Polysialyltransferase-1 Autopolysialylation Is Not Requisite for Polysialylation of Neural Cell Adhesion Molecule*

Brett E. Close, Kevin Tao, and Karen J. Colley‡

From the Department of Biochemistry and Molecular Biology, University of Illinois College of Medicine, Chicago, Illinois 60612

Polysialyltransferase-1 (PST; ST8Sia IV) is one of the α2,8-polysialyltransferases responsible for the polysialylation of the neural cell adhesion molecule (NCAM). The presence of polysialic acid on NCAM has been shown to modulate cell-cell and cell-matrix interactions. We previously reported that the PST enzyme itself is modified by α2,8-linked polysialic acid chains in vivo. To understand the role of autopolysialylation in PST enzymatic activity, we employed a mutagenesis approach. We found that PST is modified by five Asn-linked oligosaccharides and that the vast majority of the polysialic acid is found on the oligosaccharide modifying Asn-74. In addition, the presence of the oligosaccharide on Asn-119 appeared to be required for folding of PST into an active enzyme. Co-expression of the PST Asn mutants with NCAM demonstrated that autopolysialylation is not required for PST polysialyltransferase activity. Notably, catalytically active, non-autopolysialylated PST does not polysialylate any endogenous COS-1 cell proteins, highlighting the protein specificity of polysialylation. Immunoblot analyses of NCAM polysialylation by PST, synthesizing a longer polysialic acid chain than STX (37). Our laboratory has previously reported that both the PST and STX enzymes are modified by α2,8-linked polysialic acid chains when expressed in COS-1 cells (38), a process termed autopolysialylation (39). These autopolysialylated polysialyltransferases localized to the Golgi, the cell surface, and were found soluble in the extracellular space. In addition, our data suggested that these autopolysialylated polysialyltransferases were the only polysialylated proteins expressed in COS-1 cells (38). We hypothesize that the polysialic acid chains modifying PST and STX found at the cell surface and in the extracellular space could modulate cell-cell and cell-matrix interactions in a manner similar to those modifying polysialylated NCAM.

Recent in vitro studies on autopolysialylated PST indicate that PST is modified by at least four Asn-linked oligosaccharides, with the number of polysialic acid chains unknown (39). It was also suggested that autopolysialylation of PST was a requirement for enzymatic activity toward NCAM (39). Here, we report on the carbohydrate modifications of PST and identify the requirements for autopolysialylation of the enzyme, as well as the requirements for polysialylation of NCAM by PST. Mutational analysis of human PST revealed that PST is modified by five Asn-linked oligosaccharides. The majority of the PST, polysialyltransferase-1 (ST8Sia IV); V5 Ab, anti-V5 epitope tag antibody; OL.28 Ab, anti-polysialic acid antibody; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; BME, β-mercaptoethanol; CHO, Chinese hamster ovary; STX, polysialyltransferase X (ST8Sia II).

* This work was supported in part by National Institutes of Health Research Grant GM48134 (to K. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Established Investigator of the American Heart Association. To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Illinois College of Medicine, 1819 W. Polk St., M/C 536, Chicago, IL 60612. Tel.: 312-996-7756; Fax: 312-413-0364; E-mail: karenc@uic.edu.

* The abbreviations used are: NCAM, neural cell adhesion molecule;
polysialic acid of autopolyesialylated PST resides on the Asn-linked oligosaccharide modifying Asn-74. Our data also establish that the minimum requirement for autopolyesialylation of PST is an Asn-linked oligosaccharide on Asn-74. Mutants of PST shown to be non-autopolyesialylated by immunofluorescence, pulse-chase, and immunoblot analyses were co-expressed with full-length and soluble NCAM to determine if autopolyesialylation was a requirement for enzymatic activity. Results from three separate experimental methodologies show that autopolyesialylation of PST is not a prerequisite for enzymatic activity. Both soluble and membrane-bound forms of NCAM were polyesialylated when co-expressed with a full-length non-autopolyesialylated form of PST. Comparison of the extent of NCAM polyesialylation by non-autopolyesialylated and non-autopolyesialylated PST suggests that the NCAM is polyesialylated to a higher degree by autopolyesialylated PST. However, we conclude that autopolyesialylation of PST is not a prerequisite for NCAM polyesialylation.

EXPERIMENTAL PROCEDURES

Materials

Tissue culture media and reagents, including Dulbecco’s modified Eagle’s medium (DMEM), Opti-MEM I, Lipofectin, and oligonucleotides were purchased from Life Technologies, Inc. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Nih-3T3 cell membranes were purchased from Schleicher & Schuell. SuperSignal West Pico chemiluminescence reagent was obtained from Pierce. Protein molecular mass standards (myosin, 203 kDa; β-galactosidase, 109 kDa; bovine serum albumin, 78 kDa; ovalbumin, 46.7 kDa; carbonic anhydrase, 34.5 kDa) were purchased from Bio-Rad. The cDNA for human PST was a kind gift from Dr. Minoru Fukuda (Burnham Institute, La Jolla, CA). The QuikChangeTM site-directed DNA mutagenesis kit and Pfu DNA polymerase were purchased from Stratagene (La Jolla, CA). Anti-V5 epitope tag antibody was purchased from Invitrogen Corp. (Carlsbad, CA). Murine NCAM-Fc cDNA was a generous gift from Nancy Kedersha (Brigham and Women’s Hospital, Boston, MA). Mouse anti-NCAM (human CD56) antibodies were purchased from Caltag Laboratories (South San Francisco, CA). Sequenase version 2.0 DNA sequencing kit was purchased from U. S. Biological Corp. DNA purification kits were obtained from Qiagen (Valencia, CA). Protein A-Sepharose and [α-32P]dATP for DNA sequencing were obtained from Amersham Pharmacia Biotech. 5'-Express protein labeling mix was purchased from NEN Life Science Products. Fluorescein isothiocyanate (FITC)-conjugated and horseradish peroxidase-conjugated goat anti-mouse antibodies were purchased from Jackson Laboratories (West Grove, PA). Other chemicals and reagents were obtained from Sigma and Fisher Scientific (Hanover Park, IL).

Methods

Site-directed Mutagenesis of PST-V5 cDNA—Consensus N-glycosylation site Asn residues in full-length PST-V5 cDNA (38) were mutated to Ser using the QuikChangeTM site-directed mutagenesis system per manufacturer’s protocol (Stratagene). Listed are the primers used for mutation of the Asn codon to the Ser codon, with the mutating codon underlined: mutant 1, CGGTACCTTGGTTCAGGACCGGATGCAAAATGATCTTTCTTGTCC; mutant 2, AAGGTTGAGAAAUTCTCCTTCTTTGTGC and GCAAGACAGGAGGA-GATTTCACCACTC; mutant 3, GCCGAGAATTCTACCTGATCATCAG- GATTCACAG and CTATACTAGATGGAATTCTAGATG; mutant 4, TTTGGAGGCTTTCGATCCGAGAGTGACAG and CTGTCGCTCGTGAGTTAAAAGCTC; mutant 5, TTTCTCATGCTGTCGACAGCTTGTG and CAAAGGAGAATGTCGAGGAC- TGAGAAA; mutant 6, N3278, TATAGTACATTTCTCCGGACCCTAC and TCTCTAGGAGCAGGGAAAGAATTGC; mutant 7, CAGATGTATA- TTGGAGGAGGATCTCAGGACCCAGACGCATG and GCGATGACGAGCAG- TGCTATAAATCTG. Each mutation was confirmed using the Sequenase version 2.0 DNA sequencing kit (U. S. Biological Corp.) and the appropriate sequencing primer.

Transfection of COS-1 Cells with Mutant PST-V5 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, Inc. Thirty microliters of Lipofectin and 20 μg of wild type or mutant PST-V5 plasmid DNA in 3 ml of Opti-MEM I + 55 μg β-mercaptoethanol were used for transfection of each 100-mm tissue culture plate. Three microliters of Lipofectin and 0.5 μg of plasmid DNA in 300 μl of Opti-MEM + 55 μg β-mercaptoethanol were used for transfection of each coverslip.

Immunofluorescence Localization of Mutant PST-V5 Proteins—COS-1 cells were plated on glass coverslips, transfected with wild type or mutant PST-V5 cDNA, and processed for immunofluorescence microscopy as described previously (40). Briefly, cells were treated with either 0.1% Triton X-100 in internal staining or 5% paraformaldehyde to visualize cell surface staining. Anti-V5 epitope tag antibody was diluted 1:100 and the OL.28 anti-poly-sialic acid antibody, FITC-conjugated secondary antibodies, goat anti-mouse IgG and goat anti-mouse IgM, were diluted 1:200 in 5% normal goat serum/PBS blocking buffer prior to use. Coverslips were mounted on glass slides using 20 μl of mounting medium (15% (w/v) Vinol 205 polyvinyl alcohol, 33% (v/v) glycerol, 0.1% azide in PBS, pH 8.5). Cells were visualized and photographed using a Nikon Axiohot microscope equipped with epifluorescence illumination and a 60× oil immersion Plan Apochromat objective.

Metabolic Labeling of Cells and Immunoprecipitation of PST-V5 Proteins—Following transfection of COS-1 cells with wild type or mutant PST-V5 cDNA and expression of these proteins in the cells for 18 h, 10-mm coverslips were removed and washed with cysteine/methionine-free DMEM for 1 h. After incubation, this medium was replaced with 3.5 ml of fresh cysteine/methionine-free DMEM containing 100 μCi/ml 35S-Express protein labeling mix (NEN Life Science Products). Cells were incubated with the radiolabel for 1 h at 37 °C in a 5% CO2 incubator. After labeling, medium was removed and cells washed, the labeled proteins were chased for either 0 h (cell lysates) or 6 h with 4 ml of unlabeled DMEM, 10% PBS. Cells were washed with 10 ml of PBS and lysed in 1 ml of immunoprecipitation buffer 2 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS). After 6 h of chase, cell medium was collected, debris removed by centrifugation, and the supernatant frozen overnight at −20 °C.

The PST-V5 mutant and wild type proteins were immunoprecipitated from the cell lysates and 6-h chase media using 2 μg of anti-V5 epitope tag antibody and protein A-Sepharose (Amersham Pharmacia Biotech), as described previously (41). However, 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% BME and directly loaded into the gel wells. Immunoprecipitation beads were separated on 7.5% separating and 5% stacking SDS-polyacrylamide gels (42). Radiolabeled proteins were visualized by fluorography using 10% 2,5-diphenyloxazolone in dimethyl sulfoxide (43), and gels were exposed to Kodak BioMax MR film at −80 °C.

Immunoprecipitation and Immunoblot of PST-V5 Proteins—COS-1 cells were transfected with wild type or mutant PST-V5 cDNA as described before. Following transfection, the 3 ml of transfected mixture was removed, and 4 ml of DMEM, 10% FBS was added to the cells and the cells incubated for 18 h at 37 °C in a 5% CO2 incubator. Cell medium was collected, debris removed by centrifugation, and the supernatant frozen overnight at −20 °C. Cells were washed with 10 ml of PBS, removed by scraping and pelleted by centrifugation. Cells were lysed on ice by addition of 250 ml of TBS, 1% Triton X-100 for 30 min. Cellular debris was removed by centrifugation, and the supernatant frozen overnight at −20 °C. Cells were washed with 10 ml of PBS, removed by scraping and pelleted by centrifugation. Cells were lysed on ice by addition of 250 ml of TBS, 1% Triton X-100 for 30 min.

Cellular debris was removed by centrifugation, and the supernatant frozen overnight at −20 °C. Cells were washed with 10 ml of PBS, removed by scraping and pelleted by centrifugation. Cells were lysed on ice by addition of 250 ml of TBS, 1% Triton X-100 for 30 min.

Cellular debris was removed by centrifugation, and the supernatant frozen overnight at −20 °C. Cells were washed with 10 ml of PBS, removed by scraping and pelleted by centrifugation. Cells were lysed on ice by addition of 250 ml of TBS, 1% Triton X-100 for 30 min.

Cellular debris was removed by centrifugation, and the supernatant frozen overnight at −20 °C. Cells were washed with 10 ml of PBS, removed by scraping and pelleted by centrifugation.
polysialic acid antibody and anti-mouse NCAM IgG, 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 Co-expressing Mutant PST-V5 Proteins—In duplicate, COS-1 cells plated on 100-mm tissue culture plates were co-transfected with wild type and mutant PST-V5 and NCAM-Fc plasmid DNA at a ratio of 4:1, respectively (20 μg: 5 μg). For the first set of plates, 7 ml of DMEM, 10% FBS was added following transfection and the cells incubated for 18 h at 37 °C in a 5% CO2 incubator. After incubation, cells were radiolabeled for 1 h with 100 μCi/ml [35S]cysteine-methionine mix as described above. After labeling, medium was removed and cells washed, the labeled proteins were chased for 3 h with 4 ml of unlabeled DMEM, 10% FBS. Cell medium was collected, debris centrifuged down, and the supernatant frozen overnight at −20 °C. The medium was thawed at room temperature and precleared with 50 μl of a 50% slurry of Sepharose CL-6B for 1 h by end-over-end rotation at 4 °C. The Sepharose CL-6B beads were removed by centrifugation and the supernatant transferred to a clean tube. Soluble NCAM-Fc was precipitated from the medium by addition of 50 μl of a 50% slurry of protein A-Sepharose and end-over-end rotation at 4 °C. The precipitated proteins were separated on 7.5% SDS-polyacrylamide gels and radiolabeled protein bands visualized by fluorography. The molecular mass marker shown is 46.7 kDa (ovalbumin).

RESULTS

After finding that PSt is autopolsialylated in vivo (38), we wondered how autopolsialylation is related to polysialyltransferase activity. To investigate this question, we needed to know where polysialic acid was located on the PST protein in order to eliminate it and analyze the activity of the non-autopsialylated protein using NCAM as a substrate. In addition, if we found that PST was polysialylated on a limited number of Asn-linked oligosaccharides, this would suggest specificity in the autopolsialylation event and make it more reasonable to use PST as a model protein to investigate the protein sequence and carbohydrate structure requirements for its polysialylation.

PST Is Modified by Five Asn-linked Oligosaccharides—To determine which Asn-linked oligosaccharides of PST are polysialylated, we first needed to establish which Asn-linked oligosaccharide attachment sites in the PST sequence are used. Human PST (33) has six potential Asn-linked glycosylation sites in the PST sequence (Fig. 1A), with a seventh being the disallowed Asn-Pro/Thr sequence (Asn-191). Asn residues in each consensus glycosylation site were mutated to Ser as described under "Experimental Procedures." Ser was chosen to replace Asn, based on previous observations with Asn replacements in the ST6Gal I protein in which an isosteric Gln replacement generated a protein that was misfolded and retained in the endoplasmic reticulum (ER), while a Ser replacement led to a Golgi-localized and active sialyltransferase. COS-1 cells expressing wild type and mutant PST proteins were metabolically labeled with 35S-Express protein labeling mix for 1 h in order to detect predominantly non-autopsialylated species (38). V5 epitope-tagged enzymes were immunoprecipitated from cell lysates using the anti-V5 epitope tag antibody and separated on SDS-polyacrylamide gels.

Typically, a single non-autopsialylated Asn-linked oligosaccharide contributes approximately 3–4 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 3–4 kDa smaller than the wild type protein. Analysis of wild type glycosylated, but non-autopsialylated, PST by SDS-polyacrylamide gel electrophoresis revealed that the enzyme migrated with an apparent molecular mass of 61 kDa, which corresponds to the expected molecular mass of human PST modified by the V5 epitope tag and five Asn-linked oligosaccharides (Fig. 1B). Individual mutations of Asn residues 50, 74, 119, 204, and 219 to Ser resulted in proteins with reduced molecular mass (~58 kDa) in comparison to wild type PST (Fig. 1B, compare WT to N50S, N74S, N119S, N204S, and N219S). This demonstrated each of these Asn residues is modified by an oligosaccharide in the wild type PST protein. In contrast, no change in molecular mass occurred when Asn-191 or Asn-327 were converted to Ser, indicating that these resi-
Ser resulted in proteins that migrated like the wild type enzyme on SDS gels (Fig. 2A, mutants 1, 4, and 5). In contrast, mutation of Asn-2 to Ser resulted in a dramatic reduction in the high molecular mass form of the enzyme (Fig. 2A, mutant 2), and mutation of Asn-3 to Ser completely eliminated the high molecular mass form (Fig. 2A, mutant 3).

We confirmed that the polydisperse, high molecular mass forms of wild type and mutant PST proteins are α2,8-linked polysialylated proteins in a parallel immunoblot experiment (Fig. 2B). Proteins were expressed in COS-1 cells, immunoprecipitated from unlabeled cell medium, and were analyzed by immunoblotting with the OL.28 anti-polysialic acid antibody that is specific for α2,8-linked polyNeu5Ac of 5 units or longer.3 Wild type PST and the mutant proteins lacking the oligosaccharides on Asn-1, -4, and -5 were reactive with the OL.28 anti-polysialic acid antibody (Fig. 2B, WT and Mutants 1, 4, and 5). In contrast, the level of autopolysialylation of the mutant protein lacking the oligosaccharide on Asn-2 was dramatically reduced relative to that of wild type PST (Fig. 2B, Mutant 2), and no polysialic acid was detected on the mutant protein lacking the oligosaccharide on Asn-3 (Fig. 2B, Mutant 3). Was this observed decrease or absence in the autopolysialylation of these mutants due to a lower level of their expression? Lesser amounts of both mutant 2 and mutant 3 were found in the cell medium relative to the other PST proteins. However, the intracellular levels of both proteins are comparable to those of the other PST proteins (see Fig. 1B) and all of the analyzed proteins are localized in the Golgi apparatus (see Fig. 3). This indicates that the mutant 2 and mutant 3 proteins are secreted at a lower rate relative to the other PST proteins, rather than being expressed at lower levels. It is also unlikely that we have grossly underestimated the polysialylation of these proteins since the OL.28 immunofluorescence microscopy of permeabilized COS-1 cells expressing these proteins confirms these results (Fig. 3). In sum, these data show that the oligosaccharides on Asn-2 and -3 are required for the full autopolysialylation of PST.

These data suggest several possibilities. First, the oligosaccharides on Asn-2 and/or Asn-3 could be required for proper protein folding and therefore their elimination leads to inefficient transport out of the ER to the Golgi apparatus. This is highly unlikely, since we observe low molecular mass forms of the mutant enzymes in the cell medium. Second, these oligosaccharides could be sites for addition of polysialic acid. Third, the elimination of the oligosaccharides at Asn-2 and/or Asn-3 could lead to a conformationally altered protein that lacks polysialyltransferase activity or is not an effective substrate for autopolysialylation.

Wild Type and Mutant PST Enzymes Localize to the Golgi Apparatus—To determine whether elimination of the oligosaccharides on Asn-2 and -3 caused misfolding of the PST proteins and their retention in the ER, wild type and mutant PST proteins were localized in COS-1 cells using indirect immunofluorescence microscopy. The anti-V5 epitope tag antibody was used to localize the proteins internally, and the OL.28 anti-polysialic acid antibody was used to detect polysialic acid, diagnostically of autopolysialylation, at the cell surface and internally (38). Wild type PST was localized to the Golgi apparatus in COS-1 cells (Fig. 3, V5 Ab, Internal), as were all the Asn mutants (Fig. 3, V5 Ab, Internal). This observation excludes the possibility that elimination of the oligosaccharides at Asn-2 and -3 leads to improper protein folding and impaired ER to Golgi transport. Cells transiently expressing wild type PST and Asn mutants were stained with the OL.28 antibody to localize

---

3 K. Kitajima, unpublished results.
autopolysialylated PST. Both wild type and PST mutants 1, 4, and 5 were expressed internally and on the cell surface in their autopolysialylated forms (Fig. 3, OL.28 Ab, Internal and Surface; WT and Mutants 1, 4, and 5). Conversely, the surfaces of cells expressing mutants 2 and 3 were not immunoreactive with the OL.28 antibody (Fig. 3, OL.28 Ab, Surface; Mutants 2 and 3). However, polysialic acid was localized to the Golgi apparatus in a very small percentage of cells expressing mutant 2 (Fig. 3, OL.28 Ab, Internal; Mutant 2). In addition, no polysialic acid was observed in the Golgi apparatus of cells expressing mutant 3 (Fig. 3, OL.28 Ab, Internal; Mutant 3). These data correlate well with the observation that the level of autopolysialylation of secreted mutant 2 was dramatically lower than wild type PST (Fig. 2B, Mutant 2) and that no autopolysialylation was observed on the secreted mutant protein lacking Asn-3 (Fig. 2B, Mutant 3).

The Presence of Asn-74 (Asn-2) Is Sufficient for PST Autopolysialylation—In order address the second possibility and determine whether the oligosaccharides on Asn-2 and Asn-3 are modified with the polysialic acid chains, a series of mutants retaining single Asn-linked glycosylation sites were made (the “alone” series). Mutants retaining either Asn-1, Asn-2, Asn-3, Asn-4, or Asn-5 were efficiently transported to the Golgi apparatus (data shown only for 2 alone mutant; Fig. 4B, V5 Ab, Internal). Immunoblot analysis and immunofluorescence microscopy experiments using the OL.28 anti-polysialic acid antibody revealed that only the Asn-2 alone mutant was detectably polysialylated. The 2 alone mutant was expressed at comparable levels to wild type PST (Fig. 4A, 2 Alone, cell lysate); however, it was not cleaved and secreted as efficiently as wild type PST (Fig. 4A, 2 Alone, cell medium). It was found in a polysialylated form in the Golgi (Fig. 4, B, 2 Alone; and C, OL.28 Ab, Internal), at the cell surface (Fig. 4C, OL.28 Ab, Surface), and in the cell medium (Fig. 4B, 2 Alone, M). Our data establish that there is polysialic acid present on the oligosaccharide modifying Asn-2 (Asn-74) of human PST and that an Asn-linked oligosaccharide at this site is sufficient for autopolysialylation of PST. Notably, the presence of Asn-3 alone and its attached oligosaccharide was not sufficient for autopolysialylation of the enzyme (data not shown). This may suggest that elimination of the Asn-3 oligosaccharide may alter the conformation of the protein and lead to its inactivity.

Elimination of the Oligosaccharide on Asn-119 (Asn-3) Results in the Inability of PST to Polysialylate NCAM and Itself—To address the third possibility, that elimination of Asn-3 and its attached oligosaccharide cause a conformational alteration of PST rendering it an inactive enzyme or incompetent substrate, two double Asn mutants were made. Replacement of both Asn-2 and Asn-3 with Ser (mutant 2.3) resulted in expression of a properly localized (Fig. 3, Mutant 2.3, V5 Ab), non-autopolysialylated form of the PST protein (Fig. 2, A and B, Mutant 2.3; Fig. 3, mutant 2.3, OL.28 Ab). This finding was expected from the previous observations that the single replacements of Asn-2 and Asn-3 led to the expression of underautopolysialylated or non-autopolysialylated proteins (Fig. 2A, mutants 2 and 3). Unexpectedly, replacement of both Asn-3 and Asn-4 with Ser (mutant 3.4) resulted in expression of a correctly localized (Fig. 3, Mutant 3.4, V5 Ab) and autopolysialylated form of the PST protein (Fig. 2, A and B, Mutant 3.4; Fig. 3, Mutant 3.4, OL.28 Ab). Elimination of the oligosaccharide on Asn-4 in addition to the oligosaccharide on Asn-3 restored the autopolysialylation of the enzyme. These observations suggest that the additional mutation of Asn-4 and elimination of an oligosaccharide at this site corrects a conformational problem in the Asn-3 mutant that leads to its inactivity or incompetence as a substrate.

PST Autopolysialylation Is Not a Requirement for Polysialylation of NCAM—Next, we wanted to determine whether the lack of glycosylation of Asn-3 results in an inactive enzyme, and whether autopolysialylation of PST is a prerequisite for NCAM polysialylation. We co-expressed soluble NCAM-Fc with wild type and mutant PST proteins in COS-1 cells. Expressing cells were radiolabeled for 1 h and chased with unlabeled medium for 3 h, and soluble NCAM-Fc was precipitated from the medium with protein A-Sepharose as described under “Experimental Procedures.” The NCAM-Fc monomer migrates on SDS-polyacrylamide gels with an apparent molecular mass of 180 kDa, while polysialylated NCAM-Fc appears as a polydisperse band, with a molecular mass ranging from 180 kDa to above 200 kDa (Fig. 5A). We confirmed that the polydisperse appearance of NCAM-Fc reflected polysialylation by immunoblotting NCAM-Fc with the OL.28 anti-polysialic acid antibody (Fig. 5B). As expected, co-expression of wild type PST with NCAM-Fc resulted in the polysialylation of NCAM-Fc (Fig. 5, A and B, WT). Likewise, co-expression of Asn mutants 1, 4, and 5 (all autopolysialylated like wild type PST) with NCAM-Fc also resulted in the polysialylation of NCAM-Fc (Fig. 5, A and B, Mutants 1, 4, and 5). The Asn-2 mutant protein, which was modified by much reduced levels of polysialic acid, could polysialylate NCAM-Fc. However, the lower apparent molecular mass of NCAM-Fc modified by this mutant (Fig. 5B, Mutant 2) suggested that PST autopolysialylation may impact the length or number of polysialic acid chains added to NCAM-Fc. Strikingly, the Asn-3 mutant protein could not polysialylate
PST Autopolysialylation and Enzymatic Activity Requirements

**Fig. 4.** The presence of Asn-2 alone is sufficient for PST autopolysialylation. A, wild type (WT) and 2 alone mutant PST-V5 proteins were transiently expressed in COS-1 cells, metabolically labeled with 35S-Express protein labeling mix for 1 h, and immunoprecipitated from 0-h chase cell lysates and 6-h chase medium with the anti-V5 epitope tag antibody. The immunoprecipitated samples were electrophoresed on 7.5% SDS-polyacrylamide gels and radiolabeled protein bands visualized by fluorography. B, wild type and Asn-2 alone mutant PST-V5 enzymes were immunoprecipitated from COS-1 cell lysates and medium after overnight expression and subjected to immunoblot analysis with the OL.28 anti-polysialic acid antibody as described under “Experimental Procedures.” Molecular mass markers are as follows: 203 kDa, myosin; 109 kDa, β-galactosidase; 78 kDa, bovine serum albumin; 46.7 kDa, ovalbumin. C, the localization of Asn-2 alone mutant was analyzed by indirect immunofluorescence microscopy using the anti-V5 epitope tag antibodies (Internal Staining) and OL.28 anti-polysialic acid antibodies (Internal and Surface Staining). Immunofluorescence was visualized using a Nikon Axioptot fluorescence microscope and a 60× oil immersion Plan Apochromat objective. Original magnification, ×750.

NCAM-Fc (Fig. 5, A and B, Mutant 3) or itself (Fig. 2B, Mutant 3). The mutation of Asn-4 in combination with Asn-3 (mutant 3.4) rescues the ability of the enzyme to polysialylate NCAM-Fc (Fig. 5, A and B, Mutant 3.4) and itself (Fig. 2B, Mutant 3.4). These data again suggest that a single mutation of Asn-3 to Ser, and the elimination of an oligosaccharide at this site, causes a conformational change in the protein that abolishes its ability to polysialylate NCAM and itself. Most significant was the finding that mutant 2.3, which was shown to be non-autopolysialylated (Fig. 2B, Mutant 2.3), was still able to polysialylate NCAM-Fc in our co-expression assay (Fig. 5, A and B, Mutant 2.3). This suggests that the conformational changes induced by removing an Asn-linked oligosaccharide from site 3 (Asn-119) could be compensated for by removing Asn-linked oligosaccharides at either site 2 (Asn-74) or site 4 (Asn-204) to regenerate the active enzyme. In sum, these data establish that autopolysialylation of PST is not a prerequisite for its polysialylation of NCAM and that the glycosylation state of PST is critical for its activity as a polysialyltransferase.

**DISCUSSION**

In this work, we present the results of our studies on the carbohydrate modifications of the PST polysialyltransferase and their effect on enzymatic activity. Using a site-directed mutagenesis approach, we have shown that human PST is modified with five Asn-linked oligosaccharides (Fig. 1B). The oligosaccharide on Asn-2 is the major site of polysialic acid addition since its elimination in the Asn-2 mutant significantly decreased polysialylation relative to wild type PST (Fig. 2). In addition, when PST mutants containing only one Asn-linked glycosylation site were analyzed, only the Asn-2 alone mutant could support any detectable polysialylation (Fig. 4). Elimination of the oligosaccharide on Asn-3 completely abolished autopolysialylation (Fig. 2) and polysialylation of NCAM (Fig. 5). However, when Asn-2 or -4 were mutated in combination with Asn-3, this rescued the enzyme’s ability to polysialylate NCAM (mutants 2.3 and 3.4) and itself (mutant 3.4). These results suggest that elimination of Asn-3 and its oligosaccharide cause a conformational change in PST that leads to its inactivity. Finally, co-expression autopolsialylated and non-autopolysialylated PST mutant proteins with NCAM-Fc revealed that the Asn-2 mutant, with extremely low levels of polysialic acid, and the double Asn-2.3 mutant, which completely lacks polysialic acid, are still able to polysialylate NCAM, although at lower levels than wild type PST (Fig. 5). These results demonstrate that autopolsialylation of PST is not required for, but does enhance, the polysialylation of NCAM.

Our laboratory previously reported that both of the polysialyltransferases capable of polysialylating NCAM, namely PST and STX, are autopolsialylated on Asn-linked oligosaccharides (38). In this report, we establish that the oligosaccharide on Asn-74 (Asn-2) is the major site of polysialic acid addition since its elimination in the Asn-2 mutant significantly decreased polysialylation relative to wild type PST (Fig. 2). In addition, when PST mutants containing only one Asn-linked glycosylation site were analyzed, only the Asn-2 alone mutant could support any detectable polysialylation (Fig. 4). Elimination of the oligosaccharide on Asn-3 completely abolished autopolysialylation (Fig. 2) and polysialylation of NCAM (Fig. 5). However, when Asn-2 or -4 were mutated in combination with Asn-3, this rescued the enzyme’s ability to polysialylate NCAM (mutants 2.3 and 3.4) and itself (mutant 3.4). These results suggest that elimination of Asn-3 and its oligosaccharide cause a conformational change in PST that leads to its inactivity. Finally, co-expression autopolsialylated and non-autopolysialylated PST mutant proteins with NCAM-Fc revealed that the Asn-2 mutant, with extremely low levels of polysialic acid, and the double Asn-2.3 mutant, which completely lacks polysialic acid, are still able to polysialylate NCAM, although at lower levels than wild type PST (Fig. 5). These results demonstrate that autopolsialylation of PST is not required for, but does enhance, the polysialylation of NCAM.
enzymes were metabolically labeled with 35S-Express protein labeling mix for 1 h and chased with unlabeled medium for 3 h. NCAM-Fc protein was precipitated from the chase medium with protein A-Sepharose beads and separated on 5% SDS-polyacrylamide gels. The radiolabeled proteins were visualized by fluorography. B, to confirm the presence of poly-sialic acid on NCAM-Fc, a parallel co-expression experiment was performed without metabolic labeling. NCAM-Fc was precipitated from the medium after an overnight expression and immunoblotted with the OL.28 anti-poly-sialic acid antibody. Molecular mass markers are as follows: 203 kDa, myosin; 109 kDa, b-galactosidase. The symbol denotes the control transfection of NCAM-Fc.

4 J. Dykstra, B. E. Close, and K. J. Colley, unpublished observations.

This suggests that the Asn-3 mutant protein is not grossly misfolded because it is localized in Golgi and secreted as a soluble form from the cells like the wild type enzyme (Figs. 2 and 3). Alternatively, the oligosaccharides themselves could be involved in the catalytic mechanism of the polysialyltransferase. However, it is not clear how this would occur. Nevertheless, Muhlenhoff and colleagues (39) have demonstrated that the polysialic acid chains of PST are not preformed on the enzyme and transferred to NCAM.

Muhlenhoff et al. (39) suggested that autopoly-sialylation of the PST enzyme is a prerequisite for enzymatic activity. Using a protein A-PST chimeric protein, Muhlenhoff et al. (39) observed that incubation of protein A-PST isolated from CHO 2A10 cells, which are polysialic acid-negative due to a defect in the PST-1 gene, with 14C-labeled CMP-sialic acid resulted in autopoly-sialylation of the enzyme in vitro. This autopoly-sialylated protein A-PST chimera was capable of polysialylation of NCAM. However, while agalacto-PST from Lec 8 CHO cells (45) was unable to polysialylate NCAM, asialo-PST isolated from Lec 2 CHO cells (46, 47) retained the ability to polysialylate NCAM, albeit at a reduced level (39). Muhlenhoff et al. (39) concluded that autopoly-sialylation of PST was required for enzymatic activity. However, their in vitro data clearly show residual poly-sialylation activity of the asialo-PST enzyme. The data presented in this paper clearly demonstrate that autopoly-sialylation is not a requirement for poly-sialylation of NCAM by PST. Our pulse/chase and immunoblot analyses have definitively shown that Asn mutant 2.3 is not autopoly-sialylated (Fig. 2), yet still retains the ability to polysialylate NCAM-Fc (Fig. 5). We conclude that, while autopoly-sialylation of PST is not absolutely required for the poly-sialyltransferase activity of PST, it does appear to enhance either the number or length of polysialic acid chains added to NCAM. In our assay, NCAM-Fc polysialylated by Asn mutant 2 or Asn mutant 2.3 migrated with a reduced molecular mass relative to NCAM-Fc polysialylated by wild type PST (Fig. 5). Although we are unable to attribute the difference in molecular mass to either a decrease in the number of polysialic acid chains or their length, it is clear that poly-sialylation of PST does enhance the overall poly-sialylation of NCAM.

If autopoly-sialylation of PST is not necessary for enzymatic activity, what could be its purpose? One suggestion is that it stabilizes the PST enzyme as extensive glycosylation is thought to do for other proteins (48). We have shown that autopoly-sialylated PST expressed in COS-1 cells is secreted into the medium in a soluble form (38). This autopoly-sialylated, soluble form of PST could be immunoprecipitated intact from the medium 12 h after secretion. Another role suggested by our results is that PST autopoly-sialylation increases the efficiency NCAM polysialylation by the enzyme. NCAM-Fc polysialylated by wild type PST has a larger molecular mass than NCAM-Fc polysialylated by the non-autopoly-sialylated Asn-2.3 mutant or the less autopoly-sialylated Asn-2 mutant (Fig. 5B, compare WT to Mutants 2 and 2.3). Could autopoly-sialylation of PST increase its processivity on the growing polysialic acid chain? In the same way that a DNA polymerase binds to a template and polymers hundreds of bases of DNA in succession, PST could bind to its glycoprotein substrate and polymerize sialic acids in a processive manner. If autopoly-sialylation plays a role in processivity, for example, by stabilizing the interaction of PST with its glycoprotein substrate, then we would expect that an increased percentage of non-autopoly-sialylated PST may “fall off” the growing polysialic acid chain, requiring the reloading of the enzyme back onto the oligosaccharide chain in order for elongation to continue. This may result in a larger population of NCAM molecules that are modified by shorter polysialic acid chains. This could be especially noticed with the soluble NCAM-Fc due to its possibly decreased residence time in the Golgi relative to the membrane-associated form. Alternatively, autopoly-sialylation of PST could increase the efficiency of the initial binding of sugar nucleotide donor and/or glycoprotein acceptor. A decrease in efficiency of any of these steps could lead to a lower degree of NCAM polysialylation.

Our laboratory previously reported that the majority of the polysialylated material expressed by COS-1 cells transfected...
with PST cDNA represented autopolysialylated polysialyltransferase (38). A small amount of polysialylated material remaining in the supernatants of immunodepleted COS-1 cell lysates was shown to be immunoreactive with the anti-V5 antibody, indicating that it was inefficiently immunoprecipitated autopolysialylated PST (38). We now can confirm that the only polysialylated protein expressed by COS-1 cells transiently transfected with PST is the autopolysialylated polysialyltransferase itself. The Asn mutant 2.3 was shown to be non-autopolysialylated but still active in NCAM polysialylation (Figs. 2B and 5B). When COS-1 cells expressing Asn mutant 2.3 are probed with the OL.28 anti-polysialic acid antibody to detect polysialic acid, no staining is observed internally or on the cell surface (Fig. 3). This conclusively demonstrates that there are no endogenous substrates for PST in COS-1 cells. Furthermore, this result indicates that the polysialic acid staining observed when the other Asn mutants are expressed in COS-1 cells can be entirely attributed to autopolysialylation of PST (Figs. 3 and 4C). These results highlight the protein specificity of polysialylation by PST and lead to important questions about what protein and carbohydrate signals are recognized by the polysialyltransferases.

Acknowledgments—We thank Dr. Minoru Fukuda for the kind gift of the PST cDNA. We also thank Dr. Genevieve Rougon for the generous gift of the NCAM-Fc cDNA and Dr. Nancy Kedersha for the kind gift of NCAM 140 cDNA. We also thank Tracy Bohrer and Jiyan Ma for helpful discussion and suggestions.

REFERENCES

1. Troy, F. (1992) Glycobiology 2, 5–23
2. Inoue, S., and Iwaski, M. (1978) Biochem. Biophys. Res. Commun. 83, 1018–1023
3. Kitazume, S., Kitajima, K., Inoue, S., Troy, F. A. II, Cho, J.-W., Lennarz, W. J., and Inoue, Y. (1994) J. Biol. Chem. 269, 22712–22718
4. Roth, J., Kempf, A., Reuter, G., Schauer, R., and Gehring, W. (1992) J. Biol. Chem. 267, 673–675
5. Robbins, J. B., McCracken, G. H. J., Orskov, F., and Orskov, I. (1991) Adv. Neuroblastoma Research 10, 596–608
6. Muhlenhoff, M., Eckhardt, M., and Gerardy-Schahn, R. (1998) Curr. Opin. Cell Biol. 10, 558–564
7. Kojima, N., Yoshida, Y., Kurosawa, N., Lee, Y.-C., and Tsuji, S. (1995) FEBS Lett. 360, 1–4
8. Eckhardt, M., Muhlenhoff, M., Bethe, A., Koopman, J., and Gerardy-Schahn, R. (1995) Nature 373, 715–718
9. Yoshida, Y., Kojima, N., and Tsuji, S. (1995) J. Biol. Chem. 270, 17619–17622
10. Livingston, B. D., and Paulson, J. C. (1992) J. Biol. Chem. 267, 9965–9971
11. Muhlenhoff, M., Eckhardt, M., and Gerardy-Schahn, R. (1998) Curr. Top. Cell Biol. 8, 558–564
12. Zuber, C., Lackie, P. M., Catterall, W. A., and Roth, J. (1992) J. Biol. Chem. 267, 9965–9971
13. Edelman, G. M., and Crosslin, K. L. (1991) Annu. Rev. Biochem. 60, 155–190
14. Finne, J., Finne, U., Deagostini-Bazin, H., and Gordon, C. (1988) Biochem. Biophys. Res. Commun. 112, 482–487
15. Roth, J., Taatjies, D. J., Bitter-Suermann, D., and Inoue, Y. (1994) J. Biol. Chem. 269, 6943–6950
16. Rutishauser, U., Watanabe, M., Silver, J., Troy, F. A., and Vinr, E. R. (1985) J. Cell Biol. 101, 1842–1849
17. Troy, F. A., and Rosenberg, A. (1995) Glycobiology Science 771–777