Neuronal Differentiation-dependent Expression of the Disialic Acid Epitope on CD166 and Its Involvement in Neurite Formation in Neuro2A Cells*

Chihiro Sato‡§, Tsukasa Matsuda‡, and Ken Kitajima‡§¶

From the ‡Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University and the ¶Department of Animal Sciences, Division of Organogenesis, Nagoya University Bioscience Center, Nagoya 464-8601, Japan

Received for publication, June 18, 2002, and in revised form, August 28, 2002
Published, JBC Papers in Press, September 15, 2002, DOI 10.1074/jbc.M206046200

We previously demonstrated that α2,8-linked disialic acid (diSia) residues occur in several glycoproteins of mammalian brains (Sato, C., Fukuoka, H., Ohta, K., Matsuda, T., Koshino, R., Kobayashi, K., Troy, F. A., II, and Kitajima, K. (2000) J. Biol. Chem. 275, 15422–15431). The role of the diSia epitope on these glycoproteins is not known, whereas the importance of the diSia epitope on gangliolipids is well documented in neurite formation. In this study, we demonstrated that the diSia epitope (Neu5Acα2 → 8Neu5Acα2 → 3Gal) on glycoproteins, but not on gangliolipids, is involved in neurite formation in a mouse neuroblastoma cell line, Neuro2A, based on the following lines of evidence. First, the amount of diSia epitope on glycoproteins increased during retinoic acid-induced neurite formation. Second, retinoic acid treatment primarily increased the diSia epitope on a 100-kDa glycoprotein. We identified this protein as CD166 (SC1), an immunoglobulin superfamily cell adhesion molecule involved in neurite extension. Third, a monoclonal antibody against the diSia epitope specifically inhibited neurite formation of the diSia epitope on CD166.

Gangliosides are sialic acid-containing glycolipids that are abundant in brains (1, 2). α2,8-Linked disialic acid (diSia)-containing and trisialic acid-containing gangliosides (b and c series) in neuronal cells are considered to have an important role in neurite formation. Exogenous GD3 and GQ1c induce neurite sprouting and extension in a murine neuroblastoma cell line, Neuro2A, and several other types of neuronal cells (3–6), the diSia epitope either of the glycoprotein or the glycolipid might have an important role in some steps of neurite formation. Thus, the diSia epitope on gangliosides endogenously when neurite formation is induced by retinoic acid (5, 6). These observations led us to hypothesize that the diSia residue might be linked to glycoproteins and that such an epitope might be involved in neurite differentiation of Neuro2A cells.

Until recently, there has been little attention paid to the presence of di/oligoSia residues on glycoproteins, whereas the polysialic acid (polySia) chain (degree of polymerization ≥ 8) has been well studied in the neural cell adhesion molecule NCAM (7, 8) as well as in fish polysialoglycoprotein (9). PolySia in NCAM is involved in neural cell migration, axonal growth and path finding, synaptogenesis, and synaptic functions associated with learning and memory (8). Using recently developed highly sensitive techniques (10–12), we demonstrated that glycoproteins containing di/oligoSia groups with up to seven sialic residues occur in brain (10), and some other tissues (13–15) occur more frequently than previously recognized. It is thus hypothesized that these di/oligoSia moieties on glycoproteins have important functions similar to those proposed for gangliosides (10, 16).

Therefore, we searched for the diSia-containing glycoproteins in Neuro2A cells. In this study, we demonstrated the presence of the diSia epitope on glycoproteins, most prominently on O-linked glycans(s) of the 100-kDa glycoprotein (100-kDa-gp) during neurite formation, while this epitope was deficient in gangliolipids. We further identified the 100-kDa-gp as CD166 (SC1), a member of the immunoglobulin supergene family that is involved in heterophilic and homophilic interactions during neuritogenesis (17). The antibody against the diSia epitope inhibited neurite extension, indicating the importance of the diSia epitope of this glycoprotein in retinoic acid-induced neuronal differentiation. Considering these results together with the critical roles of diSia-containing gangliosides following exogenous addition to or expression in Neuro2A cells (3–6), the diSia epitope on the glycoprotein or the glycolipid might have an important role in some steps of neurite formation. Thus, the diSia epitope on gangliosides might share a common function with that of glycoproteins. We further demonstrated that the α2,8-sialyltransferase (ST8Sia III) is expressed in the Neuro2A cells and that its mRNA expression increases during neurite formation. Therefore, ST8Sia III is suggested to be the enzyme responsible for the biosynthesis of the diSia epitope on O-linked gangliosides. This finding is important because it shows a physiologic importance of ST8Sia III in the neuronal system.

EXPERIMENTAL PROCEDURES

Materials—NANase II (α2,3- and α2,6-specific sialidase) was purchased from Toyobo (Tokyo, Japan). Clostridium perfringens sialidase, acetylthiocholine, 5,5'-dithiobis-(2-nitrobenzoic acid), and BW284C51...
were purchased from Sigma. *Aerobacter ureafaciens* sialidase was purchased from Nacalai Tesque (Kyoto, Japan). BCA protein assay kit was purchased from Pierce (Pierce, IL). 1,2-Diamino-3,4-methylenedioxybenzene was purchased from Dojindo (Kumamoto, Japan). Peptide-N-glycanase was purchased from Takara (Takara, Japan). ECL reagents were purchased from Amersham Biosciences. Polyvinylidine difluoride (PVDF) membrane (Immobilon P) was a product of Millipore (Bedford, MA). Prestained molecular marker was purchased from Bio-Rad. Peroxidase-conjugated goat anti-mouse IgG + IgM was purchased from American Medical Systems (American Medical Systems, Inc., West Chester, PA), respectively. Peroxidase-conjugated rabbit anti-goat antibody was purchased from Santa Cruz Inc. (Santa Cruz, CA) and Cappel (West Chester, PA), respectively. Peroxidase-conjugated anti-hamster IgM antibody was purchased from EY Laboratories (San Mateo, CA).

**Cell Culture and Induction of Neuronal Differentiation**—Murine neuroblastoma Neuro2A cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 0.5 mg/ml of streptomycin sulfate, 2% fetal bovine serum. The culture medium was routinely changed by washing with PBS. The cell lysates were treated with 2% bovine serum albumin in PBS and incubated with the primary antibodies (10 µg/ml for S2-566 and 30 µg/ml for 12E3) at 4 °C for 20 h. After washing with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG + IgM (0.4 µg/ml) at 37 °C for 30 min. After washing with PBS, the cells were observed under a fluorescent microscope (Olympus X-71) or with a confocal microscope (Olympus X-71; by guest on July 23, 2018http://www.jbc.org/Downloaded from).

**Induction of Neuritogenesis** —Briefly, to estimate neuritogenesis quantitatively, the differentiated cells were incubated at 25 °C for 8 h in 0.125 M phosphate buffer (pH 8.2), the membranes were incubated at 25 °C for 30 min. The enzyme reaction was stopped by the addition of 10 µl of 10 M sodium acetate buffer (pH 5.5).

**Preparation of the Glycoprotein and Lipid Fractions** —The glycoprotein and lipid fractions, delipidation of the cells were performed by the fluorometric C7/C9 analysis as described previously (11). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG + IgM was purchased from Seikagaku Co. (Tokyo, Japan). Goat anti-CD166 antibodies after being desalted with Sephadex G-50 chromatography were purchased from Sigma and Nacalai Co. (Kyoto, Japan). BCA protein assay kit was purchased from Bio-Rad, and 50 ng/ml of retinoic acid (Sigma) was used as a template for PCR. Both sense and antisense primers (25 pmol each) were used as described previously (11). The concentration of proteins was determined by the BCA protein assay kit or by the measurement of absorbance at 280 nm.

**Preparation of the Glycoprotein and Lipid Fractions** —For preparation of the glycoprotein and lipid fractions, delipidation of the cells was carried out as described previously (25, 26). The resulting precipitate and the organic phases were used as the glycoprotein and glycolipid fractions, respectively. The lipid fraction was dried and applied to a DEAE-Sephadex A-25 column (CH3COO− form, chloroform/methanol/water = 30:60:8; 1 ml). The acidic lipid fraction was eluted with 5 vol of methanol containing 0.3 M CH3COONa and analyzed by the fluorometric C5/C6 analysis and TLC immunostaining (19) with the anti-diSia antibody. The lipid fraction was delipidated with 2% bovine serum albumin and then with 0.6 M of NaHCO3, as described previously (21). The acetylenyl-linesterase was also measured as described (22). Briefly, to the 40 µl of cell homogenates (see “SDS-PAGE and Immunostaining”) were added 50 µl of 2 mol M, 5′-dithiothreitol (2-nitrobenzozic acid) in 0.1 M Tris-HCl (pH 8.0) and 10 µl of 5 mM acetylthiocholine in 0.1 M Tris-HCl (pH 8.0) and incubated at 25 °C for 30 min. The enzyme reaction was stopped by the addition of 10 µl of 1 M β-ME/28/455, and absorbance at 405 nm was measured.

**Chemical Analysis** —Sialic acids were quantified by the fluorometric analysis using the α-keto acid-specific reagent 1,2-diamino-3,4-methylenedioxybenzene (23, 24). The internal sialic acids were quantitated by a 1,5-fold longer chain of the same mRNAs, respectively. The purity of the cDNAs was determined by the BCA protein assay kit or by the measurement of absorbance at 280 nm.

**Preparation of the Glycoprotein and Lipid Fractions** —The cells were harvested—0.4 days after neuronal differentiation, washed with 10 mM sodium phosphate buffer (pH 7.2), 0.15 M NaCl (PBS), and homogenized in PBS containing 1% Triton X-100, 1 mM ethylenediaminetetraacetic acid magnesium chloride, and 0.5% mercaptoethanol and placed at 60 °C for 20 min. The samples were then electrophoresed on 10% polyacrylamide gels and electrophoretically transferred to a 0.2 µm nitrocellulose membrane. The membranes were incubated with the primary antibody, S2-566 (0.5 µg/ml), or 12E3 (6.0 µg/ml) at 4 °C for 16 h. As the secondary antibody, the peroxidase-conjugated anti-mouse IgG + IgM (0.4 µg/ml) was used, and the color development was carried out as described (10).

**Immunofluorescence Microscopy** —Neuro2A cells were grown on coverslips. For immunofluorescence, the culture medium was retained, and the cells were fixed with 3% paraformaldehyde at 25 °C for 8 min, followed by washing with PBS. The cells were then blocked with 2% bovine serum albumin in PBS and incubated with the primary antibodies (10 µg/ml for S2-566 and 30 µg/ml for 12E3) at 4 °C for 20 h. After washing with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG + IgM (0.4 µg/ml) at 37 °C for 30 min. After washing with PBS, the cells were observed under a fluorescent microscope (Olympus X-71), respectively. The morphology of the cells was observed under the microscope, and photographs were taken by a ×100 magnification. The length of neurites was measured as described above (see “Cell Culture and Induction of Neuronal Differentiation”).

**Preparation of the Glycoprotein and Lipid Fractions** —The cells were seeded at 1.0 × 105/well in a 24-well plate and incubated for 24 h. The culture medium was changed to Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum and 20 µl of retinoic acid and with or without 0.1 µg/ml of monoclonal antibody S2-566 or 2A11 (day 0). The cells were incubated for 1–4 days. The culture medium was routinely changed every 2 days. The morphology of the cells was observed under the microscope, and photographs were taken at ×1000 magnification. The length of the neurites was measured as described above (see “Cell Culture and Induction of Neuronal Differentiation”).

**Preparation of the Glycoprotein and Lipid Fractions** —The recombinant mouse CD166 (20 µg/hamster) together with Freund’s complete adjuvant was intraperitoneally injected into the Syrian hamster (female, 6 weeks old). The animals were boosted twice with the protein (20 µg/hamster) mixed with Freund’s incomplete adjuvant every 2 weeks. Blood was collected 1 week after the last boost, and the serum was prepared as described (10).

**Preparation of the Glycoprotein and Lipid Fractions** —Immuno precipitation—Bälve’s mouse brain homogenate was prepared as described previously (10) and was subjected to immunoprecipitation using the hamster anti-recombinant mouse CD166 (see above) and S2-566 antibodies. After incubation with protein G-Sepharose as described previously (21), the mice were immunoprecipitated with rabbit anti-mouse IgM antibodies (Seikagaku Co.).

**Preparation of the Glycoprotein and Lipid Fractions** —Antibody Treatment—The cells were seeded at 1.0 × 105/well in a 24-well plate and incubated for 24 h. The culture medium was changed to Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum and 20 µl of retinoic acid and with or without 0.1 µg/ml of monoclonal antibody S2-566 or 2A11 (day 0). The cells were incubated for 1–4 days. The culture medium was routinely changed every 2 days. The morphology of the cells was observed under the microscope, and photographs were taken at ×100 magnification. The length of the neurites was measured as described above (see “Cell Culture and Induction of Neuronal Differentiation”).

**Preparation of the Glycoprotein and Lipid Fractions** —The following degenerate oligonucleotide primers for mouse proteins were used: STSsia I (accession number X84235, nucleotides 48–1014), 5′-CATGCTGATCTCGAGAATTCTC-3′ and 5′-CCGGCGGATTTTGGTGAAGAT-3′; STSsia II (X83562, nucleotides 48–1107), 5′-CTGCTGGTUTTCCTACCTAT-3′ and 5′-GCCGAGTCCTACGTAAGAT-3′; STSsia III (X80502, nucleotides 7–1101), 5′-AATGCGAAATGGCCCGAGT-3′ and 5′-ATGCACTTGGATAGCGAC-CT-3′; STSsia IV (X86000, nucleotides 72–1061), 5′-AATACGCCAGA-CTTGAGAC-3′ and 5′-CCTGTTGCTAGTGGTGAGC-3′; and GAPDH (M23599, nucleotides 5–1015), 5′-AAGAGGTTGACAGGGAAGAT-3′ and 5′-TTCGAGGCTAGTGGTGAC-3′. The PCR was performed at the following conditions: 5 min at 94 °C, 40 cycles were carried out on a thermal cycler; each cycle consisted of a 30 s denaturation step at 94 °C, an annealing step at 55°C for 30 s, and a 1-min extension step at 72 °C. The PCR products were separated on a 1% agarose gel and blotted onto Hybond-N membranes (Amersham Biosciences). The membranes were then probed with the indicated digoxigenin-labeled cRNAs (STSsia I, II, III, IV).
IV, and V and GAPDH; nucleotides, 48–1014, 46–1107, 7–1101, 72–1061, 176–1140, and /H11002 5 to 1013, respectively) that had been cloned from the mouse adult brain mRNAs for ST8Sia I–V and GAPDH and sequenced (14).

RESULTS

Chemical Detection of the /H9251 2,8-linked Neu5Ac Residues on Glycoproteins of Neuro2A Cells during Neurite Formation—

Neuro2A cells can be differentiated into neuron-like cells with neurites after addition of 20 μM of retinoic acid (4, 20). Morphologic changes such as neurite sprouting and extension started 1 day after treatment with retinoic acid (Fig. 1a). The proportion of differentiated cells to the total cell number also increased (Fig. 1b), and at day 4, 90% of the cells had neurites. The length of the neurite also increased as the retinoic acid incubation period increased (Fig. 1c). Acetylcholinesterase activity, which is a marker enzyme for the cholinergic neuron-like differentiation of Neuro2A (5, 21), also increased (Fig. 1d).

The Neuro2A cells were harvested every day after retinoic acid treatment (days 0–4), and the glycoprotein fraction (completely delipidated cell lysate) was analyzed for the amount of internal Neu5Ac (C9(Neu5Ac)) and terminal Neu5Ac (C7(Neu5Ac)) residues using fluorometric C7/C9 analysis. The amount of internal Neu5Ac in the glycoprotein fraction increased (Fig. 2) with the increase in acetylcholinesterase activity (Fig. 1d) and with the morphologic changes (Fig. 1a–c).

The amount of terminal Neu5Ac residues also increased, although it reached a plateau by day 2. These results suggest that the extensive formation of α2,8-linked di/oligoNeu5Ac occurs at day 2 and later. The acidic glycolipid fractions at days 0–4 were also analyzed by fluorometric C7/C9 analysis, and no internal Neu5Ac was detected (Table I). These chemical results indicate that the α2,8-linked Neu5Ac structure on the glycoprotein(s) of Neuro2A increases in a differentiation-dependent manner.

Immunoochemical Detection of the diSia Epitope, Neu5Acα2→8Neu5Acα2→3Gal, on Neuro2A Cells during Neurite Formation—To identify the diSia epitope-containing molecule(s), Neuro2A cells of days 0–4 were subjected to SDS-PAGE/Western blotting with the anti-diSia antibody S2-566, which recognizes Neu5Acα2→8Neu5Acα2→3Gal (10), and the anti-oligo/polySia antibody 12E3, which recognizes (Neu5Ac)n (n ≥ 5) (18). The diSia epitope was prominently detected at 100 kDa, and the amount of the diSia epitope on the 100-kDa glycoprotein increased as the neuronal differentiation proceeded (Fig. 3a). In contrast, there was no immunostained band with 12E3 (Fig. 3b). Thus, the oligo/polySia (degree of polymerization ≥ 5) does not occur in glycoproteins in Neuro2A cells. The acidic lipid fraction of Neuro2A cells was not immunostained on the high performance thin layer chromatography/immunostaining, indicating the absence of
that the diSia epitope is on lane 3. On the other hand, with NANase II, which cleaves the S2-566 stain of the 100-kDa-gp band disappeared (Fig. 5, S2-566 (0.51 µg/ml) was then subjected to the immunostaining with the anti-diSia antibody SDS-PAGE and electroblotted on the PVDF membrane. The membrane cated day after the incubation. The cell homogenate was separated by SDS-PAGE and analyzed. The molecular masses of the standard proteins are shown on the left of the panels. To right of each panel, the closed arrowhead represents the 100-kDa-gp, and the open arrowheads represent 58, 53, 48, 44, and 38-kDa glycoproteins.

diSia-containing gangliosides like GD3 (data not shown). This is consistent with previous reports that Neuro2A cells contain negligible amounts of GD3 and other b series gangliosides (5, 6, 21, 27). The immunoblotting results were consistent with the immunofluorescence of Neuro2A cells (Fig. 4). Immunofluorescence of the Neuro2A cells before (day 0) and after neuronal differentiation (day 4) was examined using S2-566 and 12E3. The cell surface was clearly immunostained with S2-566 at day 4 when the cells were fully differentiated (Fig. 4). On the other hand, there was no staining with 12E3. The fluorescence of S2-566 at day 4 was much more intense than that at day 0. These results indicate that the expression of the diSia epitope on the cell surface increased during the retinoic acid-induced differentiation of the Neuro2A cells. This is consistent with the results of the fluorometric C7/C9 analysis (Table I) and the immunoblotting of S2-566 (Fig. 3a).

Characterization of the diSia Epitope of the 100-kDa Glycoprotein in Neuro2A Cells and Mouse Adult Brains—To further characterize the diSia epitope of the 100-kDa-gp, we examined the sensitivity of the stain to the linkage-specific sialidase treatments (Fig. 5, lanes 1-3). With A. ureafaciens sialidase, which cleaves a2→3, a2→6, and a2→8 Neu5Ac linkages, the S2-566 stain of the 100-kDa-gp band disappeared (Fig. 5, lane 3). On the other hand, with NANase II, which cleaves a2→3 and a2→6 linkages, the S2-566 stain remained (Fig. 5, lane 2). These results confirmed that the diSia structure of the 100-kDa-gp contains the Neu5Aca2→8Neu5Ac linkage. Furthermore, S2-566 staining of the 100-kDa-gp was resistant to the peptideN-glycanase treatment (Fig. 5, lane 4), suggesting that the diSia epitope is on O-glycan chain(s). The migration of the S2-566 stain of the 100-kDa-gp did not change under non-reducing conditions (Fig. 5, lane 5), indicating that the 100-kDa-gp has no intermolecular disulfide bonds. These results indicated that the 100-kDa-gp contains the Neu5Aca2→8Neu5Ac linkage(s). The 100-kDa-gp was also detected in mouse brain homogenates by S2-566, together with five other components at 58, 53, 48, 44, and 38 kDa (Fig. 3a). The 100- and 58-kDa glycoproteins were prominently stained in mouse adult brain. These observations were previously described (10). In contrast, the 12E3 epitope was not observed except for a greater than 180-kDa NCAM as reported previously (28, 29). The S2-566 stain of the 100-kDa-gp derived from mouse adult brain had the same properties (shown in Fig. 5) as that derived from the Neuro2A

### Table I

| Incubation period | Terminal Neu5Ac | Internal Neu5Ac | Terminal Neu5Ac | Internal Neu5Ac |
|-------------------|----------------|----------------|----------------|----------------|
| 0 days            | 4250           | 107            | 14.6           | ND             |
| 1 day             | 4590           | 172            | 16.3           | ND             |
| 2 days            | 6560           | 204            | 19.1           | ND             |
| 3 days            | 7160           | 238            | 26.2           | ND             |
| 4 days            | 7500           | 293            | 30.8           | ND             |

**Fig. 3.** SDS-PAGE/Western blotting of the Neuro2A cell homogenates during differentiation. Neuro2A cells were cultured in the medium containing 20 µM of retinoic acid and harvested on the indicated day after the incubation. The cell homogenate was separated by SDS-PAGE and electroblotted on the PVDF membrane. The membrane was then subjected to the immunostaining with the anti-diSia antibody S2-566 (0.51 µg/ml) (a) and the anti-oligo/polySia antibody 12E3 (6.0 µg/ml) (b). The homogenates of the Neuro2A cells on days 0–4 and the mouse adult brain (5 µg protein/lane) were analyzed. The molecular masses of the standard proteins are shown on the left of the panels. To right of each panel, the closed arrowhead represents the 100-kDa-gp, and the open arrowheads represent 58, 53, 48, 44, and 38-kDa glycoproteins.

**Fig. 4.** Immunofluorescence of the differentiated Neuro2A cells using the anti-diSia and anti-oligo/polySia antibodies. The cells before (day 0) and 4 days after (day 4) the retinoic acid treatment were fixed with 3% paraformaldehyde and immunostained with the anti-diSia (S2-566) (10 µg/ml) and anti-oligo/polySia (12E3) (30 µg/ml) antibodies as described under “Experimental Procedures.” The photos of immunofluorescence of Neuro2A cells are shown. Bar, 10 µm.

**Fig. 5.** Immunostaining of the Neuro2A cell homogenates with the anti-diSia antibody S2-566. The Neuro2A cell homogenate of day 2 was subjected to SDS-PAGE under reducing conditions and blotted to the PVDF membranes. The membrane was untreated (lane 1) or treated with NANase II (a2,3- and a2,6-specific sialidase, 0.50 unit/ml) (lane 2), A. ureafaciens sialidase (a2,3-, a2,6-, and a2,8-specific sialidase, 0.50 unit/ml) (lane 3), or peptideN-glycanase (lane 4) (25 milliunits/ml) for 20 h prior to the immunostaining with S2-566 (0.51 µg/ml) as described under “Experimental Procedures.” The homogenates of Neuro2A cells from day 2 were separated on the SDS-PAGE under the nonreducing conditions and blotted on the PVDF membrane. The membrane was subjected to the immunostaining with S2-566 (lane 5).
cells (data not shown). These results together suggest that the 100-kDa-gp of mouse adult brain is the same glycoprotein as that of Neuro2A cells.

Identification of the 100-kDa Glycoprotein as CD166—The results described above as well as our previous results (10) clearly indicate that the 100-kDa-gp is the diSia-containing glycoprotein in mouse adult brain. Notably, it was previously suggested that rat lymphocytes also express the diNeu5Ac-containing glycoprotein at 100 kDa (30). We thus hypothesized that the 100-kDa-gp was a common diSia-containing glycoprotein between brain and lymphocytes. One such candidate glycoprotein was CD166 (SC1), because CD166 is a common glycoprotein between brain and thymus, and the molecular mass is 95–100 kDa under either reducing or nonreducing conditions (17). To determine whether the 100-kDa-gp is CD166, we developed antisera by immunizing hamsters with recombinant mouse CD166. The recombinant glutathione S-transferase-CD166 protein was immunostained with commercially available goat anti-CD166 antibodies that specifically recognize the CD166 but could not immunoprecipitate the CD166 glycoprotein (data not shown). The obtained hamster anti-mouse CD166 antibodies immunoprecipitated mouse CD166. Immunoprecipitated CD166 was immunostained with S2-566, and inversely immunoprecipitated S2-566 epitope-containing 100-kDa-gp was immunostained with the hamster anti-mouse CD166 antibodies (Fig. 6). The 100-kDa-gp of differentiated Neuro2A cells was also identified as CD166 in the same way (data not shown). These results clearly indicate that the Neu5Acα2 → 8Neu5Acα2 → 3Gal epitope-containing 100-kDa-gp is CD166.

Effect of the Anti-diSia Antibodies on Neurite Outgrowth in Neuro2A—To evaluate the importance of the diSia epitope in neuritogenesis, the cells were incubated with or without the anti-diSia monoclonal antibodies S2-566 and 2A11. The 2A11 recognizes Neu5Acα2 → 8Neu5Acα2 → 6Glc (Glc is required). The neurite lengths at days 0–4 were measured. There were obvious differences in neurite length between the cells incubated with S2-566 and those with 2A11 at days 3 and 4, although there were very few differences on days 1 and 2 (Fig. 7). Neurite extension was inhibited with 0.1 μg/ml of S2-566, whereas no inhibition was observed with 2A11, even at concentrations of up to 5 μg/ml. In the same way, there was no inhibition with the anti-oligo/polySia antibody 12E3 (data not shown). The inhibitory effects of the antibodies on neurite extension appear to be correlated with the reactivity of these antibodies to the 100-kDa-gp (Figs. 3 and 4). The increase of the α2,8-linked Neu5Ac compared with terminal Neu5Ac (Fig. 2), which indicates extensive formation of the diNeu5Ac epitope, is coincidental with the inhibitory effects of the neurite outgrowth by S2-566 (Fig. 7). These results indicate that the Neu5Acα2 → 8Neu5Acα2 glycosidically linked to the 3-position of the Gal residue is important for the neurite outgrowth, especially at later stages.

Expression of the α2 → 8-Sialyltransferase mRNAs during Neurite Formation of Neuro2A Cells—To understand the biosynthesis of the diSia structure of the glycoprotein, we analyzed the Neuro2A cells during differentiation for the expression of mRNAs for the five known α2,8-sialyltransferases using reverse transcription-polymerase chain reaction. STSsia I (31) and STSsia V (32) use glycolipids as substrates and STSsia II (or STX) (33, 34) and STSsia IV (or PST) (35) use glycoproteins, especially NCAM. STSsia III synthesizes the diSia structure both on glycoproteins and glycolipids in vitro (36). Only STSsia III was expressed in Neuro2A cells at every stage of neuronal differentiation (Fig. 8a). No other STSsia mRNAs were detected, even using highly sensitive digoxigenin-labeled probes (data not shown). Consistent with this observation, it was previously reported that Neuro2A cells express only STSsia III before neuronal differentiation, although there is no description for the expression after differentiation (37). These results are consistent with the observation that Neuro2A cells do not express any b or c series gangliosides but express the diSia structure on CD166. Semi-quantitative analysis of the STSsia III mRNA was performed, and its expression level increased 4.5-fold at day 1 and 1.7-fold at day 4 after neuronal differentiation (Fig. 8b). These results suggest that STSsia III is the enzyme responsible for the formation of the diSia on CD166 and that the increase of the diSia epitope on CD166 is due to the up-regulation of STSsia III expression.

**DISCUSSION**

The relation between neuronal differentiation and the expression of gangliosides is well studied in Neuro2A cells (3–6). Neuro2A cells differentiate into cholinerger neuron-like cells following retinoic acid treatment (3, 4). Neurotigenesis can be induced in the absence of retinoic acid by exogenous addition of the diSia epitope (Neu5Acα2 → 8Neu5Acα2 → 3Gal)-containing gangliosides (GD3 and the b series) (4) or by forced expression of GD3 and the b series gangliosides on the cells by transfection with GD3 synthesize cDNA (5, 6). These results strongly suggest the importance of cell surface expression of the diSia epitope in neurotigenesis. It was previously demonstrated, however, that the parental Neuro2A cells do not express GD3 or b series gangliosides (5, 6). In the present study, the differentiated Neuro2A cells also did not express these
Differentiation-dependent Expression of Disialic Acid Epitope

gangliosides. Therefore, we suspect that the diSia epitope is expressed on glycoproteins, but not glycolipids, during neuritogenesis.

Chemical and immunochemical analyses unequivocally showed that the diSia epitope, but not the oligo/polySia epitope \((\alpha_2 \rightarrow \delta)\) Neu5Ac, \(n \leq 5\), was expressed in several glycoproteins, primarily in the 100-kDa-gp in Neuro2A cells (Figs. 3 and 4), whereas the diSia epitope was not expressed on glycolipids. Using immunoprecipitation experiments, we identified the 100-kDa-gp to be the CD166 that is expressed in Neuro2A cells as well as in mouse adult brains. The CD166 is a membrane-spanning glycoprotein belonging to the immunoglobulin superfamily that is also named SC1/BEN/DM-GRASP (38–40). The CD166 is a cell adhesion molecule that has homophilic binding activity as well as heterophilic binding activity to CD6, 30-kDa protein, and NgCAM (17, 38, 41). In chicken brain, CD166 might be involved in neurite extension of spinal motor neurons (42). In mouse, CD166 is also involved in the path finding and/or fasciculation of specific cranial sensory nerve fibers (43).

Anti-CD166 antibody inhibits neurite extension (39, 41). Thus, CD166 is closely associated with neurite formation. Our finding that the diSia epitope was present on CD166 raises the possibility that the diSia epitope is involved in the CD166-associated path finding and fasciculation. In fact, the following lines of evidence support the possibility and suggest that the diSia epitope on CD166 is important for neurite formation of Neuro2A cells. First, expression of the diSia epitope on CD166 increases during retinoic acid-induced differentiation (Fig. 4). Second, the increase of diSia on CD166 is concomitant with the morphologic and biochemical changes in neuronal differentiation of the Neuro2A cells (Figs. 1–4). Third, the anti-diSia antibody S2-566 (specific to Neu5Acα2 → 8 Neu5Acα2 → 3Gal sequence; the Gal residue is necessary) (10) inhibited neurite extension (Fig. 7), whereas there was no inhibition by the 2A11 antibody (recognizing Neu5Acα2 → 8 Neu5Acα2 → 6Glc sequence; the Glc residue is necessary) (19) or the anti-oligo/polySia antibody 12E3. Notably, the inhibitory effect of the S2-566 appears to be associated with later stages of neurite formation rather than the earlier stages (Fig. 7). It remains to be determined how the antibody differentially inhibits later stages of neurite extension. It is interesting to note, however, that the later stages correspond to the state in which cells actively express the diSia on glycoproteins (Fig. 2).

At present, it is not clear how the diSia epitope on CD166 is important for neurite formation, but the diSia epitope on CD166 might influence neurite formation in a different way than polySia on the NCAM does. The polySia on NCAM is considered to be involved in the inhibition of neurite outgrowth through its anti-adhesive effect on the NCAM-mediated cell adhesion. On the other hand, the diSia epitope on CD166 might be involved in neurite outgrowth possibly through the facilitating effect of the diSia on CD166-mediated homophilic adhesion or heterophilic interaction with associative counterpart molecules that specifically recognize the diSia epitope, like siglecs (sialic acid-binding immunoglobulin-like lectins) (44), as suggested by the fact that the anti-diSia antibody specifically blocked neurite extension (Fig. 7). Therefore, discovery of associative counterpart molecule(s), which specifically recognize the Neu5Acα2 → 8 Neu5Acα2 → 3Gal sequence, might help to determine the underlying mechanism for this phenomenon. In this regard, siglec 11 has most recently been cloned and is expressed not only in macrophages of various tissues, like liver Kupffer cells, but also in brain microglia. This siglec recognizes the α2,8-linked sialic acid specifically, although a murine orthologue has not yet been found (45). We are now searching for the diSia-binding factor and the mechanism of neurite extension through the diSia epitope.

In Neuro2A cells, the diSia epitope is exclusively linked to glycoproteins but not to glycolipids. In mouse brain, however, the diSia epitope is present in both glycoproteins and glycolipids, which are often colocalized on the same cells. Therefore, the diSia epitope can be regarded as the common glycocone in brain. Whether the diSia epitope on either or both glycoproteins and glycolipids is of biological importance is an interesting question. A recent report that mice deficient in GD3 synthase (ST8Sia I) genes can develop normally (46) is suggestive in this regard. These mice cannot synthesize the disialylated b series gangliosides. We do not know that they can express the diSia epitope on glycoproteins like CD166, but it is possible that these mice express the diSia epitope on glycoproteins, because ST8Sia III is responsible for the synthesis of this epitope (Fig. 8). The diSia epitope in glycoproteins might compensate for the roles of the diSia-containing gangliosides. With respect to the common glycocones, (Neu5Gc)GD1c and the diNeu5Gc-containing 100-kDa glycoprotein are colocalized in rat T cells. In the case of rat T cells, the common glycocone is Neu5Gcα2 → 8 Neu5Gc and the diNeu5Gc epitope on either glycolipids or the glycoproteins might be functional in the CD4-GD1c epitope-mediated T cell activation (30). Notably, CD166 (also called an activated leukocyte cell adhesion molecule) is also expressed on activated T cells and epithelia (17). The diNeu5Gc-containing 100-kDa glycoprotein in thymus is also CD166.² The functional importance of CD166 and the diSia epitope in thymus is not yet known. Taken together, it appears that the common glycocone has common roles in brain and thymus, and elucidation of the functions of the common glycocones in those tissues is underway in our laboratory.

ST8Sia III is the enzyme responsible for the synthesis of the diSia epitope during differentiation in Neuro2A cells (Fig. 8). The absence in the Neuro2A cells of ST8Sia I and ST8Sia V, which are both responsible for the synthesis of the diSia epitope on glycoproteins, is consistent with the fact that Neuro2A cells have no GD3 or b or c series gangliosides. Neuro2A cells do not express ST8Sia II (or STX) or ST8Sia IV (or PST), which is responsible for the formation of the polySia structure on glyco-

² C. Sato, T. Matsuda, and K. Kitajima, manuscript in preparation.
proteins, especially on NCAM. This is consistent with the fact that there is a negligible amount of polysialylated NCAM in the Neuro2A cells (Fig. 4). The expression of ST8Sia III mRNA is 1.4–1.7-fold up-regulated after treatment of the cells with retinoic acid. This up-regulation of ST8Sia III mRNA expression is consistent with the increase in the amount of internal Neu5Ac residues as well as in the intensity of S2-566 immunostaining on CD166. In the 5′-flanking regions, ST8Sia III has an AP-2 site that is able to respond to retinoic acid is implicated in the regulation of neural development and myeloid cell differentiation (47, 48). The expression of ST8Sia III does not lead to in vivo synthesis of GD3 in the Neuro2A cells, although ST8Sia III has the ability to synthesize the diSia epitope on glycolipids in vitro (36). The physiologic substrate of ST8Sia III has long remained unknown, until our recent finding that adipoQ (ACRP30 or adiponectin), which is a cytokine secreted from adipose tissue (49, 50), is a substrate of ST8Sia III (14). ST8Sia III is expressed in adipose tissue and up-regulated during adipocyte differentiation (14). AdipoQ, which is secreted exclusively from 3T3-L1 cells, contains the diSia epitope on its O-linked glycan chain (14). In CD166, the diSia epitope appears to be attached to the O-linked glycan chain(s) (Fig. 5). Thus, ST8Sia III might be a disialic acid synthase that is involved in the synthesis of diSia-containing O-linked glycoproteins in various tissues.

REFERENCES

1. Nagai, Y., and Iwamori, M. (1996) in Biology of the Sialic Acids (Rosenberg, A., ed) pp. 197–214, Plenum Press, New York
2. Sharon, N., and Lis, H. (1997) in Glycoscience (Gaubius, H.-J., and Gabius, S., eds) pp. 133–189, Chapman & Hall, Weinheim, Germany
3. Leiden, R. W. (1984) J. Neurosci. Res. 12, 147–159
4. Tsuji, S., Yamashita, T., Tanaka, M., and Nagai, Y. (1988) J. Neurochem. 50, 414–423
5. Kojima, N., Kurosawa, N., Nishi, T., Hanai, N., and Tsuji, S. (1994) J. Biol. Chem. 269, 30451–30456
6. Liu, H., Kojima, N., Kurosawa, N., and Tsuji, S. (1997) Glycobiology 7, 1067–1076
7. Troy, F. A., II (1996) in Biology of the Sialic Acids (Rosenberg, A., ed) pp. 95–144, Plenum Press, New York
8. Brune, J. L., and Rutishauser U. (2000) in Molecular and Cellular Glycobiology (Fukuda, M., and Hinsgaul, O., eds) pp. 116–132, Oxford University Press, New York
9. Inoue, Y. (1992) in Polysialic Acids (Roth, J., Rutishauser, U., and Troy, F. A., II, eds) pp. 171–182, Birkhäuser Verlag, Basel, Switzerland
10. Sato, C., Fukuhara, H., Kato, K., Matsuda, T., Koshino, R., Kohayashi, K., Troy, F. A., II, and Kitajima, K. (1998) Anal. Biochem. 261, 191–197
11. Sato, C., Inoue, S., Matsuda, T., and Kitajima, K. (1999) Annu. Rev. Neurosci. 22, 102–109
12. Sato, C., Kitajima, K., Inoue, S., and Inoue, Y. (1998) J. Biol. Chem. 273, 2575–2582
13. Sato, C., Yasukawa, Z., Honda, N., Matsuda, T., and Kitajima, K. (2001) J. Biol. Chem. 276, 28849–28856
14. Nakanaka, S., Sato, C., Kitajima, K., Katagiri, K., Irie, S., and Yamagata, T. (2001) J. Biol. Chem. 276, 33657–33664
15. Sato, C., and Kitajima, K. (1999) Trends Glycosci. Glycotechnol. 11, 371–390
16. Sato, C., and Kitajima, K. (1999) Trends Glycosci. Glycotechnol. 11, 371–390
17. Bowen, M. A. (2002) in Leukocyte Typing VII (Mason, D., ed) pp. 894–895, Oxford University Press, Oxford
18. Sato, C., Kitajima, K., Inoue, S., Seki, T., Troy, F. A., II, and Inoue, Y. (1995) J. Biol. Chem. 270, 18923–18928
19. Ohta, K., Sato, C., Matsuda, T., Toriyama, M., Vacquier, V. D., Lennarz, W. J., and Kitajima, K. (2000) Glycoconj. J. 17, 205–214
20. Riboni, L., Prinetti, A., Bassi, R., Caminiti, A., and Tettamanti, G. (1995) J. Biol. Chem. 270, 26868–26875
21. Hasegawa, T., Yamaguchi, K., Wada, T., Takeda, A., Itoyama, Y., and Miyagi, T. (2000) J. Biol. Chem. 275, 8007–8015
22. Doctor, B. F., Toker, L., Roth, E., and Silman, I. (1987) Anal. Biochem. 166, 399–403
23. Nakamura, M., Sato, C., Yamaguchi, M., Takemori, Y., and Ohkura, Y. (1987) J. Biol. Chem. 262, 867–892
24. Sato, C., Kitajima, K., Inoue, S., Seki, T., Troy, F. A., II, and Inoue, Y. (1995) J. Biol. Chem. 270, 18923–18928
25. Folch-Pi, J., Arsove, S., and Meath, J. A. (1951) J. Biol. Chem. 195, 288–297
26. Livingstone, B. D., and Paulson, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10967–10971
27. Sato, C., and Kitajima, K. (1999) in Molecular and Cellular Glycobiology (Fukuda, M., and Hinsgaul, O., eds) pp. 116–132, Oxford University Press, New York
28. Sekine-Aizawa, Y., Omori, A., and Fujita, S. C. (1998) FEBS Lett. 432, 135–139
29. Burrns, F. R., von Kannen, S., Guy, L., Raper, J. A., Kamholz, J., and Chang, S. (1991) Nature 355, 535–545
30. Pourquoi, O., Hallonet, M. E. R., and De Louarin, N. M. (1992) J. Neurosci. 12, 1548–1557
31. Dellenbergs, A. P., Cheng, S. (1996) J. Cell Biol. 133, 657–666
32. Dellenbergs, A. P., Cheng, S. (1995) Dev. Biol. 169, 65–75
33. Sekine-Aizawa, Y., Omori, A., and Fujita, S. C. (1998) Eur. J. Neurosci. 10, 2810–2820
34. Crocker, P. R., and Varri, A. (2001) Trends Immunol. 22, 337–342
35. Angata, T., Kerr, S. C., Greaves, D. R., Varri, N. M., Crocker, P. R., and Varri, A. (2002) J. Biol. Chem. 277, 24466–24474
36. Kawai, H., Allende, M. L., Wada, K., Kimura, S., Kagechika, H., Shudo, K., Yoda, M., Nakano, Y., Tebe, K., Nagai, K., Kimura, S., Tomita, M., Froegel, O., and Kadowaki, T. (2001) Nature 408, 2096–2099
