A Functional Dermatan Sulfate Epitope Containing
Iduronate(2-O-sulfate)α1-3GalNAc(6-O-sulfate) Disaccharide
in the Mouse Brain

**Demonstration Using a Novel Monoclonal Antibody Raised Against
Dermatan Sulfate of Ascidia nigra**

Received for publication, March 18, 2005
Published, JBC Papers in Press, April 22, 2005, DOI 10.1074/jbc.M503036200

Oversulfated chondroitin sulfate (CS), dermatan sulfate (DS), and CS/DS hybrid structures bind growth factors, promote the neurite outgrowth of hippocampal neurons in vitro, and have been implicated in the development of the brain. To investigate the expression of functional oversulfated DS structures in the brain, a novel monoclonal antibody (mAb), 2A12, was generated against DS (An-DS) from ascidian *Ascidia nigra*, which contains a unique iD disaccharide unit, iduronic acid (2-O-sulfate)α1–3GalNAc(6-O-sulfate), as a predomina

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The abbreviations used are: CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; GAG, glycosaminoglycan; PG, proteoglycan; An-DS, dermatan sulfate from the ascidian *A. nigra*; Hr-DS, dermatan sulfate from ascidian *H. roretzi*; 2S, 2-O-sulfate; 4S, 4-O-sulfate; 6S, 6-O-sulfate; IdUA, l-iduronic acid; HexUA, hexuronic acid; ΔH

Chondroitin sulfate (CS)1 and dermatan sulfate (DS), as well as heparan sulfate (HS), are glycosaminoglycans, which are synthesized as carbohydrate side chains covalently attached to a core protein of proteoglycan (PG) (for reviews, see Ref. 1–3). CS/DS-PGs are present at cell surfaces and in the extracellular matrices of most tissues, and are significant components in the mammalian brain, where they participate in neural development by regulating neuronal adhesion and migration, neurite formation, and axonal guidance (for reviews, see Ref. 4–8). The backbone of CS and DS consists of repeating disaccharide units of GlcUA-GalNAc- and IdoUA-GalNAc-, respectively, and hybrid chains composed of both units in varying proportions also exist (9). These disaccharide units are modified during chain elongation by specific sulfotransferases at C-2 of GlcUA/IdoUA and/or C-4 and/or C-6 of GalNAc in various combinations, thereby producing characteristic sulfation patterns and enorous structural diversity for CS, DS, and CS/DS hybrid chains. Recent studies have shown that the GlcUA/IdoUA ratio and sulfation pattern of the brain CS/DS change during development (10–12), and subpopulations of the brain CS and DS chains with distinct structures play different roles in neuritogenesis (13–15). In addition, oversulfated CS and DS chains from various marine organisms promoted neurite outgrowth in murine hippocampal neurons in vitro (16–19).

Brain CS/DS chains contain small yet significant amounts of oversulfated disaccharides (11, 14, 20). For example, embryonic
pig brain-derived CS/DS (E-CS/DS) chains contain 1.7% D and/or iD units and 0.9% E units (GlcUA-GalNAc(4S,6S))(11), where 4S and 6S represent 4-O- and 6-O-sulfate, respectively. We recently identified that these rare oversulfated disaccharides, in addition to IdoUA-containing disaccharides, are critical structural elements for the neuritogenic activity of CS/DS (11, 15). Meanwhile, Tschida et al. (21) showed that the CS chains of appican, which is a CS-bearing form of amyloid precursor protein, produced by cultured glioma cells contain 14.3% E units, suggesting that some brain PGs may carry a cluster of oversulfated CS disaccharides. However, the presence of oversulfated DS structures in the mammalian brain has not been rigorously characterized or explored.

The DS chains from the body of ascidian A. nigra (An-DS) consist of a major (~80%) iD unit (IdoUA(2S)-GalNAc(6S)) in addition to a minor (~20%) iC unit (IdoUA-GalNAc(6S)), where 2S represents 2-O-sulfate (22). The iD unit-containing structure is unique, and has not been detected in mammalian tissues. However, An-DS binds various growth factors including hepatocyte growth factor (23), fibroblast growth factor 2 (FGF2), pleiotrophin (PTN), midkine (MK), and heparin-binding epidermal growth factor, and promotes neurite outgrowth toward hippocampal neurons in vitro (18), implying biological functions for the iD-containing DS structures. CS/DS hybrid chains isolated from shark skin also exhibited marked binding activity toward various growth factors including those described above and also strong neuritogenic activity, and contain small yet significant proportions of various disulfated disaccharide units including D or iD units (19).

To study the structure-function relationship of CS/DS chains and possible biological functions of iD-containing structures during the development of the brain, we generated a new monoclonal antibody (mAb) 2A12 against An-DS in the present study. Immunohistochemistry and immunocytochemistry using mAb 2A12 were performed, and two minimal decasaccharides recognized by this antibody were isolated. Interestingly, mAb 2A12 inhibited neurite growth, and the two decasaccharides inhibited the binding of several growth factors and neurotrophic factors to E-CS/DS. These results indicate that iD-containing DS structures are present in the hippocampus and cerebellum of the mammalian brain, and appear to play specific roles in the development of the central nervous system.

**EXPERIMENTAL PROCEDURES**

**Materials**—The ascidian A. nigra (Chordata-Tunicata) was collected in Angra dos Reis, Rio de Janeiro, Brazil. Postnatal day 7, adult (7 weeks old), and pregnant dY mice were purchased from SLAC Inc. (Shizuoka, Japan). CS-A from whale cartilage, CS-B from porcine skin, CS-C and CS-D from shark cartilage, CS-E from squid cartilage, chondroitinases ABC, AC-I, AC-II, and B, and mAbs CS-56 and MO-225 were purchased from Seikagaku Corp. (Tokyo, Japan). Actinase E was purchased from Kaken Pharmaceutical Co. (Tokyo, Japan). Anti-neurofilament antibody was provided by A. Faissner, Ruhr University, Germany. Alkaline phosphatase (GDNF) and brain-derived neurotrophic factor (BDNF) from R&D Systems, MN, and recombinant human-glial cell-derived neurotrophic factor (BDNF) and brain-derived neurotrophic factor (BDNF) from R&D Systems, were used. The reactivity of antibodies with various GAG subtypes was tested using ELISA as described previously (15). Briefly, various GAG preparations were individually biotinylated and immobilized on the plates. Wells were blocked with 1% bovine serum albumin (BSA), and incubated with hybridoma supernatants or ascidian fluid containing antibodies followed by alkaline phosphatase-conjugated goat anti-mouse Ig(G+M) or IgM. The secondary antibody was detected using p-nitrophenyl phosphate, and the absorbance was measured at 415 nm.

**Antibody-linked Immunoblotting Assay (ELISA)**—If not specified, all the steps were performed at room temperature. For antibody screening and the evaluation of substrate specificity, streptavidin-coated plates were used. The reactivity of antibodies with various GAG subtypes was tested using ELISA as described previously (15). Briefly, various GAG preparations were individually biotinylated and immobilized on the plates. Wells were blocked with 1% bovine serum albumin (BSA), and incubated with hybridoma supernatants or ascidian fluid containing antibodies followed by alkaline phosphatase-conjugated goat anti-mouse Ig(G+M) or IgM. The secondary antibody was detected using p-nitrophenyl phosphate, and the absorbance was measured at 415 nm. Negative controls received no An-DS and/or primary antibody. When mAb 473HD and commercial anti-CS mAbs, CS-56 and MO-556, were used, an additional antibody was employed for detection.

For the inhibitory ELISA, a certain amount (0.5 μg) of GAGs or the oligosaccharide fractions generated by digestion with chondroitinase ABC (see below) was incubated with mAb 2A12 (as ascidic acid, diluted 400-fold) in a volume of 50 μl at room temperature for 1 h before being applied to the plate. The inhibition was calculated from the reduced absorbance relative to that obtained from a control incubation without GAG.

To characterize the 2A12 epitopes in An-DS, which contains a disaccharide repeating region and an oligosaccharide-peptide linkage region, the purified An-DS (12 μg) was incubated with chondroitinase ABC (10 mIU), chondroitinase B (4 mIU), or a mixture of chondroitinases AC-I (4 mIU) and AC-II (4 mIU) in their appropriate buffers at 37 °C for 2 h. Incubation with heat-inactivated chondroitinase ABC was run as a control. The digests were added to a Maxisorp plate in a 0.1 M sodium bicarbonate buffer, pH 9.2. After incubation at 4 °C for 16 h, the plate was washed with phosphate-buffered saline (PBS), followed by the steps described above. Absorbance was recorded 2 h after the addition of p-nitrophenyl phosphate.

**Preparation and Selection of Antibodies**—Monoclonal antibodies were obtained by immunization of BALB/cA mice with a peptide preparation obtained from A. nigra. Briefly, the immunogen was injected subcutaneously into the back of mice at a dose of 200 μg/injection every 2 weeks, and after the third injection the serum was screened against highly purified An-DS using ELISA. Spleen B lymphocytes of the positive mice after the fifth injection were isolated and fused with myeloma cells. The fused hybridoma cells were cultured, and the culture supernatant was screened by ELISA using biotinylated An-DS as
antigen. Four positive clones (2A12, 3G11, 4B5, and 5F4) were selected. Each clone was injected intraperitoneally into mice to obtain ascitic fluid, which was used for evaluating substrate specificity. Among the antibodies selected, mAb 2A12 is of particular interest because of its high specificity and unique staining pattern in the mouse brain (see below).

An aliquot of 2A12 was purified using a mouse IgM purification kit according to the manufacturer’s directions. The purified antibody was quantified with a BCA protein assay kit and used for assaying the inhibition of the neurite growth of cultured embryonic hippocampal neurons (see below).

**Immunohistochemistry**—P7 and adult dDy mice were anesthetized, and the brains were dissected, immediately frozen with dry ice, and kept at −80 °C. Within 2–4 days of the dissection, the frozen brains were cut into sections 12 μm thick, dehydrated by heating at 60 °C for 1 h, and stored at −80 °C for use. For immunostaining with the antibodies, the brain sections were fixed with acetone/methanol (1:1) and rehydrated with distilled water. The sections were then treated sequentially with the following solutions: 1) 2.5% hydrogen peroxide in PBS (10 mM, pH, 7.4) for 30 min; 2) 1% BSA, 4% normal goat serum in PBS for 60 min; 3) primary antibody in 1% BSA/PBS (diluted 200-fold for 2A12 and 100-fold for CS-56) at 4 °C overnight; 4) biotinylated anti-mouse IgM (μ chain) (8 ng/ml) in 1% BSA/PBS for 60 min; 5) Vectastain ABC solution in PBS (200-fold) for 60 min; and 6) 0.06% diaminobenzidine, 0.03% hydrogen peroxide in PBS (1:1) (DAB; pH 7.6). Finally, sections were fixed with a series of ethanol solutions and mounted with a xylene-based mounting medium. As a control, mouse IgM was used as a primary antibody. To confirm the specificity of the staining with the antibodies, brain sections were pretreated with chondroitinase ABC protease-free (2 μIU/section) to remove CS and DS, and then processed for immunostaining as described above.

**Cell Culture**—Dried oligosaccharide mixture was first mixed with 1–2 μl of (Arg-Gly)15 (10 pmol) and then 1 μl of gentisic acid (1 mg/ml) (24). Each mixture was spotted on a plate for MS analysis using a mixture of (Arg-Gly)15 and gentisic acid as a control. The analysis was run in a positive mode using the HCD1001 method according to the manufacturer’s instructions. The MS spectra were recorded on a Voyager DE-EP-Pro (PerSeptive Biosystems, Framingham, MA) using the linear mode.

**Fragmentation of An-DS and Analysis of Disaccharide Composition**—An-DS was isolated and purified from the bodies of A. nigra (see "Experimental Procedures"), with a yield of 0.12% from the dried tissue. The average molecular mass of this preparation was 6.3 × 10^4 as evaluated by gel filtration (data not shown) according to a reported method (30). An analysis of disaccharide composition showed DΔ disaccharide to be the predominant unit (76.6 mol%) with DΔ, DΔ, and DΔ units (Table I) as minor components, which is consistent with a report by Pavão et al. (22). Interestingly, although An-DS is...
Anti-dermatan Sulfate Antibody Reacting with a Brain Epitope

TABLE I
Disaccharide composition of the DS from ascidian A. nigra

The DS chains (An-DS) isolated from the bodies of A. nigra was extensively digested with chondroitinase ABC. The unsaturated disaccharides obtained were identified and quantified by anion-exchange HPLC as described under "Experimental Procedures." The values represent the means of those obtained from two separate experiments.

| Unsaturated disaccharides | Mol % |
|---------------------------|-------|
| ΔA: ΔHexUA-GalNAc         | 1.1   |
| ΔB: ΔHexUA-GalNAc(4S)     |       |
| ΔC: ΔHexUA-GalNAc(6S)     | 21.4  |
| ΔD: ΔHexUA(2S)-GalNAc(6S) | 76.6  |
| ΔE: ΔHexUA-GalNAc(4S,6S)  |       |
| ΔF: ΔHexUA(2S)-GalNAc(4S,6S) | 0.9  |

* Not detected.

The DS chains (An-DS) isolated from the bodies of A. nigra was extensively digested with chondroitinase ABC. The unsaturated disaccharides obtained were identified and quantified by anion-exchange HPLC as described under "Experimental Procedures." The values represent the means of those obtained from two separate experiments.

Composed of almost exclusively, if not completely, IdoUA-containing disaccharides, it was totally resistant not only to chondroitinase AC-I but also to chondroitinase B (Fig. 1).

Selection and Evaluation of the Specificity of Anti-An-DS mAb 2A12—Monoclonal Abs were raised against An-DS in mice, and four mAbs were selected, among which 2A12 is of particular interest because of its high specificity (see below). In this study, only mAb 2A12 was characterized. The reactivity of 2A12 toward various GAG species was analyzed using ELISA (Fig. 2A), in which biotinylated GAGs were individually immobilized onto a streptavidin-coated plate. 2A12 specifically reacted with An-DS, but not any other GAGs tested including CS-A, CS-B, CS-C, CS-D, CS-E, CS-H, and HS (DS from ascidian Halocynthia roretzi, which contains iB as a predominant disaccharide unit), or heparin. It should be emphasized that 2A12 discriminated iD (IdoUA(2S)-GalNAc(6S))-containing An-DS from other oversulfated CS and DS including CS-D, which is rich (20–21%) in D units (GlcUA(2S)-GalNAc(6S)) (16, 18). This specificity was confirmed by inhibitory ELISA, in which only soluble An-DS signifi cantly inhibited the binding of 2A12 to immobilized An-DS (data not shown). To investigate whether 2A12 recognizes the disaccharide repeating region or the oligosaccharide-peptide linkage region of the intact An-DS, An-DS was incubated with various chondroitinases individually, and subjected to ELISA using a Nunc Maxisorp plate, which has a greater ability to adsorb the linkage region than the disaccharide repeating region. As shown in Fig. 2B, treatment of An-DS with chondroitinase ABC completely abolished the reactivity with 2A12, suggesting that 2A12 recognized a structure embedded in the disaccharide repeating region rather than the linkage region of An-DS. Incubation with chondroitinase AC-I, AC-II, or B had no effect on the reactivity of 2A12 with An-DS, supporting the notion that An-DS was resistant to these enzymes (Fig. 1). The mAb 2A12 was identified as IgM by ELISA, in which only anti-mouse IgM, but not anti-mouse IgG or IgA, bound to 2A12 (data not shown).

The reactivity of mAb 2A12 with E-CS/DS was also examined. Compared with mAbs 473HD (13), CS-56 (31), and MO-225 (32), all of which recognize A–D-tetrasaccharide-containing structures (33), 2A12 exhibited much less yet significant reactivity toward E-CS/DS (Fig. 2C), which is probably because of the low abundance of the unique 2A12 epitope in the E-CS/DS chains (see below). mAb 2A12 also reacted with DS isolated from shark skin (data not shown), the D or iD content of which was 3% (19). These results suggest that the 2A12 epitope may be present in certain animal tissues besides the body of the ascidian.

Immunohistochemical Detection and Localization of the 2A12 Epitope in the Mouse Brain—To further verify the expression of 2A12 in the hippocampus, dissociated hippocampal neurons from an embryonic mouse were doubly stained with antibodies against the neuronal markers (anti-MAP2 and anti-NF) and 2A12. As shown in Fig. 4, cultured hippocampal neurons were stained positive with 2A12. The cell body and most neurites showed immunoreactivity to 2A12. Notably, expression of the 2A12 epitope is stronger in the neurite-sprouting regions than other regions of the cell body.

Detection of the 2A12 Epitope at the Surface of Hippocampal Neurons—To investigate the expression of 2A12 in the hippocampus, dissociated hippocampal neurons from an embryonic mouse were doubly stained with antibodies against the neuronal markers (anti-MAP2 and anti-NF) and 2A12. As shown in Fig. 4, cultured hippocampal neurons were stained positive with 2A12. The cell body and most neurites showed immunoreactivity to 2A12. Notably, expression of the 2A12 epitope is stronger in the neurite-sprouting regions than other regions of the cell body.

Effects of 2A12 on the Neurite Growth of Hippocampal Neurons—Hippocampal neurons formed multiple neurites after a 24-h culture on a substratum containing E-CS/DS and P-ORN at a density of 10,000 cells/cm² (15). In the absence of E-CS/DS chains in the substratum, a higher cell density (2.5–5-fold) and a longer culture period (2–3-fold) are required for the neurons to form elaborate neurites (Fig. 5A) compared with cells grown on a substratum containing E-CS/DS chains. To investigate
whether the 2A12 epitope at the hippocampal neuronal surface is involved in the process of neurite formation and growth, 2A12 was added to the culture medium. 2A12 significantly suppressed neurite growth of the neurons grown on a substrate containing P-ORN only (Fig. 5B). This observation was confirmed by a statistical morphometric analysis, which revealed that 2A12 markedly inhibited the formation of neurites (Fig. 5C) and decreased the total length of neurites per cell (Fig. 5D) at concentrations over 50 μg/ml. Control IgM showed no such activity even at a concentration of 200 μg/ml (Fig. 5C and D). In contrast, squid cartilage CS-E-induced neurite outgrowth, which is mediated through a yet unidentified mechanism but not mediated by PTN unlike E-CS/DS-induced neurite outgrowth (15), was not influenced by 2A12 at a concentration of 100 μg/ml (data not shown), indicating that the inhibitory activity of the antibody was specific and was not a cytotoxic effect.

Characterization of the Minimal Structure Required for the Recognition by 2A12—In view of the findings that the 2A12 epitope is expressed by hippocampal neurons and may be involved in the formation and growth of neurites, we characterized the minimal structure of An-DS required for the recognition by 2A12.

**Fig. 2.** Characterization of the substrate specificity of mAb 2A12. A, the reactivity of mAb 2A12 with various GAG species was examined using ELISA, where authentic GAGs and Hr-DS, the DS preparation from the ascidian H. roretzi, which contains iB as a predominant unit, were included. Biotinylated GAGs (2 μg each) were individually immobilized to wells of a streptavidin-coated plastic plate, and processed for incubation with a primary antibody, mAb 2A12 (diluted 400-fold), followed by incubation with alkaline phosphatase-linked goat anti-mouse IgG (5,000-fold). The secondary antibody was detected using p-nitrophenyl phosphate as a substrate. This assay was performed three times, and the results are shown as the mean ± S.D. B, the effects of treatments with various chondroitinases (CSases) on the reactivity of An-DS with mAb 2A12 were evaluated. An-DS (12 μg) was incubated with CSase ABC (10 μIU), CSase B (4 μIU), or a mixture of CSases AC-I (4 μIU) and AC-II (4 μIU) at 37 °C for 2 h, and then each digest was added to the well of a Nunc Maxisorp plate in a 0.1 M sodium bicarbonate buffer, pH 9.2. After incubation at 4 °C for 16 h, wells were washed with PBS and processed for ELISA as described above. In the negative control, An-DS was omitted. The positive control received An-DS and inactivated chondroitinase ABC. C, the reactivity of the embryonic pig brain-derived CS/DS chains (E-CS/DS) with mAb 2A12 and with three other anti-CS antibodies, CS-56, MO-225, and 473HD, was compared using ELISA as described as above. The negative control did not receive mAb 2A12. Note that mAb 2A12 exhibited much weaker yet significant reactivity with E-CS/DS than the other three anti-CS antibodies. *, 0.01 < p < 0.05, significant difference from the control.

**Fig. 3.** Immunohistochemical detection and location of the 2A12 epitope in the mouse brain. Sagittal brain sections from P7 (panel A) and adult (panel E) mice were stained with mAb 2A12 as described under “Experimental Procedures.” The anti-CS mAb CS-56 was run for a comparison (panels B and F). mAb 2A12 barely stained the tissue after pretreatment of the sections from P7 mice with either chondroitinase ABC (panel C) or chondroitinase B (panel D). Elimination of the epitope by these enzymes was also observed in the adult tissues (data not shown). Solid arrows indicate the regions that were stained by mAb 2A12. P, pyramidal cell layer; GrDG, granular cell layer of the dentate gyrus; WM, white matter; GL, granular cell layer. Note that the border (open arrows) between the CA1 region and the dentate gyrus was stained with CS-56, but not with 2A12.

whether the 2A12 epitope at the hippocampal neuronal surface is involved in the process of neurite formation and growth, 2A12 was added to the culture medium. 2A12 significantly suppressed neurite growth of the neurons grown on a substrate containing P-ORN only (Fig. 5B). This observation was confirmed by a statistical morphometric analysis, which revealed that 2A12 markedly inhibited the formation of neurites (Fig. 5C) and decreased the total length of neurites per cell (Fig. 5D) at concentrations over 50 μg/ml. Control IgM showed no such activity even at a concentration of 200 μg/ml (Fig. 5C and D). In contrast, squid cartilage CS-E-induced neurite outgrowth, which is mediated through a yet unidentified mechanism but not mediated by PTN unlike E-CS/DS-induced neurite outgrowth (15), was not influenced by 2A12 at a concentration of 100 μg/ml (data not shown), indicating that the inhibitory activity of the antibody was specific and was not a cytotoxic effect.

Characterization of the Minimal Structure Required for the Recognition by 2A12—In view of the findings that the 2A12 epitope is expressed by hippocampal neurons and may be involved in the formation and growth of neurites, we characterized the minimal structure of An-DS required for the recogni-
The merged staining pattern is shown in C. Note that mAb 2A12 stained neurites and a restricted region of the cell body carrying multiple neurites. Scale, 50 μm.

An anti-dermatan Sulfate Antibody Reacting with a Brain Epitope

Fig. 4. Hippocampal neurons were stained with mAb 2A12. Hippocampal neurons from E15.5 mice were cultured on a P-ORN-coated coverslip for 36–48 h. Cells were fixed and doubly stained with antibodies for neuronal markers (anti-MAP2 and anti-NF) (A) or mAb 2A12 (B). The merged staining pattern is shown in C. Note that mAb 2A12 stained neurites and a restricted region of the cell body carrying multiple neurites. Scale, 50 μm.

An anti-dermatan Sulfate Antibody Reacting with a Brain Epitope

Fig. 5. Effects of mAb 2A12 on the neurite growth of hippocampal neurons in culture. mAb 2A12 was added to the culture medium at various concentrations 2 h after the seeding of E16.5 mouse hippocampal cells on a P-ORN substrate. After a 60-h culture, the cells were fixed and immunostained with anti-MAP2 and anti-NF. Representative images of the cell morphology are shown in the absence (A) or presence (B) of 100 μg/ml mAb 2A12. C and D, effects of 2A12 on neurite growth were evaluated by measuring the percentage of neurite-bearing cells (C) and the total length of neurites per cell (D). Mouse IgM (100 μg/ml) was run as a control. The values represent the mean ± S.E. of those obtained from three independent experiments in triplicate. *, 0.01 < p < 0.05; **, 0.001 < p < 0.01, significant difference from the values obtained in the experiments without exogenous antibody. Scale, 50 μm.

sensitive to chondroitinase ABC, but not to chondroitinase AC-I, AC-II, or B. Treatment of An-DS 10-b with chondroitinase ABC generated only ΔD, suggesting that the decasaccharide has the structure ΔHexUA/2Sα1→3GalNAc(6S)β1→4IdoUA/2Sα1→3GalNAc(6S)β1→4IdoUA/2Sα1→3GalNAc(6S)β1→4IdoUA/2Sα1→3GalNAc(6S) (ΔD-iD-iD-iD-iD). Treatment of An-DS 10-a with chondroitinase ABC generated ΔC and ΔD units in a molar ratio of 1:3:9. To locate the iC unit in the An-DS 10-a sequence, the reducing end of the oligosaccharide was labeled with 2AB, and the labeled oligosaccharide was treated with chondroitinase ABC and then with Δhexuronate-2-sulfatase. Chondroitinase ABC generates a 2AB-attached unsaturated tetrasaccharide irrespective of the structure of the parent oligosaccharide (27), and Δhexuronate-2-sulfatase removes a sulfate group only from the C-2 position of a ΔHexUA located at the non-reducing terminus (29). As shown in Fig. 7, Δhexuronate-2-sulfatase treatment caused the position where the 2AB-labeled unsaturated tetrasaccharide generated by digestion with chondroitinase ABC of the labeled An-DS 10-a was eluted to shift, suggesting that a sulfate group was removed from the non-reducing end of the tetrasaccharide. Such a shift was also observed for the 2AB-labeled unsaturated tetrasaccharide derived from An-DS 10-b (data not shown). However, Δhexuronate-2-sulfatase had no effect on the sulfate group at

tion by this antibody. Intact An-DS was partially degraded by chondroitinase ABC treatment, and the digests were fractionated by gel filtration. The effluent fractions were collected as indicated in Fig. 6A, and the molecular mass of each fraction was determined by MALDI-TOF MS analysis. To evaluate which fraction contained the minimal structure required for recognition by 2A12, an equal amount of each fraction was tested as an inhibitor against the reactivity of 2A12 with im-

An exchange HPLC, and two main oligosaccharides (An-DS 10-a and 10-b) were isolated (Fig. 6C). The molecular masses of An-DS 10-a and 10-b were 2613.0 and 2697.0 (Table II), respectively, indicating that the former is a decasaccharide with nine sulfate groups and the latter a decasaccharide with 10 sulfate groups. These two components exhibited comparable inhibitory effects on the reactivity of 2A12 to An-DS (Fig. 6D), suggesting that they are both recognized by the antibody. To precisely determine the sequences of both oligosaccharides, enzymatic treatment and an analysis of disaccharide composition were conducted. The results are summarized in Table II. Both were
Fig. 6. Determination of the minimal size of An-DS for the recognition by mAb 2A12. A, gel filtration chromatography of partially degraded An-DS. An-DS was partially digested with chondroitinase ABC, and the digest was subjected to gel filtration on a column of Superdex Peptide (10 × 300 mm). Fractions were collected as indicated by bars and arrows. Sizes of resolved peaks were determined by MALDI-TOF MS analysis, and are indicated by numbers of constituent monosaccharides: 2–12, di to dodecasaccharides. B, an equal amount (0.5 μg) of each fraction obtained by gel filtration was tested by ELISA as an inhibitor against the reactivity of mAb 2A12 with immobilized An-DS. Note that the decasaccharide was the smallest fraction to show significant inhibitory activity. C, the decasaccharide fraction was separated by anion-exchange HPLC as described under “Experimental Procedures” to isolate two main components, 10-a and 10-b, * a baseline shift. D, the two purified decasaccharides were subjected to an inhibitory ELISA as described above. Note that 10-a and 10-b showed similar inhibitory activity. The values in B and D represent the mean ± S.D. of those obtained from two independent experiments.

TABLE II

Properties of the two main decasaccharide components isolated from An-DS after digestion with chondroitinase ABC

Each component was subjected to analyses of molecular mass, enzymatic digestibility by various chondroitinases (CSases), and disaccharide composition as described under “Experimental Procedures.”

| Components | Molecular mass | Digestibility | CSase ABC | CSase AC-I/II | Compositions | Deduced structures | Recognition by 2A12 |
|------------|----------------|---------------|-----------|---------------|--------------|-------------------|-------------------|
| An-DS 10-a | 2613.0         | +             | –         | –             | ΔC:ΔD = 1.0:3.9 | ΔD-iD-iD-iD-iC    | +                 |
| An-DS 10-b | 2697.0         | +             | –         | –             | ΔD (> 96.4%)  | ΔD-iD-iD-iD-iC    | +                 |

* The apparent molecular mass determined by MALDI-TOF MS.
* Plus (+) and minus (−) indicate sensitivity to the enzyme indicated.
* ΔC and ΔD stand for HexUA-GalNAc(6S) and HexUA-GalNAc(6S) respectively.
* C and D stand for IdoUA-GalNAc(6S) and IdoUA-GalNAc(6S), respectively.
* Plus (+) indicates positive inhibitory activity against the binding of mAb 2A12 to immobilized An-DS in ELISA as described in the legend to Fig. 2.

Effects of the 2A12 Epitope on the Binding of Growth Factors and Neurotrophic Factors with E-CS/DS—Growth factors and neurotrophic factors play important roles in regulators of cell fate and the formation of neurons (34, 35). Our previous results have demonstrated that PTN and a subpopulation of E-CS/DS chains co-operate to promote the outgrowth of neurite from hippocampal neurons in vitro (15). Ovulsulfated disaccharides and IdoUA-containing disaccharides of the E-CS/DS chains are required for binding PTN and inducing the outgrowth of neurites. In the present study, to understand the mechanism through which 2A12 inhibited the neurite growth, An-DS 10-a and 10-b were used as inhibitors of the interaction of various growth factors and neurotrophic factors with immobilized E-CS/DS using a BIAcore system. Fig. 8 shows that, besides growth factors, two neurotrophic factors, GDNF (a member of the transforming growth factor-β superfamily) and BDNF (a member of the neurotrophin family), bound to E-CS/DS. In all the cases tested, An-DS 10-a and 10-b exhibited inhibitory activity that was comparable with or stronger than that of the intact E-CS/DS chains (Fig. 8). Interestingly, although An-DS 10-a and 10-b differ in the structure of the disaccharide unit at their reducing end only, the latter showed 5- and 3-fold stronger inhibition than the former against the binding of PTN or MK with E-CS/DS, respectively, suggesting an important role for the reducing end in the binding of oligosaccharides to these particular growth factors. These results suggest that mAb 2A12 may directly mask the functional binding sites for various growth factors and neurotrophic factors, thereby inhibiting the signaling of these receptors.

In addition, the Kₐ values of the binding of BDNF and GDNF with E-CS/DS were estimated to be 102.1 and 28.9 nM, respectively, which were comparable with those for the interaction of BDNF (Kₛ = 254 nM) and GDNF (Kₛ = 24 nM) with immobilized bovine intestinal mucosa HS (30). Considering that HS appears to be required for GDNF signaling (36), the comparable affinity for the binding of GDNF with HS and E-CS/DS may suggest that HS chains in the brain also act as a co-receptor for GDNF-like HS.
from the D unit (Fig. 2) demonstrated here that mAb 2A12 discriminated the iD unit of a low abundance and the lack of a specific enzyme differ-
ential abilities. The presence of iD in mammalian tissues has been implied (13, 33), but not clearly demonstrated because
mal structures. The presence of iD in mammalian tissues has been implied (13, 33), but not clearly demonstrated because
of its disaccharide sequence (33), whereas 2A12 recognizes iD-containing decasaccharides as minimal structures. The presence of iD in mammalian tissues has been implied (13, 33), but not clearly demonstrated because of a low abundance and the lack of a specific enzyme differ-
entiating the iD unit from the D unit. It was unambiguously demonstrated here that mAb 2A12 discriminated the iD unit from the D unit (Fig. 2A and Table II) and will be a useful tool with which to study the distribution of the iD-containing structures.

Significant yet small proportions (typically, 1.5–5%) of D and/or iD have been found in the mouse brain (20), pig brain (11), and some isolated neuronal CS-PGs, such as DSD-1-PG/ phosphacan (12, 14) and versican (37). Notably, mAb 2A12 showed reactivity with E-CS/DS (Fig. 2C) and stained restricted regions of the mouse brain (Fig. 3) and hippocampal neurons (Fig. 4), indicating the expression of iD-containing DS structures in the mammalian brain. Interestingly, pretreatment of the brain sections with chondroitinase ABC or B, largely abolished the staining by 2A12 (Fig. 3, C and D), suggesting that the 2A12 epitope in the mouse brain is embedded in DS domains rich in iD units. Although the 2A12 epitopes in the brain may contain several consecutive iD units as in the An-DS cycle, the possibility cannot be excluded that the 2A12 minimum epitope may be a decasaccharide with a core iD unit flanked by other disaccharides, or an oligosaccharide in which multiple iD units are scattered, considering the finding that An-DS and its decasaccharides were resistant to the action of chondroitinase B (Fig. 1 and Table II). The isolation of 2A12-reactive structures from the brain-derived CS/DS chains will help clarify the structure of this unique epitope.

Immunohistochemical staining (Fig. 3) using mAb 2A12 revealed a highly specific spatiotemporally regulated expression in the mouse brain. In contrast to a strong and widespread expression of the CS-56 epitope consistent with a recent report by Maeda et al. (12), the expression of the 2A12 epitope was restricted to the cerebellum and hippocampus in the early postnatal period. The distinct staining patterns of these two antibodies were probably attributable to their different specificities: the CS-56 epitope includes the A–D (GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S)) tetrasaccharide-containing octasaccharide sequences (33), whereas 2A12 recognizes iD-containing structures as discussed above. It is noteworthy that CS-56 strongly stained the border between the CA1 region and gyrus dentate in both developing and mature mouse hippocampus, whereas the 2A12 epitope was absent in both situations. Given the notion that the expression of the CS-56 epitope in this region inhibits the outgrowth of axons from the CA1 region to the dentate (38), it is conceivable that the 2A12

**DISCUSSION**

In this study, we characterized a novel IgM mAb 2A12, which was raised against the DS from ascidian *A. nigra* and recognized a unique oversulfated DS epitope structure present in the mouse brain. The antibody specifically reacted with An-DS, but not any other typical CS/DS variants or heparin, as evaluated by ELISA (Fig. 2A), suggesting that the essential residues are 2-O-sulfated α-IdoUA and 6-O-sulfated β-GalNAc, which are joined together to form →4IdoUA(2S)-
α1→3GalNAc(6S)β1→ and →3GalNAc(6S)β1→4IdoUA(2S)α1→. The An-DS decasaccharides were the smallest oligosaccharides to inhibit the binding of mAb 2A12 to An-DS, and larger oligosaccharides showed stronger inhibition (Fig. 6B), suggesting that not only the iD unit but also size is important for the recognition by this antibody. Two decasaccharides, ΔD-id;Δ-D-idΔ-D-iD and Δ-D-idΔ-D-iC, which were isolated from the products of the partial enzymatic digestion of An-DS with chondroitinase ABC, inhibited the binding of 2A12 to immobilized An-DS in the ELISA, indicating that mAb 2A12 recognizes iD-containing decasaccharides as minimal structures. The presence of iD in mammalian tissues has been implied (13, 33), but not clearly demonstrated because of a low abundance and the lack of a specific enzyme differentiating the iD unit from the D unit. It was unambiguously demonstrated here that mAb 2A12 discriminated the iD unit from the D unit (Fig. 2A and Table II) and will be a useful tool with which to study the distribution of the iD-containing structures.

Significant yet small proportions (typically, 1.5–5%) of D and/or iD have been found in the mouse brain (20), pig brain (11), and some isolated neuronal CS-PGs, such as DSD-1-PG/
epitope plays a distinct role from the CS-56 epitope in axon guidance. In addition, the spatiotemporal expression of the 2A12 epitope in the developing cerebellum and hippocampus suggests that this epitope may play a role in the development of these two particular regions of the central nervous system, which was supported by the findings that the cultured embryonic hippocampal neurons were markedly stained with 2A12 and that the addition of this antibody to the culture medium inhibited the growth of neurites in the hippocampal neurons. In biosynthesis, the iD unit is generated by the 6-O-sulfation of GalNAc, epimerization of 3-O-GlcUA to 3-O-IdoUA, and 2-O-sulfotransferase can transfer sulfate to both GlcUA and IdoUA (40). Biosynthetic regulation of the expression of the iD unit by 6-O-sulfotransferases, 2-O-sulfotransferase, and CS-epimerase may play critical roles in the development of the hippocampus and cerebellum.

CS/DS chains have recently been implicated in regulating the signaling of various growth factors, such as PTN (12, 41, 42), MK (43, 44), hepatocyte growth factor (45), and FGF7 (46), because the 6-O-sulfation of GalNAc precedes the epimerization and 2-O-sulfation (39), and 2-O-sulfotransferase can transfer sulfate to both GlcUA and IdoUA (40). Biosynthetic regulation of the expression of the iD unit by 6-O-sulfotransferases, 2-O-sulfotransferase, and CS-epimerase may play critical roles in the development of the hippocampus and cerebellum.

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Anti-dermatan Sulfate Antibody Reacting with a Brain Epitope

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