CA). The cDNA for human ST8Sia II/STX was a kind gift from Dr. John Lowe (University of Michigan, Ann Arbor). Murine NCAM-Fc cDNA was a generous gift from Dr. Genevieve Rougon (CNRS, Marseilles, France). Full-length NCAM 140 cDNA (human) and the OL.28 Ab hybridomas (mouse IgM) were gifts from Nancy Kedersha (Brigham and Women's Hospital, Boston, MA). The OL.28 Ab is specific for α 2,8-linked polyNeu5Ac of 5 units or longer (Close *et al.*, 2000; Sato *et al.*, 2000). The QuikChange site-directed DNA mutagenesis kit and Pfu DNA polymerase were purchased from Stratagene (La Jolla, CA). V5 Ab (mouse IgG) was purchased from Invitrogen. Sequenase version 2.0 DNA sequencing kit was purchased from United States Biochemical (Cleveland, OH). DNA purification kits were obtained from Qiagen (Valencia, CA). Protein-A Sepharose and $\alpha^{35}\text{S}$ -dATP for DNA sequencing were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). ^{35}S -Express protein labeling mix was purchased from Perkin Elmer Life Sciences (Boston, MA). Fluorescein isothiocyanate (FITC)-conjugated and horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies were purchased from Jackson Laboratories (West Grove, PA). Other chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO) and Fisher Scientific (Hanover Park, IL).

Site-directed mutagenesis of ST8Sia II/STX cDNA

Consensus N-glycosylation site Asn residues in full-length V5-tagged ST8Sia II/STX cDNA (Close and Colley, 1998) were mutated to Ser or Gln using the QuikChange site-directed mutagenesis system according to the manufacturer's protocol (Stratagene). The following primers were used for mutation of the Asn codons to the Ser codons, with the mutating codon in bold:

- Mutant 0, GCTGAAGTTGTAATAT**TCCGGCTCCTCATCAC** and GTGATGAGGAGCC**GGATATTACA**ACTTCAGC
- Mutant 1, GTTGTTGACAGAAGTT**TCCGAA**AGCATCAAGC and GCTTGATGCTTT**TCCGAA**ACTTCTGTCAACAAC
- Mutant 2, CCAAATGGAGACAT**TCCCAGACGCTCTCTC** and GAGAGAGCGTCT**GGGAATGTCTCCATT**TGG
- Mutant 3, GACAGCACCATGT**TCCGTGTCCCAGAACC** and GTTCTGGGACAC**GGACATGGTGTCTGTCTC**
- Mutant 4, GAGGACTTGGTCT**TCCGCCACGTGGCG** and CGCCACGTGG**CGGAGACCAAGTCCTC**
- Mutant 5, GCACAGCCTCT**TCCGGCAGCATCCTG** and GGATGCTGCC**GGAGAGGCTGTGCAG**.

The following primers were used for mutation of the Asn codons to the Gln codons, with the mutating codon in bold:

- Mutant 2, CCAAATGGAGACAT**CAACAGACGCTCTCTC** and GAGAGAGCGTCT**GTTGATGTCTCCATT**TGG
- Mutant 4, GAGGACTTGGT**TCCAAGCCACGTGGCG** and CGCCACGTGG**CTTGGACCAAGTCCTC**
- Mutant 5, GCACAGCCT**CCAAGGCAGCATCCTG** and GGATGCTGCC**TTGGAGGCTGTGCAG**.

Each mutation was confirmed using the Sequenase version 2.0 DNA Sequencing kit (United States Biochemical) and the appropriate sequencing primer.

Transient transfection of COS-1 cells with mutant ST8Sia II/STX cDNAs

COS-1 cells maintained in DMEM, 10% FBS were plated on 100-mm tissue culture plates or 12-mm glass coverslips and grown in a 37°C, 5% CO₂ incubator until 50–70% confluent. Lipofectin transfections were performed according to the protocols provided by Life Technologies, as described previously (Close *et al.*, 2000).

Immunofluorescence localization of mutant ST8Sia II/STX proteins

COS-1 cells were plated on glass coverslips, transiently transfected with wild-type or mutant V5-tagged ST8Sia II/STX cDNA, and processed for immunofluorescence microscopy as described previously (Colley *et al.*, 1992; Close *et al.*, 2000). V5 Ab was diluted 1:100; the OL.28 Ab, FITC-conjugated secondary antibodies, goat anti-mouse IgG, and goat anti-mouse IgM, were diluted 1:200 in 5% normal goat serum/phosphate buffered saline (PBS) blocking buffer prior to use.

Metabolic labeling of cells and immunoprecipitation of ST8Sia II/STX proteins

Following transient transfection with wild-type or mutant V5-tagged ST8Sia II/STX cDNA and expression of these proteins for 18 h, COS-1 cells plated in 100-mm dishes were labeled for 1 h with 100 $\mu\text{Ci/ml}$ ^{35}S -Express protein labeling mix (Perkin Elmer Life Sciences) and chased for either 0 h (cell lysates only) or 6 h as described previously (Close *et al.*, 2000). The

mutant and wild-type V5-tagged ST8Sia II/STX proteins were then immunoprecipitated from the cell lysates and 6 h chase media using 2 µg of V5 Ab and protein A-Sepharose (Amersham Pharmacia Biotech) as previously described (Colley *et al.*, 1989; Close *et al.*, 2000). To avoid breakdown of the polysialic acid, the boiling step was omitted and the immunoprecipitation beads were resuspended in 50 µl of Laemmli sample buffer containing 5% β-mercaptoethanol (BME) and directly loaded into the gel wells. Immunoprecipitated proteins were separated on 7.5% separating/3% stacking SDS–polyacrylamide gels (Laemmli, 1970). Radiolabeled proteins were visualized by fluorography using 10% 2,5-diphenyloxazole in dimethyl sulfoxide (Bonner and Lasky, 1974), and gels were exposed to Kodak BioMax MR film at –80°C.

Immunoprecipitation and immunoblot of ST8Sia II/STX proteins

COS-1 cells were transiently transfected with wild-type or mutant V5-tagged ST8Sia II/STX cDNA as previously described (Close *et al.*, 2000). Eighteen hours posttransfection, cell medium was collected. The V5-tagged ST8Sia II/STX mutant and wild-type proteins were immunoprecipitated from the cell lysates and media and electrophoresed as described above. Following electrophoresis, proteins were subjected to immunoblotting as previously described (Close *et al.*, 2000). OL28 Ab (IgM) was used at a 1:500 dilution, and the HRP-conjugated secondary antibody, goat anti-mouse IgM, was used at a 1:8000 dilution.

Indirect immunofluorescence microscopy of COS-1 cells coexpressing full-length NCAM and mutant ST8Sia II/STX proteins

COS-1 cells maintained in DMEM, 10% FBS were plated on 12-mm glass coverslips and grown in a 37°C, 5% CO₂ incubator until 50–70% confluent and essentially transfected as previously described (Close *et al.*, 2000). For each coverslip, the ratio of V5-tagged mutant ST8Sia II/STX to NCAM 140 plasmid DNA transfected was 1:1 (0.5 µg:0.5 µg). Cells were fixed and processed for immunofluorescence microscopy as previously described (Close *et al.*, 2000). Primary antibodies (V5 Ab and OL28 Ab) and secondary antibodies (FITC-conjugated goat anti-mouse IgG and goat anti-mouse IgM) were diluted 1:200 in blocking buffer (5% normal goat serum in PBS) prior to use.

Precipitation and immunoblot analysis of soluble NCAM-Fc from COS-1 cells coexpressing mutant ST8Sia II/STX proteins

In duplicate, COS-1 cells plated on 100-mm tissue culture plates were transiently cotransfected with wild-type and mutant V5-tagged ST8Sia II/STX and NCAM-Fc plasmid DNA at a ratio of 4:1, respectively (20 µg:5 µg). For the first set of plates, cells were radiolabeled for 1 h with 100 µCi/ml ³⁵S-Express protein labeling mix (Perkin Elmer Life Sciences) and chased for 3 h with 4 ml unlabeled DMEM, 10% FBS as previously described (Close *et al.*, 2000). NCAM-Fc was precipitated from the medium using protein-A Sepharose, and the precipitated proteins were separated on 5% separating/3% stacking SDS–polyacrylamide gels after the addition of Laemmli sample buffer containing 5% BME and incubation of the protein A-Sepharose beads at 65°C for 5 min. This heating step was added to eliminate the formation of NCAM-Fc

dimers. Gels were processed for fluorography as described above. For the second set of plates, unlabeled NCAM-Fc was precipitated from the medium 18 h posttransfection as described above. The precipitated NCAM-Fc proteins were subjected to immunoblotting with OL28 Ab as described above.

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Abbreviations

BME, β-mercaptoethanol; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; NCAM, neural cell adhesion molecule; OL28 Ab, anti-polysialic acid antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; V5 Ab, anti-V5 epitope tag antibody.

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