Degree of Polymerization (DP) of Polysialic Acid (PolySia) on Neural Cell Adhesion Molecules (N-CAMS)

DEVELOPMENT AND APPLICATION OF A NEW STRATEGY TO ACCURATELY DETERMINE THE DP OF polySIA CHAINS ON N-CAMS*

Received for publication, August 9, 2005, and in revised form, September 8, 2005. Published, JBC Papers in Press, September 19, 2005, DOI 10.1074/jbc.M508762200

Daisuke Nakata and Frederic A. Troy II

From the Department of Biochemistry and Molecular Medicine, University of California School of Medicine, Davis, California 95616

α2,8-Linked polysialic acid (polySia) is a structurally unique anti-adhesive glycoprotein that covalently modifies N-linked glycans on neural cell adhesion molecules (N-CAMS). These sugar chains play a key role in modulating cell-cell interactions, principally during embryonic development, neural plasticity, and tumor metastasis. The degree of polymerization (DP) of polySia chains on N-CAM is postulated to be of critical importance in regulating N-CAM function. There are limitations, however, in the conventional methods to accurately determine the DP of polySia on N-CAM, the most serious being partial acid hydrolysis of internal α2,8-ketosidic linkages that occur during fluorescent derivatization, a step necessary to enhance chromatographic detection. To circumvent this problem, we have developed a facile method that combines the use of Endo-β-galactosidase to first release linear polySia chains from N-CAM, with high resolution high pressure liquid chromatography profiling. This strategy avoids acid hydrolysis prior to chromatographic profiling and thus provides an accurate determination of the DP and distribution of polySia on N-CAM. The potential of this new method was evaluated using a nonpolysialylated construct of N-CAM that was polysialylated in vitro using a soluble construct of ST8Sia II or ST8Sia IV. Whereas most of the oligosialic acid/polySia chains consisted of DPs ~50–60 or less, a subpopulation of chains with DPs ~150 to ~180 and extending to DP ~400 were detected. The DP of this subpopulation is considerably greater than reported previously for N-CAM. Endo-β-galactosidase can also release polySia chains from polysialylated membranes expressed in the neuroblastoma cell line, Neuro2A, and native N-CAM from embryonic chick brains.

α2,8-linked polysialic acid (polySia) is a unique linear homopolymer of sialic acid (Sia) that is widely distributed in nature, being expressed as the capsular polysaccharide on bacterial pathogens, including neuroinvasive Escherichia coli K1 and Neisseria meningitidis Gp B (reviewed in Refs. 2 and 3). The glycoprotein is also expressed on glycoproteins in fish and sea urchin eggs, amphibians, animal and human brains, and a variety of human cancers (3–14). The major carrier protein of polySia in mammalian cells is the neural cell adhesion molecule, N-CAM, in which polySia extends tri- and tetraantennary N-linked glycan chains (12–14). The α-subunit of the sodium channel in rat brain is also reported to be polysialylated (15), as are the two mammalian polysialyltransferases (polySTs), ST8Sia II (STX) (16) and ST8Sia IV (PST) (17, 18). Whereas both STX and PST are autopolysialylated in transfected cells (16–20), this post-translational modification may be of questionable biological significance, since it does not appear to occur during neural development in the embryonic chick brain (21). O-Linked polySia chains also covalently modify N-CAM (22, 23) and are expressed on an unidentified protein in human breast cancer cells (24). Recently, polySia was reported on O-linked glycan chains that covalently modify the CD36 molecule in human milk (25) and as α2,9-linked polySia chains in a sea urchin sperm glycoprotein (26, 27).

The mechanism of polysialylation of N-CAM has been studied extensively. The amount of polySia changes dramatically during embryonic development (28–30), where it mediates homophilic and heterophilic interactions between N-CAM-expressing cells (9, 31–33). The posttranslational polysialylation of N-CAM decreases N-CAM-dependent cell adhesion and migration, and in this sense, polySia functions as an antiadhesive glycoprotein. With the exception of polySia-N-CAM expression during neural development, the biological function of polysialylation in eukaryotes remains largely unknown (3, 4, 35). It was recently reported, however, that the muscle-specific domain in an N-CAM isoform was polysialylated and that this modification correlated with myoblast differentiation (22).

In vertebrates, STX and PST can independently catalyze synthesis of polySia (17–19), although the two enzymes may act cooperatively (36–38). These transferases are usually expressed at high levels in embryonic tissues, principally brain and kidney, and at lower levels in adult tissues. An important exception to this paradigm is the persistent level of expression in regions of the brain showing neural plasticity, including the hippocampus, hypothalamus, dentate gyrus, and olfactory bulb (22, 39–41). Both PST and STX are highly conserved in their primary amino acid sequence, showing 59% identity. They differ, however, in their temporal expression patterns in different tissues, in their mRNA expression levels and in the apparent chain length of their in vitro synthesized polySia chains (28–30, 37, 38, 42).

Although chicken and mouse N-CAM both have six potential N-glycosylation sites, only two of the three sites in the fifth Ig-like domain appear to be polysialylated (23, 37). A triantennary glycan containing two polySia chains with degrees of polymerization (DPs) ~14–27 are expressed in embryonic chick brain N-CAM (43). This is an important...
finding, because it shows that more than one glycan chain can be polysialylated, which is in contrast to the popular schematic diagrams often depicting polySia on N-CAM as a single, long chain (18, 36, 42). It has also been reported that some of the polysialylated tri- and tetraantennary chains on embryonic chick and mouse brain N-CAMs can be sullated on the most distal GlcNAc residues (44–47).

It has been postulated, but not proven, that the DP of polySia on N-CAM may be a critically important structural feature regulating N-CAM function by influencing cell-cell, cell-substratum and polySia-antipolySia antibody interactions (32, 35, 48). Whereas there is considerable structural information on the protein and N-linked oligosaccharides on N-CAM (44–47), there is, with one exception (43), a paucity of information about how the DP may change during neuronal development in those regions of the brain showing neural plasticity or in human cancer cells expressing polysialylated N-CAM. To ultimately understand the relationship of DP to biological function, it is imperative that we be able to accurately determine the DP and to determine how this changes during N-CAM-mediated processes. To achieve this goal, it is necessary to develop new methods to accurately determine the full length of polySia chains on N-CAM and not simply to determine the DP of the major subset of chains that have already undergone some acid hydrolysis during fluorescent derivatization (43, 49). Therefore, the objective of this study was to develop a new strategy to determine the DP of polySia on N-CAM without first subjecting the chains to acid hydrolysis before high resolution HPLC profiling.

EXPERIMENTAL PROCEDURES

Materials—A full-length construct of STX (pcDNA3.1-STXsV5His), a soluble form of (pcDNA3.1-STXsV5His) fused with either V5 and His6 epitope tags at the C terminus and a soluble Fc-N-CAM (mouse homologue) were kindly provided by Dr. K. J. Colley (University of Illinois, Chicago, IL). The soluble mouse homologue of Fc-N-CAM was originally developed by Dr. Genevieve Rougon (Marseille, France). Anti-polySia antibody (12F8) was purchased from BD Biosciences (San Jose, CA). 2-Aminoacridin (AMAC), 1,2-diamoino-4,5-methylenedioxybenzene (DMB), and 3′- or 6′-sialyllactose were purchased from Sigma. Endo-β-galactosidase (Endo-β-Galase) from Escherichia freundii was purchased from V-LABS, Inc. (Covington, LA). The DNA-Pac PA-100 anion exchange column was purchased from Dionex Corp. (Sunnyvale, CA). The TSK-gel ODS-120T reverse phase column was purchased from Tosho (Tokyo, Japan). Colominic acid was purchased from Nakarai Tesque (Kyoto, Japan).

Construction of an Expression Plasmid of a Soluble Construct of STX with N-terminal V5 and His6 Tags—A DNA fragment encoding for a truncated form of STX and lacking the 46-amino acid sequence at the N terminus was amplified by PCR using pcDNA3.1-STXsV5His as a template and the following primers: 5′-TGCTCGAGATGGTGATGGTGATGATGAC-3′ and 3′-CTTACATAGCAAATCTAA-3′. The DNA fragment coding V5 and His6 epitope tags subcloned into pGEM-T Easy vector was removed by digestion with XhoI and EcoRV and ligated into pcDNA3.1-STXs through the same site (pcDNA3.1-V5HisSTXs).

Expression and Purification of the Soluble Form of STX—The soluble form of STX, designated STX(s), was prepared by transfection of the expression plasmid, pcDNA3.1-V5HisSTXs, into COS7 cells. Transfection was carried out according to Qiagen protocol for Superfect. After 24–36 h, the culture medium containing the secreted enzyme was collected. The enzyme was purified using Ni2+-nitrilotriacetic acid-agarose beads (Qiagen) as follows. To 10 ml of culture medium containing STX(s) were added 50 μl of 1.0 M Tris-HCl (pH 8.0) and 200 μl of Ni2+-nitrilotriacetic acid-agarose beads. After incubation at 4 °C for 2 h, the Ni2+-nitrilotriacetic acid-agarose beads were washed with 5 volumes of 20 mM Tris-HCl (pH 8.0) containing 5 mM imidazole, 500 mM NaCl, and 10% glycerol. After sequentially washing with 3 volumes each of 10, 30, and 60 mM imidazole, STX(s) was eluted with 3 volumes of 20 mM Tris-HCl (pH 8.0) containing 200 mM imidazole, 500 mM NaCl, and 10% glycerol. The 200 mM imidazole fraction was concentrated, and the buffer was changed to 50 mM MES (pH 6.1) containing 10% glycerol using a YM-30 Millipore filter. SDS-PAGE/silver stain and Western blotting with an anti-V5 mAb was used to analyze STX during the purification procedure.

Purification of Fc-N-CAM—The cell culture medium of COS7 cells transfected with the Fc-N-CAM expression plasmid was applied to a Protein A-Sepharose column (1 ml, 1.5 × 0.6 cm) equilibrated with 20 mM Tris-HCl (pH 8.0) and washed with 10 ml of 20 mM Tris-HCl containing 150 mM NaCl. Proteins bound to Protein A-Sepharose were eluted with 6 ml of ImmunoPure Gentle Ag/Ab elution buffer (Pierce). The eluate was collected and dialyzed against 20 mM Tris-HCl (pH 8.0) using a Slide-A-Lyzer 10K dialysis cassette (Pierce), according to the manufacturer’s protocol, and concentrated using a YM-50 Millipore filter. SDS-PAGE and Comassie Blue staining were used to determine the purity of the Fc-N-CAM.

Construction of Full-length PST Form with V5 and His6 Tags at the N Terminus—A DNA fragment encoding for full-length form of PST was amplified by PCR using pcDNA3.1-PSTsV5His as a template and the following primers: 5′-TAGATATCGGCCCTCGAGATGGTGATGGTGATGAC-3′ and 3′-TGGATATCATGGGTAAGCCTATCCCTAACCC-3′. The amplified DNA fragment was subcloned into pGEM-T Easy vector (Promega). The inserted fragment was removed by digestion with XbaI and EcoRV and ligated into pcDNA3.1-PSTsV5His through the same site (pcDNA3.1-PST). The DNA fragment coding for V5 and His6 epitope tags was amplified by PCR using pcDNA3.1 plasmid as a template and the following primers: 5′-TGCTCGAGATGGTGATGGTGATGATGAC-3′ and 3′-TTAGGAAGAG-3′. The amplified DNA fragment was subcloned into pGEM-T Easy vector (Promega). The inserted fragment was removed by digestion with XbaI and EcoRV and ligated into pcDNA3.1-PSTsV5His through the same site (pcDNA3.1-V5HisPST). The DNA fragment coding for V5 and His6 epitope tags was amplified by PCR using pcDNA3.1 plasmid as a template and the following primers: 5′-TGCTCGAGATGGTGATGGTGATGATGAC-3′ and 3′-TGCTCGAGATGGTGATGGTGATGAC-3′. The amplified DNA fragment was subcloned into pGEM-T Easy vector (Promega) and concentrated using a YM-50 Millipore filter. SDS-PAGE and Comassie Blue staining were used to determine the purity of the Fc-N-CAM.

Reconstituted in Vitro PolyST assay—Incorporation of Neu5Ac from CMP-Neu5Ac into Fc-N-CAM as the exogenous acceptor substrate was carried out by a modification of the polyST assay developed earlier in our laboratory (29). All incubation mixtures contained 50 mM MES buffer (pH 6.1), 10 mM MnCl2, 1 mM CMP-Neu5Ac, purified Fc-N-CAM (0.6 μg of protein), and STX(s) (3 μg of protein) in a total volume of 15 μl. After incubation at 37 °C for 2 h, polysialylation of N-CAM was qualitatively assessed by SDS-PAGE/Western blot analysis both before and after treatment with endo-N-acylneuraminidase (Endo-N) or Endo-β-Galase using either anti-polySia or anti-N-CAM mAbs. A portion of the incubation mixture was also subjected to DP analysis, as described below. In contrast to the polyST activity in embryonic chick brain membranes, STX activity in the reconstituted assay was not influenced by dithiothreitol.
Determination of the DP of PolySia: Release of the PolySia Chains from FcN-CAM by Endo-β-Galase—To determine the DP of the polySia chains synthesized by the polysialyltransferases on FcN-CAM in the reconstituted in vitro assay, the polysialylated N-CAM product described above was first purified by adsorption onto Protein A-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C. After washing the beads with 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 10 μl of beads were incubated with 0.4 units of Endo-β-Galase in 40 μl of 50 mM ammonium acetate buffer, pH 5.9, containing 0.5% CHAPS at 37 °C for 12 h. An identical incubation mixture without Endo-β-Galase was included as a control. After incubation, the beads were sedimented by centrifugation (15,000 × g/∼30 s), washed with distilled water, and the supernatant fractions were combined. The supernatant fractions containing the polySia chains released by Endo-β-Galase were subjected to high resolution separation on a HPLC anion exchange column, as described below. The amount of Sia in each fraction was then quantitatively determined using the DMB method (50, 51). The washed beads were also subjected to SDS-PAGE/Western blot analysis and blotted using the anti-polySia antibody, 12F8. When compared with the minus enzyme control, this provided an independent assessment of the efficacy of Endo-β-Galase release of the polySia chains from FcN-CAM. The reaction conditions developed here did not result in hydrolysis of the internal α2,8-sialyl linkages, as assessed by a second control incubation mixture wherein an authentic sialyloligomer of DP 15 was incubated under identical conditions (see “Results”).

We also discovered that inclusion of the CHAPS detergent in the incubation mixture was essential for the Endo-β-Galase-catalyzed release of polySia chains from embryonic chick brain membranes. These chains were more resistant to release by Endo-β-Galase than those synthesized in vitro. CHAPS had only a modest effect on release of polySia from the polysialylated FcN-CAM. This finding suggests that there may be some structural (e.g., sulfated GlcNAc residues) and/or conformational differences between the two different forms of polysialylated N-CAM.

Identification of Galactose as the 2-Aminoacridion (AMAC) Derivative in the PolySia Chains (DP 120–130) Released from N-CAM by Endo-β-Galase—PolySia chains released from the N-linked glycans on N-CAM by Endo-β-Galase would be expected to have a galactosyl residue at their reducing termini (Fig. 1). To verify the presence of Gal, polySia chains that eluted from the HPLC column with a DP 120–130 were isolated, hydrolyzed in 0.1 M trifluoroacetic acid at 80 °C for 30 min and evaporated to dryness in vacuo. Derivatization with AMAC was carried out by a modification of a previously described procedure (52). Briefly, the dried samples were resuspended in 10 μl of distilled water to which was added an equal volume of 5 mM AMAC in 15% acetic acid and 2 volumes of 1 M NaCNBH3 in Me2SO. After incubation at 50 °C for 2 h, the reaction mixture was again evaporated to dryness, and the sample was dissolved in distilled water and analyzed by HPLC on a TSK-gel ODS-120T column (4.6 × 250 mm). As a control, 10 μl of 5 mM galactose was derivatized with AMAC under identical conditions, and the resulting HPLC profile was used to determine the chromatography position of Gal-AMAC. The AMAC derivatives were excised at 420 nm, and emission was recorded at 528 nm.

Fluorometric HPLC Analysis of DMB-derivated PolySia—As a control for the size of polySia chains released from N-CAM by Endo-β-Galase, DMB derivatization of a polydisperse mixture of oligo- and polysia (colominic acid) from Nakarai Tesque was carried out as previously described (49). To 5 μl of a 20 mg/ml solution of these sialyloligopolymers in distilled water (on ice) was added an equal volume of 40 mM trifluoroacetic acid containing 7 mM DMB, 18 mM Na2S2O4, 1.0 mM 2-mercaptoethanol, and 20 mM trifluoroacetic acid. The reaction mixture was incubated at 10 °C for 12, 24, and 48 h. After DMB derivatization, one-quarter volume of 1.0 M NaOH was added to neutralize the solution, and the samples were frozen at −80 °C until HPLC analyses.

Anion exchange HPLC chromatography of DMB-derivatized colominic acid and undervirified polySia chains released from N-CAM by Endo-β-Galase was carried out on a Shimadzu preparative HPLC system (model XC-8A) using a DNA Pac PA-100 column (4 × 250 mm) and a Shimadzu spectrofluorometric detector (model RF-10AXL). The liquid chromatograph was equipped with a Shimadzu system controller (model SCI-10A) with a diode array detector (model SPD-M10A). The column was eluted with a convex gradient of ammonium acetate (0–2.0 M; pH 8.0) at a flow rate of 1 ml/min. Under these conditions, the concentration of ammonium acetate at 0, 5, 15, 20, 35, 45, and 182.5 min was 0, 0, 20, 25, 32.5, 40, 62.5, and 100% (2.0 M), respectively. After 182.5 min, the column was then eluted with 5.0 M ammonium acetate (up to 190 min). The elution profile for DMB-derivatized colominic acid was monitored by fluorescence (excitation, 373 nm; emission, 448 nm).

High Resolution Profiling of PolySia Chains Released from FcN-CAM Using DNA Pac PA-100—The polySia chains released from polySia-FcN-CAM by Endo-β-Galase were separated using the same high resolution HPLC procedure as described above for the fluorometric HPLC analysis of DMB-derivatized colominic acid. For elution of the extended polySia chains containing DP values greater than those present in colominic acid (DP > 115), the column was first eluted with 5 ml each of 3 M and then 5 M ammonium acetate (from 182.5 until 212.5 min). One hundred thirty-five fractions (1.5 ml each) were collected for each sample between 10 and 212.5 min. Each fraction was evaporated to dryness in a SpeedVac concentrator. The column profile of polySia was determined by pooling every five fractions, which were then hydrolyzed to free Neu5Ac with 0.1 N trifluoroacetic acid at 80 °C for 30 min. After evaporation in the SpeedVac concentrator, the amount of Neu5Ac in each fraction was quantitatively determined by the DMB/fluorometric HPLC assay, as described below. The DNA Pac PA-100 column was regenerated between each run by washing with 10 ml of 5 M ammonium acetate.

Establishment of Neuro2A Cells Stably Expressing the Full-length Construct of PST—The mouse neuroblastoma cell line, Neuro2A, was transfected with pcDNA3.1-V5HisPSTf by Superfect Reagent (Qiagen) and exposed to selection medium containing 550 μg/ml Geneticin (Invitrogen) for 2 weeks by replacing the medium every 3 days. Pools of selected Neuro2A cells stably expressing the full-length form of PST were cloned by the limited dilution method. To confirm expression of PST, the presence of polySia-NCAM in membranes from these cells was determined by SDS-PAGE/Western blot analyses using the anti-polySia antibody, 12F8. Membranes isolated from the cloned Neuro2A cells expressing polySia-NCAM were also used to establish that Endo-β-Galase could release these chains from a native biological membrane, and their DP was determined by high resolution HPLC profiling, as described below under “Results and Discussion.”

Quantitative Determination of Neu5Ac in HPLC Fractions Using the DMB-Fluorometric Method—After the polySia chains released from N-CAM by Endo-β-Galase had been separated by high resolution HPLC on the DNA Pac PA-100 column, the amount of Sia in each pooled fraction was quantitatively determined by fluorometric HPLC after DMB derivatization. Authentic Neu5Ac was used as the control for calibration of fluorescence intensity versus the amount of Sia in each column fraction. For these determinations, an equal volume of 40 mM trifluoroacetic acid containing 7 mM DMB, 18 mM Na2S2O4, 1.0 mM 2-mercaptoethanol, and 20 mM trifluoroacetic acid was added to each
Degree of Polymerization of Polysialic Acid on N-CAM

FIGURE 1. Model of oligo-/polysialylated N-glycan of N-CAM: The C7/C9 method for DP analysis and the structural complexity introduced by multiantennary chains. Schematic representation of the triantennary N-glycan structure of N-CAM showing variable oligo-/polysialylation on each antenna. Neither the C7/C9 method of DP analysis nor cleavage of the glycan by Endo H (arrow 1) or PNGase F (arrow 2) can provide the DP of individual polySia chains unless the chains are first released from the N-glycan. It is also not possible with the C7/C9 method to differentiate between two chains of DP 50 each and a single chain of DP 100, unless the chains are first released from the N-glycan. Endo-β-Galase can release individual oligo-/polySia chains from the N-glycan (open triangle), thus permitting their DP to be determined by high resolution HPLC profiling, as described.

RESULTS AND DISCUSSION

The C7/C9 Method for DP Analysis and the Structural Complexity Introduced by Multiantennary Chains of Oligo-/PolySia on N-CAM—Studies to determine the DP of the polySia capsule on neuroinvasive E. coli K1 were the first to show that these linear chains consisted of >200 Sia residues (53). This was achieved by sequential periodate oxidation and borohydride reduction of Uri-14C-radiolabeled chains that selectively converted the nonreducing Neu5Ac residue to the 7-carbon analogue, 5-acetamido-3,5-dideoxy-L-arabino-2-heptulosonic acid (Neu5Ac7), a method originally described by van Lenten and Ashwell (54). After complete hydrolysis, the ratio between Neu5Ac7 and Neu5Ac was then used to calculate the DP. The C7/C9 method is not applicable, however, for determining the DP of polySia on N-CAM, because the method requires free, linear chains. As shown in Fig. 1, application of the method to a mixture of mono-, di-, oligo-, and polysialylated multiantennary N-linked glycans would reveal only an average DP, based on the total net negative charge that reflects all of the Sia residues on the different chains. The C7/C9 method thus markedly underestimates the true DP when carried out on multiantennary N-glycans.

Importantly, the C7/C9 method cannot differentiate between a chain with a DP of 100 and two chains, each with a DP of 50. C7/C9 analysis of shorter oligo-/polySia chains also results in similar ambiguities, as shown in Fig. 1. As a consequence of this limitation, several alternate strategies have been developed for DP analysis of N-CAM that have provided estimates ranging from 40 to >90 Sia residues (19, 38, 42, 49, 55–57).

Whereas chemical methods of analysis employing fluorescent derivatization with DMB have provided the most promising approach (46, 49, 56, 57), they underestimate chain length because of the intramolecular self-cleavage of internal Sia residues resulting from the lability of α2,8-ketosidic linkages to even mildly acidic conditions (58). Unfortunately, acidic conditions (pH ~1.9) are required for DMB fluorescent tagging, a prerequisite for enhanced sensitivity required for chromatographic detection in the femtomol to picomol range. After DMB derivatization, the partially hydrolyzed fluorescent chains are then commonly profiled by various anion exchange chromatographic methods, and their DP is estimated based on their elution position (43, 49, 56, 57).

Conventional Methods and the New Strategy Developed to Determine the DP of PolySia Chains on Multiantennary N-Glycans Where the Chains are First Released by Endo-β-Galase before High Resolution HPLC Profiling—In addition to the limitations imposed by partial acid hydrolysis of polySia chains before HPLC profiling, other potential problems can obscure an accurate determination of the DP of polySia chains on N-CAM. In the conventional strategy for DP analysis, as schematized in Fig. 2A, sialylated multiantennary N-linked glycans are often first released from the protein with either N-acetylgalactosaminidase (Endo-H) or protein N-glycanase F (PNGase F) (19, 38, 42, 55–57).

Since individual polySia chains are not released by either hydrolyase, a composite mixture of mono-, di-, oligo-, and polysialylated tri-/tetraantennary N-glycans are released and profiled. Thus, it is not possible to accurately determine the DP of individual chains based on their HPLC elution profiles, since such a mixture represents multiply sialylated chains with varying DPs and not the DP of individual polySia chains. The multiantennary complexes of N-glycans also call into question the validity of chain lengths based on SDS-PAGE gel mobility, since it is often misinterpreted that slower gel mobility is correlated with increased DP (38, 42). Similarly, the conclusion that N-CAM N-glycans containing more polySia chains are thus longer (38) is also misleading, since the intensity of Western blot staining is not a reliable predictor of chain length. Despite the shortcoming of PNGase F for DP determinations, the enzyme is useful for releasing N-linked glycans from N-CAM for structural determination by mass spectrometry (45–47).

Although in vitro radiolabeling (Fig. 2A) is an alternate method that avoids acid hydrolysis (53), it is of limited value in the absence of procedures to generate linear chains for either high resolution HPLC or application of the C7/C9 method. Radiolabeling is also not practical for in vivo labeling of polySia in animal models, including embryonic chick embryos, mice, or human tumors.

Because of these problems and limitations, we sought to develop a new strategy to determine the DP of polySia that avoids acid hydrolysis before HPLC profiling. As schematized in Fig. 2B, this has been achieved by combining the use of Endo-β-Galase to first release individual polySia chains from N-CAM, with high resolution HPLC profiling. After the chains are separated, we then capitalized on the high sensitivity of the DMB method to quantitatively determine the amount of Neu5Ac in each HPLC fraction. To evaluate the potential relevance of this strategy, a nonpolysialylated construct of N-CAM4 was polysialylated in a reconstituted in vitro system by a soluble construct of STX or PST and analyzed. The results provide an accurate determination of the distribution.
and DP of the parent polySia chains on N-CAM and have revealed extended chain lengths considerably longer than reported previously, as described below.

**Affinity Purification of the Soluble Construct of STX (ST8Sia II)**—The soluble form of STX(s) was prepared in COS7 cells that were transfected with the expression plasmid, pcDNA3.1-V5HisSTXs. After growth for 24 h, the culture medium containing the secreted form of STX(s) was collected, and the enzyme was purified using a Ni$^{2+}$-chelating column, as described under “Experimental Procedures.” Fig. 3A shows the silver stain and Western blot profiles for the affinity-purified STX(s) after SDS-PAGE.

Two different molecular sizes of the enzyme were detected with apparent molecular masses of 55 and 68 kDa. Since the molecular mass estimated from the primary amino acid sequence of STX(s) with the N-terminal tag was ~40 kDa, these data suggested that both forms of the enzyme were probably modified by posttranslational glycosylation.

**Structure and Glycosylation Status of STX(s)**—To determine whether the 55- and 68-kDa forms of STX(s) were glycosylated, sialylated, or polysialylated, the purified enzyme was treated with either PNGase F, exosialidase II (*Arthrobacter ureafaciens*), or Endo-N before SDS-PAGE. After PNGase F treatment, a single band with a molecular mass of ~40 kDa was detected by the anti-V5 antibody (Fig. 3B, lane 4), compared with the predicted size based on the primary sequence, this shows that both forms of the enzyme were N-glycosylated. Exosialidase treatment alone resulted in the disappearance of the 68-kDa form of the enzyme, with no change in the mobility of the 55-kDa species (Fig. 3B, lane 2). This indicated that the difference in molecular mass between the 55- and 68-kDa forms of the enzyme was due to sialylation of the 68-kDa species. Neither the 55- nor 68-kDa forms of the enzyme appeared to be polysialylated, however, since both showed the identical SDS-PAGE mobility after pretreatment with Endo-N (Fig. 3B, lane 3).

These results show that STX(s) was expressed as two different sized N-linked glycoproteins: a 55-kDa species that is glycosylated but not sialylated and a 68-kDa species that is both glycosylated and sialylated. That the 68-kDa sialylated form was not polysialylated also shows that our STX(s) construct with the V5 tag in the N terminus did not catalyze its own polysialylation (autopolysialylation), as was reported to occur in STX with a C terminus V5 tag (16, 17). Angata *et al.* also reported that a recombinant PST expressed in insect cells possessed polysialyltransferase activity, yet was not autopolysialylated (59). Further, we found that there was no difference in the PAGE mobility between the reduced and nonreduced forms of STX(s), suggesting that our soluble construct did not form dimers or oligomers through disulfide bonds. A similar conclusion was reached for a soluble construct of PST when expressed in recombinant baculovirus-infected insect cells (59). This finding is in contrast to the dimeric form of the polySTs found in embryonic chick
brains and suggests that dimerization is not an essential requirement for polysialylation (21).

**Polysialyltransferase Activity of STX(s) in the in Vitro Reconstituted Assay**—To determine whether our STX(s) construct could catalyze the *in vitro* polysialylation of N-CAM, a reconstituted system was developed in which the purified enzyme was incubated in the presence and absence of purified FcN-CAM for 2 h at 37 °C. As shown in Fig. 4 (lane 2), anti-polySia antibody immunoreactivity with an apparent molecular mass >200 kDa was detected by SDS-PAGE. Such polydispersity in this high *M*<sub>r</sub> region of the gel is consistent with that expected for polysialylated N-CAM (8). In contrast, no polySia was detected in the control sample incubated in the absence of FcN-CAM (Fig. 4, lane 1), even in the region expected for autopolylysialylated STX(s) (>68 kDa).

These results established two points. First, our STX(s) construct is capable of catalyzing synthesis of polySia chains *in vitro* using FcN-CAM as an exogenous acceptor substrate. This shows that STX(s) does not need to be intercalated into the membrane to catalyze polymerization of extended polySia chains. Second, it shows that our STX(s) construct did not undergo autopolylysialylation. This latter finding provides further evidence that STX autopolylysialylation is not an obligatory requirement for N-CAM polysialylation. There has been some controversy regarding this point. Studies by Gerardy-Schahn and co-workers (18, 20) reported that STX and PST both underwent "autopolylysialylation" and that this post-translational modification was required for polysialylation of N-CAM. In contrast, studies by Coley and co-workers (60) and Angata et al. (59) showed that autopolylysialylation was not a prerequisite for N-CAM polysialylation, a conclusion supported by our present findings.

**Acid Lability of Internal α2,8-Ketosidic Linkages in PolySia to DMB Derivatization**—Conventional methods to determine the DP of oligo-/polySia on N-CAM often use HPLC profiling on ion exchange columns with radiolabeled (38, 42) or fluorescent detection or high performance anion exchange chromatography with pulsed electrochemical detection (43, 49, 56, 57, 61). In both cases, separation is based on the net negative charge density of the chains. The lower resolution of the elution profile at higher salt concentrations poses a limitation that makes it difficult to determine the exact length of chains with DPs >30–40 (57). Zhang et al. (62) were the first to report that polySia chains with DPs >90 could be separated by the high performance anion exchange chromatography-pulsed electrochemical detection method. DPs >40 were also reported using this method for polySia chains liberated from embryonic chick brain N-CAM, even after some acid hydrolysis inherent in the DMB derivatization method (61). This finding suggested that even longer polySia chains may actually be present on N-CAM. Importantly, the above findings demonstrated the sensitivity and applicability of the high performance anion exchange chromatography-pulsed electrochemical detection method for determining minimum DP values and established that polySia chains in the embryonic chick brain can exceed ~90 Sia residues. Subsequent studies by Inoue and colleagues showed an enhanced resolution of longer polySia chains on a Dionex DNA PAC PA-100 anion exchange column after DMB derivatization (43). It became evident during the course of these earlier studies, however, that the DMB method used for fluorescent derivatization of the reducing terminus resulted in some hydrolysis of the parent polySia chains, thus rendering it impossible to accurately determine the DP of the parent polySia chains on N-CAM (43, 61, 63). Further studies reported that presumed full-length polySia chains were selectively released from N-CAM, even under the acidic conditions required for DMB derivatization (pH ~1.9; at 10 °C for 48 h). This, it was concluded, was because hydrolysis of the Siaα2,3- and Siaα2,6-Gal linkages that attach polySia chains to the core N-linked oligosaccharides on N-CAM were hydrolyzed 2.5–4-fold faster than internal α2,8-sialyl linkages (49). This conclusion was based, however, on pseudo-first order rate constants of hydrolysis that compared under strongly acidic conditions, either 0.02 M trifluoroacetic acid at 50 °C or 0.1 M trichloroacetic acid at 80 °C, the trisaccharides Neu5Aca2,3-Galβ1,4-Glc- and Neu5Aca2,6-Galβ1,4-Glc with the disaccharide, Neu5Aca2,8-Neu5Ac. It was further assumed in these studies that every α2,8-ketosidic linkage in polySia was hydrolyzed at the same rate. Since this conclusion and assumption seemed contrary to the known acid lability of internal α2,8-ketosidic linkages in polySia that Manzi et al. (58) showed was caused by a general acid-catalyzed intramolecular self-cleavage of Sia units, we reevaluated the stability of polySia to DMB derivatization under conditions reported to cause minimum chain degradation, viz. 20 mM trifluoroacetic acid (pH 1.9) at 10 °C for 48 h (43).

Fig. 5 shows the high resolution HPLC profile of DMB-derivatized oligo-/polySia (colominic acid) on a DNA PAC PA-100 column after DMB derivatization (20 mM trifluoroacetic acid) at 10 °C for 12, 24, and 48 h.

Compared with the 12-h profile (Fig. 5A), it is evident that some hydrolysis of the internal α2,8-linkages had occurred by 24 h (Fig. 5B), which was even more extensive after 48 h of labeling (Fig. 5C). Hydrolysis was particularly evident for the higher DPs (~30–80), as can be seen by comparing the 12 and 24 h profiles with the 48 h profile. Concomitant with the loss in the higher DPs was an increase in the population of smaller sialyl oligomers (DPs <10). TABLE ONE summarizes the percentage of each family of DPs remaining after 12, 24, and 48 h of DMB derivatization.

This quantitative determination shows the extent of hydrolysis during DMB derivatization and reveals extensive hydrolysis of the α2,8-ketosidic linkages in both the shorter and longer polySia chains. This is particularly noteworthy after 48 h. To further evaluate the selective acid labilization of interketosidic linkages and its impact on DP values, we compared the hydrolysis profiles of polySia with defined DPs of 16–22, 29–38, and 80–89 with hydrolysis during DMB derivatization in 20 mM trifluoroacetic acid at 4 °C for 24 h. As shown in Fig. 6, only 7% hydrolysis occurred with DPs 16–22 (Fig. 6A), whereas 16% hydrolysis occurred with the family of DPs 29–38 (Fig. 6B). Remarkably, 61% hydrolysis occurred within the polySia chains ranging in DP from 80 to 88 residues (Fig. 6C).
These results thus confirmed that the internal α2,8-ketosidic linkages in the longer polySia chains were more readily hydrolyzed than those in the shorter sialyl oligomers. This finding is consistent with the conclusion reached earlier by Manzi et al. (58) that the longer chains are more sensitive to acid hydrolysis than shorter chains. Two important conclusions follow from these results. First, an accurate determination of the DP of polySia cannot be determined by DMB derivatization, even when carried out at low temperature and under the optimal conditions reported to minimize hydrolysis. Second, because of the acid hydrolysis that occurs during DMB derivatization, any DP value obtained by this method will represent a lower or minimum DP from what is actually present in the parent chain, and this underestimate is greater in chains with higher DPs. As a consequence of our finding that DMB derivatization cannot provide an accurate determination of the DP of polySia chains, we sought an alternative strategy to release polySia from N-CAM that avoided acid hydrolysis before their high resolution profiling, as described below.

Release of Linear PolySia Chains from Polysialylated N-CAM by Endoβ-Galase—Based on the above findings, it became evident that for an accurate determination of the DP of polySia chains attached to the N-linked glycans of N-CAM, a new strategy would have to be developed. One such strategy would be to first release the linear polySia chains from

FIGURE 5. Acid lability of internal α2,8-ketosidic linkages in polySia to DMB derivatization. The stability of polySia to DMB derivatization was determined in 20 mM trifluoroacetic acid at 10 °C for 12 h (A), 24 h (B), and 48 h (C). For this experiment, 200 μg of colominic acid were derivatized, and 50 μg of this solution were subjected to anion exchange HPLC on a DNAPac PA-100 Dionex column, as described under “Experimental Procedures.” DP values for the polySia chains are given above the column fractions, which were detected with a fluorescent detector (excitation, 373 nm; emission, 448 nm). The insets in each panel show a magnification of the higher DP values for each time point.
the N-glycan under nonhydrolytic conditions before their separation by high resolution HPLC profiling, as schematized in Fig. 2B. To achieve this goal, we postulated that linear polySia chains might be selectively released from the N-linked oligosaccharide core of polysialylated FcN-CAM by Endo-β-Galase. The enzyme from E. freundii specifically cleaves endo-β-galactosyl linkages in both type 1 (Sia\(^{2,3}\)-Gal) and type 2 (Sia\(^{2,3}\)-Gal) lactosamine structures. Both type 1 and type 2 structures are reported to be present in the tri- and tetraantennary glycans of embryonic chick brain N-CAM (44). As shown in Fig. 1, it would thus be expected that linear polySia chains terminating in a galactosyl residue at the potential reducing terminus would be released by Endo-β-Galase treatment of polySia-N-CAM.

To test this idea experimentally, FcN-CAM was polysialylated in vitro with STX(s), collected by adsorption on Protein A-Sepharose beads, and treated with Endo-β-Galase, as described under “Experimental Procedures.” After enzyme digestion, the supernatant fraction containing the released polySia chains was collected, and the beads were washed with distilled water. The supernatant fraction was subjected to DP analysis, as described below, and the beads were subjected to SDS-PAGE/Western blot analysis to monitor the extent of depolyssialylation. As shown in Fig. 7 (lane 2), Endo-β-Galase was effective in releasing all of the polySia chains from FcN-CAM, as no immunoreactivity was seen with the anti-polySia mAb. In contrast, strong immunoreactivity was observed in the Endo-β-Galase minus control (lane 1) in the high Mr region of the gel expected for polysialylated N-CAM.

### Stability of PolySia to Conditions for Endo-β-Galase Release of PolySia Chains from FcN-CAM

To determine whether polySia was hydrolyzed under the reaction conditions developed for their Endo-β-Galase-catalyzed release from N-CAM, a DMB-derivatized polySia was incubated under the identical reaction conditions used for their release from FcN-CAM (100 mM AcOH/NH\(_4\)OAc, pH 5.9, containing 0.5% CHAPS) for 4 and 12 h at 37°C. As shown in Fig. 8, the HPLC profiles were identical at 0, 4, and 12 h, indicating that no hydrolysis of the α2,8-sialyl linkages had occurred.

Importantly, no shorter sialyloligomers with DP<15 were observed, which would have been expected had there been any chain degradation under these incubation conditions.

### DP Analysis of Linear PolySia Chains Released from Polysialylated N-CAM by Endo-β-Galase

FcN-CAM was polysialylated by STX(s) in the in vitro reconstituted polyST assay, as described under “Experimental Procedures.” Linear polySia chains released by Endo-β-Galase were subjected to high resolution HPLC separation on an anion exchange DNA Pac PA-100 column using a gradient of ammonium acetate. Column fractions (1.5 ml) were collected from 10 to 212 min, and the ammonium acetate was removed under vacuum.

In Fig. 9A, the total amount of Sia in each of the pooled five fractions was quantitatively determined by the DMB method after hydrolysis with 0.1 M trifluoroacetic acid. The four insets show the amount of Sia in each of the individual fractions corresponding to fraction numbers 1–5 (DP = 2–5), 71–75 (DP = 150–150), fractions 86–90 (DP = 180 ± 2), and fractions 106–110 (DP = 400 ± 6). Fig. 9B shows the relative amount of polySia in each fraction, which was determined by dividing the total amount of Sia in each fraction (from Fig. 9A) by the average DP for that fraction. The DP values for fractions 1–50 (DP = 2–90) in Fig. 9 were determined by comparison with the profile of DMB-derivatized colominic acid (Fig. 9C). DP values greater than ~90 present in fractions 51–115 (DP = 90–190) were estimated from the calibration curve shown in Fig. 9D. This plot shows the relationship between the DP of DMB-colominic acid (as retention time), the amount of oligo-/polySia in each column fraction (as fluorescence), and the gradient concentration of NH\(_4\)OAc. Prior to this work, no DP standards for polySia chains with DP >~90 were available for study.

Several conclusions can be drawn from these findings. First, it is evident that STX(s) is capable of synthesizing a mixture of polydisperse polySia chains on N-CAM with DPs ranging from ~2 and extending to ~400 in the reconstituted STX(s)/FcN-CAM assay system. Second,
Our preliminary studies with polysialylation of FnC-CAM by PST(s) revealed similar HPLC profiles as shown in Fig. 9 for STX(s), including the subpopulation of higher DP chains. Based on their HPLC profiles, for example, it was not possible to distinguish between the DP of polySia chains synthesized by STX(s) and PST(s). This finding is in contrast to that reported earlier by Angata et al. (38) and Kitazume-Kawaguchi et al. (42), who reported that PST synthesized longer chains than STX. As noted above, however, an accurate determination of chain length cannot be made on the basis of SDS-PAGE mobilities or HPLC profiling of polysialylated N-linked glycans after their release from N-CAM by PNGase F.

**Endo-N Treatment of Higher DPs**—To verify that the extended chains determined to be DP ~120–132 by DNA-Pac PA-100 column chromatography (Fig. 9A) were polySia, the column fraction corresponding to these DPs was collected, desalted, treated with Endo-N, and rerun on the same DNA-Pac PA-100 column. The limit digestion products of this depolymerase were shown previously to be DPs 1–6, thus making Endo-N a diagnostic enzyme for detecting and characterizing α2,8-linked polySia chains (65). As shown in Fig. 10A, ~85% of the Sia appeared as oligoSia with DPs 5 and 6. A control sample of 1 μg of colominic acid that was treated under the identical conditions also yielded the expected oligoSia profile (Fig. 10B).

On the basis of these findings, we conclude that the long polySia chains synthesized by STX(s) in the reconstituted polyS antigen assay and eluting from the DNA-Pac PA-100 column as extended chains were indeed polymers of polySia. Further, the extended polySia chains were also shown to contain Gal, based on detection of the Gal-AMAC derivative, as described under “Experimental Procedures” (results not shown).

**Endo-β-Galase Release and DP Determination of PolySia Chains from Polysialylated Membranes of Neuro2A Cell**—The applicability of our method to release polySia chains from biological membranes by Endo–β-Galase and to determine their DP by HPLC profiling was tested using Neuro2A cells. This mouse neuroblastoma cell line, which lacks polySia, was stably transfected with a full-length construct of PST, and from polysialylated membranes. We have also demonstrated that Endo–β-Galase can catalyze the release of polySia chains from COS7 cells co-transfected with FcNCAM and either PST or STX and from polysialylated NCAM expressed on embryonic chick brains.

In summary, the key aim of this study was to develop a new strategy to accurately determine the DP of polySia chains on N-CAM that avoided the acidic conditions inherent in the conventional DMB derivatization methods. Successful completion of this goal was achieved by using Endo–β-Galase to first release individual polySia chains from the N-glycans of N-CAM under nonhydrolytic conditions and then to determine their DP by high resolution profiling on a DNA-Pac PA-100 anion exchange column.
By quantitatively determining the amount of Sia in each column fraction after profiling by DMB derivatization, an enhanced sensitivity was obtained that was proportional to the number of Sia residues in each chain, since each monomeric unit was fluoroscently labeled. As shown in Fig. 9, an unexpected finding was the subpopulations of quite homogeneous chains with DPs in the range 150–180 and even extending up to DP 400. Whereas the number of chains that exceeded DP 140 amounted to ~3% of the total, the uniqueness of this higher subpopulation of chains was also seen in the chains released from Neuro2A cells (Fig. 11). It is also noteworthy that in the reconstituted system, the soluble constructs of STX and PST both have the capability to polymerize polySia chains that are remarkably longer than the DP 20 or 40 reported previously (38, 42). These data also show an overall polydisperse distribution of chains, which is contrary to the unimodal distribution of chains reported by other investigators using the acidic conditions of DMB derivatization (43, 49, 57).

Our strategy leading to the development of this method was to reduce the complexity of the problem when using the embryonic chick or rat brain models. The reason for this was that our initial studies had shown that we could release some of the polySia chains from polysialylated N-CAM, but we could not release them all. We therefore chose to use a simpler system consisting of soluble constructs of STX or PST and FcN-CAM as the exogenous acceptor substrate. Thus, whereas it is clear from our findings that Endo-β-Galase can catalyze the release of polySia chains from N-CAM expressed on both polysialylated recombinant and native membranes, we do not fully understand why we can release all of the polySia chains from some polysialylated N-CAMs (e.g. Fig. 7, lane 2) but not others, and this remains a relevant point for future investigation.

FIGURE 9. DP analysis of linear polySia chains released from polysialylated FcN-CAM by Endo-β-Galase. The DP of the linear polySia chains synthesized by STX(s) and released from FcN-CAM by Endo-β-Galase was determined by high resolution HPLC separation on an anion exchange DNA-Pac PA-100 column (run from 10 to 212 min), as described under "Experimental Procedures." Each five fractions were pooled, and the total amount of Sia in the pooled fractions was quantitatively determined by DMB derivatization, after hydrolysis of each separated fraction in 0.1 N trifluoroacetic acid (A). The insets show the DP for fraction numbers 1–5 (DP 2–5), 71–75 (DP 150 ± 2), 86–90 (DP 180 ± 2), and 106–110 (DP 400 ± 6). The relative amount of polySia in each fraction was determined by dividing the total amount of Sia in each fraction by the average DP for that fraction (B). C, the column profile of DMB-derivatized colominic acid that serves as a control for the DPs in fractions 1–50 (DPs 2–90). The ammonium acetate calibration curve (D) was used to approximate those polySia chains with DPs >90 present in fractions 51–115 (DP 90 to ~190). The DP value of ~400 was estimated from the relationship between the ammonium acetate gradient and the DP shown at the top of A.
with Endo-β-Galase activity. Importantly, however, since the major aim of this study was to develop an experimental strategy to determine the DP of polySia on N-CAM, the fact that some, but not all of the chains may be released from different N-CAMs does not preclude a successful completion of our goal, since the maximum DP observed after HPLC profiling still represents a minimum DP. Whereas some of the non-released chains could be longer or shorter, we simply do not know why, but that does not negate the DP findings.

An important corollary of this study was the finding that soluble constructs of STX or PST are capable of synthesizing full-length polySia chains, which means that the enzymes do not need to be intercalated into the membrane to catalyze the presumed processive mechanism of chain polymerization. What signals might regulate chain initiation, polymerization, and termination and how these processes might be influenced by the topological orientation of the transferases and their association with the fibronectin-like domains of N-CAM within the lumen of the Golgi (23, 34) are also important questions that await future studies. The outcome of such studies may have relevance to neural development and plasticity and perhaps to a better understanding of the malignant potential of those metastatic human cancer cells that express the polySia glycoptope (4).

Acknowledgment—We thank Daisuke Kato for technical support during the earlier phase of this study.

REFERENCES

1. Nakata, D., and Troy, F. A. (2004) Glycobiology 14, 1055
2. Troy, F. A. (1992) Glycobiology 2, 5–23
3. Troy, F. A. (1995) in Biology of the Sialic Acids (Rosenberg, A., ed) pp. 95–144, Plenum Press, New York
4. Troy, F. A. (2004) in Encyclopedia of Biological Chemistry (Lennarz, W. J., and Lane, M. D., eds) Vol. 3, pp. 407–414, Academic Press, Inc., New York
5. Kitajima, K., Inoue, S., Inoue, Y., and Troy, F. A. (1988) J. Biol. Chem. 263, 18269–18276
6. Kitaumi, 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
7. Becker, C. G., Becker, T., and Roth, J. (1993) Cell Tissue Res. 272, 289–301
8. Vinr, E. R., McCoy, R. D., Vollger, H. W., Willson, N. C., and Troy, F. A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1971–1975
9. Scheidegger, E. P., Lackie, P. M., Papay, J., and Roth, J. (1994) Lab. Invest. 70, 95–106
10. Hildebrandt, H., Becker, C., Gliuer, S., Roos, H., Gerardy-Schahn, R., and Rahmann, H. (1998) Cancer Res. 58, 779–784
11. Tanaka, F., Ohara, Y., Nakagawa, T., Kawanami, Y., Miyahara, R., Li, M., Yanagihara, K., Nakayama, J., Fujimoto, J., Enaka, K., and Wada, H. (2000) Cancer Res. 60, 3072–3080
12. Finne, J. (1982) J. Biol. Chem. 257, 11966–11970
13. Rothbard, J. B., Brackenbury, R., Cunningham, B. A., and Edelman, G. M. (1982) J. Biol. Chem. 257, 11064–11069
14. Finne, J., Finne, U., Deagostini-Bazin, H., and Goridis, C. (1983) Biochem. Biophys. Res. Commun. 122, 482–487
15. Zuber, C., Lackie, P. M., Catterall, W. A., and Roth, J. (1992) J. Biol. Chem. 267, 9965–9971
16. Close, B. E., and Colley, K. J. (1998) J. Biol. Chem. 273, 34586–34593
17. Close, B. E., Tao, K., and Colley, K. J. (2000) J. Biol. Chem. 275, 4484–4491
18. Mühlenhoff, M., Eckhardt, M., Bethe, A., Frosch, M., and Gerardy-Schahn, R. (1996) EMBO J. 15, 6943–6950
19. Angata, K., Suzuki, M., McAuliffe, J., Ding, Y., Hindsougl, O., and Fukuda, M. (2000) J. Biol. Chem. 275, 18594–18601
20. Mühlenhoff, M., Manegold, A., Windfuhr, M., Gotza, B., and Gerardy-Schahn, R. (2001) J. Biol. Chem. 276, 34066–34073
21. Reyaso-Paz, S., Pham, D.H., Zhang, L., Sato, C., Kitajima, K., and Troy II, F. A. (2001) Glycoconj. J. 18, 151
22. Suzuki, M., Angata, K., Nakayama, J., and Fukuda, M. (2003) J. Biol. Chem. 278, 49459–49468
23. Close, B. E., Mendiratta, S. S., Geiger, K. M., Broom, L. J., Ho, L. L., and Colley, K. J. (2003) J. Biol. Chem. 278, 30879–30885
24. Martin, C. M., Kedersha, N. L., Drapp, D. A., Tsui, T. G., and Colley, K. J. (1996) Glycobiology 6, 289–301
Degree of Polymerization of Polysialic Acid on N-CAM

25. Yabe, U., Sato, C., Matsuda, T., and Kitajima, K. (2003) J. Biol. Chem. 278, 13875–13880
26. Inoue, S., Poongodi, G. L., Suresh, N., Jennings, H. J., and Inoue, Y. (2003) J. Biol. Chem. 278, 8541–8546
27. Miyata, S., Sato, C., Kitamura, S., Toriyama, M., and Kitajima, K. (2004) Glycobiology 14, 827–840
28. Kurosawa, N., Yoshida, Y., Kojima, N., and Tsuji, S. (1997) J. Neurochem. 69, 494–503
29. Sevigny, M. B., Ye, J., Kitazume-Kawaguchi, S., and Troy, F. A. II (1998) Glycobiology 8, 857–867
30. Ong, E., Nakayama, J., Angata, K., Reyes, L., Katsuyama, T., Arai, Y., and Fukuda, M. (1998) Glycobiology 8, 415–424
31. Rutishauser, U., Watanabe, M., Silver, J., Troy, F. A., and Vimr, E. R. (1985) J. Cell Biol. 101, 1842–1849
32. Acheson, A., Sunshine, J. L., and Rutishauser, U. (1991) J. Cell Biol. 114, 143–153
33. Rutishauser, U., and Landmesser, L. (1996) Trends Neurosci. 19, 422–427
34. Mendiratta, S. S., Sekulic, N., Lavie, A., and Colley, K. J. (2005) J. Biol. Chem. 280, 32340–32348
35. Seidenfaden, R., Krauter, A., Schertzinger, F., Gerardy-Schahn, R., and Hildebrandt, H. (2003) Mol. Cell. Biol. 23, 5908–5918
36. Angata, K., and Fukuda, M. (2003) Biochimie (Paris) 85, 195–206
37. Angata, K., Suzuki, M., and Fukuda, M. (1998) J. Biol. Chem. 273, 28524–28532
38. Angata, K., Suzuki, M., and Fukuda, M. (2002) J. Biol. Chem. 277, 36808–36817
39. Seki, T., and Arai, Y. (1991) Anat. Embryol (Berl.) 184, 395–401
40. Seki, T., and Arai, Y. (1993) Neurosci. Res. 17, 265–290
41. Seki, T., and Rutishauser, U. (1998) J. Neurosci. 18, 3757–376642
42. Kitazume-Kawaguchi, S., Kabata, S., and Arita, M. (2001) J. Biol. Chem. 276, 15696–15703
43. Inoue, S., and Inoue, Y. (2001) J. Biol. Chem. 276, 31863–31870
44. Kudo, M., Kitajima, K., Inoue, S., Shiokawa, K., Morris, H. R., Dell, A., and Inoue, Y. (1996) J. Biol. Chem. 271, 32667–32677
45. Wahrer, M., Geyer, H., von der Ohe, M., Gerardy-Schahn, R., Schachner, M., and Geyer, R. (2000) Biochimie (Paris) 85, 207–218
46. Geyer, H., Bahr, U., Liedtke, S., Schachner, M., and Geyer, R. (2001) Eur. J. Biochem.
268, 6587–6599

47. Liedtke, S., Geyer, H., Wahrer, M., Geyer, R., Frank, G., Gerardy-Schahn, R., Zähringer, U., and Schachner, M. (2001) Glycobiology 11, 373–384
48. Häyrinen, J., Haseley, S., Talaga, P., Mühlenhoff, M., Finne, J., and Vliegenthart, J. F. (2002) Mol. Immunol. 39, 399–411
49. Inoue, S., Lin, S. L., Lee, Y. C., and Inoue, Y. (2001) Glycobiology 11, 759–767
50. Hara, S., Takemori, Y., Yamaguchi, M., Nakamura, M., and Ohkura, Y. (1987) Anal. Biochem. 164, 138–145
51. Hara, S., Yamaguchi, M., Takemori, Y., Nakamura, M., and Ohkura, Y. (1986) J. Chromatogr. 377, 111–119
52. Lehman, M. A., and Gao, N. (2003) Glycobiology 13, 1–3
53. Rohr, T. E., and Troy, F. A. (1980) J. Biol. Chem. 255, 2332–2342
54. van Lenten, L., and Ashwell, G. (1971) J. Biol. Chem. 246, 1889–1894
55. Livingston, B. D., Jacobs, J. L., Glick, M. C., and Troy, F. A. (1988) J. Biol. Chem. 263, 9443–9448
56. Lin, S. L., Inoue, Y., and Inoue, S. (1999) Glycobiology 9, 807–814
57. Inoue, S., and Inoue, Y. (2001) Biochimie (Paris) 83, 605–613
58. Manzi, A. E., Higa, H. H., Diaz, S., and Varki, A. (1994) J. Biol. Chem. 269, 23617–23624
59. Angata, K., Yen, T. Y., El-Battari, A., Macher, B. A., and Fukuda, M. (2001) J. Biol. Chem. 276, 15369–15377
60. Close, B. E., Wilkinson, J. M., Bohrer, T. J., Goodwin, C. P., Broom, L. J., and Colley, K. J. (2001) Glycobiology 11, 997–1008
61. Lin, S. L., Inoue, Y., Kitazume-Kawaguchi, S., Lennarz, W. J., Sevigny, M. B., Troy, F. A., and Inoue, S. (1999) in Sialobiology and Other Novel Forms of Glycosylation (Inoue, Y., Lee, Y. C., and Troy, F. A., eds) pp. 81–87, Gakushin Publishing Co., Osaka, Japan
62. Zhang, Y., Inoue, Y., Inoue, S., and Lee, Y. C. (1997) Anal. Biochem. 250, 245–251
63. Inoue, S., Lin, S. L., and Inoue, Y. (2000) J. Biol. Chem. 275, 29968–29979
64. Nakagawa, H., Yamada, T., Chien, J. L., Gardas, A., Kitamikado, M., Li, S. C., and Li, Y. T. (1980) J. Biol. Chem. 255, 5955–5959
65. Hallenbeck, P. C., Yu, F., and Troy, F. A. (1987) Anal. Biochem. 161, 181–186