Developmental stage-dependent expression of an α2,8-trisialic acid unit on glycoproteins in mouse brain

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The monoclonal antibody mAb.A2B5 is a marker for the detection of oligodendrocyte progenitor cells that differentiate into type-2 astrocytes and oligodendrocytes. It is also a useful antibody for separating these cells from other lineage populations. The epitope of this antibody is considered to be the gangliosides GT3 and GQ1c. In this study, we sought to define more precisely the structure of the epitope. Accordingly, we chemically synthesized defined oligosialic acid structures linked to phosphatidylethanolamine and bovine serum albumin and used these to determine the antigenic specificity. mAb.A2B5 recognized the Neu5Acα2→8Neu5Acα2→8Neu5Acα2→ structure on both glycolipids and glycoproteins. We then examined whether the mAb.A2B5 epitope exists on glycoproteins in developing mouse brains. Western blot analyses revealed the expression of four glycoproteins reactive with the mAb.A2B5, and their expression was dependent on the stage of neuronal development. All the immunoreactivity in these glycoproteins with mAb.A2B5 disappeared after sialidase treatment and were resistant to chloroform/methanol extraction. These epitopes were also detected in brain homogenates from both GD3 synthetase-null and GD3/GD2 synthetase double null mice. These findings show that the α2,8-trisialic acid (triSia) unit recognized by mAb.A2B5 resides not only on gangliosides but also on glycoproteins in developing mouse brain. We postulate that the triSia structure on glycoproteins may be involved in oligodendrocyte differentiation, similar to the case with the α2,8-triSia structure on gangliosides. Real time polymerase chain reaction analysis of the developmental expression of all known ST8Sia genes, which are responsible for the biosynthesis of α2,8-linked Sia residues, showed that ST8Sia III gene expression correlated with expression of the triSia epitope. We suggest that ST8Sia III is the principal sialyltransferase responsible for synthesis of the α2,8-triSia units on glycoproteins.

Keywords: GT3/mAb.A2B5/neural stem cell/oligodendrocyte/oligosialic acid

Introduction

Sialic acids (Sia) are a family of 9-carbon carboxylated sugars comprising nearly 50 members that are derivatives of N-acetylneuraminic acid (Neu5Ac), N-glycolyneuraminic acid and deaminoneuraminic acid (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) (Angata and Varki 2002; Schauer 2004). Due to their net negative charge at physiological pH, they act to mediate ligand–receptor and cell–cell interactions. Mice deficient in a UDP-GlcNAc 2-epimerase/ManNAc kinase are embryonic lethal (Schwarzkopf et al. 2002). Sia are sometimes linked to each other to form polymerized chains consisting of oligo- and polysialic acid (polySia). Oligo–polySia chains in nature usually have a degree of polymerization (DP) ranging from 3 to >400 Sia residues (Sato and Kitajima 1999; Nakata and Troy 2005; Drake et al. 2008). Polysialylated NCAM is found most commonly on neural cell adhesion molecules (N-CAM) in embryonic brain and in specific regions of the adult brain where neurogenesis persists (Seki and Arai 1991, 1993). The polySia chains on N-CAM regulate cell–cell communication during development by their anti-adhesive effect (Troy 1996; Bonfanti 2006; Rutishauser 2008). We recently demonstrated that a brain-derived neurotrophic factor dimer binds directly to polySia to form a large complex with an Mr greater than 2000 kDa under physiological conditions and that it functions as a reservoir for neurotrophins (Canato et al. 2008). Such multifunctional polySia structures are widely used as markers of adult-neurogenesis, although the biological significance of their presence in adult brain is not as well understood as in embryonic brain. AntipolySia antibodies are available for immunopanning of neuron-lineage cells that contain polysialylated NCAM from embryonic stem cells (Schmandt et al. 2005).

In contrast to polySia, shorter oligomers of α2,8-linked disialic acid (diSia and triSia) chains with DP of two and three Sia residues are common structural units of gangliosides. In particular, b-series gangliosides (diSia-containing glycolipids) are involved in various biological processes, including cell adhesion (Nicoll et al. 2003; Avril et al. 2006), cell differentiation (Kojima et al. 1994), signal transduction (Okada et al. 2002; Susuki et al. 2007) and surface expression of stage-
specific antigens (Yanagisawa and Yu 2007). The biological importance of di/triSia units on gangliosides has been confirmed in GD3 synthetase knockout (KO) mice (Okada et al. 2002) and GD3/GD2 synthetase double KO (DKO) mice (GM3 only mice; Inoue et al. 2002). Little attention, however, has been paid to the occurrence and functions of such short sialyl oligomers on glycoproteins (Sato 2004). Glycoproteins containing diSia groups occur in nature more frequently than previously recognized (Sato et al. 2000), as revealed by newly improved analytical methods to detect di- and oligoSia structures (Sato et al. 1998, 1999, 2000). Several glycoproteins had been reported earlier to contain the diSia structure (Fukuda et al. 1985; Zhang et al. 1997; Fox et al. 2001). It has been hypothesized that the functions of these di- and oligoSia moieties on glycoproteins are similar to those proposed for gangliosides. The diSia structures on glycoproteins are involved in neurite outgrowth of neuroblastoma cells that contain negligible amounts of b-series gangliosides (Sato et al. 2002). Compared with b-series gangliosides, knowledge of the function of c-series gangliosides, including GT3 and GQ1c (Eisenbarth et al. 1979; Kasai and Yu 1983; Saito et al. 2001; Ngamukote et al. 2007), is limited, although there is a specific antibody for detecting these gangliosides, designated monoclonal antibody mAb.A2B5 (Eisenbarth et al. 1979). mAb.A2B5 has been used to detect triSia-containing gangliosides in brains and extra-neural tissues, including kidney and liver (Saito and Sugiyama 2000).

Fig. 1. Chemically synthesized sialyl compounds. A. Sialyl compounds (1, 8-aminooctyl 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosonic acid, monoSia; 2, 8-aminooctyl 5-acetamido-8-O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid, diSia; 3, 8-aminooctyl 5-acetamido-8-O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid-triSia) used for the synthesis of neosialoglycoprotein. B. oligo/polyNeu5Ac-PE (neosialoglycolipids).
mAb.A2B5 is a well-described antibody that was originally prepared against chicken embryo retina cells (Eisenbarth et al. 1979). It is widely used as a marker of rat glial precursor cells (Raff et al. 1983), known as O-2A progenitor cells (Dubois-Dalcq 1987), which differentiate into type-2 astrocytes and oligodendrocytes (Yanagisawa and Yu 2007). In practice, mAb.A2B5 is utilized as a reagent for selecting glial precursor cells from among neural stem cells in mouse, rat and human tissues (Stallcup and Beasley 1987). Cells from human glioblastomas that express the mAb.A2B5 epitope have properties resembling cancer stem cell (Soeda et al. 2009; Tchoghndjian et al. 2010). The occurrence and function of the triSia glycoconjugate on glycoproteins, however, remain unknown because of the lack of a triSia-recognizing antibody. The possible functions of triSia on gangliosides also remain unknown. In this study, we sought to determine if mAb.A2B5 could be used to specifically detect the triSia-containing glycoconjugate in glycoproteins. A previous study suggested the occurrence of A2B5 epitopes on glycoproteins in human head and neck squamous cell carcinoma (Bolot et al. 2003). However, this study was too preliminary to confirm the suggestion. Accordingly, we determined the antigenic specificity of mAb.A2B5 using chemically defined α2,8 sialyl structures and discovered that this antibody was specific for the detection of the Neu5Acα2,8Neu5Acα2,8Neu5Ac glycoconjugate on both gangliosides and glycoproteins. Using this antibody, we have identified at least four triSia-containing glycoproteins in developing mouse brain. Further, we have suggested ST8SialI as the principal sialyltransferase responsible for synthesis of the α2,8-triSia epitope, using real-time polymerase chain reaction (PCR).

**Results**

The antigenic specificity of mAb.A2B5 is considered to be GT3 or GQ1b (Kasai and Yu 1983; Saito et al. 2001), although the precise specificity, including the requirement of the lipid-linked moiety, reducing terminal Gal residue or additional sialyl residues, is not known. To determine the exact antigenic specificity of mAb.A2B5, we first prepared neoglycoproteins using chemically synthesized sialyloligomeric structures (Figure 1). To effectively introduce sialyloligomers into a carrier protein, we conjugated an amino group to the sialyloligomer at the reducing terminus with a spacer (Tanaka et al. 2006, 2008). With these compounds, monosialyl, α2,8-linked di-sialyl and α2,8-linked trisialyl residues were successfully conjugated with bovine serum albumin (BSA) using the cross-linker N-(m-maleimidobenzoyloxy) succinimide (MBS). Neoglycoproteins were then subjected to Sephacryl S-300 chromatography (Figure 2A) to confirm the conjugation wherein the cross-linked compound eluted before the native BSA. The purified neoglycoproteins were further confirmed by silver staining (Figure 2B). Concomitant with the disappearance of the native BSA protein band was the appearance of high-molecular weight, polydisperse bands (Figure 2B, lanes 1–3). The molecular mass of these bands changed depending on the DP. The monosialyl, disialyl and trisialyl residues were successfully conjugated to BSA at ratios of 8.7:1, 8.1:1 and 4.5:1, respectively ((Neu5Ac)n-BSA=mass:mol), as determined by quantifying the amount of Sia. These sialylneoglycoproteins were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot analysis using mAb.A2B5. This antibody recognized the triSia-containing neoglycoprotein but not monoSia- or diSia-containing BSA (Figure 2C). This finding indicates that mAb.A2B5 recognition starts with a DP3, and recognition does not require a reducing terminal Gal linked to a Sia residue.

To better understand the exact antigenic specificity of mAb.A2B5 with respect to the DP, a phosphatidylethanolamine...
dipalmitoyl-conjugated sialyloligomer (Neu5Ac)n-PE was prepared with a reducing terminal Neu5Ac that was linearized by reductive amination (Sato et al. 1995) and analyzed for mAb.A2B5 reactivity. OligoSia-PE was first used as a test antigen (mixture of DP 2-10) and mAb.A2B5 recognized this antigen but not monoSia-PE (Figure 3A, circle vs square). To confirm the DP required for the recognition of mAb.A2B5, we synthesized lipidated oligomers using oligoSia with a DP ranging from 2 to 5 and used these as test antigens. The antibody did not react with (Neu5Ac)2,3-PE (DP=1–2; reducing terminus was acyclic by reductive amination), but did react with (Neu5Ac)3-PE (DP=3), although the reactivity was dramatically decreased toward (Neu5Ac)5-PE, based on ELISA (Figure 3B). The antibody showed only slight reactivity against polySia (Figure 3B, polySia). This finding is consistent with previous immunohistochemical findings that the mAb.A2B5 epitope does not overlap with polySia-NCAM (Dietrich et al. 2002). The α2,8-linked oligo/polySia is labile and can degrade to shorter sialyloligomers (Kitazume et al. 1992; Manzi et al. 1994), making it difficult to obtain and retain a uniform DP. Oligomerized Sia structures readily degrade under acidic conditions. (Sia)n in solution can degrade to (Sia)2 ∼ (Sia)n−1 during reductive amination, although the majority of the sample retains the starting DP. While the oligoSia can be separated by anion-exchange chromatography, degradation can occur even when stored in the cold, and during ELISA analysis. Therefore, to accurately measure the DP, a thin layer chromatography (TLC)-immunostaining assay was carried out because the sample could be analyzed immediately after conjugation and chromatography. We developed (Neu5Ac)3-PE on the TLC plate and immediately performed immunoblotting with mAb.A2B5. Only (Neu5Ac)4-PE (DP=3) was detected (Figure 3C) while (Neu5Ac)5-PE (DP=2) and (Neu5Ac)6-PE (DP=4) were not recognized by mAb.A2B5. These results clearly show that mAb.A2B5 specifically reacts with the Neu5Acα2→8Neu5Acα2→8Neu5Ac structure. To determine if this antibody recognized the α2,8-triSia sequence within glycoproteins, we tested it against an embryonic pig brain homogenate. This homogenate was subjected to SDS–PAGE/ western blotting analysis using mAb.A2B5 and mAb.12E3. mAb12E3 recognizes (Neu5Ac)n, where n is ≥5 (Sato et al. 1995, 2000). mAb.12E3 also recognized the embryonic form of pig brain NCAM as a polydisperse band at >200 kDa, consistent with previous results (Figure 4, −Endo-N, IB: 12E3). In contrast, mAb.A2B5 did not recognize embryonic NCAM (Figure 4, −Endo-N, IB: A2B5) confirming earlier results based on histochemical analysis (Dietrich et al. 2002). The western blotted membrane was then digested with endo-N-acetylneuramidase.
minidase (Endo-N), which cleaves polySia internally to produce oligoSia residues (DP<5) (Hallenbeck et al. 1987, Kitajima et al. 1988). Concomitant with the depolymerization of the polySia chains on embryonic NCAM was the loss of mAb.12E3 immunoreactivity (Figure 4, Endo-N+, IB: 12E3), showing that there were no (Neu5Ac)n, n ≥ 5 structures in NCAM after Endo-N treatment. The reactivity of mAb.A2B5, however, appeared after Endo-N digestion (Figure 4, Endo-N+, IB: A2B5), indicating that the majority of the Endo-N treated embryonic NCAM had oligoSia residues with DP <5. This finding is consistent with the above conclusion that mAb.A2B5 recognizes the α2,8-triSia sequence in glycoproteins.

mAb.A2B5 is widely used as a marker for GT3 because it recognizes the α2,8-triSia sequence on this ganglioside. The ability of this antibody to recognize the triSia structure in glycoproteins has not been carefully examined. To study this problem, we prepared homogenates from developing embryonic mouse brains from day (E) 7 to 8 weeks postnatally and analyzed them for expression of the triSia-containing glycoprotein by western blot analysis using the mAb.A2B5 antibody. For comparison with NCAM, the major carrier glycoprotein of polySia, the anti-oligo/polySia antibody, 12E3 and an anti-NCAM antibody (H.28) were also used. The developmental profiles of polySia and NCAM were the same as that reported previously (Weinhold et al. 2005) (Figure 5A, IB: 12E3 and NCAM). Polysialylation at E14 increased until birth, then showed a dramatic decrease after birth. By 8 weeks, nearly all polysialylation had disappeared. The NCAM immunostaining profile was similar to the 12E3 immunostaining, although polysialylation of NCAM had disappeared while only the adult forms of NCAM (140 kDa and 180 kDa) were immunostained. By contrast, immunodetection with mAb.A2B5 was completely different, as it showed immunoreactivity with glycoproteins with molecular masses of 260 kDa, 160 kDa, 120 kDa and 80 kDa. Expression of the 260-kDa band at E14 was upregulated until P0 and then decreased after birth. This staining disappeared in adult brain, and a 280-kDa band appeared in the adult at 8 weeks. The 160-kDa band remained relatively unchanged during development. Expression of the 120-kDa band increased until postnatal day 0 (P0), and the molecular mass of the band gradually increased to 130 kDa after birth. The 80-kDa band decreased after birth. Thus, all mAb.A2B5-immunostainings changed depending on the developmental stage (Figure 5, IB: A2B5). It was therefore important to determine if NCAM expressed the mAb.A2B5 epitope, although it has been reported that the immunostainings of polySia-NCAM and mAb.A2B5 do not overlap (Dietrich et al. 2002). Accordingly, we immunoprecipitated NCAM from mouse brain and immunostained it with mAb.A2B5 and mAb.12E3. The immunoprecipitated NCAM showed strong immunostaining with mAb.12E3 (Figure 5B, IP: NCAM, IB: 12E3), but no immunostaining with mAb.A2B5 (Figure 5B, IP: NCAM, IB: A2B5). This finding establishes that mAb.A2B5 immunostaining described above was from expression of the α2,8-triSia sequence expressed on glycoproteins other than NCAM, a new finding that has not been previously reported.

To confirm that the immunoreactive bands were sialylglycoconjugates, they were treated with exosialidase because the Endo-N sialidase does not act on short sialyoligomers (Hallenbeck et al. 1987, Kitajima et al. 1988). The mAb A2B5 immunostaining disappeared after 1 h of exosialidase treatment (Figure 6A), showing that the triSia structure was responsible for the immunostaining. Gangliosides were then extracted from the brain homogenate with chloroform/methanol (C:M=2:1 and 1:2) to exclude the possibility that the mAb. A2B5 immunoreactivity did not come from gangliosides (Sato et al. 2000). When the C:M extracts were analyzed by western blotting using mAb.A2B5, the immunostaining pattern remained unchanged (Figure 6B), providing further evidence that the triSia glycotope is expressed on glycoproteins. To demonstrate that the mAb.A2B5 immunoreactivity was a direct result of the binding of this antibody to glycoproteins, we used brain homogenates derived from GD3 synthetase KO mice and GD3 synthetase and GD2 synthetase DKO mice. GD3 synthetase KO mice cannot synthesize GT3 (Okada et al. 2002) and GD3/GD2 synthetase DKO mice have only GM3 as a ganglioside component (Inoue et al. 2002). When these homogenates were immunostained with mAb.A2B5, four immunoreactive bands with molecular masses of 260 kDa, 160 kDa, 120 kDa and 80 kDa were observed. The immunoreactive staining profile was the same as that of the ddy mice (Figure 6C), indicating that the immunostainings were results of mAb.A2B5 binding to glycoproteins. These data show that the mAb.A2B5 epitope includes triSia-containing glycoproteins that are present in mouse brain and that expression of the epitope changes during brain development.

To investigate the sialyltransferase(s) responsible for the α2,8-trisialylation of glycoproteins, we carried out real-time PCR. Among the six α2,8-linked sialyltransferases, ST8Sia II (STX), ST8Sia III, ST8Sia IV (PST) and STSia VI have enzymatic activity toward glycoproteins (Takashima 2008). ST8Sia II and ST8Sia IV are well-studied polysialyltransferases that catalyze NCAM polysialylation (Angata et al. 2002). By comparison, ST8Sia I and ST8Sia V catalyze diand trisialylation of gangliosides. The sialyltransferase(s) responsible for synthesis of triSia on glycoproteins is not known. To determine the expression levels of these α2,8-linked sialyltransferases, we used specific primers for the six α2,8-sialyltransferases and cDNAs from developing mouse brain prepared from the same samples used for the western blot analysis (Figure 5). The expression levels of ST8Sia I and ST8Sia V (Figure 7A, ST8Sia I and ST8Sia V) were recipro-
Expression of ST8Sia II (STX) was upregulated at E17 before polySia expression peaked (Figure 7A, ST8Sia II). These results are consistent with those reported previously (Galuska et al. 2008). ST8Sia III (Yoshida et al. 1995) and ST8Sia VI (Takashima et al. 2002; Teintenier-Lelièvre et al. 2005) catalyze di-oligosialylation on glycoproteins. The copy number of ST8Sia VI was extremely small (Figure 7A, ST8Sia VI) compared with that of the other sialyltransferases, indicating that the amount of the glycotope that is synthesized by this enzyme may be relatively small, although the kinetic parameters are unknown. The copy number of ST8Sia III (Figure 7A, ST8Sia III) was nearly the same as that of ST8Sia I, and the expression pattern was also developmentally regulated. We then compared the immunostaining levels of polySia and triSia (Figure 7C) with mRNA expression (Figure 7B). The relationship between triSia expression and mRNA expression of ST8Sia III was nearly identical to that of polySia expression and ST8Sia II expression, strongly indicating that ST8Sia III may be the principal enzyme responsible for α2,8-trisialylation of glycoproteins.

Discussion

DiSia and triSia epitopes are well known in glycolipids. However, we recently reported that the diSia epitope is not restricted to b-series gangliosides but also present on glycoproteins (Sato et al. 2000; Yasukawa et al. 2006). For these studies, we used an anti-GD3 antibody mAb.S2-566, because we demonstrated that it recognizes the Neu5Acα2→8Neu5Acα2→3Gal structure on both glycolipids and glycoproteins, although some other anti-GD3 antibodies only recognize the disialyl gangliosides, but...
GT3 or related c-series gangliosides (Kasai and Yu 1983; Saito et al. 2001). However, the precise antigenic specificity of the antibody for DP of Sia residues, requirement of Gal and recognition of sialyloligomer on glycoproteins remain unknown. In this study, we focused on these points.

To determine the exact antigenic specificity of mAb.A2B5, we synthesized oligoNeu5Ac-PE structures and new sialyl compounds of defined chemical structures in antibody binding assays to determine specificity. Naturally occurring gangliosides sometimes contain small amounts of contaminants after purification, and such contaminants can lead to conflicting results. In the case of sialyloligomers, which are important structures on gangliosides, our new method makes it possible to chemically synthesize several different sialyl structures with well-defined DPs and with reactive groups at their reducing terminal end (Tanaka et al. 2006, 2008). Sialyloligomers with DPs ≥4, however, remain difficult to synthesize. Using these newly synthesized sialyloligomers, we clearly showed that the glyco- tope of this antibody is Neu5Acα2→8Neu5Acα2→ Neu5Acα2. This result indicates that mAb.A2B5 only requires the triSia structure (neither tetra nor greater DP) and that the Gal structure is not required for immunoreactivity. This is the new information on the specificity of mAb.A2B5. In general, it can be problematic to determine the DP requirement because of the degradation of the oligo/polysialylation as a result of the lability of the oligo/polySia structures, particularly under acidic conditions (Kitazume et al. 1992; Manzi et al. 1994). To define the DP requirement precisely, we finally used TLC immunostaining because it has an advantage of separating lipitated oligosialyl structures. The periodate treatment that destroys the nonreducing terminal end of α,2,8-linked sialyloligomeric structures completely abolished the reactivity with mAb.A2B5 (Figure 3D). These results indicate that the conformation of the triSia structure is unique and that mAb.A2B5 requires such a unique conformation in the periphery of the glycoconjugates based on the fact that the periodate oxidized (Sia)₄-PE, and sialyloligomeric structures with DP ≥4 did not react with mAb.A2B5, despite the fact that they contain the (Neu5Ac)₃ sequence within their chains. In addition, the minimum requirement of mAb.OL28 is (Neu5Ac)₃ and higher DPs. This antibody can also react with polySia-NCAM similar to the antipolySia antibodies, indicating that OL.28 can react with the helical structure of polySia at the distal region (Sato et al. 2000). In contrast, mAb.A2B5 could not react with polySia-NCAM, suggesting that the internal and distal structures in the polySia chain may have different conformations than the A2B5-reactive triSia unit. It is well established that the conformational structure of polySia depends on the DP (Brisson et al. 1992). In this study, we focused especially on the DP of the sialyloligomer. The previous reports showed that mAb.A2B5 can recognize GT3, irrespective of the presence and absence of 9-O-acetyl group (Dubois et al. 1990). The triSia unit in 9-O-acetyl GT3 might be the same unique conformation. Using mAb.A2B5, Ngamukote et al. (2007) examined glycolipid fractions from mouse brain at several different stages of

not diSia on glycoproteins. In case of c-series gangliosides, mAb.A2B5 is a widely used marker of O-2A progenitor cells (Yanagisawa and Yu 2007) and its epitope is considered to be GT3 or related c-series gangliosides (Kasai and Yu 1983; Saito et al. 2001). However, the precise antigenic specificity of the

![Fig. 6. Characterization of the mAb.A2B5 epitope by western blotting. A. Sialidase treatment. The brain homogenates were treated with (+) or without (−) sialidase at 37°C for 1 h. The samples were subjected to SDS–PAGE/western blotting with mAb.A2B5 (1 μg/mL). B. Chloroform/methanol treatment. Mouse brain homogenates from 3 days after birth were subjected to SDS–PAGE and electroblotted onto PVDF membranes. The membranes were then extracted with (+) or without (−) chloroform/methanol as described in the Materials and methods. The membranes were then incubated with mAb.A2B5 (1 μg/mL; IB: A2B5) or IgM (1 μg/mL; IB: IgM) and visualized. C. Immunostaining of mouse brain from GD3 synthetase KO mice and GD3/GD2 KO mice. Mouse brain homogenates (16 weeks after birth; lane 1, ddy mice; lane 2, GD3/GD2 synthetase KO mice; lane 3, GD3 synthetase KO mice) were subjected to SDS–PAGE/western blotting with mAb.A2B5 (1 μg/mL; IB: A2B5).](http://glycob.oxfordjournals.org/)

![Fig. 7. Real-time PCR of mRNA for α2,8-sialyltransferases. A. cDNAs from the same mouse brain used for western blotting as shown in Figure 5 and subjected to real-time PCR analysis for α2,8-sialyltransferase mRNAs. The fluorescent PCR products amplified using specific primers (Table 3) were semi-quantitated by measuring the fluorescence using an iCycler iQ Real-Time PCR Detection System. The copy number of each sialyltransferase in the brain samples was calculated based on the amount of each control sialyltransferase plasmid. B. Summary of expression of mAb for sialyltransferases during brain development (ST8SiaI, □; ST8SiaII, ○; ST8SiaIII, ▲; ST8SiaV, ▲; ST8SiaV, □; ST8SiaIV, ■). C. Expression of mAb.12E3 epitope (polySia-NCAM) and mAb.A2B5 epitope on glycoproteins. Immunostained bands observed in Figure 5 were densitometrically measured and normalized based on the staining of the internal standard, GAPDH. The relative amount of 8-week staining was set to 1.0. (○, mAb.12E3 staining/GAPDH staining; ■, mAb.A2B5/GAPDH staining).](http://glycob.oxfordjournals.org/)

mAb A2B5 binds to triSia on glycoproteins in brain.

A

ST8SiaI

ST8SiaII

ST8SiaIII

ST8SiaIV

ST8SiaV

ST8SiaVI

B

C

E14 E17 P0 P3 P7 P10 3W 8W

day

copy number (10^6/ml)

E14 E17 P0 P3 P7 P10 3W 8W

day

relative value

copy number (10^8/ml)

copy number (10^8/ml)

copy number (10^6/ml)

copy number (10^6/ml)
development. They found that GT3 and more complex gangliosides, possibly GQ1c, were detected in the developing mouse brain and that GQ1c expression persisted in brains between E12 and the adult stage, although expression of GT3 was significantly decreased during development. This observation was consistent with the expression level of mRNA for ST8Sia I (GD3 synthetase), which can also synthesize GT3 (Nakayama et al. 1996). We postulated that triSia-containing glycoproteins may be present in the brain because we found earlier the presence of diSia-containing glycoproteins in brain and serum (Sato et al. 2002; Yasukawa et al. 2006). To test our hypothesis, glycoproteins were immunostained after SDS–PAGE and western blotting using mouse brain homogenates at several developmental stages ranging from E14 to 8 weeks after birth. With the anti-triSia antibody, we observed four major bands that were not removed by chloroform/methanol extraction and were sensitive to sialidase treatment. These four glycoproteins had molecular masses of 260, 160, 120 and 80 kDa, and their expression levels changed during development. Further proof that the α2,8-triSia sequence was expressed on glycoproteins was provided by studies using gene knockout mice to investigate the sialyltransferase(s) responsible for di/triSia synthesis on gangliosides. These mice also showed mAb.A2B5-immunoreactive glycoprotein bands. Taken together, these results clearly show that the mAb.A2B5 epitope, Neu5Acα2,8Neu5Acα2,8Neu5Ac, is expressed on both gangliosides and at least four distinct glycoproteins. In practice, mAb.A2B5 is a widely used marker of O-2A progenitor cells (Dubois-Dalcq 1987) as well as a cancer stem cell marker (Soeda et al. 2009; Tchoghadjian et al. 2010). Some of these A2B5 epitopes might come from the triSia-containing glycoproteins found in the developing mouse brain in this study. Further studies to characterize the newly identified triSia-containing glycoproteins are currently underway in our laboratories. It is interesting to note that autoantibodies against GT3 are found in ~30% of newly diagnosed type 1 diabetic subjects, although GT3 was not observed at detectable levels by high performance TLC or high performance liquid chromatography (HPLC) in human pancreas or isolated islets (Misasi et al. 1997). We thus hypothesize that the immunogen for these autoantibodies may have come from the α2,8-triSia structure expressed on glycoproteins that we have identified and described above.

ST8Sia III and ST8Sia VI are two candidate sialyltransferases for di/oligosialylation on glycoproteins since they are known to catalyze di/oligosialylation in vitro (Sato et al. 2001, Takashima et al. 2002, Teintenier-Lelièvre et al. 2005). While ST8Sia VI can synthesize the diSia structure on glycoproteins (Takashima et al. 2002), the sialyltransferase responsible for synthesis and amounts of the α2,8-triSia structure has not yet been reported. Based on the mRNA expression patterns of the sialyltransferases and comparison with ST8Sia II, we considered ST8Sia III as likely candidate sialyltransferase for synthesis of the α2,8-triSia structure on glycoproteins. To further examine this possibility, we transfected the six sialyltransferase genes into several cell lines and immunostained the cells with mAb.A2B5. Not only the ST8Sia I gene, but also other sialyltransferase genes, including ST8Sia III enhanced cell staining, although staining was faint compared to that of the cells transfected with ST8Sia I gene. These results thus suggest that more than one α2,8-sialyltransferases may be involved in synthesis of the A2B5 epitope on the cell surface. Faint immunostaining of the transfected cells may also indicate that trisialylation occurs on a limited number of cell surface glycoproteins. Thus, trisialylation may be a protein-specific modification similar to the polysialylation on NCAM (Rutishauser 2008). It will therefore be important in our future studies to characterize the carrier proteins expressing the triSia glycotope and to confirm the sialyltransferase(s) that is/are responsible for trisialylation of glycoproteins. Recently, studies in zebrafish showed that gene knockdown of ST8SiaIII by morpholino oligonucleotides induced abnormal segment boundary formation (Bentrop et al. 2008). This abnormality could be due to deficiencies in the level of the triSia (or diSia) structure on glycoproteins. Differential immunostaining of these tissues with our different anti/oligosialic acid antibodies may provide further insight to better understand trisialylation on glycoproteins and gangliosides on the cell surface.

Materials and methods

Materials

Mouse monoclonal antibody A2B5 was purchased from Japanese Collection of Research Bioreresources bank (Osaka, Japan). mAb.12E3 (IgM) that recognizes (Neu5Ac)n (n ≥ 5) (Sato et al. 1995) was generously gifted by Dr. Tatsunori Seki (Tokyo Medical University, Tokyo, Japan). Rat monoclonal antibody H.28 was generously gifted by Dr. Rita Gerardy-Schahn (Hannover Medical School, Hannover, Germany). Colominic acid was from Nakalai (Kyoto, Japan). Enhance chemiluminescence (ECL) reagents were purchased from GE (Piscataway, NJ). Polyvinylidene difluoride (PVDF) membrane (Immobilon P) was from Millipore (Bedford, MA). Prestained molecular weight marker was purchased from Bio-Rad (Hercules, CA). DdY mice were obtained from Japan SLC Co. (Hamamatsu, Japan), MBS was purchased from Thermofisher Scientific (Rockford, IL). All of the animal procedures were performed according to the guidelines for the care and use of experimental animals of Nagoya University.

Chemical analysis

Sia was quantitated by the resorcinol (RCH) method (Svennerholm 1957) and the 1,2-diamino-4,5-methylenedioxybenzene fluorometric HPLC method (Hara et al. 1987). Protein was quantitated by bicinechonic acid assay (Thermo Fisher Scientific, Rockford, IL).

Synthesis of amino-terminal introduced Sia

The samples were synthesized as described (Tanaka et al. 2006, 2008) and confirmed by nuclear magnetic resonance (NMR) spectrometry as described below. 8-Amino-octyl 5-acetamido-3,5-dideoxy-o-glycofera-o-galacto-2-nonulopyranosyl acid (Figure 1A-1) -[α]28D = −4.47 (c 0.60, H2O); 1H NMR (400 MHz, D2O) δ 3.55–3.85 (m, 8H, CH2), 3.43 (dt, 1H, OC=H2), 3.45 (dt, 1H, OC=H2), 3.43 (dt, 1H, OC=H2), J = 7.3 Hz, J = 7.2 Hz), 2.93–3.00 (m, 2H, NCH2), 2.71 (dd, 1H, H-3eq., J3eq.,= 4.3 Hz, Jgen = 12.1 Hz), 2.01 (s, 3H, Ac), 1.51–1.63 (m, 5H, H-3ax., OC=CH2, NCH2(CH2)2), 1.30 (m, 8H, aliphatic); 13C NMR (100 MHz, D2O, Aceton-d6) δ 175.9, 174.3, (101.5, 92.4, 91.3, 83.3, 80.8, 54.6, 42.6, 35.4, 28.2, 27.2, 26.0, 25.0, 24.0, 23.0, 22.0, 21.0, 20.0, 19.0, 18.0, 17.0, 16.0, 15.0, 14.0, 13.0, 12.0, 11.0, 10.0, 9.0, 8.0, 7.0, 6.0, 5.0, 4.0, 3.0, 2.0, 1.0, 0.0 ppm.
threitol at r.t. for 1 h was precipitated with trichloroacetic acid in 8 M urea and injected to a Mono Q HR5/5 (0.5 × 5 cm, Pharmacia, Sweden) column. Preparation of oligo/polySia

Saline (PBS)) to obtain the BSA-conjugated Sia oligomer. (m, 13H), 3.40 (dt, 1H, OC\_3364, 2935, 1600, 1470, 1029, 615 (cm\^-1)).

\[
J_{\text{gem}} = 3.70, 13C NMR (100 MHz, D2O, Acetone-d6) \delta 175.7, 175.7, 174.1, 174.1, (102.0, 101.2, anomeric), 79.5, 74.9, 73.6, 73.5, 72.5, 70.5, 69.2, 68.7, 65.6, 63.6, 63.5, 62.5, 62.5, 53.3, 52.7, 52.7, 41.4, 40.3, 29.5, 28.7, 26.7, 23.5, 26.1, 25.6, 23.2, 23.2, 22.9; IR (ESI-TOF) calcd for C_{30}H_{54}N_{3}O_{17} [M+H]^+ 728.3453, found 728.3453.

Chemically synthesized \(\alpha\),8-linked mono-, di- and trisialic acid (0.5 \(\mu\)mol) was dissolved in 0.01 M phosphate buffer (pH 7) and 500 \(\mu\)L of MBS (3.14 mg) in dimethylformamide was added. After incubation at room temperature (r.t.) for 30 min, MBS was removed with dichloromethane. BSA (0.0385 \(\mu\)mol in 8 M ura–0.2 M Tris–HCl (pH 8.6)) incubated with dithiothreitol at r.t. for 1 h was precipitated with trichloroacetic acid and re-dissolved with 6 M ura–0.02 mM phosphate buffer (pH 7.0) and was added to MBS-bound Sia and incubated at r.t. for 3 h. The reaction mixture was then applied to the Sephadex S-300 chromatography (1.3 × 100 cm, phosphate buffered saline (PBS)) to obtain the BSA-conjugated Sia oligomer.

**Preparation of oligo/polySia**

Mild acid hydrolysates of colominic acid (1 mg) were subjected to a Mono Q HR5/5 (0.5 × 5 cm, Pharmacia, Sweden) anion-exchange column and separated on a JASCO HPLC system. The sample was loaded on a column and eluted with 20 mM Tris–HCl (pH 8.0), followed by NaCl gradient (0–20 min, 0 M; 20–60 min, 0–0.25 M; 60–100 min, 0.25–0.35 M; 100–110 min, 0.35–1 M; 110–120 min, 1 M) in 20 mM Tris–HCl (pH 8.0). The flow rate was 500 \(\mu\)L/min, and fractions were monitored by a UV detector (UV, JASCO, Japan) at wavelength 210 nm. \(\alpha\)–2–8-Linked homooligo/polyNeu5Ac (DP 2–8) were pooled and desalted with Sephadex G-25 column (1.2 × 65 cm, ddw).

**Synthesis of oligo/polyNeu5Ac-PE**

Lipidation was carried out as described previously based on reductive amination (Sato et al. 1995). Oligo/polyNeu5Ac with DP = 2–5 or colominic acid (100 \(\mu\)g as Sia) in 10 \(\mu\)L of water was incubated at 60°C for 2 h with phosphatidylethanolamine dipalmitoyl (PE, 450 \(\mu\)g) dissolved in 90 \(\mu\)L of a mixture of chloroform/methanol (1:2, v/v). One hundred micrograms of sodium cyanoborohydride in 10 \(\mu\)L of methanol was added and further incubated at 60°C for 16 h. The solution was applied to Sep-Pack and the column was washed with water and the lipidated samples were eluted with methanol. The product was spotted on the TLC plate (Kiesel gel 60, Merck, Darmstadt, Germany), developed in 1-propanol/25% NH4OH/water (6:1:2.5, v/v/v) for 12 h and visualized by the resorcinol reagent.

**Antibody binding assay**

Antibody binding to a series of oligo/polyNeu5Ac-PE was determined using the ELISA method (Sato et al. 1995). For the ELISA test, oligo/polyNeu5Ac-PE was serially diluted in ethanol (19–75 pmol/well as respectively) on plate. After dry-up, the wells were then blocked with 1% BSA/PBS at 37°C for 1 h and incubated with the mAb.A2B5 (50 ng/well) at 4°C overnight. The antibody binding was detected using peroxidase-conjugated goat antimouse (IgG + IgM) antibody. Periodate treatment was performed as described (Sato et al. 1995).

**TLC immunostaining**

(\(\text{Neu5Ac})_n\)-PE was spotted on the TLC plate (Polygram G, Merck), developed in 1-propanol/25% NH4OH/water (6:1:2.5, v/v/v) for 12 h. The plate was then coated with a 0.4% polyisobutylmethacrylate solution. The plate was overlaid by mAb. A2B5 and then peroxidase-conjugated antimouse IgG+M. The reacted band was visualized with ECL reagent.

**SDS–PAGE**

Mouse brain samples (stage: E14, E17, P0, P3, P7, P10, 3W, 8W) from ddy and brain samples (1 year) from GD3 synthetase knockout mice and GD3/GD2 synthetase knockout mice were homogenized on ice in PBS containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (1 \(\mu\)g/mL aprotinin, 2 \(\mu\)M leupeptin, 1 \(\mu\)M pepstatin, 1 mM ethylenediamine tetra-acetic acid). The homogenates or protein samples were dissolved in Laemmli buffer (Sato et al. 2000) and placed at 65°C for 15 min. After about 20 \(\mu\)g protein for brain homogenates and 0.5 \(\mu\)g protein for neoglycoprotein sample/lane were electrophoresed on the 7% polyacrylamide gels, silver staining or immunoblotting on PVDF membrane was performed.

925


**Table 1. Primer sequences used in real-time PCR**

<table>
<thead>
<tr>
<th>Name of enzyme</th>
<th>Primer sequence</th>
<th>Accession number</th>
<th>PCR product size (bp)</th>
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</thead>
<tbody>
<tr>
<td>ST8Sia I</td>
<td>F 5'-TGCCCCATCCTTTTTGCTATGAC-3'</td>
<td>BC030647</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>R 5'-CTTCAAATGCCAGCTGGAAGG-3'</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>ST8Sia II</td>
<td>F 5'-GGCTGTGGCAGGAGATTG-3'</td>
<td>BC096546</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>R 5'-GGCATACTGGTGAGCAGCC-3'</td>
<td></td>
<td>88</td>
</tr>
<tr>
<td>ST8Sia III</td>
<td>F 5'-CCCTCTGATTTTATGCT-3'</td>
<td>BC075645</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'-CGGGAACATGTCACATCG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST8Sia IV</td>
<td>F 5'-AGGCCCTGGTGAATGTCAC-3'</td>
<td>BC060112</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5'-TCATTGGTAGAAACTTGAG-3'</td>
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<td></td>
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<tr>
<td>ST8Sia V</td>
<td>F 5'-CCAGAGAATGGCCCTACTA-3'</td>
<td>BC034855</td>
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</tr>
<tr>
<td></td>
<td>R 5'-CTGTTGATTCCTCTCCCG-3'</td>
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<td></td>
</tr>
<tr>
<td>ST8Sia VI</td>
<td>F 5'-GGCAGCAGAAAGATATG-3'</td>
<td>AB059554</td>
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<tr>
<td></td>
<td>R 5'-CCACCTGGGTGGCTTCT-3'</td>
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<tr>
<td>GAPDH</td>
<td>F 5'-CTGGACTGACACACTGCTAG-3'</td>
<td>BC082592</td>
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</tr>
<tr>
<td></td>
<td>R 5'-AGTCTCTGGTGGTCAGTG-3'</td>
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<td></td>
</tr>
</tbody>
</table>

**Immunostaining**

The PVDF membrane was blocked with PBS containing 0.05% Tween 20 and 1% BSA at 25°C for 1 h. The membrane was incubated with the primary antibody, A2B5 (1 μg/mL), 12E3 (0.5 μg/mL) or H28 (1 μg/mL) at 4°C for overnight. As the secondary antibody, peroxidase-conjugated antimouse IgG antibody (1/3000 diluted) or peroxidase-conjugated antirat IgG antibody (1/5000 diluted) or secondary antibody, peroxidase-conjugated antimouse IgG+M, was immunoblotted using antipolySia antibody (12E3) and mAb.A2B5.

**Exo- and endosialidase and chloroform/methanol treatments of the blotting membrane**

Tissue homogenates were electrophoresed and transferred to the PVDF membrane as described above. The membrane was washed with the glycisodebuffer, and the membrane was then treated with Clostridium perfringens exosialidases (10mU/mL) in 50 mM sodium acetate buffer (pH 5.5) or endosialidase, Endo-N (0.9 mM/L) in 50 mM Tris–HCl (pH 8.0) at 37°C for 1 h. In case of chloroform/methanol (C/M) treatment, the PVDF membrane was soaked briefly with methanol and blocked with BSA.

**Immunoprecipitation**

Mouse brain homogenates (about 1 mg/mL as protein, 100 μL) were pretreated with 100 μL of 1:1 solution of protein G-Sepharose at 4°C for 2 h and resins were removed. The pretreated mouse homogenate was incubated with protein G-Sepharose (100 μL) coupled with rat anti-NCAM antibody (H.28) at 4°C for 16 h. The resins were washed four times with PBS and subjected to SDS–PAGE/western blotting. The immunoprecipitate was immunoblotted using antipolySia antibody (12E3) and mAb.A2B5.

**Real-time RT-PCR**

Total RNAs were prepared from each of mouse brain samples (stage: E14, E17, P0, P3, P7, P10, 3W, 8W) with TRIZOL reagent as described previously (Sato et al. 2002). The first strand cDNAs were synthesized, and then the quantitative real-time PCR was performed using primers as shown in Table 1 (0.5 pmol each) and SYBR GreenER qPCR SuperMix for iCycler premix® (Invitrogen, CA). PCR products were analyzed by the iCycler iQ real-time PCR analyzing system (Bio-Rad, CA). Every sample was measured in triplicate, and the gene expression levels were expressed with S.D.

**Statistics**

Values in the ELISA and real-time PCR experiments were obtained based on the triplicated experiments. All data in these experiments were shown with S.D.

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We thank Ms. Shino Matsumoto for preparing the materials.

**Conflict of interest statement**

We declare no conflict of interest.

**Abbreviations**

BSEA, bovine serum albumin; diSia, disialic acid; DP, degree of polymerization; ECL, enhanced chemiluminescence; MBS, N-(m-maleimidobenzoyloxy) succinimide; Neu5Ac, N-acetylneuraminic acid; NMR, nuclear magnetic resonance; oligoSia, oligosialic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PE, phosphatidylethanolamine; polySia, polysialic acid; PVDF, polyvinylidene difluoreide; PCR, polymerase chain reaction; r.t., room temperature; SDS, sodium dodecyl sulfate; Sia, sialic acid; TLC, thin layer chromatography; trSiSa, trisialic acid.

**References**


