

Expression Cloning and Characterization of NSIST, a Novel Sulfotransferase Expressed by a Subset of Neurons and Postsynaptic Targets

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Synapses are distinguished by localized concentrations of specific proteins, many of which bear the marks of posttranslational processing such as glycosylation and sulfation. One strategy to elucidate this posttranslational tailoring is to identify the enzymes that create these modifications. Monoclonal antibody 3B3 recognizes a carbohydrate-containing epitope expressed on dystroglycan and other constituents of *Torpedo* electric organ synaptic membranes. We used mAb 3B3 in an immunofluorescence-based expression-cloning method and isolated a cDNA clone conferring mAb-3B3 immunoreactivity to transfected COS cells. The deduced polypeptide has a predicted molecular weight of 51 kDa, a type II transmembrane topology, and four potential N-linked glycosylation sites. The polypeptide, which we term NSIST (nervous system involved sulfotransferase), shows extensive, although not complete, homology to a chondroitin-6-sulfotransferase and limited homology to other sulfotransferases. In NSIST-transfected COS cells, ³⁵SO₄ incorporation and chondroitin-sulfate-like immunoreac-

tivity are increased. *In vivo*, NSIST occurs as a single 2.4 kb transcript abundant in *Torpedo* electric organ, moderately expressed in spinal cord and electric lobe, and undetectable in non-neural tissues. Immunohistochemistry shows that NSIST is expressed in a punctate distribution in the innervated portion of electrocytes. In the CNS, NSIST-like immunoreactivity is localized within the somas of motor neurons and neurons of the electromotor nucleus, whereas mAb-3B3 immunostaining is associated with cell surfaces and neuropil. Neuronal NSIST is therefore likely to exert its effects extracellularly; although NSIST is synthesized by neurons, its product, the 3B3 epitope, is found outside neuronal cell bodies. Our evidence indicates that NSIST participates in nervous system specific posttranslational modifications, perhaps including those at synapses.

Key words: synaptic differentiation; posttranslational processing; chondroitin sulfate; sulfotransferase; *Torpedo*; electric organ; CNS

The establishment and preservation of a synapse demands concerted interplay between the presynaptic neuron and its target (Sanes and Scheller, 1997). This latter cell bears the postsynaptic membrane, a discrete subcellular domain harboring the diverse yet unique array of molecular components supporting rapid and reliable synaptic transmission. Creating and maintaining a synaptic specialization requires modulatory input contributed by both presynaptic and postsynaptic cells and occurring within the context of numerous molecular pathways.

One such mechanism entails the posttranslational modification of synaptic components by selective addition of carbohydrate moieties to them. Such modifications provide structural and functional diversity with a concomitant high degree of specificity. In general, neurons make good use of this molecular diversity; in the CNS, the attachment of carbohydrates to specific surface proteins is a critical determinant for *cis*- and *trans*- interactions among cells (Schachner and Martini, 1995) and probably underlies as-

pects of process outgrowth and structural plasticity exhibited by neurons (Emerling and Lander, 1996; Fryer and Hockfield, 1996).

Several lines of evidence indicate a pivotal role for carbohydrates in neuromuscular synaptogenesis. *N*-acetylgalactosaminyl-terminated saccharides are selectively localized to neuromuscular junctions, where they are thought to modulate synaptic differentiation (Martin and Sanes, 1995). A key synthetic enzyme, *N*-acetylgalactosaminyl transferase, also has a synaptic localization (Scott et al., 1990). Additional synapse-specific carbohydrates include a glycosylation-dependent epitope of entactin (Chiu and Ko, 1994) as well as certain heparan sulfate proteoglycans (Anderson et al., 1983; Bayne et al., 1984; Sanes et al., 1986). Finally, muscle-derived chondroitin sulfate proteoglycans (CSPGs) are likely to play a central role in acetylcholine receptor (AChR) clustering (Mook-Jung and Gordon, 1995; Bowen et al., 1996).

Sulfation often acts in concert with glycosylation to create the unique structural features associated with discrete physiological functions of the parent molecule. For example, sulfation and glycosaminoglycan chain elongation are thought to be coupled in muscle cells, and such linkage may be important for AChR clustering on myotubes (Bowen et al., 1996). Thus, glycosylation and sulfation are ideally suited to play a major cooperative role in modifying and maintaining synaptic specializations.

One strategy for examining the posttranslational processing of synaptic proteins entails identifying and characterizing the particulate enzymes. In the present study, we used the knowledge that mAb 3B3, previously developed in this laboratory (Bowe et

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al., 1994), recognizes a sialic acid-containing epitope borne by a group of membrane-associated synaptic glycoproteins in *Torpedo* electric organ, among them α - and β -dystroglycan. We used an immunofluorescence-based expression-cloning strategy to identify an enzyme that is essential for creating this epitope. The enzyme has strong homologies to a chondroitin-6-sulfotransferase (C6ST; Fukuta et al., 1995), which can also catalyze the sulfation of sialyl lactosamine oligosaccharides (Habuchi et al., 1997). Because this enzyme is enriched in the *Torpedo* nervous system, we have named it NSIST (nervous system involved sulfotransferase). Furthermore, we show that NSIST is selectively expressed by *Torpedo* motor neurons and that its product, the 3B3 epitope, is associated with neuronal surfaces and neuropil in the CNS. Thus, NSIST is a novel sulfotransferase that is likely to function within the nervous system, in which it could execute specific structural and functional modifications of synaptic components.

MATERIALS AND METHODS

Expression cloning. An oligo-dT-primed cDNA library was prepared from *Torpedo* electric organ polyA mRNA and ligated into the *Bst*XI cloning site of the expression vector pCMX (Davis et al., 1991). Recombinants in the library had an average insert size of 1.5 kb. Approximately 1 μ g of the library was transfected using DEAE-dextran (Sambrook et al., 1989) into COS cells plated onto single-welled chamber slides (Nunc, Roskilde, Denmark; $\sim 3.0 \times 10^5$ cells per slide). Two days later, intact cultures were immunostained with mAb 3B3 and brightly stained cells collected with a glass micropipette. For technical reasons, this harvest included ~ 5 –10 neighboring cells.

Plasmid DNA was extracted from each harvest by incubation for 1 hr at 55°C in 200 μ l extraction buffer (100 mM EDTA, 0.5% SDS, 10 mg/ml proteinase K, and 10 mg/ml tRNA in 10 mM Tris, pH 8.0), purified, amplified in *Escherichia coli*, and retransfected into COS cells. Clonal cDNA was obtained after two or three rounds of repeated selection and enrichment.

Antibodies. Monoclonal antibody 3B3 was produced and characterized as described in Bowe et al. (1994) and used as an undiluted hybridoma supernatant or as purified IgG, with similar results. Monoclonal antibody CS-56 (anti-chondroitin sulfate) was obtained from Sigma (St. Louis, MO). Anti-agrin antibody 6D4 was used as an undiluted hybridoma supernatant (Fallon et al., 1985).

For production of the anti-NSIST antisera, a synthetic peptide corresponding to the region TPPKKGTEKFP of the NSIST sequence (Fig. 1) was synthesized bidirectionally so that a cyst-gly linker could be added at either end, and the resulting mixture was then conjugated to purified protein derivative (PPD). Polyclonal antisera were produced in rabbits (CoCalico); antisera RG211 and RG212 were produced by two different rabbits that had been immunized with the same peptide mixture.

Immunostaining of COS cells. For mAb 3B3 immunostaining, transfected COS cell monolayers were washed in HEPES-buffered minimal essential medium (MEM-H), blocked in MEM-H containing 1% bovine serum albumin and 10% normal horse serum, and incubated in mAb3B3 followed by rhodamine-conjugated horse anti-mouse IgG₃ (1:100; Fisher Scientific, Houston, TX). Cultures were fixed in 100% methanol for 5 min at -20°C , air-dried, and mounted in Citifluor (Pella, Redding, CA). Slides were viewed with rhodamine optics on a Zeiss Axioplan microscope.

For anti-NSIST immunostaining, transfected cells were first permeabilized in 100% methanol for 5 min at -20°C , then blocked and treated as above (but with 10% normal goat serum). Cells were then incubated with RG211 antiserum or nonimmune serum (1:100), followed by fluorescein-conjugated goat anti-rabbit Ig (1:100).

Immunostaining of frozen sections. Cryostat sections (12 μ m) of fresh-frozen *Torpedo* electric organ, electric lobe, spinal cord, and skeletal muscle were fixed for 5 min at room temperature in 1% paraformaldehyde in PBS, rinsed in PBS, and blocked for 1 hr in PBS containing 1% nonfat dry milk and 10% normal goat or horse serum (depending on the secondary antibody used). Sections were incubated in mAb 3B3, mAb 6D4 (undiluted or 1:10 hybridoma supernatant), RG212 (1:500), or nonimmune control serum diluted in PBS containing 1% NFD, 1%

| | |
|--|------|
| TTCTTACTGCTTACCGAAATTAATACGACTCACTATAGGGAGACCCAGCTTCTAGAGATC | 60 |
| CCTCGACCTCGAGATCCATTGTGCTCTAAAGCTGGCAGTGGAGCGAGTCGGTGGATCCC | 120 |
| CTCCCTCCGGGCTGCATCGTCCGACAGTGTTCGGGAAAGAAGGCACATCCAGGCC | 180 |
| TAGATCTGCACCGAGCTGCTCGCCTGGTGATGTGAATTCATCTGCTCCCTCACACGT | 240 |
| CGAGCACAGTGTGTGGCGGAGGACTGGAGAAATGCTGATGTGACCTTTGGCTTACCG | 300 |
| GGAGACTGGCCGAGCGCTGGCATGGGAGCAGGCTCACAGAAATCTCTGACGTACCAATTGAG | 360 |
| AAGCAACGCTCTTACCTAGGACATCCCGTGCAGCTCTGAGTCAGGAGTCCAGCCCTCA | 420 |
| AAGCCAGTAGCAATGAAATGAGAAGCAAATATGCAATATTCTGTTTTGTGTGTGCT | 480 |
| M K M R S K Y A I I L F F V V A | 16 |
| CTTGTGATCATTTGAAAAAGAAAGAACATAATTTCAAGGGTGTCTGACAAGTTTACACTG | 540 |
| <u>L V I I</u> E K E R N I I S R V S D K F T L | 36 |
| AAATTCACACAGCGGAATCTGTGGCCCAACACAGGATTTCTGCCAGATCTCTGACG | 600 |
| K F P H A E S V A P <u>N N T</u> I S A R S L T | 56 |
| AAGAATACTCCCTGCTGCCCACTCAGTCTGCCCTGGAAGCTGCTAAAGGCCCGCA | 660 |
| K <u>N N S</u> L L A N S V A A V T G G L L K A R | 76 |
| AGAAGCTACTCTGCTACTGCAAGCGCAGCCAGCAGTGTGAGGAAAGTCTTGAAGGCC | 720 |
| R S Y S S L Q S A A S S D V R K V L K G | 96 |
| CGAAGCACTTGTGTGATGGCGACTACCAGGACTGGTCTCTCTTTTGGGGCAGTTTC | 780 |
| R K H L L L M A T T R T G S S S F V G E F | 116 |
| TTCAACCAGAACACGACATCTTCTACCTGTTTGAACCCGCTGTCGACGTTGGAGAAGCG | 840 |
| F N Q N N D I F Y L F E P L W H V E K T | 136 |
| GTCACCTTTGAGCCGGTGGAAATGAACGCTGTGGCTTCTCCATTTTACCOCGATGTG | 900 |
| V T F E P G G M N A V A S S I A I Y R D V | 156 |
| GTGCGCAGTGTGATGTGGCGACTGTACACACTGGAATAATTTCCATTTCCCGATGGCC | 960 |
| V Q Q L M L C D L Y T L E N F L F P M A | 176 |
| GACCCCATCTCACAGGCATCTTCCGTCGAGGCTCCAGCAAGTCTTGTGCGAGGGA | 1020 |
| D R H L T G I L F R R G S S K S L C E G | 196 |
| GAGGTTTGCACACCACCTAAGAAGGCGGACCGAGAAGTTTCCCTGTCGGTACCGGAC | 1080 |
| E V C T P P K K G G T E K F P C R L R D | 216 |
| TGTGGCTCCTCAATTTGACCCCTGGCCACTAGGCACTGCTCAAAGCAGCAGCTGGCC | 1140 |
| C G L L N L T L A T Q A C L K Q K H V A | 236 |
| ATAAAGCCGTCCTCCCTGAGGCACTGGAGTGTCTCCGCGCCACTGTGGAGGACTTCAGG | 1200 |
| I K T V P L R Q L E F L R P L V E D F R | 256 |
| ATCAACCTGAAGATCATTCAGTGTGGTGCAGCCAGGCGGCTGGCTTCAAGGATG | 1260 |
| I N L K I I Q L V R D P R A V L A S R M | 276 |
| GTGGCTTCCCGCAGCAAGTACAATGCGTGAAGAAGTGGGCCAACGAAGGAGAGTGGC | 1320 |
| V A F P S K Y N A W K K W A N E G R V P | 296 |
| GACGACGACGAGGTCGGCAAGATCCGAGGGAATGCGAGAATCCCGGGCCACCGCACAA | 1380 |
| D D D E V G K I R G N C E N L R A T A Q | 316 |
| CTTGGCCTCAGTCAGCCCTTGGTGAAGGATCGCTTCTGCTGATGCGTTACGAGGAC | 1440 |
| L G I S Q P P W L K D R F L L M R Y | 336 |
| ATTGCGTGGAGCCCGTGAAGAGGCGCAGGAGATGTACCGCTTTTCCGGCATCCCATG | 1500 |
| I A L E P V K R A Q E M Y R F S G I P M | 356 |
| ACACCCGAGGTCAAAGTGGATCTACGAAAACACCAAGTTTCCAAGGCCAGCAACAAC | 1560 |
| T P E V K K W I Y E N T Q V S K A S N N | 376 |
| ATCTACTCAACGCACAAAATCTCTCGGAGCAGITTTGAGAAGTGGCGGCTTGGTCTGCC | 1620 |
| I Y S T H K I S S E Q F E K W R L G L P | 396 |
| TTCAAGATGCCAGAGTGTCCCAACAGGTTGTGACGCGCAATGAAACTGTTCCGGCTAC | 1680 |
| F K I A R V V Q Q V C G P A M K L M R Y | 416 |
| AAGTTGGTGAAGGACGCGACCAACCTTGGCAACAGGCTGTCGACCGCTGAGGAGAAGCAG | 1740 |
| K L V K D A A T L A <u>N R S</u> A S L L E N R | 436 |
| AACTTTTGATAAACCCTAACCTCGGAAGAAGTTGCAATCGTCTTCGAGCAGCCGAAGCAT | 1800 |
| N F W I T | 441 |
| GAGAGATAAACTTGAATCTTCTTGGACCTTCTACAGAAGTCCCAAGCCAAATACGACATC | 1860 |
| TCCTGGATGGATGTGCAATGGCCTGACCAGGACCCACAGGACAAAACAGTTGCTTGGAC | 1920 |
| AATGGAATCACAAAATGAAGCACACAATGAGGGATTTATTTTAAAGGAAAAGATTAT | 1980 |
| TTTTATGATGATTTTATAAAGATTTAGCATGTTGAGGCTAGATGGGAAACAGGGTTG | 2040 |
| CGAAGATCTTA | 2052 |

Figure 1. Nucleotide and deduced amino acid sequence of NSIST. The putative transmembrane domain/signal sequence is underlined with a double solid line. Potential N-linked glycosylation sites are underlined with single solid lines. The amino acid sequence used to generate a synthetic peptide antigen is underlined with a dotted line. This sequence has been deposited in GenBank, accession number AF079875.

RESULTS

Expression cloning of NSIST

Previous work in this laboratory showed that mAb 3B3 recognizes a carbohydrate-containing epitope abundant in *Torpedo* electric organ synaptic membranes (Bowe et al., 1994). We reasoned that we could use mAb 3B3 as a tool to clone the enzyme(s) responsible for creating this epitope. In preliminary immunofluorescence experiments we found that mAb 3B3 did not bind to untransfected COS cells. Thus, we used mAb 3B3 in an antibody-based expression-cloning scheme to screen COS cells that had been transfected with a cDNA library derived from *Torpedo* electric organ. This method allowed us to select for particular subcellular localizations (e.g., cell surface or intracellular) of the expressed epitopes.

First-round transfection of COS cells with electric organ library yielded approximately one positive cell per 3×10^6 total cells plated. Positive cells showed bright, punctate, surface immunolabeling with mAb3B3 (see Fig. 5A). After two additional rounds of plasmid selection and amplification we obtained a clonal cDNA that, when transfected into COS, resulted in many 3B3-positive cells per slide. Based on our characterization and on sequence homology (see below), we have named the product of this clone NSIST.

Sequence analysis of NSIST indicates that it encodes a sulfotransferase

The nucleotide and deduced polypeptide sequences of NSIST are shown in Figure 1. The cDNA contains 2052 nucleotides, with an open reading frame beginning at a putative translation initiation site. This open reading frame encodes 441 amino acid residues and is likely to be complete because it is flanked by in-frame stop codons and untranslated, noncoding sequence.

The deduced amino acid sequence predicts a protein with a molecular weight of 51 kDa. A putative signal peptide of 14 amino acids begins at residue 7; this hydrophobic region constitutes the sole predicted transmembrane domain. In addition, the protein sequence includes four potential sites of N-linked glycosylation as well as a cysteine-enriched central region.

Database searches using the BLAST algorithm (Altschul et al., 1990) revealed that the deduced NSIST polypeptide sequence has varying degrees of homology to at least three distinct sulfotransferases. NSIST shows the strongest homology to C6ST, cloned from chick chondrocytes (Fukuta et al., 1995). Overall, the two amino acid sequences are 58% identical (Fig. 2A). The NSIST and C6ST sequences show 78% identity over a 33-amino acid domain near the amino terminal. There is a 200-amino acid central region with 69% identity, and the N-terminal 137 amino acids of the two polypeptides are 60% identical. In contrast, one domain of NSIST (amino acids 44–106) is only 17% identical to the C6ST sequence.

NSIST also shows a more limited homology to other sulfotransferases (Fig. 2B). Two regions of 20 and 60 amino acids within the NSIST sequence have weak but consistent identity (35 and 17%, respectively) with a bacterial lipooligosaccharide sulfotransferase (Roche et al., 1991) as well as another family of sulfotransferases that include alcohol and hydroxysteroid sulfotransferases (50 and 23%; Rikke and Roy, 1996). The second of these regions also shows 38% identity to part of a plant flavonol sulfotransferase (Varin et al., 1992). Taken together, these homologies suggest that NSIST is a sulfotransferase, a possibility that we sought to test further (see below).

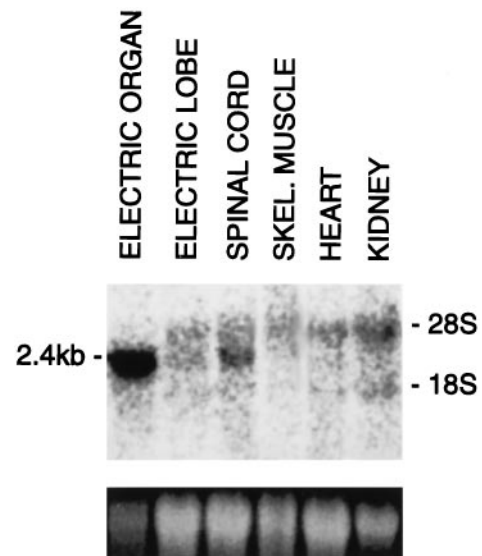


Figure 3. NSIST mRNA expression in adult *Torpedo* tissues. Each lane of the Northern blot contains 10 μ g of total RNA from electric organ, electric lobe with surrounding midbrain, spinal cord, skeletal muscle, heart, or kidney. A single NSIST transcript was detected with an estimated size of 2.4 kb. The transcript is most abundant in electric organ, moderately expressed in electric lobe and spinal cord, and not detected in the other tissues shown. Similar results were obtained in three experiments from different animals. The bands at 28S and 18S are likely to represent nonspecific binding to ribosomal RNA. Ethidium bromide staining of the RNA gel (bottom panel) shows that loading is similar across lanes, with the exception of a slight underloading for electric organ.

NSIST mRNA is abundant in particular regions of the *Torpedo* nervous system

We next determined the size and tissue distribution of the NSIST transcript in *Torpedo*. As shown in Figure 3, NSIST is expressed as a single 2.4 kb mRNA. Transcript expression varies for different tissues: levels are highest in electric organ, with lower levels in spinal cord and electric lobe. The signal for electric organ is likely to represent an underestimate of actual transcript levels because of slight underloading of the RNA from that tissue. We did not detect any signal in the other tissues examined, including skeletal muscle, heart, and kidney. Thus, NSIST mRNA is selectively expressed in the nervous system. We obtained comparable results in experiments using mRNA from three different rays. Furthermore, a band of comparable size was observed when total RNA from NSIST-transfected COS cells was similarly probed (data not shown).

The NSIST protein is tightly associated with membranes of *Torpedo* electric organ

To characterize the NSIST protein, we raised polyclonal antisera against a synthetic peptide corresponding to part of the central cysteine-rich sequence (Fig. 1). Two antisera, RG211 and RG212, were used in immunoblotting and immunostaining to reveal NSIST's tissue and subcellular localization, biochemical properties, and relationship to the 3B3 epitope in electric organ and COS cells.

Immunoblotting of membrane fractions from *Torpedo* electric organ (Fig. 4A, lanes 1, 3) showed that NSIST migrates with an apparent molecular weight of 136 kDa. The band appears slightly fuzzy, consistent with glycosylation of the core protein at one or more of the predicted sites. NSIST was resistant to extraction from these membrane fractions at pH 11 (Fig. 4A, lane 2),

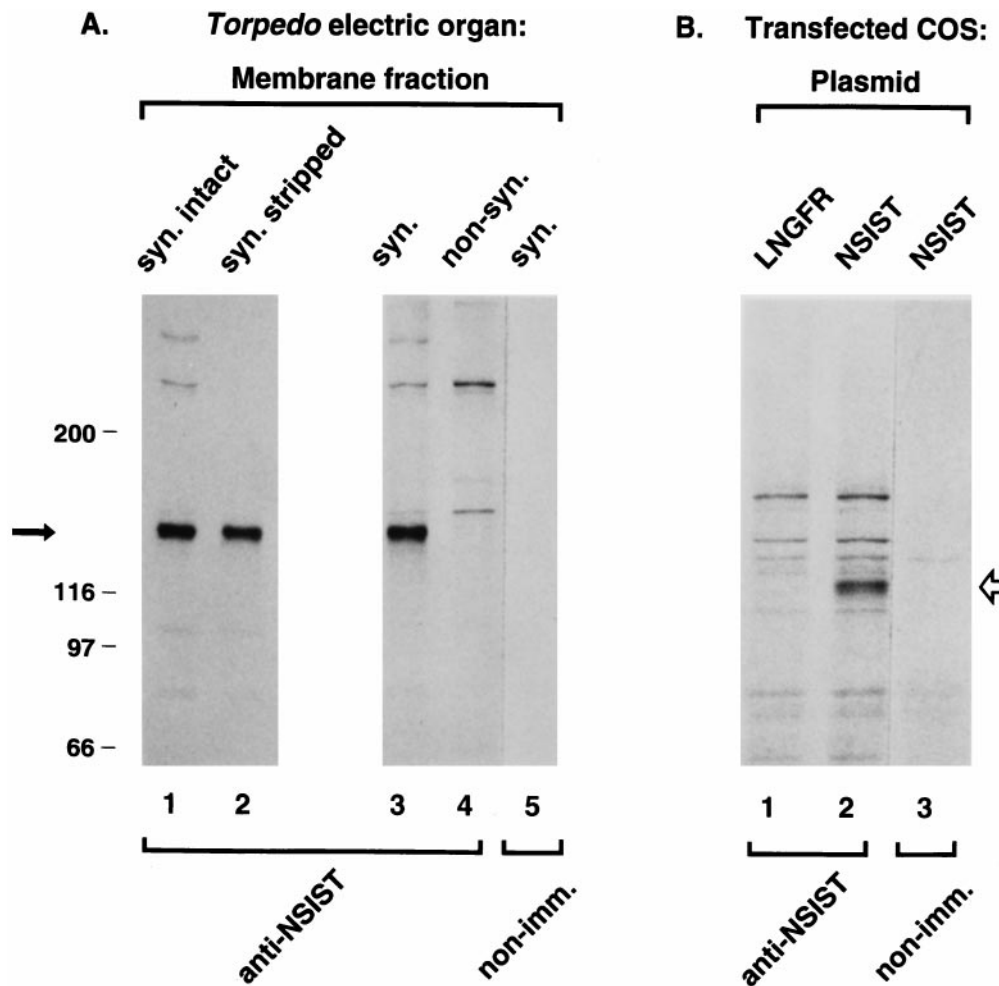


Figure 4. Characterization of native and recombinant NSIST polypeptides. *A*, *Torpedo* electric organ membrane fractions enriched in either synaptic or nonsynaptic components were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with anti-NSIST antiserum RG212 (lanes 1–5). Membrane fractions enriched in synaptic components were used intact (lanes 1, 3, 5) or after extraction at pH 11 (lane 2). A single prominent polypeptide of ~136 kDa is revealed (solid arrow), which persists after high pH extraction of the membranes. The polypeptide is not detected in membrane fractions enriched in nonsynaptic components (lane 4) or in control synaptic fractions probed with nonimmune serum (lane 5). *B*, Membranes from COS cells transfected with cDNA encoding NSIST (lane 2) or an irrelevant polypeptide (*LNGFR*, lane 1) and probed with anti-NSIST antiserum RG211. A major polypeptide of ~118 kDa is only detected in cells transfected with NSIST cDNA (open arrow). This polypeptide was not visualized when blots of NSIST-transfected cells were probed with nonimmune serum (lane 3).

suggesting that it is tightly membrane-associated. Finally, NSIST is abundant in fractions enriched in synaptic components as compared with those enriched in nonsynaptic components (Fig. 4*A*, lane 4). However, because these membrane fractions are isolated on the basis of sedimentation in sucrose gradients (Neubig et al., 1979), this selective association should not be taken to indicate that NSIST is uniformly associated with AChR-rich, postsynaptic membranes; rather, the NSIST-containing membranes may also include certain copurifying Golgi and/or post-Golgi membranes. Indeed, our immunolocalization indicates that NSIST is associated with only a subpopulation of membranes in the innervated portion of the electrocytes.

Characterization of recombinant NSIST

To better understand the nature of NSIST, we next characterized its expression in transfected COS cells. Figure 4*B* shows an immunoblot of membranes from COS cells transfected with cDNA encoding NSIST (lane 2) or an irrelevant polypeptide (*LNGFR*, lane 1), probed with the RG211 antiserum. A polypeptide migrating at ~118 kDa was detected only in the NSIST-transfected cells. This polypeptide also has the slightly diffuse appearance characteristic of glycosylated proteins.

To determine the relationship of NSIST to the mAb 3B3 epitope, we double labeled transfected COS cells with anti-NSIST antiserum RG211 and mAb 3B3 (Fig. 5). mAb 3B3, but not RG211, labeled unpermeabilized cells, indicating that the mAb3B3 epitope is extracellular. On the other hand, NSIST

immunoreactivity was only detected in permeabilized cells. To compare the subcellular distributions of these antigens in same cell, intact cells were first immunostained with mAb 3B3, then permeabilized and labeled with anti-NSIST. Figure 5 shows that mAb 3B3 labels the cell surface in a punctate pattern, whereas anti-NSIST shows bright internal staining. Under any conditions tested, no staining of untransfected cells was observed with either reagent.

Notably, only a subset of the mAb-3B3-positive COS cells expressed NSIST. Counts of the stained cells showed that, although labeling of the positive cells was well above the threshold of detectability, approximately threefold more cells were positive for mAb 3B3 than for anti-NSIST. The fact that more cells stain for mAb 3B3 than for anti-NSIST suggests that the product(s) of NSIST, and/or NSIST itself, are secreted. Furthermore, these findings support the hypothesis that the NSIST cDNA encodes an enzyme responsible for producing an essential part of the mAb 3B3 epitope.

The above data indicate that NSIST is an enzyme that modifies endogenous COS cell substrates to create the 3B3 epitope; moreover, the sequence homology of NSIST suggests that this activity involves sulfation. To test this proposal, we transfected COS cells with plasmids encoding either NSIST or an irrelevant polypeptide (*LNGFR*) and measured incorporation of $^{35}\text{SO}_4$. For both membrane and cytosolic COS fractions, counts were 8–10% higher for NSIST- than for *LNGFR*-transfected samples. These differences

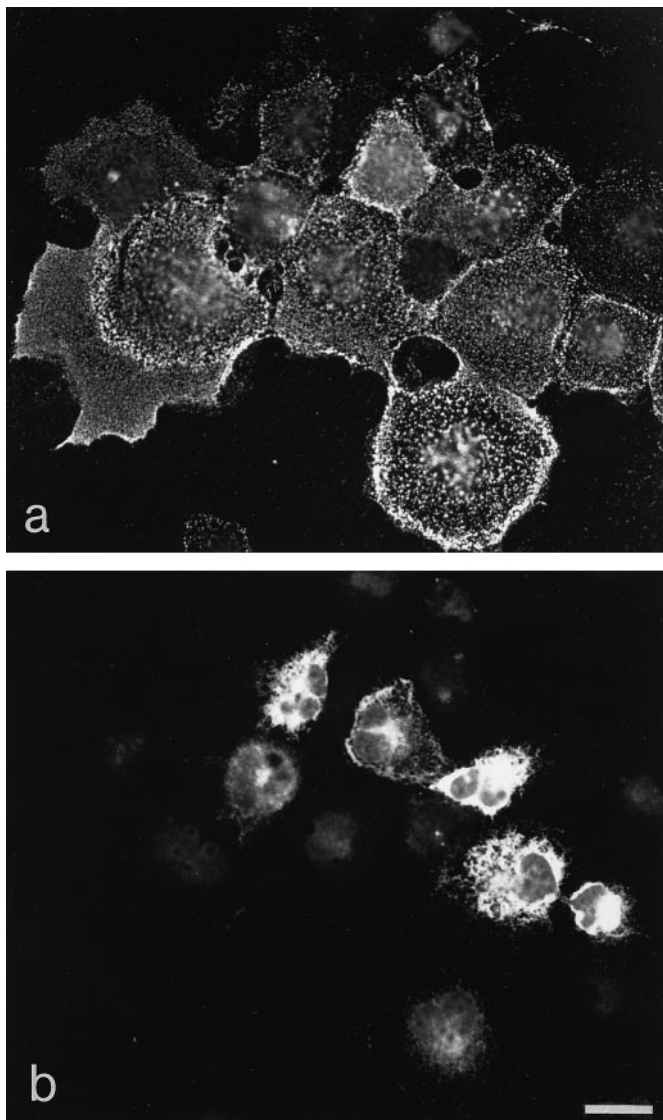


Figure 5. NSIST-transfected COS cells show different staining distributions for NSIST and mAb 3B3. Living NSIST-transfected COS cells were incubated with mAb 3B3, then fixed, permeabilized and double labeled with anti-NSIST antiserum RG211. Two distinct staining patterns emerge, with mAb 3B3 labeling the transfected cell surfaces in a punctate manner (*a*) and RG211 showing bright internal staining of only a subset of the mAb-3B3-positive cells (*b*). Transfected cells labeled with nonimmune serum show no staining (data not shown). Scale bar, 20 μ m.

indicate that NSIST expression in COS cells is accompanied by elevated sulfate incorporation into membranous as well as cytosolic fractions.

Chondroitin sulfate-like immunoreactivity is increased in NSIST-transfected COS cells

The finding that NSIST has a high degree of homology to a C6ST suggests that NSIST may modify CSPGs. To investigate this possibility we used mAb CS-56, an antibody that recognizes chondroitin sulfate (CS), to probe immunoblots of transfected COS membrane fractions. Figure 6 shows that CS-56 labels a broad polydispersed band spanning ~60–200 kDa in NSIST-transfected COS membranes. There were also several discrete polypeptides in this region that showed increased CS-56 immunoreactivity in transfected cells. This finding indicates that CS is

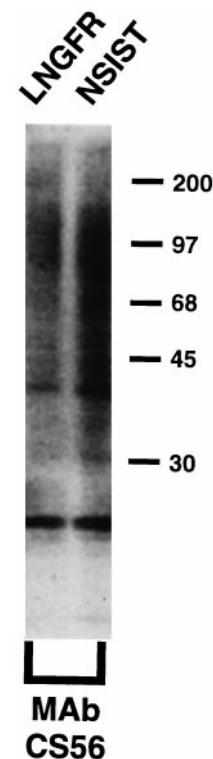


Figure 6. Transfection with NSIST cDNA increases mAb CS-56 immunolabeling to COS cell membrane fractions. COS cells were transfected with NSIST cDNA or with an irrelevant cDNA (*LNGFR*). Cell membranes were separated by SDS-PAGE on a 5–15% gradient gel, transferred to nitrocellulose, and probed with anti-CS mAb CS-56. A broad band spanning ~60–200 kDa is prominent in the NSIST-transfected sample, but only weakly visible in membranes from the nontransfected cells. Some additional bands are also specifically labeled in the NSIST lane. There was no labeling when irrelevant IgM was used as the primary antibody (data not shown).

a substrate for NSIST. Furthermore, the labeling of additional bands in the NSIST lane suggests that the enzyme may act at other structurally related sites, perhaps including the 3B3 epitope (see Discussion).

Immunolocalization of NSIST in the nervous system

We next investigated the cellular localization of NSIST in the intact nervous system. In *Torpedo* electric organ, NSIST-like immunoreactivity is concentrated in large puncta (Fig. 7*A,C*). Finely stained filaments extending away from the electrocyte membranes were also occasionally observed. Double labeling of AChRs with rh-BTx (Fig. 7*B*) shows that NSIST-rich puncta are associated with the innervated and, to a lesser extent, the noninnervated portions of the electrocytes and are likely to be intracellular. No staining was observed if nonimmune serum was substituted for anti-NSIST antiserum (Fig. 8*A*). MAb 3B3 labeling, in contrast to the NSIST immunoreactivity, included cell surfaces as well as fine granular material that filled the spaces between the tiers of electrocytes (Fig. 7*D*).

In sections of spinal cord, we observed NSIST immunolabeling of a compact cluster of large neuronal cell bodies in the ventral horn (Fig. 9). No labeling was detected in control sections (Fig. 9*B*). The size and location of the NSIST-stained cells indicates that they are motor neurons. The staining is intracellular and is concentrated in small patches throughout the cytoplasm. Much of this staining is likely to represent material in the Golgi apparatus,

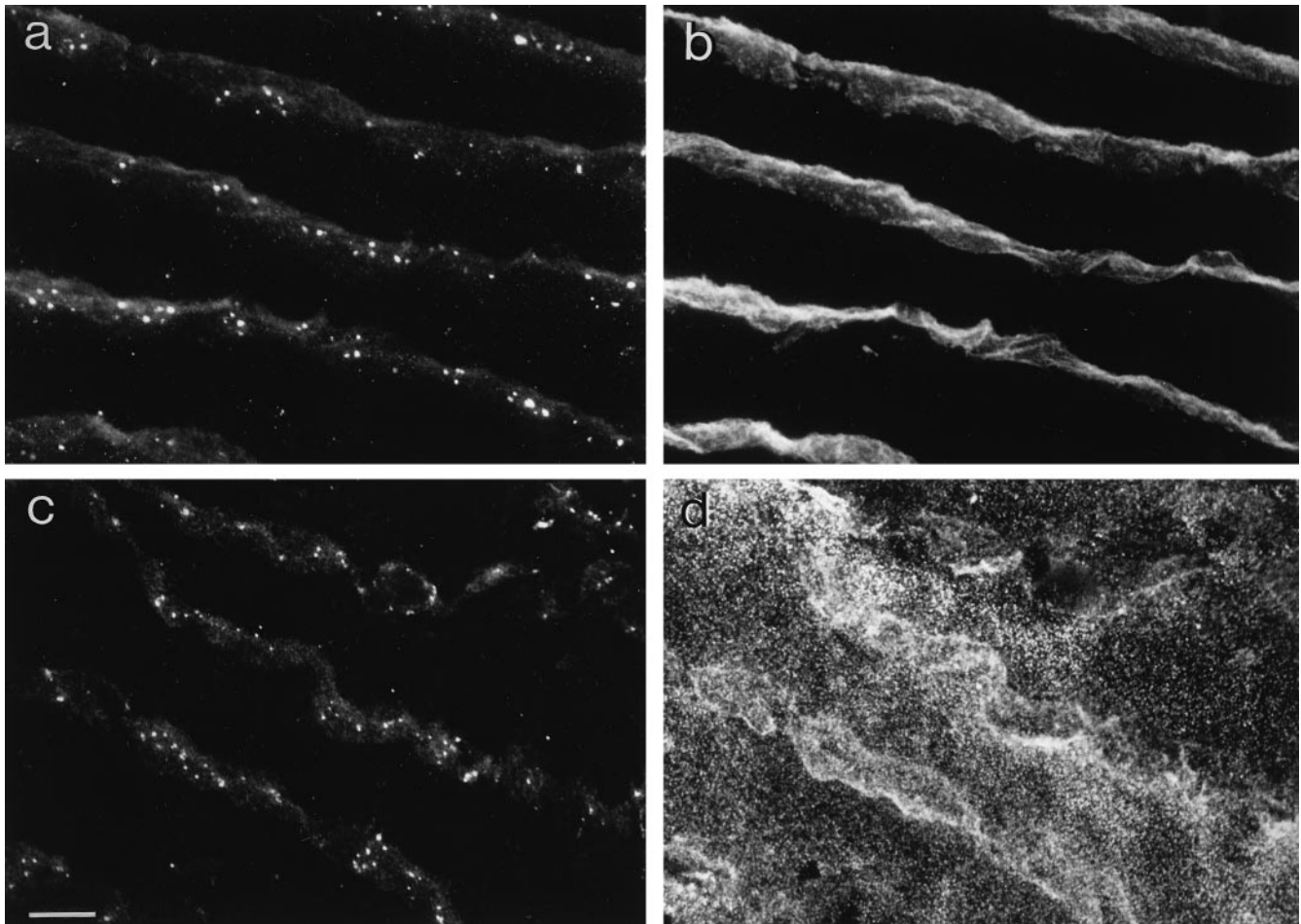


Figure 7. NSIST and the mAb 3B3 epitope have distinct distributions in *Torpedo* electric organ. Frozen sections from *Torpedo* electric organ were double labeled with anti-NSIST antiserum RG212 (*a*) and rh-BTx (*b*) or with RG212 (*c*) and mAb 3B3 (*d*). NSIST-like immunoreactivity (*a*, *c*) is distributed in large puncta, most of which are associated with the innervated portion of the electrocytes. The latter is identified by bright staining with rh-BTx (*b*). The mAb 3B3 epitope is associated with electrocyte surfaces (*d*) and is also found between the tiers of cells. Scale bar, 20 μ m.

because the NSIST staining pattern was indistinguishable from that observed for agrin (data not shown), a protein known to be concentrated in this subcellular compartment in these cells (Magill-Solc and McMahan, 1988).

We also examined the electromotor nucleus of the electric lobe, which contains the neurons innervating the electric organ. In coronal sections of electric lobe, we observed strong anti-NSIST immunostaining within neuronal cell bodies (Fig. 10*A*). No immunolabeling was detected in control sections (data not shown). As in the spinal cord motor neurons, this staining has a patchy cytoplasmic distribution suggestive of an association with Golgi membranes.

3B3 immunostaining has a unique distribution in the electric lobe

We next wanted to know whether the product of the activity of NSIST was also expressed in the CNS. Monoclonal antibody 3B3 immunolabeling reveals marked extracellular staining in the electromotor nucleus (Fig. 10*B*). There is punctate staining on the surfaces of neuronal cell bodies, best seen as the focal plane is changed. In addition, another prominent form of staining consists of a reticular pattern in the parenchyma between neurons. Taken together, these distributions strongly suggest that glycoconjugates modified by NSIST are associated with the surfaces of

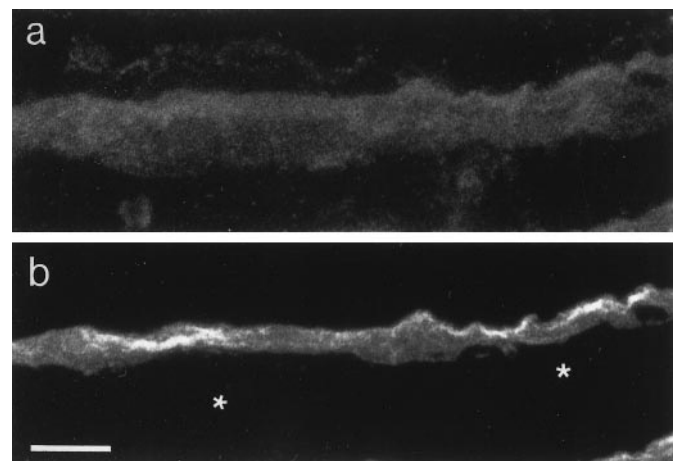


Figure 8. Controls for the specificity of *Torpedo* electric organ immunostaining. Sections of electric organ were incubated with nonimmune rabbit serum (*a*) under the same conditions used in Figure 7. The innervated portion of a segment of electrocyte is revealed by rh-BTX staining (*b*). The lower boundary of the noninnervated portion of the electrocyte is indicated by the asterisks in *B*. In contrast to the specific signal observed for anti-NSIST RG212 immunoreactivity (Fig. 7), no specific immunolabeling was observed under these control conditions. Scale bar, 20 μ m.

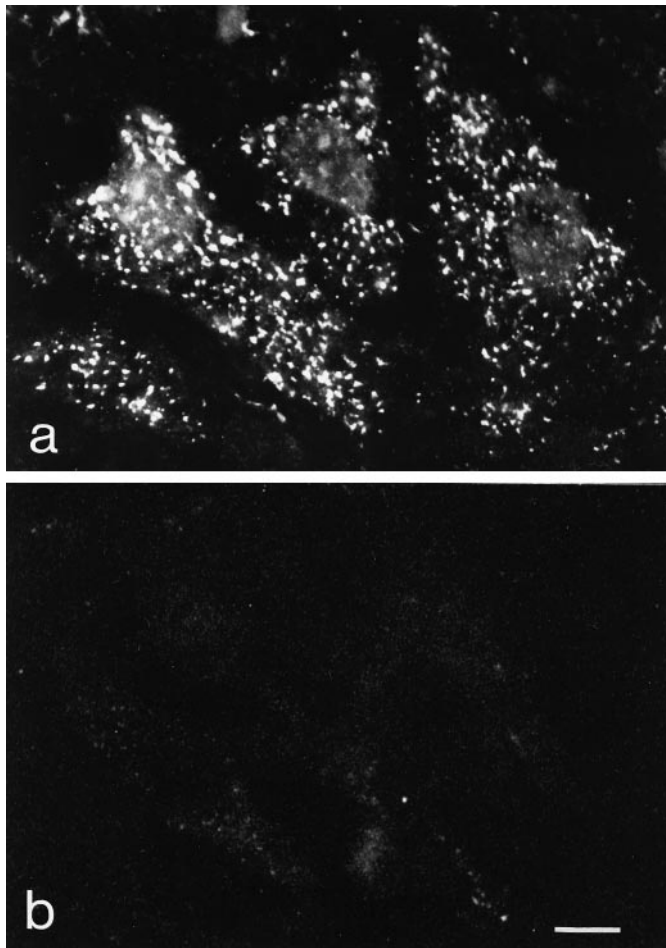


Figure 9. NSIST is distributed within the cell bodies of motor neurons. Transverse cryostat sections of *Torpedo* spinal cord were stained with anti-NSIST antiserum RG212 (*a*). RG212 labeling revealed an extensive, patchy, cytoplasmic distribution of NSIST within motor neuron cell bodies. The staining pattern suggests an association with the Golgi apparatus. A similar section labeled with nonimmune serum (*b*) shows no staining. Scale bar, 20 μ m.

neuronal cell bodies, as well as the extracellular matrix and neuropil in the CNS.

DISCUSSION

Posttranslational processing occurs for many synaptic components; however, a full understanding of such molecular modification requires characterizing the pertinent synthetic enzymes. We sought to elucidate the synthesis of the synapse-associated 3B3 epitope, known to contain sialidase-sensitive carbohydrate constituents. Using mAb 3B3 as an expression-cloning probe, we isolated a novel cDNA clone encoding a polypeptide that is enriched in the nervous system of *Torpedo*. This polypeptide has sequence homology to several sulfotransferases, including a chondroitin sulfotransferase. The polypeptide, NSIST, is abundantly expressed by motor neurons and neurons of the electromotor nucleus. Moreover, the cytoplasmic localization of NSIST differs markedly from the extracellular distribution of the 3B3 epitope. NSIST is the first sulfotransferase shown to be expressed by a specific set of neurons. Furthermore, the distinct subcellular localization of neuronal NSIST together with the localization of its product(s) in the neuropil, suggests a role for NSIST in

regulating cell–cell interactions, particularly at synapses, through the posttranslational processing of specific neuronal constituents.

Analysis of the deduced amino acid sequence of NSIST provides several clues about the structure of the polypeptide. The single 14-amino acid hydrophobic domain near the N terminus (Fig. 1*A*) is characteristic of a type II transmembrane-associated protein in which the signal sequence is retained as a Golgi retention signal (Wickner and Lodish, 1985). Our finding that NSIST resists extraction at pH 11 also suggests that this hydrophobic domain is part of the mature polypeptide. The cationic amino acid residues flanking the transmembrane domain are typical of type II transmembrane proteins. Notably, this transmembrane domain is shorter than the canonical type II domain, which contains at least 17 amino acids (Klein et al., 1985). A similarly short transmembrane domain has been observed for the chicken C6ST by Fukuta et al. (1995), who further found that the cleavage site for generating a soluble form of the C6ST falls within this domain.

Our evidence indicates that the NSIST product(s) are secreted in cultured cells. Double labeling of transfected COS cells with anti-NSIST antiserum and mAb 3B3 showed internal NSIST immunoreactivity but external mAb 3B3 labeling. Furthermore, many more cells were positive for mAb 3B3 than for RG211, indicating that the product(s) of the activity of NSIST may be secreted and able to bind remote sites. Alternatively, it is possible that a secreted form of NSIST, not detectable with our antisera, could modify substrates on the surface of nontransfected cells. In fact, the cleaved, soluble form of C6ST is fully active *in vitro* (Habuchi et al., 1993). Whatever the mechanism, the predominantly intracellular expression of the enzyme and the extracellular localization of its product are in good agreement with their distribution in the nervous system (see below).

NSIST itself is likely to be a glycoprotein. The predicted polypeptide contains four potential N-linked glycosylation sites. One or more of these sites probably bears carbohydrate side chains: the polypeptide shows a diffuse appearance on immunoblots, and the apparent molecular weight of *Torpedo* NSIST as judged by SDS-PAGE is 136 kDa, whereas the predicted molecular weight is 51 kDa. This difference is unlikely to be attributable to alternative splicing, because NSIST in transfected COS cells has an apparent molecular weight of 118 kDa, although the transfected insert encodes a polypeptide with the predicted molecular weight of 51 kDa. It is more likely that the discrepancies between the deduced and observed molecular weights are largely attributable to the decreased SDS-binding (and thus slower mobility) characteristic of many glycoproteins (Deyst et al., 1995; Radeke et al., 1987). It is also possible that NSIST is subject to additional, as yet unknown, posttranslational modifications.

Several lines of evidence indicate that NSIST is a sulfotransferase. First, the features of the transmembrane domain of NSIST noted above are characteristic of this class of enzymes; moreover, NSIST contains significant additional homologies to several sulfotransferases. Second, $^{35}\text{SO}_4$ incorporation was increased in both membrane and cytosolic fractions from NSIST-transfected COS cells. In fact, the 8–10% increase that we measured is likely to represent an underestimate, because only a fraction of the cells picked up and expressed transfected plasmid. Finally, chondroitin sulfate expression is increased in NSIST-transfected COS cells as judged by immunolabeling with mAb CS-56, which recognizes some sulfate-containing moieties of CS (Avnur and Geiger, 1984).

NSIST is likely to modify other substrates in addition to CS. The mAb-3B3 epitope, which is created by NSIST, is sensitive to

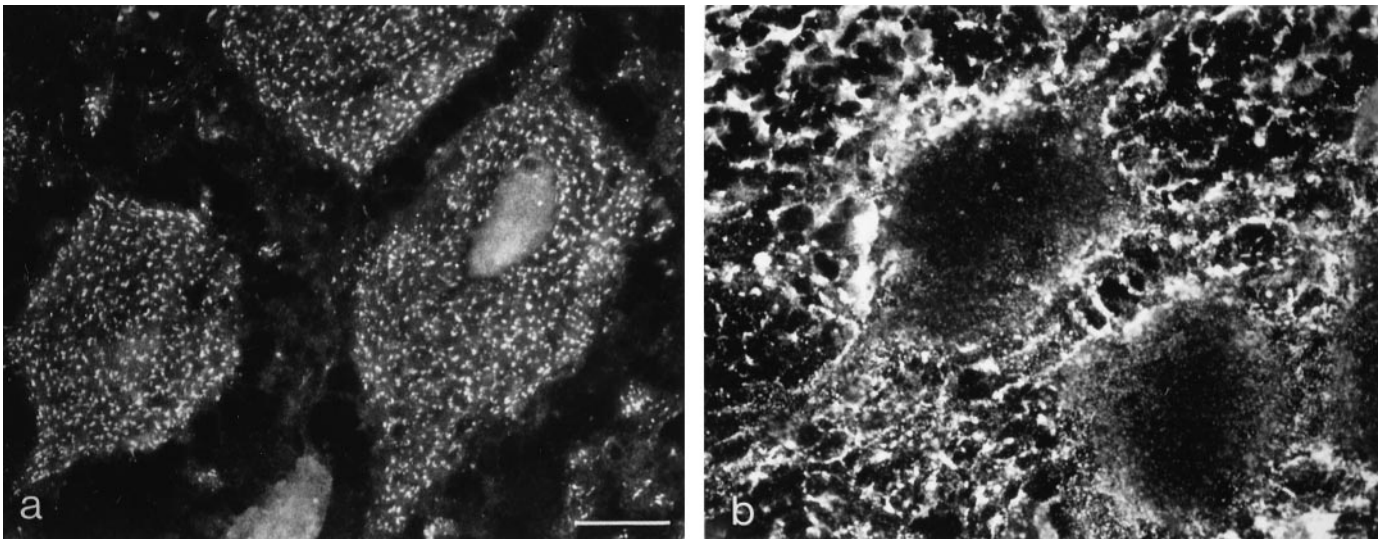


Figure 10. NSIST and the mAb-3B3 epitope have distinct localizations in neurons and neuropil of the *Torpedo* electromotor nucleus. Cryostat sections containing the electromotor nucleus of the *Torpedo* electric lobe were stained with anti-NSIST antiserum RG212 (*a*); neighboring sections were immunostained with mAb 3B3 (*b*). RG212 shows abundant patchy cytoplasmic staining throughout the neuronal cell bodies in the electromotor nucleus, indicating an association with the Golgi apparatus. No staining was seen in control sections. The 3B3 epitope has a punctate distribution on the surfaces of these cell bodies. In addition, between the cell bodies there is bright 3B3 immunostaining associated with the neuropil of the electromotor nucleus. Scale bar, 20 μ m.

sialidase, but not chondroitinase, digestion (Bowe et al., 1994). This epitope is thus likely to include sulfated moieties in close proximity to sialic acid. Such apposition is akin to that seen for the HNK-1 epitope, which contains both sialic acid and sulfated oligosaccharides (Field et al., 1992). Notably, C6ST can transfer sulfate to position 6 of the galactose residue in three known molecular contexts: (1) *N*-acetylgalactosamine (GalNAc) in CS (Habuchi et al., 1993); (2) galactose in keratan sulfate (Sugumaran et al., 1995; Habuchi et al., 1996); and (3) galactose in sialyl lactosamine oligosaccharides (SiaAc α 2-3Gal β 1-4GlcNAc; Habuchi et al., 1997). Taken together with the data presented here, it seems likely that NSIST may act in more than one context to produce unique sulfate-containing moieties. One such substrate may be α -dystroglycan, which bears abundant mAb-3B3 epitopes and which contains Sia α 2-3Gal β 1-GlcNAc as its major sialylated *o*-glycosidically linked oligosaccharide and is likely to be sulfated (Bowe et al., 1994; Smalheiser and Kim, 1995; Chiba et al., 1997).

Although NSIST is structurally related to the C6ST synthesized by chick chondrocytes, it seems likely to be a distinct enzyme. In addition to its remarkably selective expression in nervous tissue, one domain of NSIST shows greatly reduced homology to C6ST (Fig. 2*A*). It is possible that this domain is important for substrate specificity. Indeed, in preliminary experiments we have observed that COS cells transfected with C6ST do not express the mAb 3B3 epitope (B. McKechnie, M. Nastuk, O. Habuchi, and J. Fallon, unpublished observations).

To our knowledge NSIST is the first sulfotransferase demonstrated to be synthesized by neurons. A galactocerebroside sulfotransferase involved in myelin sulfatide synthesis is localized to oligodendrocyte and Schwann cell Golgi membranes (Benjamins et al., 1982; Tennekoon et al., 1983), but this enzyme lacks sequence homology to NSIST (Honke et al., 1997). A glucuronyl glycolipid sulfotransferase is enriched in gray matter (Chou and Jungalwala, 1993) and may be involved in creating the HNK-1 epitope. However, its homology to NSIST cannot be assessed, because the primary structure of this sulfotransferase is unknown.

In *Torpedo* electric organ, NSIST is expressed predominantly in the synaptic portion of the electrocyte. This distribution is consistent with the idea that the products of NSIST play a role at synapses. Indeed, the 3B3 epitope is selectively expressed in postsynaptic membrane fractions from this tissue (Bowe et al., 1994). NSIST is also enriched in these fractions. However, our immunolocalization data indicate that this enzyme is probably not a component of the AChR-rich, postsynaptic membranes, but rather of intracellular membranes that cofractionate with them. The punctate immunostaining observed in the electrocytes is similar to that observed by Jasmin et al. (1992) for Rab6p, which is a component of Golgi and post-Golgi vesicles in these cells. The clearly Golgi-like distribution seen in motor neurons is also consistent with such an intracellular localization. On the other hand, some carbohydrate-modifying enzymes are expressed on the postsynaptic membrane (see below). In this regard it will be important in future work to determine the localization of NSIST at the ultrastructural level.

A potential role for NSIST in the CNS is suggested by its localization to the neurons of the electromotor nucleus. Here, NSIST is distributed in an array that includes the Golgi apparatus, indicating that these neurons synthesize the enzyme. On the other hand, mAb 3B3 labels the surfaces of these neurons as well as the neuropil between them. Thus, the products of NSIST (or NSIST itself), may be locally secreted, allowing for involvement in cell-cell interactions intrinsic to the CNS. This latter possibility may be relevant to the finding that specific subsets of neurons in the mammalian CNS express a perineuronal CSPG (Lander et al., 1997). Furthermore, cultured cortical neurons produce an entirely extracellular, surface-associated CSPG that may constitute a neuronally derived component of the extracellular matrix (Lander et al., 1998).

The expression of NSIST in *Torpedo* motor neurons provides a mechanism for synapse-specific sulfation at the neuromuscular junction. In this regard, it is noteworthy that synaptic CS has been implicated in the formation of neuromuscular junctions. C2 muscle cells require surface CS to produce spontaneous AChR

clusters in culture (Mook-Jung and Gordon, 1995). The S27 variant of C2 cells, which exhibits reduced incorporation of sulfate into proteoglycans (Gordon and Hall, 1989), are unable to form spontaneous or agrin-induced AChR clusters (Gordon et al., 1993). Notably, S27 cells can form AChR clusters when cocultured with motor neurons (Gordon et al., 1993). Thus, the production of NSIST by motor neurons could distinguish synaptic from extrasynaptic CS through differential sulfation, an interesting possibility given the view that the degree of sulfation of synaptic CS is probably important to its function (Mook-Jung and Gordon, 1995; Bowen et al., 1996). Although we did not observe elevated NSIST levels at neuromuscular junctions, it is noteworthy that a specialized Golgi apparatus exists just under the postsynaptic membrane (Ralston et al., 1993; Antony et al., 1995). It is thus feasible that NSIST is also selectively expressed at junctional nuclei in muscle. Although undetectable in our Northern blots, any NSIST mRNA associated with this relatively small population of nuclei should be readily demonstrable with *in situ* hybridization.

A selective activity of NSIST at synapses would add an intriguing dimension to the molecular interplay of synaptogenesis. We would expect this sulfotransferase to act in coordination with other enzymes responsible for posttranslational processing. Notably, a specific postsynaptic localization has been demonstrated for GalNAc-terminated carbohydrates (Martin and Sanes, 1995) as well as for a muscle-derived GalNAc transferase (Scott et al., 1990). Furthermore, coordinated expression has been observed for a GalNAc transferase and its companion sulfotransferase in several tissues, including brain (Dharmesh et al., 1993). NSIST, as the first sulfotransferase shown to be synthesized by specific types of neurons, is a promising candidate to participate in synapse-specific sulfation of particular carbohydrate moieties. In this light, it will be important to show whether NSIST interacts with enzymes such as GalNAc transferase in the coordination of synapse-specific posttranslational processing.

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