A select group of mammalian proteins have been shown to possess α2,8-polysialylated oligosaccharide chains. The best studied of these proteins is the neural cell adhesion molecule (NCAM). Polysialylation of NCAM has been shown to decrease NCAM-dependent and independent cell adhesion. PST (ST8Sia IV) and STX (ST8Sia II) are the two polysialyltransferases responsible for NCAM polysialylation. Recent studies revealed that PST itself is autopolysialylated in vitro (Muhlenhoff, M., Eckhardt, M., Bethe, A., Frosch, M., and Gerdardy-Schahn, R. (1996) *EMBO J.* 15, 6943–6950). Here we report studies on the biosynthesis and localization of the PST and STX polysialyltransferases. Both PST and STX are expressed as high molecular mass, polydisperse forms that are associated with the cell and found soluble in the medium. Analysis of these high molecular mass forms by glycosidase digestion and serial immunoprecipitation/immunoblot experiments demonstrated that PST and STX are autopolysialylated in vitro. Indirect immunofluorescence microscopy and immunoprecipitation analyses demonstrated that autopolysialylated PST and STX are localized in the Golgi, on the cell surface, and in the extracellular space. The cell surface and extracellular localization of these polysialylated polysialyltransferases suggest that their polysialic acid chains, like those of NCAM, may modulate cell interactions.

Polysialic acid is a linear homopolymer of N-acetyl or N-glycolyl neuraminic acid (Neu5Ac or Neu5Ge) occurring in α2, 5-, 8-, or 9-ketosidic linkages (reviewed in Ref. 1). Polysialic acid has been found widely distributed throughout nature, from the capsular polysaccharide of neuroinvasive bacteria, such as *Escherichia coli* K1 (2), to unfertilized rainbow trout eggs and the jelly coat of sea urchin eggs (3, 4), to human tumors and tissues (1, 5). While there has been a great deal of research on bacterial polysialylation, eukaryotic, especially mammalian, protein polysialylation has been the focus of intense investigation in recent years.

Although polysialic acid has been found throughout the taxonomic spectrum, only the oligosaccharides of a few mammalian proteins bear this modification. While polysialic acid has been found on the α-subunit of the rat brain voltage-sensitive sodium channel (6) and unidentified proteins in breast cancer and basophilic leukemia cell lines (7), the most abundant carrier of polysialic acid is neural cell adhesion molecule (NCAM) (8). Polysialylated NCAM has been observed in developing brain (9), kidney (10), heart, and muscle (11). It is widely postulated that the polysialylation of NCAM oligosaccharides during the development of the nervous system and other organ systems in the embryo and neonate leads to a general decrease in cell adhesion (12–17). It is believed that the presence of cell surface polysialic acid disrupts the homophilic binding properties of NCAM and facilitates cellular migration, neurite outgrowth, and synaptic plasticity (12–17). Interestingly, polysialylated NCAM is also reexpressed on some metastatic cancers such as neuroblastoma (18), small cell lung carcinoma (19), and the highly metastatic kidney tumor, Wilm's tumor (5). As in development, cell surface-expressed polysialylated NCAM is thought to increase the migration of cancer cells, thereby enhancing their metastatic potential (18, 20–23).

Two recently cloned polysialyltransferases have been shown to be responsible for the polysialylation of NCAM. The first, STX (ST8Sia II), is a type II membrane protein with a predicted molecular mass of 42.5 kDa and six potential N-linked glycosylation sites. STX has been cloned from rat (24), mouse (25), and human (26) sources and was shown to have polysialyltransferase activity toward N-linked oligosaccharide structures (25–27). The second polysialyltransferase, PST (ST8Sia IV), is also a type II membrane protein with a predicted molecular mass of 41.2 kDa. PST has five potential N-linked glycosylation sites, with four reportedly being used (28). PST was cloned from human (29), hamster (30), mouse (31), rat (32), and chicken (2) sources and was also shown to have polysialyltransferase activity toward N-linked oligosaccharides (30, 31, 34). Interestingly, recent in vitro studies on PST revealed that the PST enzyme is itself modified by polysialic acid and that this modification may be important for activity (28).

In light of the observation that PST is polysialylated in vitro, no published data to date have shown in vitro polysialylation of STX or in vivo polysialylation of either enzyme. Here we report the *in vivo* autopolysialylation of both PST and STX. Pulse-chase immunoprecipitation analyses revealed that both enzymes are associated with the cell and are found as high molecular mass, soluble forms in the extracellular space. Glycosidase digestion demonstrated that the high molecular mass forms of PST and STX are due to modification of the enzymes’ complex, N-linked oligosaccharides. This modificati-

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‡ The abbreviations used are: NCAM, neural cell adhesion molecule; PST, polysialyltransferase (ST8Sia IV); STX, sialyltransferase X (ST8Sia II); OL,28, anti-polysialic acid antibody; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PNGase F, peptide N-glycosidase F; Endo H, endo-β-N-acetylglicosaminidase H; Nm, neuraminidase; Endo N, endo-N-acetylglicosaminidase; CHO, Chinese hamster ovary cells; PBS, phosphate-buffered saline.

§ J. L. Bruses, K. G. Rollins, and U. Rutishauser, GenBank™ accession number AF008194.
tation was subsequently identified as α2,8-poly saccharide by its susceptibility to endo-α-acetylmuramidase (Endo N) digestion (35) and the immunoprecipitated proteins' reactivity with the anti-poly saccharide acid antibody, OL 28 (7), which has been shown to recognize α2,8-poly saccharide acid chains of more than 4 units3. Last, indirect immunofluorescence localization of autopoly sacylated PST and STX shows that both localize to the Golgi and the cell surface. Based on these data, we hypothesize that autopoly sacylated PST and STX, found on the cell surface and in the extracellular space, may modulate the interactions of expressing cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

Tissue culture media and reagents, including Dulbecco's modified Eagle's medium (DMEM), α-minimal essential medium, Opti-MEM I, Lipofectin, LipofectAMINE, and LipofectAMINE Plus were purchased from Life Technologies, Inc. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Nitrocellulose membranes were purchased from Schleicher and Schuell. SuperSignal chemiluminescent reagents were obtained from Pierce. Protein A-Sepharose was obtained from New England Biolabs (Beverly, MA). pcDNA3.1/V5-His vectors and anti-V5 epitope tag antibodies were purchased from Invitrogen Corp. (Carlsbad, CA). Oligonucleotides and restriction enzymes were purchased from Life Technologies, Inc. Peptide N-glycosidase F (PNGase F) was obtained from New England Biolabs (Beverly, MA). Endo-β-acetylgalactosaminidase II (Endo H) and Vibrio cholerae neuraminidase (V. cholerae Nm) were purchased from Boehringer Mannheim. Endo N was a gift from Dr. Frederic Troy II (Department of Biological Chemistry, University of California, Davis). β2-2-Express protein labeling mix and [α-32P]dATP for DNA sequencing were purchased from NEN Life Science Products. Protein A-Sepharose was obtained from Amersham Pharmacia Biotech. Fluorescein isothiocyanate-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.

**Methods**

**Construction of Epitope-tagged PST and STX—Full-length PST cDNA was obtained from Dr. Minoru Fukuda (Burnham Institute, La Jolla, CA) and full-length STX cDNA was obtained from Dr. John Lowe (University of Michigan, Ann Arbor, MI). The PST and STX cDNAs were amplified by polymerase chain reaction amplification using Vent DNA polymerase and oligonucleotide primers specific for each cDNA (GATATCCAAGATGCGCTACTAGGAGA and TCTAGACCGTCGATCCAG for PST; GATACATGCGACTCTTACCTCG for STX). These primers specifically introduced an MluI and an XhoI site at the 3'-end of each cDNA. Following restriction enzyme digestion, PST and STX cDNAs were ligated into previously digested pcDNA3.1/V5-His vector DNA to complete the construction of PST-V5 and STX-V5. Inserts were confirmed using the Sequenase version 2.0 DNA Sequencing Kit (Amersham Pharmacia Biotech) and the T7 primer TAATAGCAGTACTATA TAG (Genosys Biotechnologies, The Woodlands, TX).

**Transfection of COS-1, CHO, and Lec2 CHO Cells—COS-1, CHO, and Lec2 CHO cells maintained in DMEM, 10% FBS (COS-1) or a minimal essential medium, 10% FBS (CHO and Lec2 CHO) 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 (COS-1) and LipofectAMINE Plus (CHO and Lec2 CHO) transfections were performed according to the protocols provided by Life Technologies, Inc. Thirty microliters of Lipofectin or LipofectAMINE Plus and 20 μg of PST-V5 or STX-V5 plasmid DNA in 3 ml of Opti-MEM I plus 55 μM β-mercaptoethanol were used for transfection of each 100-mm tissue culture plate. Two microliters of Lipofectin or LipofectAMINE Plus and 0.5 μg of plasmid DNA in 250 μl of Opti-MEM plus 55 μM β-mercaptoethanol were used for transfection of each coverslip.

**Metabolic Labeling of Cells and Immunoprecipitation of Polysialyltransferase Enzymes—Following transfection of COS-1, CHO, or Lec2 CHO cells with V5 epitope-tagged PST or STX cDNA and expression of these enzymes in the cells for 18 h, 100-mm tissue culture dishes of transfected cells were incubated 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 10% CSF-10, 0.1% SSS-Express precipitation 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, cells were washed, and the labeled proteins were chased for various periods of time with DMEM, 10% FBS. Cell medium was collected, and cells were washed with 10 ml of PBS and lysed in 1 ml of immunoprecipitation buffer 2 (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.1% Nonidet P-40, 0.1% sodium deoxycholate, 0.5% SDS, 5% 2,3,6-tris-acetylglucosamine). Protein A-Sepharose was obtained from Amersham Pharmacia Biotech, as described previously (36). 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% β-mercaptoethanol and directly loaded into the gel wells. Immunoprecipitated proteins were separated on 7.5% separating, 3% stacking polyacrylamide gels (37). Radiolabeled proteins were visualized by fluorography using 10% 2,5-diphenyloxazol in dimethyl sulfoxide (38), and gels were exposed to Kodak BioMax MR film at –80 °C.

**Glycosidase Digestions of Immunoprecipitated Enzymes—** COS-1 cells expressing either PST-V5 or STX-V5 were metabolically labeled for 4 h, and radiolabeled proteins were immunoprecipitated as described above. Following the final wash, the immune complexes bound to protein A-Sepharose beads were treated with glycosidases for 18 h with shaking at 37 °C. For PNGase F, 1500 units of enzyme was added to 70 μl of distilled H2O and 30 μl of 1× reaction buffer (50 mM sodium phosphate, pH 7.5) per manufacturer's instructions. PNGase F cleaves between the innermost N-acetylgalactosamine and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins. For V. cholerae Nm digestions, 30 milliunits of enzyme was added to 70 μl of 1× reaction buffer (50 mM sodium acetate, pH 5.5, 4 mM calcium chloride, 100 μg/ml bovine serum albumin) per manufacturer's instructions. V. cholerae Nm hydrolyzes terminal N- or O-acetylated sugars that are linked via α2,8-, α2,6-, or α2,3-bonds. For Endo H digestions, a 1:10 dilution of enzyme in 20 μl Tris-HCl, pH 7.4, 0.2 M mg/ml bovine serum albumin was added to the immune complexes. Endo H cleaves α2,8-poly saccharide acid chains at random sites, requiring a minimum chain length of five sialic acid residues for activity (35). For Endo H digestions, 10 milliunits of enzyme was added to 90 μl of 1× reaction buffer (0.1 sodium citrate, pH 6.0, 0.1% bovine serum albumin, 0.2% β-mercaptoethanol) (39). Endo H cleaves on high mannose or hybrid N-linked oligosaccharide structures of glycoproteins. Digested samples were electrophoresed on a 7.5% SDS-polyacrylamide gel after the addition of 50 μl of Laemmli buffer (37) and β-mercaptoethanol to 5% final concentration. Radiolabeled proteins were visualized by fluorography, and gels were exposed to Kodak BioMax MR film at –80 °C.

**Serial Immunoprecipitation and Immunoblotting of Polysialyltransferase and Treatment of Immunoblots with Endo N—** COS-1 cells were transfected with PST-V5 or STX-V5 cDNA, and unlabeled cell lysates and medium were collected after an overnight incubation. Lysates and medium were subjected to serial immunoprecipitation by rotation of samples for 1.5 h with 0.75 μg of V5 antibody and 50 μl of a 50% slurry of protein A-Sepharose in PBS. After this incubation, the immune complexes were centrifuged, and supernatants were removed to new tubes. An additional 0.75 μg of V5 antibody and 50 μl of a 50% slurry of protein A-Sepharose in PBS were added to the supernatants, and this mixture was rotated for an additional 1.5 h. This scheme was repeated a total of four times, with the final supernatant being retained. The immune complexes were electrophoresed on 7.5% separating, 3% stacking SDS-polyacrylamide gels. Following electrophoresis, proteins were electrophoretically transferred to nitrocellulose membranes overnight at 500 mA. The membranes were processed for immunoblotting according to the manufacturer's protocol (Pierce). Anti-V5 epitope tag antibody (IgG) was diluted 1:5000, and anti-poly saccharide acid antibody, OL.28 (IgM), was diluted 1:500 in blocking buffer (5% dry milk in Tris-buffered saline, pH 8.0, 0.1% Tween 20). Anti-V5 epitope tag antibody; 2% dry milk in Tris-buffered saline, pH 8.0, for OL.28 (IgM). Anti-β2-mercaptoethanol-conjugated secondary antibodies, goat anti-mouse IgG and goat anti-mouse IgM, were each diluted 1:8000 in blocking buffer (5% dry milk in Tris-buffered saline, pH 8.0, 0.1% Tween 20). Immunoblots were developed using the SuperSignal chemiluminescence kit (Pierce) and exposed to Kodak BioMax MR film at room temperature.

Separately, aliquots (2.5% of total volume) of the final supernatants

3 K. Kitajima, unpublished results.
of cell lysates and medium from the serial immunoprecipitation were electrophoresed on a 7.5% SDS-polyacrylamide gel, and total protein was transferred to nitrocellulose membranes. The immunoblots were cut into strips, and selected strips were treated overnight at 37 °C with a 1:10 dilution of Endo N. Immunoblotting with the anti-V5 epitope tag and anti-polysialic acid antibodies was performed as described above.

**Immunofluorescence Localization of PST and STX—**Cells were plated on glass coverslips, transfected with PST-V5 or STX-V5, and processed for immunofluorescence microscopy as described previously (40). Briefly, cells were treated with either –20 °C methanol to visualize internal staining or 3% paraformaldehyde to visualize cell surface staining. Anti-V5 epitope tag antibody was diluted 1:100, and the OL.28 anti-polysialic acid antibody, fluorescein isothiocyanate-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 Axioptet microscope equipped with epifluorescence illumination and a 60 × oil immersion Plan Apochromat objective.

**RESULTS**

**PST and STX Are Modified to High Molecular Mass Sialylated Forms That Are Found Associated with the Cell and Soluble in the Extracellular Space—**In addition to their expected localization in the Golgi, many glycosyltransferases are found as soluble forms in the extracellular space and at low levels on the cell surface (41–46). To determine whether PST and STX are cleaved and secreted into the extracellular space, we performed pulse-chase immunoprecipitation analyses. PST and STX tagged with the V5 epitope at their carboxyl termini were transiently expressed in CHO cells. Cells were metabolically labeled with 35S-Express protein labeling mix for 1 h and chased with unlabeled medium for 0–12 h. V5 epitope-tagged proteins were immunoprecipitated from both cell lysates and media with the anti-V5 epitope tag antibody, and immunoprecipitated proteins were analyzed on SDS-polyacrylamide gels. An approximately 57-kDa protein was immunoprecipitated from lysates of CHO cells expressing the PST-V5 enzyme (Fig. 1A). The size of this protein corresponds well with the expected molecular mass of the full-length epitope-tagged PST-V5 (Fig. 1B) modified by four N-linked oligosaccharides (28). A smaller 52-kDa protein was immunoprecipitated from media and is likely to represent a cleaved and secreted form of the enzyme. More interestingly, we observed a heterogeneous, immunoreactive band extending from 105 to 190 kDa associated with the cell at early chase time points (1 and 3 h) and in the medium from 1 to 12 h of chase (Fig. 1A). The polydisperse appearance of this high molecular mass immunoreactive band suggests that a large proportion of the PST-V5 is highly glycosylated and most likely polysialylated.

Analysis of STX-V5 protein transiently expressed in CHO cells revealed a similar pattern of biosynthesis. A protein of 58 kDa molecular mass was observed associated with the cells, and a smaller, 53-kDa species appeared in the medium after 1 h of chase (Fig. 1C). Again, the size of the 58-kDa protein corresponds well with the calculated molecular mass of the epitope-tagged STX (STX-V5) modified by four N-linked oligosaccharides, while the size of the 53-kDa protein suggests that it is a cleaved and secreted form of the full-length enzyme. Like PST-V5, we also observed a heterogeneous band extending from 20 to 131 kDa in the medium from 1 to 12 h of chase (Fig. 1C). The polydisperse appearance of this band again suggests that the high molecular mass forms of the STX-V5 is highly glycosylated and most likely polysialylated. These results demonstrate that both PST and STX are cleaved and secreted from cells as both highly modified and less modified forms. The polydisperse appearance of the highly modified forms of PST-V5 and STX-V5 suggest that both enzymes are extensively glycosylated and probably polysialylated.

In order to determine what proportion of the molecular mass of PST-V5 and STX-V5 could be attributed to the addition of sialic acid, we expressed the PST-V5 and STX-V5 proteins in Lec2 CHO cells, metabolically labeled the cellular proteins, and immunoprecipitated the enzymes with the anti-V5 epitope tag antibody (Fig. 1, B and D). Lec2 CHO cells lack a functional CMP-NeuAc transporter and consequently show a very significant reduction in sialylation of glycoproteins and glycolipids compared with wild type CHO cells (47, 48). The PST-V5 enzyme secreted from the Lec2 cells migrated on SDS-polyacrylamide gels with a molecular mass ranging from 55 to 82 kDa (Fig. 1B). This is markedly smaller than the 105–190-kDa PST-V5 secreted from wild type CHO cells (Fig. 1A). Similarly, the STX-V5 enzyme secreted from Lec2 cells migrated on SDS-polyacrylamide gels with a molecular mass of 56–80 kDa, and this too was significantly smaller than the 70–131-kDa STX-V5 protein secreted from wild type CHO cells (Fig. 1, compare C and D). These data indicate that the expression of the high molecular mass forms of PST-V5 and STX-V5 polysialyltransferases depend upon the cells’ ability to sialylate glycoproteins.

**PST and STX Are Polyasialylated on Complex N-Linked Oligosaccharides—**The previous results suggest that the bulk of these enzymes’ molecular mass increase could be attributed to the addition of sialic acid residues to the oligosaccharide structures of PST-V5 and STX-V5. In light of Gerardy-Schahn and colleagues’ (28) demonstration that PST is autopolyasialylated in vitro, it is likely that the high molecular mass forms of the polysialyltransferases we observe are a result of their in vivo autopolyasialylation. To determine whether PST-V5 and STX-V5 are autopolyasialylated on complex N-linked oligosaccharides, we analyzed their carbohydrate structures using PNGase F, Endo H, Endo N, and V. cholerae Nm. Radiolabeled PST-V5 and STX-V5 were immunoprecipitated with the anti-V5 epitope tag antibody from CHO cell medium after 6 h of chase. The radiolabeled protein-antibody complexes were bound to protein A-Sepharose beads and then treated overnight with specific glycosidases. Treatment of soluble PST-V5 with PNGase F reduced the high molecular mass and low molecular mass soluble forms of the enzyme to two species of 40 and 36 kDa. This demonstrated that the high molecular mass form of PST-V5 possesses highly modified N-linked oligosaccharides and that the low molecular mass form of the enzyme possesses less modified N-linked oligosaccharides (Fig. 2, PST-V5, +PNGase F). The predicted molecular mass of unglycosylated PST-V5 is approximately 45 kDa, suggesting that the two species observed after PNGase F digestion of PST-V5 represent different proteolytically processed forms of PST-V5. Treatment of PST-V5 with Endo H had no effect on the high molecular mass form of the enzyme (Fig. 2, PST-V5, +Endo H). However, the low molecular mass, soluble form of PST-V5 was susceptible to Endo H digestion, indicating that this form possesses only high mannose N-linked oligosaccharides. Digestion of PST-V5 with either Endo N or V. cholerae Nm reduced the molecular mass of the enzyme to 57–101 kDa (Fig. 2, PST-V5, +V. cholerae Nm, +Endo N). The sensitivity of the high molecular mass form of the PST-V5 to Endo N, an endoglycosidase specific for α2,8-linked polysialic acid (35), demonstrates that this polysialyltransferase is indeed polyasialylated itself.

Using an identical approach, analysis of the modification of STX-V5 with these glycosidases gave similar results as those obtained for glycosidase-treated PST-V5. Briefly, PNGase F treatment of STX-V5 resulted in a single band of 37 kDa (Fig. 2, STX-V5, +PNGase F), indicating that the high molecular mass modification of STX-V5 resides on the enzyme’s N-linked oligosaccharides. Also, like PST-V5, the high molecular mass form of STX-V5 was not sensitive to treatment with Endo H,
while the low molecular mass form of the enzyme was sensitive to this endoglycosidase (Fig. 2, STX-V5, +Endo H). Digestion of STX-V5 with either Endo N or V. cholerae Nm reduced the apparent molecular mass of STX-V5 to 55–103 kDa (Fig. 2, STX-V5, +V. cholerae Nm, +Endo N). Taken together, these results demonstrate that the high molecular mass modification of STX-V5 and PST-V5 is the result of sialylation, specifically polysialylation, of the complex N-linked oligosaccharides of both enzymes.

The Majority of the Polysialylated Material Expressed by Transfected COS-1 and CHO Cells Represents the Autopolysialylated Polysialyltransferases—To provide further evidence for the in vivo autopolysialylation of PST-V5 and STX-V5 and to determine whether there are any other substrates for polysialylation expressed by COS-1 cells, we performed serial immunoprecipitation/immunoblotting analyses on cell lysates and medium fractions from STX-V5- and PST-V5-transfected COS-1 cells. The unlabeled medium and lysates of COS-1 cells expressing PST-V5 or STX-V5 were subjected to four immunoprecipitation cycles, with retention of the final supernatant. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot analysis using the

FIG. 1. Biosynthesis of PST-V5 and STX-V5 in wild type CHO and Lec2 CHO cells. CHO and Lec2 CHO cells transiently transfected with either PST-V5 (A and B) or STX-V5 (C and D) cDNAs were metabolically labeled for 1 h with 35S-Express protein labeling mix and chased with unlabeled medium for 0–12 h, and the polysialyltransferases were immunoprecipitated from cell lysates and media with the anti-V5 epitope tag antibody. The samples were separated on 7.5% SDS-polyacrylamide gels, and radiolabeled protein bands were visualized by fluorography. Molecular mass markers are as follows: 203 kDa, myosin; 109 kDa, β-galactosidase; 78 kDa, bovine serum albumin; 46.7 kDa, ovalbumin; 34.5 kDa, carbonic anhydrase. m, mock transfection.
with 35S-Express protein labeling mix, and the polysialyltransferases with either PST-V5 or STX-V5 cDNAs were metabolically labeled for 1 h

OL.28 antibody as described under “Methods.” As shown in Fig. 3, both PST-V5 and STX-V5 proteins immunoprecipitated from COS-1 cell lysates and media were immunoreactive with the anti-polysialic acid antibody, OL.28 (Fig. 3B; PST-V5 and STX-V5, +Endo N). Based on these results, it is likely that most and perhaps all of this residual polysialylated protein is inefficiently immunoprecipitated autopolsialylated polysialyltransferases.

**Autopolysialylated PST and STX Are Localized in the Golgi**

To test the possibility that there are other glycoproteins expressed in COS-1 cells that act as substrates for the polysialyltransferases, we analyzed aliquots of the final supernatant from the serial immunoprecipitations with the OL.28 and the anti-V5 epitope tag antibodies (Fig. 3B shows cell lysates only). Immunoblot analysis of the final supernatants from the medium immunoprecipitations demonstrated that the serial anti-V5 antibody immunoprecipitations had completely depleted the medium of both PST-V5- and STX-V5-expressing cells of any polysialylated, OL.28-reactive material (data not shown). Immunoblot analysis of the final supernatants from the cell lysate immunoprecipitations demonstrated that there was residual OL.28-reactive polysialylated material remaining in the final supernatant following the serial immunoprecipitations (Fig. 3B; PST-V5 and STX-V5, OL.28 antibody, −Endo N). Immunoblots of this residual polysialylated material were treated with Endo N to remove polysialic acid and then incubated with the anti-V5 antibody to determine whether this material represented unique polysialylated proteins or inefficiently immunoprecipitated V5-tagged PST or STX (Fig. 3B; PST-V5 and STX-V5, V5 antibody, +Endo N). The anti-V5 epitope tag antibody did recognize protein on the Endo N-treated PST-V5 and STX-V5 immunoblots, suggesting that polysialylation is masking the V5 epitope in solution (see serial immunoprecipitations; Fig. 3) and on the immunoblots (Fig. 3B, PST-V5 and STX-V5, V5 antibody, −Endo N). Based on these results, it is likely that most and perhaps all of this residual polysialylated protein is inefficiently immunoprecipitated autopolsialylated polysialyltransferases.

**DISCUSSION**

Previous work by Gerardy-Schahn and colleagues demonstrated that PST is autopolsialylated in vitro (28). We wondered whether both PST and STX, the two known polysialyltransferases responsible for NCAM polysialylation, are autopolsialylated in vivo and where they are localized. Immunoprecipitation analyses demonstrated that both PST and STX are found as high molecular mass forms associated with the cell and in the cell medium (Fig. 1). Glycosidase digestions demonstrated that PST and STX are α2,8-polysialylated on complex N-linked oligosaccharides (Fig. 2). The immunoreactivity of the immunoprecipitated PST and STX with the anti-polysialic acid antibody, OL.28, again confirmed that these polysialyltransferases are autopolsialylated (Figs. 2 and 3). In addition, serial immunoprecipitation analyses suggest that these enzymes represent the majority of, even the only, polysialylated proteins expressed by COS-1 cells transfected with PST-V5 or STX-V5 cDNAs (Fig. 3). Finally, indirect immunofluorescence microscopy (Fig. 4) and immunoprecipitation time courses (Fig. 1) demonstrated that PST and STX are localized not only in the Golgi but also at the cell surface and in the extracellular space.

The Endo N sensitivity and OL.28 anti-polysialic acid antibody reactivity of the PST and STX proteins expressed in CHO and COS-1 cells demonstrated that these two polysialyltransferases are polysialylated themselves. Muhlenhoff et al. (28) demonstrated that PST-1 could autocatalytically polysialylate its own N-linked oligosaccharide chains in vitro and that ter-
terminal α2,3- or α2,6-linked sialic acid was not required for autopolysialylation. In addition, this group showed that asialo-PST-1 synthesized in Lec2 cells, but not agalacto-PST-1 synthesized in Lec8 cells, maintained the ability to polysialylate NCAM in vitro. From these data, they suggest that autopolysialylation of PST-1 may be required for enzyme activity. It is not clear how they came to this conclusion. It is clear that the PST-1 expressed in wild type CHO cells synthesized more polysialylated NCAM in their in vitro assay than did the enzyme synthesized in CHO 6B2 (Lec2) cells that lacked sialic acid on its N-linked oligosaccharides. This may indicate that polysialylated PST-1 has an enhanced activity relative to the unsialylated enzyme. However, the experiments of Muhlenhoff et al. (28) suggested that this was not because the preassembled polysialic acid chains were transferred from the polysialyltransferase to the NCAM acceptor.

In our analysis of the biosynthesis and secretion of PST and STX expressed in wild type CHO and Lec2 CHO cells, we noticed differences in the extent of PST-V5 and STX-V5 polysialylation in wild type CHO cells. When expressed in CHO cells, PST-V5 appeared to be polysialylated to a greater extent than the STX-V5 protein. Similar results were observed when these proteins were expressed in COS-1 cells (data not shown). These observations and those of others (51) suggest that the two polysialyltransferases may have different limits on the length of the α2,8-polysialic acid chains that serve as their substrate. We also observed that the enzymes expressed in the Lec2 CHO cells, which lack the ability to sialylate glycoproteins, were secreted at a lower rate than the enzymes synthesized in wild type CHO cells (Fig. 1, compare A to B and C to D). Likewise, the lower molecular mass population of PST-V5 and STX-V5 proteins synthesized in wild type CHO cells that lacked polysialic acid chains remained cell-associated for longer times than did the high molecular mass polysialylated forms of the polysialyltransferases (Fig. 1, A and C). While sialylation/polysialylation was not a prerequisite for cleavage and secretion, it seemed that sialylation/polysialylation did enhance the rate of enzyme cleavage and secretion. Other researchers have noticed that N-linked oligosaccharide structures do influence protein trafficking in the cell. The addition of N-linked oligosaccharides to a growth hormone-VSV G chimeric protein allowed it to be transported out of the Golgi to the cell surface (52, 53), while the presence of oligosaccharide structures on a soluble protein can influence the polarity of its secretion (54). How the polysialylation of PST and STX is influencing the Golgi retention of these enzymes is not clear. However, these observations suggest that PST and STX polysialylation may shorten enzyme residence time in the Golgi and allow them to move more rapidly to the cell surface or another post-Golgi compartment, where they are cleaved and secreted.

The serial immunoprecipitation analyses performed in Fig. 3 show that there is a population of polysialylated enzymes that is not efficiently immunoprecipitated by the anti-V5 epitope tag antibody. After several immunoprecipitations, it appeared that we had depleted the OL.28-reactive, polysialic acid-containing material from the cell lysates of PST-V5- or STX-V5-expressing...
cells (Fig. 3A, PST-V5 and STX-V5, IP#1–4). However, the final supernatant continued to be reactive with the anti-polysialic acid antibody and with the anti-V5 epitope tag antibody only after treatment of the immunoblots with Endo N (Fig. 3B, PST-V5 and STX-V5). These results suggest that much of the residual polysialylated material in the final supernatant of the serial cell lysate immunoprecipitations represents inefficiently immunoprecipitated V5-tagged enzymes. It may be that the extent of polysialylation and/or the specific placement of polysialic acid chains lead to the masking of the V5 epitope. In addition, while we cannot completely rule out the presence of other polysialylated proteins, it seems likely that the polysialyltransferases represent the bulk of polysialylated material expressed by the transfected COS-1 cells, since the major polysialyl substrate, NCAM and the voltage-sensitive sodium channel, are not expressed in these cells (26).4

We have localized PST and STX to the Golgi and cell surface of COS-1 cells using the anti-V5 epitope tag antibody and the OL.28 anti-polysialic acid antibody (Fig. 4, A–D) and OL.28 anti-polysialic acid (E–H) primary antibodies and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibodies. Prior to staining, cells were fixed and permeabilized with methanol to visualize internal structures (A, B, E, and F) or fixed with 3% paraformaldehyde to visualize only cell surface (C, D, G, and H). Immunofluorescence was visualized using a Nikon Axiohot fluorescence microscope and a 60× oil immersion Plan Achromat objective; magnification, ×750.

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4 B. Close and K. Colley, unpublished results.

Fig. 4. PST-V5 and STX-V5 are localized in the Golgi and at the cell surface in the steady state. COS-1 cells transiently expressing either PST-V5 or STX-V5 were analyzed by indirect immunofluorescence microscopy using both the anti-V5 epitope tag (A–D) and OL.28 anti-polysialic acid (E–H) primary antibodies and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibodies. Prior to staining, cells were fixed and permeabilized with methanol to visualize only cell surface (C, D, G, and H). Immunofluorescence was visualized using a Nikon Axiohot fluorescence microscope and a 60× oil immersion Plan Achromat objective; magnification, ×750.

(55). In the adjacent intestinal goblet cells, this enzyme is found throughout most of the Golgi cisternae, soluble in mucin droplets, and at the cell surface (55). Recently, we have identified two isoforms of the ST6Gal I that differ in their localization and processing (45). The ST cys isoform is retained intracellularly in the Golgi, while the ST tyr isoform is found in the Golgi and at low levels on the cell surface and is cleaved and secreted into the cell medium (45). The cleavage and secretion of the ST6Gal I and the polysialyltransferases is not surprising and is probably not an artifact of overexpression in a tissue culture cell system, since many soluble glycosyltransferases have been detected in body fluids such as serum, colostrum, and urine (for examples, see Refs. 56–60).

It is unclear what function cell surface and soluble glycosyltransferases could be performing. It seems unlikely that these cell surface and soluble enzymes act as glycosyltransferases, since their sugar nucleotide donors are not present in the extracellular space. One possibility, previously suggested by others (56, 57, 61), is that they are utilizing their ability to bind carbohydrates and are acting as lectins that could mediate cell adhesion if correctly positioned at the cell surface. The sialoadhesins (I-type lectins) are a family of sialic acid-specific lectins that are also members of the immunoglobulin superfamily. These cell surface lectins have been demonstrated to mediate the interactions of a variety of cell types via binding of specifically linked cell surface sialic acid residues (reviewed in Refs. 33 and 62). It is possible that cell surface or soluble sialyltransferases could also mediate interactions via binding galactose or sialic acid residues. The presence of long polysialic acid chains on polysialyltransferases could negatively modulate a putative lectin-mediated cell adhesion process, as they do in NCAM-mediated cell adhesion. Alternatively, the polysialic acid chains may act as substrates to enhance a sialic acid-specific, lectin-mediated, cell adhesion process. The role of these polysialylated polysialyltransferases in mediating or modulating cell adhesion or even cell signaling processes in vivo relies on their cell surface and extracellular expression in normal cells and cancer cells. These and other investigations of how the expression of these polysialylated proteins influence cell adhesion are currently under way.
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