Characterization of the Major Core Structures of the α2→8-linked Polysialic Acid-containing Glycan Chains Present in Neural Cell Adhesion Molecule in Embryonic Chick Brains*

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To gain more insight into the possible functional significance of the core glycan chain(s) on which polysialylation takes place in polysialic acid (poly-Sia)-containing glycoproteins, the structure of the core glycans in the embryonic form of chick brain neural cell adhesion molecule (N-CAM) was examined using chemical and instrumental techniques. The following new structural features, which had not been reported by the early pioneering study by Finne (Finne, J. (1982) J. Biol. Chem. 257, 11906–11970), were revealed (Structure I). (i) Two distinct types of multiantennary N-linked glycans, i.e. tri- and tetra-antennary structures, are present; (ii) an α1-6-linked fucosyl residue is attached to the proximal GlcNAc residue of the di-N-acetylated chains; (iii) the action of GlcNAc-transferase V, which catalyzes the attachment of the β(1→6)-linked GlcNAc residue on the (1→6)-α-linked mannose (Man) arm, appears to be essential for polysialylation to occur on the core glycan chain, is suggested by the fact that the Man residue α1→6-linked to the β-linked Man residue is invariably substituted by the GlcNAc residue; (iv) both type 1 (Galβ1→3GlcNAc) and type 2 (Galβ1→4GlcNAc) glycans are present in the peripheral portion of the core glycan structure. An extended form of the type 2 chain, i.e. Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc, is also expressed on the (1→3)- and (1→6)-α-linked Man arms; (v) on average about 1.4 mol of sulfate is attached to the type 2 N-acetyllactosamine chain(s), where in the extended form the sulfate group is probably substituted at the O-3 position of the outmost GlcNAc residue, i.e. Galβ1→4(HSO3-3)GlcNAcβ1→3Galβ1→4GlcNAcβ1→Man.

It is possible that the unusual structural features identified in this study might play a role in the initiation of polysialylation and our data should facilitate future research regarding the signals that control polysialylation.

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Neural cell adhesion molecule (N-CAM) is a widely distributed cell-surface glycoprotein, which mediates and regulates various cell-cell interactions (1). Multiple molecular forms of N-CAM are now known to be expressed from a single copy gene, depending on spatiotemporal stages of the cells (1). The molecular diversity is produced by various modifications. One is due to an alternative splicing, which gives rise to three major forms of N-CAM with different membrane-anchoring modes, whose extracellular domains contain a tandem alignment of immunoglobulin-like (Ig) and fibronectin-type (III) domains (1). Modification by glycosylation, sulfation, and phosphorylation of the protein core also results in great molecular diversity and consequent functional differences (1–4). Addition of polysialic acid (polySia), which is a unique homopolymer of α2→8-linked sialic acid (Sia), is the most important modification of N-CAM. Expression of polySia chains on N-CAM is developmentally regulated and negatively affects the adhesive properties of the cells (5, 6). Polysialylation occurs on N-glycan chains in the fifth Ig domain, where two of the three N-glycosylation sites have been shown to be polysialylated (7).

Interestingly, in embryonic vertebrate brain, N-CAM is the major carrier protein of polySia chains (8). The question why N-CAM is selectively polysialylated in embryonic brains remains unelucidated. It is possible that a particular protein sequence may play a role in determining the expression of polysialyl units on certain glycoproteins, as is the case with mannose-6-phosphate-bearing lysosomal enzymes (9) and 4-O-sulfated GalNAc terminated glycoprotein hormones (10). In a recent report on polysialylation of N-CAM, the importance of the domain organization of the protein was suggested, although the involvement of a specific protein sequence determinant was not ruled out (7). Alternatively, it is also conceivable that the core glycan structure codes a signal for initiation of polysialylation. For example, the sialotransferase responsible for 4-O-sulfation of terminal GalNAc is known not to recognize the peptide sequence of the acceptor glycoprotein hormones (11). Thus, a cryptic signal, which triggers initiation of polysialylation, could be present in the core glycan chain.

We have recently demonstrated that biosynthesis of poly-

1 The abbreviations used are: N-CAM, neural cell adhesion molecule; α2→6-ST, α2→6-sialyltransferase; α2→8-ST, α2→8-sialyltransferase; α2→8-polyS, α2→8-polysialotransferase; Endo-PSSg, endolysidase- treated PSSg; Ig, immunoglobulin; FAB-MS, fast atom bombardment mass spectrometry; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; Sia, sialic acid; oligoSia, oligosialic acid; polySia, polysialic acid; PSSg, polysialyl glycopeptide fraction prepared from embryonic chick brain homogenate; TLC, thin-layer chromatography; TOCSY, total correlation spectroscopy; TBA, thioarbitu-
Sulfated Core N-Glycan Chains in N-CAM

The overall structure of \( \alpha_2 \rightarrow 8 \)-linked polySia chain-containing tri- and tetraantennary glycans present in the chick embryonic brain N-CAM molecule.

PolySia biosynthesis of polySia chains, and a series of transferases are probably involved besides those needed for core glycosylation. Much effort has been devoted to clarifying the biosynthetic mechanism of polySia chain formation in embryonic brains using partially purified enzyme preparations, and it is now known that the developmentally dependent and down-regulation of expression of enzyme activity parallels the polySia epitope expression (14, 15). Recently, several reports on expression cloning of \( \alpha_2 \rightarrow 8 \)-polySTs from different animal origins have appeared, permitting elucidation of regulatory mechanisms of polySia expression using molecular biological approaches (16–21). At least two distinct types of \( \alpha_2 \rightarrow 8 \)-polySTs were shown to exist in rat brain (20). However, it is still unclear how critical these enzymes are in the \textit{in vivo} biosynthesis of polySia chains, and nothing is known about their substrate specificities or whether such enzymes are regulated by particular protein sequences or glycan motifs.

To address these important problems, we are determining detailed structures of the glycan chains present in N-CAM molecules. In this paper we report the results of our studies on the structural determination of the polysialylated glycan chain(s) present in the embryonic form of chicken brain N-CAM. In 1982, Finne (22) reported some evidence suggesting the presence of both tri- and tetraantennary structures in the polysialylated glycan chains of N-CAM from fetal rat brains, although no further details were described. The present study has revealed several new structural features, not reported by the early pioneering work by Finne (22), which include some possible candidates for polysialylation signals.

**Preparation of Polysialyl Glycopeptide Fraction (PSgp)**

In one experiment, 100 brains (40 g) were homogenized in 120 ml of 10 mM Tris-HCl, pH 8.0, and mixed with 320 ml of methanol and 160 ml of chloroform by stirring for 30 min. After centrifugation at 14,000 rpm at 4°C for 20 min, the pellet was suspended in 300 ml of 10 mM Tris-HCl (pH 8.0)/methanol/chloroform (3:8:4, v/v), stirred for 30 min, and centrifuged. The pellet was suspended in 100 ml of ethanol and filtered to remove chloroform and methanol. The residue (about 15 g) was suspended in 380 ml of 0.1 mM Tris-HCl, pH 8.0, containing 10 mM CaCl\(_2\), and incubated under toluene with 0.2 g of Actinase E (Kaken Co., Ltd., Tokyo, Japan) at 37°C. After 24 and 48 h, 0.2 g each of Actinase E was added. After a 72-h incubation, the digest was centrifuged at 12,000 rpm at 4°C.

The supernatant prepared from 500 brains was applied to a column of DEAE-Sephadex A-25 (equilibrated with 10 mM Tris-HCl, pH 7.0), and followed by stepwise elution with 1.0 liter each of 0.2, 0.6, and 1.0 mM NaCl in the equilibration buffer. The 0.6 mM NaCl fraction was diluted with 2.0 liters of 0.1 mM Tris-HCl, pH 7.0, and applied to a DEAE-Sephadex A-25 column, eluted first with 0.2 mM NaCl in the same buffer, and next with 900 ml of a linear gradient of 0.2–0.7 mM NaCl in the same buffer. Elution profile was monitored by the TBA method (25, 26) for sialic acid. The fractions were also tested for polySia by measuring the rate of acid hydrolysis as described below. The pooled fraction positive for poly-Sia was concentrated and applied on a Sephacryl S-200 column. Molecular weight markers used were dextran (Sigma; \( M_\text{r} \), 487,000, 72,200, 39,000, and 9,400) and galactose. The polySia-positive fraction was dialyzed and subjected to DEAE-Sephadex A-25 chromatography. The column was eluted first with the equilibration buffer, and then with 900 ml of linear gradient of 0–0.7 mM NaCl in the same buffer. The pooled polySia-positive fraction was desalted.

**Preparation of Asialo-PSgp and Endosialidase-treated PSgp, Endo-PSgp**

PSgp (3.6 mg as Neu5Ac), prepared from 450 brains, was digested with 600 milliunits of \textit{Arthrobacter ureafaciens} exosialidase (Nacalai Co., Kyoto, Japan) at 37°C for 72 h (27). Sia released was removed by Sephadex G-25 gel filtration. The flow-through glycopeptide fraction was treated with 0.1 mM NaOH for 30 min, neutralized with 0.1 mM HCl, and redigested with 75 milliunits of the sialidase at 37°C for 3 h. The digest was chromatographed on a Sephadex G-50 column (eluted with 50 mM \( \text{NH}_4\text{HCO}_3\)) and the asialo-glycopeptide fraction, asialo-PSgp, was desalted. Asialo-PSgp was applied on a DEAE-Sephadex A-25 column, and eluted first with 2 ml of 10 mM Tris-HCl, pH 7.0, and next with 2 ml of 0.3 mM NaCl in the same buffer. The 0.3 mM NaCl fractions were pooled, rechromatographed on a Sephadex G-50 column, and desalted.

PSgp (4 mg as Neu5Ac) from 500 brains was digested with total of 750 milliunits of Endo-N at 37°C for 72 h in 20 mM Tris-HCl, pH 7.4. During incubation an aliquot was examined at every 12 h for the absence of polymer by TLC (see below), and the enzyme (125 milliunits each) was added to the reaction mixture. Digestion continued until no further change in chain length of the released oligoSia was confirmed after prolonged incubation with additional enzyme. Endo-PSgp was separated from oligoSia by a Sephadex G-50 column and desalted.

**Identification and Quantitation of O-Acetyl Neu5Ac in Polysia Chain of PSgp**

The oligoSia fraction obtained by Endo-N treatment of PSgp was incubated in 0.01 M trifluoroacetic acid at 70°C for 30 min and applied on a Sephadex A-25 column (equilibrated with 10 mM Tris-HCl, pH 7.0). The column was eluted with 5 ml each of 0.05, 0.2 mM NaCl in the same buffer.

**Materials**

Embryonic chick brains were excised from 3200 14-day-old embryos (amber) and stored at –80°C. Endo-N (23) and anti-poly(\( \alpha_2 \rightarrow 8 \))-linked Neu5Ac) H.46 (24) were kindly provided by Dr. Frederic A. Troy II (University of California, Davis) and Dr. John B. Robbins (National institutes of Health, Bethesda, MD), respectively.

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**Structure I.** The overall structure of \( \alpha_2 \rightarrow 8 \)-linked polySia chain-containing tri- and tetraantennary glycans in the chick embryonic brain N-CAM molecule.
buffer. The 0.05 M NaCl fraction that contained Sia was desalted and subjected to preparative TLC. The Sia fraction was spotted on a 0.2-mm-thick silica gel plate (Kiesel gel 60, Merck) and developed in 1-butanol/1-propanol/water (5:10:3, v/v/v) (28). The slit of the plate was visualized by the resorcinol method (29), and the band corresponding to O-acetylated Neu5Ac was extracted with 10% ethanol for FAB-MS measurement.

For quantifying O-Ac Neu5Ac, the oligoSia fraction was hydrolyzed as described above, and O-Ac Neu5Ac and Neu5Ac obtained by preparative TLC were analyzed by the TBA method (25, 26). Identification and quantitation of O-Ac Neu5Ac in PSgp were also made by fluorometric HPLC essentially according to the method of Hara et al. (30). A glycoprotein fraction isolated from carp eggs was used as a source for Neu5Ac. The reaction mixtures were analyzed by the TBA method (25, 26). Identification and quantitation of Neu5Ac in PSgp were also made by fluorometric HPLC essentially according to the method of Hara et al. (30).

Determination of Sia and Polysialic Acid

Sia was determined by the mild acid hydrolysis-subsequent mild methanolysis/GLC (32). The presence of polySia was analyzed by the following methods.

Acid Hydrolysis Rate Measurement—Each sample (1.6 μg as Neu5Ac) was hydrolyzed in 0.05 M trifluoroacetic acid at 80°C for 0 to 3 h and released Sia was determined by the TBA method. The rate of hydrolysis of α2,8-linked polySia is shown to be significantly slower than that of α2,3- and α2,6-linked linkages of Sia. Incubation periods necessary for completion of hydrolysis were 3 h for polyo2,8-Sia and 30–45 min for α2,3- and α2,6-sialosides (33).

Reactivity to Anti-poly(α2,8-Neu5Ac), H.46—Reactivity with H.46 antibodies was tested by the Ouchterlony double immunodiffusion method (34).

Mild Acid Hydrolysis-TLC—Sample (10 μg of Neu5Ac) was partially hydrolyzed in 0.05 M trifluoroacetic acid at 80°C for 15 min. The hydrolysate was analyzed for formation of oligoSia by TLC (see below).

TLC Analysis

Samples were spotted on 0.2-mm-thick silica gel plates (Kiesel gel 60, Merck), and developed in 1-propanol/25% ammonia/water (6: 1: 2.5, v/v). The plate was visualized by the resorcinol reagent (33).

Chemical Analysis

Sia was quantitated by the TBA method and the resorcinol method (25, 26, 29). Carbohydrate composition and amino acid analyses were carried out as previously reported (35, 36). Methylation analysis of glycopeptides and oligosaccharides was carried out according to Anumula and Taylor (37). Partially methylated alditol and hexosaminotol acetates were quantitated by GLC analysis (38). Sulfate ion was determined by the HPLC analysis of acid hydrolysate of samples. Five to 10 nmol of each sample were hydrolyzed in vacuo in 6 M HCl at 110°C for 24 h and applied to a TSK gel IC-anion PW column, which was equilibrated with 0.5 M sodium phosphate, pH 7.0, and kept in the dark at room temperature. After 6 h the reaction was stopped by adding 10 μl of 2 M ethylene glycol and stood for 30 min. The samples thus obtained were subjected to carbohydrate analysis. Asialo fetuin GP-I (40) and A-1 (41) were used as control.

Salmonella typhimurium Exosialidase Digestion

Endo-PSgp or PSgp (0.5 to 1 nmol) was digested with 0.5 unit of Salmonella typhimurium exosialidase (Takara Co., Kyoto, Japan) in 100 μl of 50 mM sodium acetate, pH 5.5, at 37°C for 30 min.

FAB-MS Spectrometry

For O-Ac Neu5Ac, the perdeuterioacetylated sample was prepared for FAB-MS (42). The perdeuterioacetylated sample was dissolved in 10 μl of methanol, and a 1-μl aliquot was added to the monothioglycerol matrix. The FAB mass spectrum was recorded using a VG Analytical ZAB-2SE FPD mass spectrometer fitted with a cesium ion gun operated at 20–25 kV. Data acquisition and processing were performed using the VG Analytical Opus software. Permethylated derivatives of Smith degradation products of Endo-PSgp were similarly analyzed by FAB-MS.

500-MHz 1H NMR Spectroscopy

One-dimensional and two-dimensional 500-MHz 1H NMR spectra were measured on a Bruker AMX-500 NMR spectrometer at 23°C. Two-dimensional TOCSY was determined using a MLEV-17 mixing sequence of 100 and 50 ms. Chemical shifts are expressed in parts/million (ppm) relative to the methyl signal of sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d4.

RESULTS

Preparation of Polysialylated Glycopeptides, PSgp

A fraction exhibiting a slow rate of hydrolysis of interketo-sidic linkages typical for that of α2–8-linked polysialic acid chains was eluted under a single sharp peak at about 0.45 M NaCl on DEAE-Sephadex A-25 chromatography of the Actinase E digest of delipidated embryonic chick brain homogenate. This fraction was eluted from a Sephacryl S-200 column in the molecular weight region from 6,500 to 20,000. The pooled fraction was subjected to DEAE-Sephadex A-25 rechromatography and named as PSgp. The isolation of PSgp was confirmed by the binding activity with H.46 antibody and susceptibility to Endo-N digestion (data not shown).

Preparation of Endo-N-digested PSgp, Endo-PSgp

Exhaustive digestion of PSgp with Endo-N gave the glycopeptide fraction (Endo-PSgp) and free oligoSia with DP ranging from 1 to 9, which were detected by TLC analysis. The Endo-PSgp, Kav = 0.24, was separated from the oligoSia fraction (Kav = 0.28).
Sulfated Core N-Glycan Chains in N-CAM

Endo-PSgp contained Asx and Ser in a molar ratio of 1:0.61 relative to 3 mol of Man (Table I) and the N-terminal amino acids of Endo-PSgp were determined to be Asx and Ser in a molar ratio of 1:0.85. These results suggested that Endo-PSgp consisted of a 1:0.85 mixture of glycosaparagine and glycopeptide having ±Ser-Ser-Asn sequence, in which the glycan chain was attached to the Asn residue. Polysialylated glycan chains have been shown to be linked to one or more glycosylated Asn residues present in the fifth immunoglobulin-like domain of N-CAM (7), and the Ser-Ser-Asn sequence is located at the second site of glycosylation. Thus, at least 46% of the polySia-glycan chains are attached to the second site. No information on the possible polysialylation of the other two glycosylation sites was provided by the above data, but our results are not inconsistent with the recent report of mutant N-CAM expression experiments on transfected cells, where it is proposed that the second and third sites are heavily polysialylated (7).

Preparation of Asialo-PSgp

Extensive removal of the Sia residues by digestion of PSgp was only attained after mild alkaline treatment, because the presence of O-Ac Neu5Ac residues in the polySia chains prevented complete digestion (see below). Asialo-PSgp, completely devoid of Neu5Ac, was obtained at $K_{av} = 0.40$ on Sephadex G-50 chromatography, and applied on a DEAE-Sephadex A-25 column. No carbohydrate component was detected in the flow-through fraction and the retarded fraction contained asialo-PSgp, thus indicating that asialo-PSgp contained some anionic residues in the glycan chains, because it was composed of neutral sugars, Ser, and Asn (Table I). Inorganic anion analysis showed the presence of 1.6 mol of sulfate ion/3.0 mol of Man residues in asialo-PSgp (Table I) and that no phosphate ion was present. Sulfate was also detected in Endo-PSgp.

Methylation Analysis of Endo-PSgp, Asialo-PSgp, and Desulfated Asialo-PSgp

The results of methylation analysis of Endo-PSgp, asialo-PSgp, and desulfated asialo-PSgp are summarized in Table II. One residue each of 2,6-Man and 3,6-Man and in total one residue of 2-Man and 2,4-Man were detected in these three samples. These results, together with the $\text{H}^1$ NMR data as shown below, are consistent with a proposed structure consisting of an almost equimolar mixture of the following two structures (Structure II).

1.4 mol of t-Gal and 4.0 mol of 3-Gal were present in Endo-PSgp. Based on the known substrate specificity of Endo-N (23), the presence of t-Gal in Endo-PSgp indicated that these Gal residues should have been non-sialylated in the intact glycan. Desialylation of Endo-PSgp resulted in the decrease of about 2 mol of 3-Gal with a concomitant increase of t-Gal, indicating that Neu5Ac residues are linked to the O-3 position of the Gal residues. About 1 mol of 3-Gal persisted on de-sialylation and subsequent desulfation of Endo-PSgp, indicating the presence of a non-sialylated and non-sulfated internal Gal residue. As shown below, this can be attributed to an extended form of the type 2 N-acetyllactosamine structure.

In Endo-PSgp and asialo-PSgp, t-Fuc was detected in the same amount as 4,6-GlcNAc, suggesting the presence of the Fuc1→6(GlcNAc4→4)GlcNAc$\rightarrow$ sequence, which was also supported by the $\text{H}^1$ NMR spectral analysis (see below). On desulfation of asialo-PSgp, 0.23 mol (or 1.4 mol for corrected value)$^a$ of 3,4-GlcNAc and 0.4 mol of 4,6-GlcNAc disappeared with a concomitant increase of 1.7 mol of 4-GlcNAc. No change occurred in 3-GlcNAc or substitutions on Gal and Man residues. The mild methanolysis used for desulfation also resulted in defucosylation, and the disappearance of 0.4 mol of 4,6-GlcNAc was accompanied by a corresponding 0.3-mol loss of t-Fuc, which was again compatible with the presence of the Fuc1→6(GlcNAc4→4)GlcNAc$\rightarrow$ sequence in asialo-PSgp. Thus, the loss of 3,4-GlcNAc and the major increase in 4-GlcNAc were attributable to desulfation, indicating that sulfate groups reside in position O-3 of GlcNAc residues.$^a$ Notably, 1–2 mol of 3-GlcNAc were detected in all three samples and were assigned to the type 1 sequence, Gal1→3GlcNAc1→, as shown below.

TABLE I

| Chemical composition of PSgp, Endo-PSgp, and asialo-PSgp |
|-----------------|-----------------|-----------------|
|                 | PSgp            | Endo-PSgp       | Asialo-PSgp     |
| Fuc             | 0.80            | 1.2             | 0.51$^a$        |
| Man             | 3.0             | 3.0             | 3.0             |
| Gal             | 6.3             | 5.4             | 5.0             |
| GalNAc          | 1.2             | 6.5             | —               |
| GlcNAc          | 6.5             | 7.1             | 6.5             |
| Neu5Ac          | 58$^b$          | 3.9             | —               |
| Asx             | 1.0$^d$         | 1.0             | ND              |
| Ser             | 3.6             | 0.61            | ND              |
| Sulfate         | ND$^c$          | 1.4             | 1.6             |

$^a$ Partial hydrolysis of Fuc residue occurred during exosialidase digestion at pH 4.8 at 37 °C for 72 h.
$^b$ ND, not determined; —, not detected.
$^c$ Neu5Ac was quantitated by the resorcinol method. The value was ranging from 33 to 175 (on average 58) among preparations.
$^d$ GlcNAc/Thr/Ala/Met/Leu/Lys (2.0:3.5:0.8:1.4:1.0:0.90:1.1, mol/mol) were also detected in PSgp.

TABLE II

| Methylation analysis of Endo-PSgp, asialo-PSgp, and desulfated asialo-PSgp |
|-----------------|-----------------|-----------------|
| Partially methylated alditol acetate | Endo-PSgp | Asialo-PSgp | Desulfated asialo-PSgp |
| 2,3,4-Tri-O-Me Fuc | 0.66            | 0.3            | —$^a$             |
| 2,3,4,6-Tetra-O-Me Gal | t-Fuc          | 1.4            | 3.4             |
| 2,4,6-Tri-O-Me Gal   | 3-Gal           | 4.0            | 1.5             |
| 3,4,6-Tri-O-Me Man   | 2-Man           | 0.53           | 0.63            |
| 3,6-Di-O-Me Man      | 2,4-Man         | 0.58           | 0.37            |
| 3,4-Di-O-Me Man      | 2,6-Man         | 0.96           | 1.0             |
| 2,4-Di-O-Me Man      | 3,6-Man         | 1.0$^f$        | 1.0$^f$         |
| 3,6-Di-O-Me GlcN(Me)Ac | 4-GlcNAc       | 3.8            | 3.1             |
| 4,6-Di-O-Me GlcN(Me)Ac | 3-GlcNAc       | 1.3            | 1.8             |
| 6-Mono-O-Me GlcN(Me)Ac | 3,4-GlcNAc    | 0.27 (1.6$^f$) | 0.2 (1.4$^f$)   |
| 3-Mono-O-Me GlcN(Me)Ac | 4,6-GlcNAc    | 0.60           | 0.4             |

$^a$ —, not detected.
$^b$ 2,4-Di-O-Me is set equal to 1.0 for each sample.
$^c$ Corrected value; see Footnote 3 in the text.
Sulfated Core N-Glycan Chains in N-CAM

Table III
Carbohydrate compositional changes of asialo-PSgp on periodate oxidation/Smith degradation experiments

| Component | Asialo-PSgp | IO_4-treated asialo-PSgp | IO_4-treated sm-asialo-PSgp |
|-----------|-------------|--------------------------|-----------------------------|
| Fuc       | 0.51        | —                        | —                           |
| Man       | 3.0         | 2.3                      | 0.4                         |
| Gal       | 5.0         | 2.2                      | 2.0                         |
| GlcNAc    | 6.9         | 6.9                      | 2.0                         |

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Additional points:
- The sulfated core N-glycan chains consist of 0.9 mol of Neu5Ac
- The terminal sequences of sialylated antennae can be considered to consist of 0.9 mol of Neu5Ac/inter-or proximal Neu5Ac ratio is 2.4:1.5 (mol/mol). Therefore, the terminal sequences of sialylated antennae can be considered to consist of 0.9 mol of Neu5Ac.0263Gal and 1.5 mol remained unchanged, indicating that the distal Neu5Ac/inter-or proximal Neu5Ac ratio is 2.4:1.5 (mol/mol). Therefore, the terminal sequences of sialylated antennae can be considered to consist of 0.9 mol of Neu5Ac.

Table IV
Carbohydrate composition and methylation analyses of IO_4-treated Endo-PSgp and Smith degradation products of Endo-PSgp

| Component | Endo-PSgp | IO_4-treated Endo-PSgp | Smith degradation products |
|-----------|-----------|------------------------|-----------------------------|
| Fuc       | 1.2       | —                      | —                           |
| Man       | 3.0^a     | 2.2                    | 0.32                        | 1.2                         |
| Gal       | 5.4       | 4.3                    | 1.1                         | 1.8                         |
| GlcNAc    | 7.1       | 7.1                    | 2.5                         | 3.4                         |
| Neu5Ac    | 3.9       | 1.5                    | —                           | —                           |
| t-Fuc     | 0.66      | ND^b                   | —                           | —                           |
| t-Man     | —         | ND                     | <0.1                        | —                           |
| 2-Man     | 0.53      | ND                     | —                           | —                           |
| 3-Man     | —         | ND                     | 0.1                         | 0.5                         |
| 2,4-Man   | 0.58      | ND                     | 0.13                        | 0.55                        |
| 2,6-Man   | 0.96      | ND                     | —                           | —                           |
| 3,6-Man   | 1.0       | ND                     | —                           | —                           |
| t-Gal     | 1.4       | ND                     | 1.8                         | 1.9                         |
| 3-Gal     | 4.0       | ND                     | —                           | 1.1                         |
| t-GlcNAc  | —         | ND                     | 0.75                        | 0.54^c                      |
| 4-GlcNAc  | 3.8       | ND                     | 0.75                        | 0.53^c                      |
| 3-GlcNAc  | 1.3       | ND                     | 0.33                        | 0.11^c                      |
| 3,4-GlcNAc| 0.27      | ND                     | —                           | —                           |
| 4,6-GlcNAc| 0.80      | ND                     | —                           | —                           |

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Additional notes:
- a ND, not determined; —, not detected.
- Values are expressed as molar ratios relative to Man in Endo-PSgp set equal to 3.0 mol. Each component sugar was quantitated by the GLC method.
- The sum of t-, 4-, and 3-GlcNAc amounts was smaller than that from the GLC analysis, possibly due to the reason described in Footnote 5.

Periodate Oxidation and Smith Degradation of Asialo-PSgp

To obtain information on the internal carbohydrate chain sequence, periodate oxidation/Smith degradation experiments were carried out. Periodate oxidation of asialo-PSgp resulted in complete destruction of Fuc and a decrease of about 3 mol of Gal (Table III), suggesting that Fuc and about 3 mol of Gal were located at the nonreducing termini, consistent with the methyllysis analysis (see above). These results are also consistent with the linkage analysis that indicated some internal 3-Gal residues, because about 2 mol of Gal remained oxidized. These Gal residues survived during Smith degradation and subsequent periodate oxidation (see IO_4-treated sm-asialo-PSgp in Table III), whereas GlcNAc residues, on the other hand, decreased (Table III), suggesting that peripheral GlcNAc residues in Man-core was partial for asialo-PSgp as well as control asialobiantennary N-glycan (41).

Periodate Oxidation and Smith Degradation of Endo-PSgp

Periodate oxidation of Endo-PSgp resulted in decrease of about 1 mol of both Gal and Man, and complete disappearance of Fuc (Table IV). The decrease of Man and Fuc was the same as for asialo-PSgp (see above). The presence of 1.1 mol of periodate oxidizable Gal confirmed the existence of unsubstituted Gal residues in Endo-PSgp, which was also indicated by the methyllysis analysis (Table II). The periodate oxidation also gave information on the chain length of oligoSia attached in Endo-PSgp. Of the 4 Neu5Ac residues on average, present in Endo-PSgp, 2.4 mol of Neu5Ac were oxidized and 1.5 mol remained unchanged, indicating that the distal Neu5Ac/inter-or proximal Neu5Ac ratio is 2.4:1.5 (mol/mol). Therefore, the terminal sequences of sialylated antennae can be considered to consist of 0.9 mol of Neu5Ac/263Gal and 1.5 mol Neu5Ac2→8Neu5Ac2→3Gal, although we can not exclude the possible occurrence of a minute amount of trisialylated Gal. The presence of the Neu5Ac2→3Gal sequence was confirmed by Salmonella typhimurium sialidase digestion as is shown below. Assuming that the core glycans on average 3.5 antennae (see Structure II), the nonreducing termini of the antennae of Endo-PSgp were estimated to contain 1.1 mol of unsubstituted Gal and 2.4 mol of sialylated Gal (see Structure III), which is consistent with the ^3H NMR data of Endo-PSgp (see below).

To obtain more information on the core glycan structure, fragment oligosaccharides obtained by Smith degradation of...
Endo-PSgp were characterized by composition and methylation analyses as well as FAB-MS spectrometry. Endo-PSgp was subjected to periodate oxidation, BH₄⁻ reduction, and acid hydrolysis, and the products were separated into neutral and acidic fractions by a DEAE-Sephadex A-25 column. On acid hydrolysis, all Sia residues were cleaved off and the sulfate group was the only acidic moiety in the products. Forty-three percent of the GlcNAc residues were recovered in the neutral fraction and 57% in the acidic (sulfated) fraction.

Positive FAB-MS of the permethylated sample gave prominent A-type ions, where \( r = \) glycerol, and \( m/z = 260 \) for Hex-HexNAc. Other A-type ions were also observed at \( m/z = 1117 \) (Hex₃HexNAc), \( 1158 \) (Hex₂HexNAc), and \( 1362 \) (HexHexNAc), presumably derived from cleavage of the di-N-acetyltetrasaccharide sequence in the glycopeptide (or Asn). \( r = \) glycerol, and \( m/z = 260 \) for Hex-HexNAc, respectively, and at \( m/z = 584 \) and 464 for (Hex-HexNAc + H)+ and Hex-HexNAc, respectively (Table V). Other A-type ions were also observed at \( m/z = 1117 \) (HexHexNAc), \( 1158 \) (HexHexNAc), and \( 1362 \) (HexHexNAc), presumably derived from cleavage of the di-N-acetyltetrasaccharide sequence in the glycopeptide (or Asn).

The results of methylation analysis of the neutral fraction were \( t\)-Gal/t-GlcNAc/4GlcNAc/3-GlcNAc and 1.0:0.75:0.75:0.38 (mol/mol), and 2,4-Man and 3-Man were almost undetectable (Table IV). Yields of 4-GlcNAc (0.75 mol), 3-GlcNAc (0.38 mol), and \( t\)-Gal (1.1 mol) indicated the presence of Gal1→4GlcNAc and Gal1→3GlcNAc in a molar ratio of 2.1:1. \( t\)-GlcNAc was suggested to come from the unsubstituted terminal Gal-GlcNAc sequence, and the formation of 0.75 mol of \( t\)-GlcNAc from 1 mol of the glycan chain was consistent with data based on methylation analysis of Endo-PSgp (Table II) and on periodate oxidation of Endo-PSgp (1.1 mol of Gal were oxidized), both showing the presence of unsubstituted Gal. The linkage between the unsubstituted Gal and GlcNAc was not determined here, but it was presumed to be exclusively 1→3, when one considers the total amount of 3-GlcNAc in Endo-PSgp (1.3 mol) and a very low yield of 3-GlcNAc (0.1 mol) in the acidic fraction (see the following paragraph). Combining all of these data together, it was concluded that Endo-PSgp contains unsulfated carbohydrate sequences as shown in Structure IV. Values in brackets represent the relative amounts of these structures in 1 mol of core glycan chain, as shown in Structure IV.

**Salmonella typhimurium Exosialidase Digestion of Endo-PSgp and PSgp**

PSgp and Endo-PSgp were digested with the Neu5Acα2→3Gal-specific sialidase, and the release of free Neu5Ac was observed from Endo-PSgp, but not from PSgp. These results confirmed the presence of the Neu5Acα2→3Gal sequence in Endo-PSgp, and its absence in PSgp.

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*Man derivatives were detected on GLC in smaller amounts that expected from the composition. On methylation analysis, yields of partially methylated sugar alditol acetates derived from glycopeptides were empirically found to be lower than those from free glycan, possibly, at least in part, because of the difficulty in permethylation of amino acid residue(s) resulting in lowering the recovery of permethylated derivatives into organic phase after permethylation.*

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*Glycopeptides (or Asn) usually give major A-type ions from cleavage between the two GlcNAc residues of the core glycan.*
\[ \text{\(^1H\) NMR of Asialo-PSgp} \]

One-dimensional \(^1H\) NMR and two-dimensional TOCSY spectra of asialo-PSgp are shown in Fig. 1 (A and B). Proton resonances were assigned based on the two-dimensional TOCSY using two different mixing times and the previously reported data (e.g., 43, 44), and their assignments are summarized in Table VI. The residue numbering is shown in Structure VI. Based on chemical shifts and coupling constants of the H-1 residues in Table VI, respectively, assigned to H-1 and H-2 of the reported data. (TOCSY using two different mixing times and the previously arising from GlcNAc in a (Fig. 1) at 4.54 ppm corresponded to that in the Gal\(\beta\)1→4GlcNAc\(\beta\)1→Man sequence, and had cross-peaks centered at 3.72 ppm (H-2, H-3, and H-4), 3.59 ppm (H-5) and 3.92 ppm (one of the H-6 signals). The signal at 4.58 ppm was assignable to H-1 of the GlcNAc residue in the Gal\(\beta\)1→3GlcNAc\(\beta\)1→Man sequence, and gave several cross-peaks at 3.78–3.85 ppm, 3.55 ppm, 3.47 ppm, and 3.97 ppm. We were not able to assign all these signals, but the data were closely consistent with the reported data for the sequence: H-1, 4.59–4.62 ppm; H-2, 3.83–3.87 ppm; H-3, 3.80–3.84 ppm; H-4, 3.55–3.58 ppm; H-5, 3.48–3.50 ppm; H-6, 3.78–3.79 ppm; H-6', 3.92–3.94 ppm. A signal at 4.60 ppm was assigned to H-1 of the GlcNAc residue in the sequence of GlcNAc\(\beta\)1→3Gal\(\beta\)1→4GlcNAc\(\beta\)1→Man. This GlcNAc H-1 is known to resonate at lower field as compared with that of the GlcNAc residue in the sequence Gal\(\alpha\)1→4GlcNAc\(\beta\)1→3Gal\(\beta\)1→Man (4.60 ppm versus 4.54 ppm; reported in Ref. 44). No signal was observed at 4.70 ppm, where the GlcNAc H-1 in the Gal\(\beta\)1→4GlcNAc\(\beta\)1→3Gal\(\beta\)1→sequence is reported to resonate (44). Considering the presence of the Gal\(\beta\)1→4GlcNAc\(\alpha\)1→3Gal\(\beta\)1→sequence in a sulfated form in asialo-PSgp, the GlcNAc residue in this sequence is suggested to be invariably sulfated. From the area intensity of the H-1 of each peripheral GlcNAc residue in asialo-PSgp, the proportion of 4-GlcNAc, 3-GlcNAc, and 3-O-sulfated GlcNAc was estimated to be 1.8:1.5:1.5 (mol/mol), being consistent with the results of methylation analysis of asialo-PSgp (Table II).

Gal—A cluster of resonance signals centered at 4.45 ppm was assigned to H-1 of unsubstituted Gal residues, which were observed to a lesser extent on the spectrum of Endo-PSgp. These signals gave cross-peaks with three groups of signals at 3.92, 3.65, and 3.54 ppm, consistent with the previously reported data for this type of Gal residue. The cross-peak connecting 4.53 and 4.18 ppm signals suggested that these were possibly assignable to H-1 and H-4 of 3-O-substituted Gal, respectively, as was previously reported.

\[ \text{\(^1H\) NMR of Endo-PSgp} \]

The one-dimensional \(^1H\) NMR and two-dimensional TOCSY spectra are shown in Fig. 2, A and B, respectively. The spectra of Endo-PSgp are closely similar to those of asialo-PSgp, and the following points are noteworthy.

A cluster of signal peaks from about 4.45 ppm in the asialo-PSgp spectrum was diminished and shifted to about 4.54 ppm (Fig. 1A versus Fig. 2A), indicating that peaks at 4.54 ppm were assignable to H-1 of the Gal residue of the NeuAc(263)Gal\(\beta\)1→sequence. The resonance signals observed at 3.55, 4.11, and 3.96 ppm were, respectively, assignable to H-2, H-3, and H-4 of the sialylated GalS. The small resonance peaks remaining at 4.45 ppm on the spectrum of oligosaccharide unit required for sponge cell adhesion (H-1, 4.816 ppm; H-2, 3.843 ppm; H-3, 4.407 ppm; H-4, 3.629 ppm; H-5, 3.514 ppm; Ref. 45).

Other resonance signals assignable to the H-1 of peripheral \(\beta\)-GlcNAc residues were observed at 4.54, 4.58, and 4.60 ppm (Fig. 1A). The GlcNAc H-1 signal at 4.54 ppm corresponded to that in the Gal\(\beta\)2→4GlcNAc\(\beta\)1→Man sequence, and had cross-peaks centered at 3.72 ppm (H-2, H-3, and H-4), 3.59 ppm (H-5) and 3.92 ppm (one of the H-6 signals). The signal at 4.58 ppm was assignable to H-1 of the GlcNAc residue in the Gal\(\beta\)1→3GlcNAc\(\beta\)1→Man sequence, and gave several cross-peaks at 3.78–3.85 ppm, 3.55 ppm, 3.47 ppm, and 3.97 ppm. We were not able to assign all these signals, but the data were closely consistent with the reported data for the sequence: H-1, 4.59–4.62 ppm; H-2, 3.83–3.87 ppm; H-3, 3.80–3.84 ppm; H-4, 3.55–3.58 ppm; H-5, 3.48–3.50 ppm; H-6, 3.78–3.79 ppm; H-6', 3.92–3.94 ppm. A signal at 4.60 ppm was assigned to H-1 of the GlcNAc residue in the sequence of GlcNAc\(\beta\)1→3Gal\(\beta\)1→4GlcNAc\(\beta\)1→Man. This GlcNAc H-1 is known to resonate at lower field as compared with that of the GlcNAc residue in the sequence Gal\(\alpha\)1→4GlcNAc\(\beta\)1→3Gal\(\beta\)1→sequence is reported to resonate (44). Considering the presence of the Gal\(\beta\)1→4GlcNAc\(\alpha\)1→3Gal\(\beta\)1→sequence in a sulfated form in asialo-PSgp, the GlcNAc residue in this sequence is suggested to be invariably sulfated. From the area intensity of the H-1 of each peripheral GlcNAc residue in asialo-PSgp, the proportion of 4-GlcNAc, 3-GlcNAc, and 3-O-sulfated GlcNAc was estimated to be 1.8:1.5:1.5 (mol/mol), being consistent with the results of methylation analysis of asialo-PSgp (Table II).
Endo-PSgp were assignable to H-1 of unsubstituted gal residues, and the groups of cross-peaks at 3.92, 3.65, and 3.53 ppm also substantiated such assignment, thus confirming the presence of the unsubstituted terminal Gal residues in Endo-PSgp suggested by methylation analysis (see above).

Three pairs of H-3 proton chemical shifts of Neu5Ac were observed and assigned, based on the previous data (46), as (H-3eq, H-3ax) = (2.75 ppm, 1.79 ppm) for the terminal Neu5Ac residue in Neu5Acα263Gal1→ structure, (2.77 ppm, 1.75 ppm) for the distal Neu5Ac residue in oligoSia, and (2.66 ppm, 1.73 ppm) for the internal Neu5Ac residue including proximal Neu5Ac residue in oligoSia (see also Structure III). The H-3 proton area intensity of the terminal and distal Neu5Ac residues was almost twice as strong as that of the proximal Neu5Ac residue, which is in good agreement with the results from the periodate oxidation experiment of Endo-PSgp (see Structure II).

Detection and Identification of O-Acetylated Neu5Ac Residue in PolySia Chain of PSgp

On exosialidase digestion of PSgp, 68% of Neu5Ac residues were released after a 26-h incubation at 37 °C, while almost all Neu5Ac were released from PSgp pretreated with mild alkali (data not shown), suggesting the presence of alkali-sensitive modification on Neu5Ac residues in PSgp. TLC of the mild acid hydrolysate of the oligoSia fraction obtained by Endo-N treat-
Sulfated Core N-Glycan Chains in N-CAM

Summary of proton chemical shifts for each carbohydrate residue in asialo-PSgp

| Residue | Proton | Chemical shift (ppm) |
|---------|--------|---------------------|
| GlcNAc-1 | H-1 | 5.03<sup>a</sup> |
| | H-2 | 3.85 |
| | H-3 | 3.77 |
| | H-4 | 3.60<sup>b</sup> |
| GlcNAc-2 | H-1 | 4.67 |
| | H-2 | 3.74 |
| | H-3 | 3.58 |
| | H-4 | 3.59 |
| | H-5 | 3.58 |
| Man-3 | H-1 | 4.77 |
| | H-2 | 5.12 |
| | H-3 | 4.20 |
| | H-4 | 4.05<sup>c</sup> |
| | H-5 | 3.91<sup>c</sup> |
| Man-4<sup>c</sup> | H-1 | 4.86 |
| | H-2 | 4.08 |
| | H-3 | 3.86 |
| | H-4 | 3.38 |
| Fuc | H-1 | 4.87 |
| | H-2 | 3.79 |
| | H-5 | 4.13 |
| | Methyl | 1.19 |
| Peripheral GlcNAcs 3-O-sulfated | H-1 | 4.75 |
| | H-2 | 3.78 |

| Residue | Proton | Chemical shifts (ppm) |
|---------|--------|---------------------|
| GlcNAc-1 | H-3 | 4.32 |
| GlcNAc-2 | H-4 | 3.70 |
| Man-3 | H-5 | 3.58 |
| Man-4 | H-1 | 4.58 |
| Fuc | H-4 | 3.55 |
| Peripheral GlcNAcs 3-O-sulfated | H-1 | 4.60 |

<sup>a</sup> 5.03 ppm for glycosamniglycine, 5.05 ppm for glycopeptide.
<sup>b</sup> No Fuc residue on GlcNAc-1.
<sup>c</sup> 4.05 ppm for 2,4-Man, 3.91 ppm for 2-Man.
<sup>d</sup> This signal resonates at the down-field as compared to the reported value (4.46 ppm, Ref. 44), possibly because the penultimate GlcNAc residue is 3-O-sulfated.

### Structure VI. The residue numbering of the core trimannosyl di-N-acetychitobiosyl structure.

This study, we have examined the structure of the polysialylated glycopeptide (PSgp) derived from 14-day embryonic chick brain, which is present faster than Neu5Ac. This spot of modified Neu5Ac amounted to as much as 10% of total Neu5Ac in the oligoSia fraction and diminished after alkali treatment of the hydrolysate. In the positive mode FAB-mass spectrum of the deuterioacetylated derivative of the modified Neu5Ac fraction, a peak corresponding to [mono-O-Ac-tri-O-deuterioAc Neu5Ac + Na]<sup>+</sup> was detected at m/z 554.

HPLC analysis of the mild acid hydrolysate of PSgp revealed the presence of 7-O-Ac (about 1%), 8-O-Ac (about 1%), 9-O-Ac (about 3%), and 7(8),9-di-O-Ac (about 1%) Neu5Ac (Fig. 3).

**DISCUSSION**

In this study, we have examined the structure of the polysialylated glycocarboxyl peptide (PSgp) derived from 14-day embryonic chick brain, which is present faster than Neu5Ac. This spot of modified Neu5Ac amounted to as much as 10% of total Neu5Ac in the oligoSia fraction and diminished after alkali treatment of the hydrolysate. In the positive mode FAB-mass spectrum of the deuterioacetylated derivative of the modified Neu5Ac fraction, a peak corresponding to [mono-O-Ac-tri-O-deuterioAc Neu5Ac + Na]<sup>+</sup> was detected at m/z 554.

HPLC analysis of the mild acid hydrolysate of PSgp revealed the presence of 7-O-Ac (about 1%), 8-O-Ac (about 1%), 9-O-Ac (about 3%), and 7(8),9-di-O-Ac (about 1%) Neu5Ac (Fig. 3).

(a) Two distinct types of multiantennary structures, i.e. tri- and tetraantennary, are present as shown in Structure II, and on average 3.5 antennae are attached to the α-Man residues.

(b) An α1-6-linked fucosyl residue is attached to the proximal GlcNAc residue of the di-N-acetychitobiosyl unit.

(c) Notably, the mannose residue α-(1→6)-linked to the β-linked Man residue of the core is invariably 2,6-di-O-substituted by the GlcNAc residues, indicating the importance of GlcNAc transferase V, which catalyzes the attachment of the β-(1→6)-linked GlcNAc residue on the (1→6)-α-linked Man arm, for polysialylation to occur on the core glycann chain.

(d) The peripheral portion of the core glycann structure was found to be more complicated than recognized previously (22) and was revealed to contain both type 1 Galβ1→3GlcNAc and type 2 Galβ1→4GlcNAc sequences. Furthermore, an extended form of the type 2 chain, Galβ1→4-GlcNAc β 1→3Galβ1→4GlcNAc, was also shown to occur. These three structures are attached on the (1→3)- and (1→6)-α-linked Man arms in the proportion of 1:1: 1.8: 0.65. The presence of type 1 and the extended form of type 2 chains are unusual structural features in the core glycann of the embryonic N-CAM, and these unique features may possibly be relevant to initiation signal(s) for polysialylation.

(e) Most interestingly, the core oligosaccharide unit contains, on average, about 1.4 mol of sulfate attached to the O-3 position of the peripheral GlcNAc residues. Sulfate groups reside exclusively in the type 2 N-acetyllactosamine chain(s) and the extended form of N-acetyllactosamine chain, where the sulfate is probably located on the outermost GlcNAc residue, i.e. Galβ1→4(HSO3)GlcNAcβ1→3Galβ1→4GlcNAcβ1→Man, as evidenced from the data based on 1H NMR measurement. No sulfation was shown to occur on the type 1 chain, as expected if one considers that sulfotransferase and β1→3-galactosyltransferase compete for the same site, i.e. the O-3 position of GlcNAc residues of the acceptor glycann core since both sulfation and type 1 galactosylation take place at the O-3 of the GlcNAc residues.

Two major oligosaccharides were isolated from periodate oxidation/Smith degradation products of Endo-PSgp: one sulfated and the other unsulfated in a molar ratio of 1.73:1.66. Useful information about the glycann core came from the structural analysis of these oligosaccharides: (i) Galβ1→4(HSO3)-GlcNAcβ1→29%); (ii) Galβ1→4(HSO3)-GlcNAcβ1→3Galβ1→4GlcNAcβ1→19%); (iii) Galβ1→4GlcNAcβ1→14%); and (iv)
Galβ1→3GlcNAcβ1→(32%), as shown in Structures III and IV. Interestingly, at least one terminal residue of the antennae was found not to be sialylated, indicating that polysialylation occurs asymmetrically on the antennae. However, the biological significance, if any, and the biosynthetic mechanism of such asymmetric polysialylation remain to be elucidated. Nevertheless, inner carbohydrate structural elements for embryonic chick brain N-CAM may possibly contribute to the regulation of elongation of polySia chain(s).

It should be noted that our results showed that, after Endo-N digestion, only mono-, di-, and trisialyl groups were left on the core glycan chain. Particular attention will thus need to be given to the elucidation of polysialylglycan structures suggested by previous experiments using anti-polySia antibodies. Recently, we have examined the antigenic specificities of various anti-polySia antibodies, some of which appear to recognize oligoSia chains as short as di- or trisialyl sequences (46).

Sulfation is acknowledged as a biologically important modification of carbohydrate residues of glycoconjugates (45, 47–51), and sulfated glycan chains are known to participate in regulation of the lifetime of serum glycoprotein hormones (47), to constitute a ligand structure for certain receptors (48, 49), and to be involved in mediation of cell-cell adhesion (45, 50, 51). In vertebrate glycoproteins, 6-O-sulfated GlcNAc, 3- or 6-O-sulfated Gal residues are frequently found (52–54) and less prevalent but still important were 4-O-sulfated GalNAc residues (10, 11, 47). The present finding of 3-O-sulfated GlcNAc residue in the glycan core chain of polysialylated N-CAM from fetal chick brain is novel and unusual in vertebrate glycoproteins, although the presence of such unusual structural elements has been documented in vertebrate proteoglycans and in invertebrate glycons, both of which are of biological importance. Thus a pentasaccharide sequence containing a 3-O-sulfated N-sulfated GlcNAc residue in heparin is a structural unit required for the binding to antithrombin III (49) and HSO₃⁻→3GlcNAcβ1→3Fuc is a part of the carbohydrate unit present in the sponge cell surface polysaccharide necessary for cell aggregation (45).

Vertebrate N-CAM molecules are known to have sulfated carbohydrate units (2, 4, 51) mostly in the HSO₃⁻→3GlcAβ1→3Gal sequence as a part of the HNK-1 antigen (4, 51). We have evidence of the presence of 3-O-sulfated GlcNAc residues in pig brain N-CAM. Thus at least two novel types of sulfotransferase involved in the biosynthesis of 3-O-sulfated GlcNAc and 3-O-sulfated GlcA residues are considered to be expressed in vertebrate brains. Glycoprotein-specific sulfotransferases are unknown at present, although a sulfotransferase catalyzing the formation of 4-O-sulfated GalNAc residues on N-CAM may possibly contribute to the regulation of elongation of polySia chain(s).

FIG. 2. Two-dimensional TOCSY spectra at 500 MHz of Endo-PSgp in D₂O at 300 K. The regions of 3.3–5.2 ppm (A) and 1.5–3.5 ppm (B) are shown. The mixing time was 100 ms. a, Gal residue in Neu5Acα2→3Galβ1→ sequence; b, GlcNAc residue in HSO₃⁻→3Galβ1→4 GlcNAcβ1→ sequence; c, unsubstituted terminal β-Gal residue; d, a cross-peak between H-3eq and H-3ax of the distal Neu5Ac residue in oligoNeu5Ac structure; e, that of Neu5Ac residue in Neu5Acα2→3Galβ1→ structure; f, that of the internal Neu5Ac residues including proximal Neu5Ac residue in oligoNeu5Ac structure (see Structure III).

FIG. 3. HPLC of mild acid hydrolysat of PSgp (A) and carp egg glycoprotein (B). 1, Neu5Ac; 2, Neu5,7Ac₂; 3, Neu5,8Ac₂; 4, Neu5,9Ac₂; 5, Neu5,7(8),9Ac₃.

Y. Ono, K. Kitajima, S. Inoue, and Y. Inoue, unpublished results.
linked glycan chains has recently been identified (11). Much remains to be learned about the mode and importance of sulfation on GlcNAc residues of the core glycan chain, and experiments testing the effect of chloride-induced deprivation of sulfate donor on expression of polySia chain are under way in our laboratory.

Finally, the polysialyl chain of N-CAM is partially (5–10%) O-acetylated on either O-7 or O-9 and both O-7 and O-9 of interchain and nonreducing terminal Neu5Ac residues. A part, if not all, of the O-acetyl derivative as revealed in HPLC analysis can be accounted for by migration of the O-acetylglycosyl chain during hydrolysis and/or the derivatization prior to HPLC, although its natural occurrence cannot be ruled out. This is the first report of the presence of O-Ac groups on the N-CAM polySia chain, although polysialylated capsular polysaccharides from certain strains of Escherichia coli K1 are known to contain O-Ac groups at O-7 or O-9 of Neu5Ac residues, and the responsible O-acetyltransferase that transfers O-acetyl group from acetyl-CoA to polySia chain with DP more than 14 has been identified (55). The partially O-acetylated polySia chain of N-CAM was sensitive to Endo-N giving rise to free oligoSia chains of DP as large as about 9, which are longer than those from unsubstituted polySia chains. Thus, the polySia chain of N-CAM is considered to be sparsely O-acetylated. Endo-N recognizes homooligos of sialic acid with minimum DP 5 (23) and can access the intervening stretch of unsubstituted Neu5Ac residues. Alternatively, some clusters of O-acetylation might occur on a distal region of the polySia chain of DP > 55 (56), leaving the proximal region susceptible to Endo-N. A plausible function of O-acetylation is a termination signal for polySia chain elongation. O-acetylation of polySia would prevent α2-8-polysialyltransferase from acting as an acceptor substrate. Recently, we proposed a concept of termination of polySia elongation based on our biosynthetic studies on polySia formation, where KDNI capping of the polySia chain on the O-linked glycan chains of trout polysialoglycoprotein was demonstrated as a stop signal for the elongation reaction (13). More recently, 9-O-sulfation and 8-O-sulfation were proposed as the most likely termination signals in polySia elongation in sea urchin egg cell surface polySialglycoprotein (57) and in sperm surface oligo/polysialoglycosphingolipids (58), respectively.

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