Heparin-binding Growth Factor, Pleiotrophin, Mediates Neuritogenic Activity of Embryonic Pig Brain-derived Chondroitin Sulfate/Dermatan Sulfate Hybrid Chains*

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Chondroitin sulfate (CS) and dermatan sulfate (DS) chains play roles in the central nervous system. Most notably, CS/DS hybrid chains (E-CS/DS) purified from embryonic pig brains bind growth factors and promote neurite outgrowth toward embryonic mouse hippocampal neurons in culture. However, the neuritogenic mechanism is not well understood. Here we showed that pleiotrophin (PTN), a heparin-binding growth factor, produced mainly by glia cells, was the predominant binding partner for E-CS/DS in the membrane-associated protein fraction of neonatal rat brain. The CS/DS chains were separated on a PTN column into unbound, low affinity, and high affinity fractions. The latter two fractions promoted outgrowth of dendrite- and axon-like neurites, respectively, whereas the unbound fraction showed no such activity. The activity of the low affinity fraction was abolished by an anti-PTN antibody or when glia cells were removed from the culture. In contrast, the high affinity fraction displayed activity under both these conditions. Hence, PTN mainly from glia cells mediated the activity of the low affinity but not the high affinity fraction. The anti-CS antibody 473HD neutralized the neuritogenic activities of both fractions. Interaction analysis indicated that the 473HD epitope and PTN-binding domains in the E-CS/DS chains largely overlap. The three affinity subfractions differed in disaccharide composition and the distribution of L-iduronic acid-containing units along the chains. Oversulfated disaccharides and nonconsecutive iduronic acid-containing units were the requirements for the E-CS/DS chains to bind PTN and to exhibit the neuritogenic activities. Thus, CS subpopulations with distinct structures in the mammalian brain play different roles in neurotogenesis through distinct molecular mechanisms, at least in part by regulating the functions of growth factors.

Chondroitin sulfate (CS)1 and dermatan sulfate (DS) proteoglycans (PGs), as well as heparan sulfate PGs (HS-PGs), have been implicated in the processes of neural development in the brain such as neuronal adhesion, migration, and neurite formation (for reviews see Ref. 1–3). CS and DS are synthesized as glycosaminoglycan (GAG) side chains of PGs and consist of repeating disaccharide units of -GlcUA-GalNAc- and -IdoUA-GalNAc-, respectively, and also exist as CS/DS hybrid chains composed of both units in varying proportions. Recent studies have demonstrated that the disaccharide composition and the GlcUA/IdoUA ratio of brain CS/DS chains change during development (4–7) and that certain CS/DS hybrid epitopes are found only in specific regions of the mammalian brain (6, 8), suggesting that CS/DS hybrid chains or their subpopulations play distinct roles in the development of the brain.

The effects of CS/DS chains on neurite formation are controversial. During development, strong immunostaining for CS is often localized at the boundaries of brain subregions such as the roof plate and midline dorsal tectum acting as barriers to migrating neurons or extending axons (9–12). In vitro, CS chains inhibit the migration of neurons and outgrowth of neurites on defined growth-promoting substrata (13–15), and the enzymatic removal of CS chains in vivo permits both axonal regeneration after nigrostriatal tract axotomy and spinal cord injury (16–18). However, tissues expressing CS do not always exclude the entry of axons, and in some cases immunostained CS coincides with developing axon pathways (19, 20). Several studies suggest that some CS-PGs and CS/DS chains promote rather than inhibit neurite outgrowth under certain conditions (21). DSD-1-PG (22), the mouse homologue of rat phosphacan,
promotes neurite outgrowth toward embryonic rat hippocampal neurons, and this effect is neutralized by the monoclonal antibody (mAb) 473HD, which recognizes the DSD-1 epitope embedded in the CS side chains (23).

Further investigations (6, 24, 25) have demonstrated the importance of the rare oversulfated disaccharide units such as Di/Di [HexaUA(2S)1–3GalNAc(6S)] and E/E [HexUA1–3GalNAc(4S,6S)] in the neuritogenic activities of CS/DS chains, which, however, seem to be excluded from the E-CS/DS chains shown here (23). Other recent studies suggest a contribution of IdoUA-containing disaccharides to the neuritogenic activities of CS/DS chains, and the importance of rare oversulfated disaccharide units such as (1→2)-GalNAc(4S,6S) in the neuritogenic activities of CS/DS hybrid chains derived from DSD-1-PG/phosphacan of neonatal mouse brain (25), embryonic pig brain (7), hagfish notochord (26), and shark skin (27). However, the mechanism underlying the neuritogenic activity of CS/DS chains is not well understood. Most interestingly, CS/DS chains (E-CS/DS) purified from embryonic pig brains promoted the outgrowth of dendrite-like neurites toward embryonic mouse hippocampal neurons and bound various growth factors, which have been implicated in neuronal regulation including neuronal adhesion (28, 29) and neuritogenesis (7). On the other hand, brain CS/DS chains bind various extracellular matrix molecules and cell adhesion molecules such as neuron-glial cell adhesion molecules, neuron-glial cell adhesion molecule-related molecules, F3/contactin, tenascin-C, etc. (1, 30).

Here we identified a specific ligand, which regulates the neuritogenic activity of CS/DS chains using affinity chromatography on an E-CS/DS-coupled matrix, as pleiotrophin (PTN). The structural characteristics of the functional E-CS/DS chains for the binding of PTN and the recognition by mAb 473HD were also clarified. CS/DS subpopulations of the embryonic pig brain with distinct structures were shown to play different roles in neuritogenesis through distinct molecular mechanisms at least in part by regulating the functions of a heparin-binding growth factor PTN.

**EXPERIMENTAL PROCEDURES**

**Materials**—Embryonic pigs (body weight 570–620 g) were purchased from a local pig-raising company, and neonatal Wistar rats and pregnant dY mice were obtained from SLC Ine (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, mouse mAb CS-56 and MO-225, amino-cellulofine gel, chondroitinase ABC, chondroitinase AC, chondroitinase AC, chondroitinase AC, Streptomyces griseus, amino cellulofine gel, chondroitinase AC, chondroitinase AC, and protein G-Sepharose 4FF were obtained from Amersham Biosciences. Streptavidin-coated 96-well plates were purchased from Nalge Nunc International (Roskilde, Denmark). Recombinant human (rh) PTN for interaction assays was purchased from RELIA Tech GmbH (Braunschweig, Germany). rhPTN for preparing an affinity column was produced and purified to homogeneity as in the case of recombinant midkine (31), and was a gift from Dr. Sakuma (Cell Signal Inc., Yokohama, Japan). Polyclonal goat anti-rh-PTN IgG and polyclonal goat anti-rh-midkine IgG were obtained from Genzyme/Techne (Cambridge, MA). Horseradish peroxidase-conjugated donkey anti-goat IgG and goat anti-mouse IgG (M) were purchased from Chemicon (Temecula, CA). Rabbit mAb 473HD (IgM) was raised against mouse brain DSD-1-PG/phosphacan as described (23), and goat anti-rat IgM was from StressGen Biotechnology Corp. (San Diego, CA). HS and heparin from bovine intestinal mucosa, a protease inhibitor mixture, poly-ornithine (P-ORN), and anti-neurofilaments were purchased from Sigma, and anti-microtubule-associated protein 2 (MAP2) was from Leico Technologies Inc. (St. Louis, MO). A Vectastain ABC kit was obtained from Vector Laboratories, and a BCA kit was from Pierce. All other chemicals and reagents were of the highest quality available.

**Preparation of E-CS/DS Chains**—The E-CS/DS chains were purified from a brain homogenate from 28 embryonic pigs and were prepared with detergent-free phosphate-buffered saline (PBS) as described previously with some modifications (7). Brieﬂy, the PBS-soluble PG fraction was digested with actinase E to degrade proteins, and the resulting GAG chains were recovered by ethanol precipitation and then dialyzed by gel filtration on a PD-10 column. The GAG mixture was treated with nitrous acid at pH 1.5 at room temperature for 45 min to remove HS followed by dialysis. The dialyzed sample was subjected to an anion-exchange chromatography on an Accell QMA Plus cartridge using a stepwise elution with 0.3 M phosphate buffers, pH 6.0, containing 0.15, 0.5, and 1.5 M NaCl. The 1.5 M NaCl-eluted fraction, which contains 90% of all CS/DS chains, was further purified for gel filtration on a Superdex 75 column as described (7). Trace amounts of free peptides were removed by C-18 hydrophobic chromatography. E-CS/DS could be completely digested with chondroitinase ABC (data not shown, confirming the purity).

**Preparation of a Membrane-bound Protein Fraction**—Neonatal (P1) Wistar rats were anesthetized, and the whole brain was dissected. Tissues (15.1 g) were mixed with 25 ml HEPES buffer (2.5 ml/g tissue), pH 7.2, containing 2.5 mM CaCl2, 1 mM MgCl2, and a protease inhibitor mixture and homogenized in a glass-Teflon Potter homogenizer. The homogenate was centrifuged at 2,000 × g for 10 min at 4 °C, and the precipitate was resuspended in a buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM CaCl2, 0.5% CHAPS, and 0.2M NaCl. The supernatant was subjected to ultracentrifugation at 105,000 × g for 1 h at 4 °C. The resulting residue was mixed with a 2% CHAPS containing 10 mM Tris-HCl buffer, pH 7.4, supplemented with 0.15 M NaCl, 5 mM EDTA and protease inhibitors and agitated at 4 °C overnight. After extraction, the concentrations of CHAPS and CaCl2 were adjusted to 0.5% and 5 mM, respectively, by addition of the Tris-HCl buffer and a 1 mM CaCl2 solution. The mixture was centrifuged at 40,000 × g for 20 min at 4 °C, and the supernatant was subjected to affinity chromatography using the CS/DS-coupled column.

**Affinity Chromatography of Membrane-bound Proteins**—The coupling of CS/DS chains to amino-cellulofine gel was carried out as described by Funahashi et al. (32). The amino-cellulofine gel (1 ml) was suspended in an aqueous E-CS/DS solution (6 mg/800 μl), and the pH was then adjusted to 4.5 with 1M HCl. The mixture was initiated by addition of 200 μl of an 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride solution (6.9 mg/200 μl, pH −4.5), and the pH was kept between 4.5 and 5.5 by intermittent addition of 2 M HCl over a 1-h period. The incubation was allowed to continue for 4 °C with gentle rotation and then stopped by addition of 3 ml of 0.1 M NaHCO3, pH 8.5. The mixture was centrifuged to recover the supernatant. The precipitated gel was washed with CHAPS and 4M urea. Likewise, an affinity chromatography was carried out using each control column. All steps were done at 4 °C.

**Amino Acid Sequence Analysis**—Fractions obtained by affinity chromatography were treated with 80% acetone to precipitate proteins, which were separated by 12.5% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane according to the method of Towbin et al. (34). Resolved proteins were stained with Coomassie Brilliant Blue R-250 for 5 min and then destained with 50% methanol for 15 min. Protein bands were excised, and the NH2-terminal amino acid sequences were determined in an Applied Biosystems Sequencer 492.

**Affinity Fractionation of E-CS/DS**—Bovine serum albumin-free rh-PTN (0.5 mg) was coupled to a Hitrap N-hydroxysuccinimide-activated affinity column (1 ml) according to the manufacturer’s protocol. To protect the GAG-binding sites of PTN from possible inactivation by the coupling, the flow rate of the PTN at rebinding was slowed down to 5 μl/min prior to the coupling. The efficiency of coupling was about 70%, as estimated by the quantification of uncoupled proteins using a BCA kit. The affinity column was equilibrated with 5 ml of 10 mM Tris-HCl buffer (buffer B), pH 7.4, containing 0.15 M NaCl after a wash with buffer B containing 2.0 mM NaCl. E-CS/DS (250 μg) was dissolved in 500 μl of buffer B containing 0.15 M NaCl and loaded onto the column. To maximize the absorbance, loading was repeated six times by recycling.
each unbound fraction. Subsequently, the column was washed stepwise with 3 ml of buffer B containing 0.15, 0.4, or 1.0 mM NaCl. To obtain enough bound material, multiple affinity fractionations were performed at 4 °C. All fractions were desalted on a PD-10 column.

**Cell Culture**—Hippocampal cells were cultured using embryonic day 16.5 (E16.5) mouse brains as described previously (23, 24). Briefly, the hippocampi were obtained by microdissection and dissociated with trypsin treatment. Dissociated cells were suspended in Eagle's modified essential medium (EMEM) containing N2 supplement and then seeded on coverslips as described below. For the culture of purified hippocampal neurons, the dissociated cells were pre-cultured in EMEM containing 10% fetal bovine serum for 3 h at 37 °C to remove more adherent non-neuronal cells (35, 36). After 3 h, the non-adherent cells (neurons) were harvested, rinsed with PBS, seeded on coverslips, and cultured in serum-free EMEM as described above.

**Neurite Outgrowth Assays**—Neurite outgrowth of hippocampal neurons from E16.5 mouse brains was assayed as described (20, 21). Briefly, plastic coverslips (10 × 10 mm) were treated with 1.5 μg/ml of P-ORN in 0.1 M borate buffer, pH 8.2, for 2 h at room temperature, then further coated with CS/DS chains at a dose of 1.0–3.0 μg/ml per coverslip in PBS at 4 °C overnight, and washed with PBS three times before cells were plated on these coverslips at 10,000 cells/cm². Blocking with mAb 473HD was performed as described (37). All steps of ELISA were performed at room temperature. Blocking with 3 ml of buffer B containing 0.15, 0.4, or 1.0 M NaCl. To obtain enough bound material, multiple affinity fractionations were performed at 4 °C. All fractions were desalted on a PD-10 column.

Surface Plasmon Resonance Analysis—Inhibition assays against the interaction of PTN or 473HD with immobilized E-CS/DS was performed using a BIAcore system (BIAcore AB, Uppsala, Sweden) according to the manufacturer's directions. Eluted E-CS/DS was immobilized onto a streptavidin-derivatized sensor chip as described previously (7). To investigate the relation between the 473HD epitope and the PTN-binding domains in E-CS/DS, the E-CS/DS-immobilized sensor chip was first saturated with repeated injections of 473HD antibody or PTN, followed immediately by an injection of PTN (100 ng) or 473HD antibody (diluted 50-fold) in 10 mM HEPES containing 0.15 mM NaCl, 3 mM EDTA, and 0.005% (w/v) Tween 20 (running buffer) onto the surface of the sensor chip. Response curves were recorded, and maximal values were used for calculation.

To investigate the structural characteristics of the PTN-binding domains in E-CS/DS, PTN (100 ng) was incubated with a wide range of concentrations of various GAGs at room temperature for 15 min in a total volume of 130 μl before being spotted to the sensor chip. Response curves were recorded, and the inhibition efficiency was calculated from the reduced values of the maximal response to that of no incubation with GAGs.

**Disaccharide Composition Analysis**—Aliquots of E-CS/DS affinity fractions corresponding to 100–500 pmol of disaccharide were exhaustively digested with chondroitinases ABC (5 μU) or AC-I (2 μU) in 30 mM NaAc buffer containing 0.4% (w/v) Triton X-100 at 4 °C with 30 μl of anti-PTN antibody (10 and 100 μg/ml) or rh-PTN (0.1 and 1.0 μg/ml) was used, it was added to the medium 2 h after the seeding. Rat IgM or goat IgG was run as a control depending on the primary antibody used. After a 24-h culture, cells were fixed with 4% (w/v) paraformaldehyde and then immunostained with anti-MAP2 and anti-neurofilament as described (25). The antibodies were detected using a Vectorstain ABC kit with 3,3'-diaminobenzidine as a chromogen. The number of primary neurites per cell, where primary neurites represent the neurites longer than the cell body. One hundred clearly isolated cells with at least one neurite longer than the cell body were chosen at random per coverslip. The results were expressed as the mean ± S.E., and the significance of differences between means was evaluated with the Student's t-test.

**Western Blotting**—Hippocampal cell or neuronal (4 × 10⁶ cells) in N2-supplemented EMEM were plated on 6-cm culture plates coated with P-ORN and then E-CS/DS or with P-ORN alone as described above. After 24 h of culture under 5% CO2 at 37 °C, the cells were harvested, rinsed with PBS, and then E-CS/DS-immobilized sensor chips were used for calculation.

**Enzyme-Linked Immunosorbent Assay (ELISA)—** E-CS/DS was biotinylated as described (37). All steps of ELISA were performed at room temperature. Biotinylated E-CS/DS in 50 μl of PBS was added to the streptavidin-coated 96-well plate (2 μg/well) and incubated for 1 h. After a wash with 200 μl of PBS containing 0.05% Tween 20 (PBS-T) three times, each well was blocked with 100 μl of 1% bovine serum albumin in PBS for 1 h. Subsequently, 50-fold diluted antibody, 473HD, CS-56, or MO-225, in PBS (50 μl) was added to the wells and incubated for 1 h. Wells were washed with 200 μl of 0.05% Tween 20, 0.15% NaCl, and 10 μl Tris-HCl buffer, pH 8.0 (TBS-T), three times and incubated with 50 μl of alkaline phosphatase-linked anti-rat IgM (diluted 1,000-fold for 473HD) or anti-mouse IgM (diluted 5,000-fold for CS-56 and MO-225) for 1 h. After the washing of plates with TBS-T, color was developed by adding 50 μl of p-nitrophenyl phosphate in 0.1 M sodium carbonate, pH 9.8, and the absorbance at 415 nm was recorded 1 h after the addition of p-nitrophenyl phosphate. As a negative control, the primary antibody was omitted. For the inhibition ELISA, 473HD (diluted 50-fold) was incubated with different concentrations of CS/DS preparations in PBS at room temperature for 1 h, and the mixture was applied to a 96-well plate. The inhibition efficiency was calculated from the reduced absorbance compared with that obtained from an incubation without GAGs.
E-CS/DS was only 90% with a lower proportion of the ΔA unit compared with a chondroitinase ABC digestion, indicating the presence of an IdoUA-containing iA unit consistent with our previous report (7).

To isolate E-CS/DS-binding proteins, an E-CS/DS-immobilized gel was prepared and mixed with the membrane-associated protein fraction from whole brains of postnatal day-1 rats. The mixture was rotated overnight and then poured into an open column. After the washing of the column with a buffer containing 0.2 mM NaCl, the bound proteins were eluted stepwise with 10 mM EDTA- and 1 mM NaCl-containing buffers and then 4 M urea (Fig. 1A). No significant band was detected in the EDTA- or 4 M urea-eluted fractions. Two significant protein bands (17 and 32 kDa) and one faint band at 28 kDa were detected in the fraction eluted from the E-CS/DS affinity column with 1 M NaCl. Neither the band at 17 nor 32 kDa was detected in the 1 M NaCl-eluted fraction from a CS-B affinity column. The 17-kDa protein was also found in the 1 M NaCl-eluted fraction from the E-CS/DS-coupled column, indicating a specific interaction between these proteins and E-CS/DS. The 17-kDa protein was also found in the 1 M NaCl-eluted fraction from a CS-B-coupled affinity column, suggesting that the binding of this protein to E-CS/DS requires an IdoUA-containing structure.

Liquid phase amino acid sequencing revealed that the NH₂ terminus of the 32-kDa protein was blocked, and the NH₂-terminal sequence obtained from the 28-kDa protein did not match any sequences reported to date (data not shown). How-ever, the NH₂-terminal amino acid sequence of the 17-kDa protein was GKKKEPKKKVKSSDAEGEXQKVY, which faithfully matched that of PTN, also known as a heparin-binding growth-associate molecule, except for the four unidentified amino acids denoted by X. This is consistent with a report that PTN is a binding partner for phosphacan, a brain extracellular matrix CS-FG in a CHAPS extract of the mouse brain (44). Reportedly, it binds the protein core of this PG and the CS side chains also. Here we demonstrated that PTN could bind to brain-derived CS/DS chains independently of the core protein and that the interaction appears to require the IdoUA-containing structures, because PTN interacted with E-CS/DS and CS-B but not CS-A.

**Demonstration of Endogenous PTN in the Hippocampal Cell Culture—PTN is a mitogenic and neuritogenic growth factor originally isolated from the brain (45). To investigate whether PTN is involved in the neuritogenic activity of E-CS/DS, the possible production of PTN in the E16.5 mouse hippocampal cell culture was first examined by immunoprecipitation. After a 24-h culture, PTN was detected in the pooled fraction of the conditioned medium and cell lysate, and culturing hippocampal

**TABLE I**

| E-CS/DS | E-CS/DS-U | E-CS/DS-L | E-CS/DS-H |
|---------|-----------|-----------|-----------|
| CSase ABC | CSase AC-I | CSase ABC | CSase AC-I |
| % mol proportion |
| 0.88 | 0.85 | 1.04 | 1.07 |

a Not detected.

b These peaks contained the ΔHexUA-GalNAc(4S,6S) unit and an unidentified component resistant to chondroitinase AC-I.

c Sulfation degree was calculated as the average number of sulfate groups/disaccharide unit.

**FIG. 1.** Affinity purification of E-CS/DS-binding proteins. A, CHAPS extract of the membrane fraction from neonatal rat brains was applied to the E-CS/DS affinity column, which was washed stepwise with 0.2 mM NaCl followed by 10 mM EDTA, 1 mM NaCl, and then 4 M urea. B, proteins in each fraction eluted from the E-CS/DS/coupled column were recovered by acetone (80%) precipitation, separated by 12.5% SDS-PAGE, and visualized by silver staining. Three protein bands at 17, 28 (faint), and 32 kDa were detected in the 1 M NaCl-eluted fraction from the E-CS/DS affinity column. The 17-kDa protein was also found in the 1 M NaCl-eluted fraction from a CS-B-coupled affinity column, suggesting that the binding of this protein to E-CS/DS requires an IdoUA-containing structure.

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Demonstration of PTN in the mouse hippocampal cell culture. Embryonic day 16.5 mouse hippocampal cells (4 × 10^6) were cultured on the substratum coated with P-ORN and then E-CS/DS or with P-ORN alone at 37 °C for 24 h. The cell layer was then solubilized with a buffer containing 1% CHAPS, and the lysate was combined with the conditioned medium and precipitated with the anti-PTN antibody or the anti-MK antibody. Each precipitate was analyzed by blotting with the anti-PTN antibody (left panel) or the anti-MK antibody (right panel), respectively. A control (right lane in the left panel) was carried out without antibodies. The symbols *–* and +*+* denote the presence and absence of each component in the substratum, respectively.

Affinity-fractionated E-CS/DS subfractions exhibit distinct neurite outgrowth-promoting activities in the hippocampal cell culture. A, the E-CS/DS chains were fractionated on a PTN column into unbound, low affinity, and high affinity fractions (E-CS/DS-U, E-CS/DS-L, and E-CS/DS-H, respectively), which were eluted with buffers containing 0.15, 0.4, and 1.0 M NaCl. The relative percentages of these three fractions are shown. B–D, E16.5 mouse hippocampal cells were cultured on the substrata coated with either one of the three fractions at doses of 1.0 and 3.0 μg per coverslip. In the case of E-CS/DS, 2 μg was coated on the coverslip for neurite outgrowth assay. After a 24-h culture, the cells were immunostained, and the cell morphology was analyzed as described under “Experimental Procedures.” Representative images (B) are shown, and the neurite outgrowth was determined as the mean length of total neurites per cell (C) and the mean number of primary neurites longer than the cell body per cell (D). The values represent the mean ± S.E. from three independent experiments. *, 0.01 < p < 0.05, and **, 0.001 < p < 0.01, significant difference from the control. Scale bar, 50 μm.

These results indicated that the neurite outgrowth-promoting materials in E-CS/DS were enriched in its PTN-bound fractions, suggesting a possible involvement of endogenous PTN in the E-CS/DS-induced neurite outgrowth. In addition, the promoting effects of the low and high affinity fractions might involve distinct molecular mechanisms, because they gave a different cell morphology.

To further examine whether endogenous PTN was involved in the E-CS/DS-induced neurite outgrowth in the hippocampal cell culture, an anti-PTN antibody was added to the culture medium. The antibody significantly suppressed the E-CS/DS-L-induced neurite outgrowth at a concentration of 1.0 μg/ml, and exhibited a stronger inhibition at a higher concentration (10 μg/ml) (Fig. 4). On the other hand, the antibody only slightly interfered with the E-CS/DS-H-induced neurite outgrowth (Fig. 4). Control IgG at 10 μg/ml did not influence the promoting effects of either E-CS/DS-L or E-CS/DS-H. Thus, it appears that endogenous PTN mediates the neurite outgrowth.
PTN Synthesized by Adherent Cells Mediates Dendrite-like Neurite Outgrowth—PTN is highly expressed in the embryonic brain, especially in the CA1 subregion of the hippocampus (47). To clarify the origin of the endogenous PTN in the hippocampal cell culture, the hippocampal cells were fractionated into non-adherent neuronal cells and adherent cells, which account for 73–86% and 14–27% of the total cell population, respectively, by preculturing in a serum-containing medium. Immunoprecipitation revealed PTN in the adherent cell fraction, but not in the neuronal cell fraction (Fig. 5A), indicating that endogenous PTN in the hippocampal cell culture was derived from the adherent cells, which mainly contained glia cells but might also include neuronal stem cells and precursor cells for neurons and glia cells.

The purified hippocampal neurons were then used for the neurite outgrowth assay. E-CS/DS-L and E-CS/DS-H induced neurite outgrowth through distinct molecular mechanisms.

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PTN Interacts with E-CS/DS-L and E-CS/DS-H with Distinct Kinetics—The different functions of PTN in the E-CS/DS-L and E-CS/DS-H-induced neurite outgrowth suggested that E-CS/DS-L and E-CS/DS-H interact with PTN in distinct ways. To examine this possibility, a quantitative kinetic analysis of the interaction of PTN with immobilized E-CS/DS-L and E-CS/DS-H was carried out using a BIAcore system. The sensorgrams are shown in Fig. 6, and the kinetic parameters obtained are summarized in Table II. PTN exhibited a higher affinity for E-CS/DS-H (Kd = 0.34 nM) than for E-CD/DS-L (Kd = 37 nM). There was no big difference in the association rate constant for the interactions of PTN with E-CS/DS-L (ka = 1.44 × 10^5 M^-1 s^-1) and E-CS/DS-H (ka = 2.66 × 10^5 M^-1 s^-1), whereas the dissociation rate constant for the release of PTN from E-CS/DS-L (kd = 5.33 × 10^4 s^-1) was 60-fold faster than that from E-CS/DS-H (kd = 9.09 × 10^-4 s^-1). These results indicate that the interaction of PTN with E-CS/DS-L is characterized by quick binding and an easy dissociation as recently reported for the interaction of PTN with CS/DS chains from shark skin (27), whereas the interaction of PTN with E-CS/DS-H is marked by quick binding and an extremely slow dissociation, reflecting the structural difference between the PTN-binding domains of these subfractions, which share a common structural element. A similar slow dissociation in the BIAcore system was observed for the interactions of PTN with CS-E (data not shown) and CS-H (hagfish notochord CS/DS chains) (26), both of which promote the outgrowth of axon-like neurites in vitro (23, 25).

Effects of mAb 473HD on the E-CS/DS-induced Neurite Outgrowth—The monoclonal antibody 473HD recognizes an epitope structure embedded in the CS side chains of DSD-1-PG/phosphacan isolated from neonatal mouse brains (23). DSD-1-PG/phosphacan promotes neurite outgrowth of hippocampal neurons from E18 rats (23) and E 16.5 mice (25), and these activities are neutralized by 473HD. Here we tested the effects of 473HD on the E-CS/DS-L- and E-CS/DS-H-induced neurite outgrowth. As shown in Fig. 7, 473HD suppressed the promoting activities of E-CS/DS-L and E-CS/DS-H in the hippocampal cell culture, suggesting that the 473HD epitope(s) is involved in the neurite outgrowth-promoting activities of both E-CS/DS-L and E-CS/DS-H.

The PTN-binding Domain and the 473HD Epitope Overlap in E-CS/DS—As described above, PTN-bound fractions, E-CS/DS-L and E-CS/DS-H, elicited neurite outgrowth, with E-CS/DS-L being more effective than E-CS/DS-H. Since the PTN-binding domain of 473HD overlaps with the E-CS/DS-variant-binding domains, these results suggest an involvement of the 473HD epitope(s) in the neurite outgrowth-promoting activities of E-CS/DS-L and E-CS/DS-H.
DS-L and E-CS/DS-H, were the biologically active subpopulations of E-CS/DS for its promotion of neurite outgrowth, and the 473HD epitope(s) in both preparations were the functional structural element for this activity, which raised the possibility that the PTN-binding domain(s) and the 473HD epitope(s) overlap in the E-CS/DS chains. To test this hypothesis, possible interactions of E-CS/DS with 473HD and two commercial anti-CS monoclonal antibodies, CS-56 (48) and MO-225 (49), were first examined by ELISA. 473HD strongly interacted with immobilized E-CS/DS, whereas CS-56 and MO-225 showed no significant interaction (Fig. 8A). The inhibition ELISA further revealed that in inhibitory activity toward the interaction between 473HD and E-CS/DS, the E-CS/DS subfractions obtained by the PTN affinity chromatography ranked in the following order: E-CS/DS-H (IC50 = 0.14 μg/ml) > E-CS/DS-L (IC50 = 0.44 μg/ml) > E-CS/DS (IC50 = 1.0 μg/ml) > E-CS/DS-U (IC50 = 17.8 μg/ml) (Fig. 8B), indicating the abundance of 473HD epitope(s) in the PTN-bound and unbound fractions in this order.
The experiments were performed three to the values obtained from the experiments without coating GAGs and followed by a wash with PBS. E16.5 mouse hippocampal cells were plated and incubated at room temperature for 2 h. After the incubation followed by a wash with PBS, E16.5 mouse hippocampal cells were plated on the coverslips and cultured for 24 h. The cells were immunostained H-induced neurite outgrowth in the hippocampal cell culture.

Effects of various GAGs on the Binding of PTN to E-CS/DS—In view of the functional importance of the PTN binding for the neurite outgrowth-promoting activity, the structural characteristics of such binding domains were investigated using various CS and HS variants in inhibition assays using a BIAcore system. As shown in Fig. 9A, heparin and HS potently inhibited the binding of PTN to immobilized E-CS/DS (IC_{50} = 0.09 and 0.16 \mu g/ml, respectively), reflecting the heparin-binding property of PTN. Oursulfated CS-E and CS-D also strongly suppressed the binding (IC_{50} = 0.13 and 0.24 \mu g/ml, respectively), and CS-C had a moderate effect (IC_{50} = 1.47 \mu g/ml). However, CS-A only slightly influenced the binding of PTN to E-CS/DS (IC_{50} > 100 \mu g/ml). Most interestingly, CS-B, which has a comparable degree of sulfation (~1.05 sulfate/disaccharide) with CS-C, exhibited strong inhibition (IC_{50} = 0.22 \mu g/ml) in this interaction. The overlaid sensorgrams obtained by inhibition assays using 1.0 \mu g/ml of each CS or HS variant are shown in Fig. 9B as examples. The results suggested that oursulfated disaccharides and/or IdoUA-containing structures in the E-CS/DS chains might be involved in the binding to PTN.

Comparison of Disaccharide Composition and Distribution of IdoUA along the E-CS/DS Chains in the Affinity Fractions—To further investigate the PTN-binding structures in E-CS/DS, the disaccharide composition and the distribution of IdoUA residues along the chains were compared among E-CS/DS-U, E-CS/DS-L, and E-CS/DS-H. The results from the analysis of disaccharide composition after digestion with chondroitinase ABC or AC-I are summarized in Table I. The contents of O and/or C6C units gradually decreased in the fractions with higher affinity for PTN, suggesting that the long and consecutive sequences containing O and/or C6C units were not directly involved in the PTN-binding structure. In contrast, increased amounts of the A unit were observed with increasing affinity, indicating a positive role for A and/or C6C units in the binding of PTN to E-CS/DS. In addition, oursulfated disaccharide units (\Delta D, \Delta B, \Delta E, and \Delta T) were recovered in the PTN-bound fractions at higher concentrations compared with those of E-CS/DS-U. Most intriguingly, \Delta D was the most abundant unit in E-CS/DS-L, whereas the \Delta E unit along with \Delta B and \Delta T was found at the highest concentrations in E-CS/DS-H. The degrees of sulfation of E-CS/DS-L (1.04 sulfate/disaccharide) and E-CS/DS-H (1.07 sulfate/disaccharide) were comparable, but significantly higher than that of E-CS/DS-U (0.85 sulfate/disaccharide), suggesting that the degrees of sulfation and the di- and/or
**FIG. 8.** The 473HD antibody epitope and the PTN-binding domains in the E-CS/DS chains overlap. **A**, interactions between immobilized E-CS/DS and anti-CS antibodies, 473HD, CS-56, and MO-225, were examined by ELISA as described under “Experimental Procedures.” **B**, in the inhibition ELISA, 50-fold diluted 473HD antibody was incubated with a wide range of concentrations of the unfractionated E-CS/DS or its subfractions, E-CS/DS-U, E-CS/DS-L, and E-CS/DS-H, at room temperature for 1 h, and each mixture was applied to the ELISA well, where E-CS/DS chains had been immobilized. The inhibition efficiency was calculated from the reduced absorbance compared with that obtained without soluble CS/DS. These experiments were performed in duplicate, and the values represent means. **C**, inhibition of the PTN binding to E-CS/DS by 473HD was analyzed by using a BIAcore system. An E-CS/DS-immobilized sensor chip was saturated with repeated injections of 473HD followed by an injection of 100 ng of PTN, and the response obtained by the injection of PTN was recorded. The responses obtained after injections of PTN with and without the prior saturation with 473HD are shown. **D**, an E-CS/DS-immobilized sensor chip was saturated with repeated injections of PTN followed by an injection of the 50-fold diluted 473HD antibody. The maximal responses obtained by injection of 473HD with and without the PTN saturation are shown. The values in **C** and **D** represent the mean ± S.D. from three independent experiments.

**FIG. 9.** Structural requirements for PTN binding in the E-CS/DS chains. **A**, inhibitory effects of various concentrations of heparin, CS-E, HS, CS-B, CS-D, CS-C, and CS-A on the binding of PTN (100 ng) to the immobilized E-CS/DS chains were analyzed using a BIAcore system. **B**, overlaid sensorgrams obtained with 1 μg/ml of various GAGs as inhibitors are shown. **C**, comparison of the distribution of IdoUA residues along the CS/DS chains of the E-CS/DS subfractions. Equivalent amounts (1 nmol as disaccharides) of E-CS/DS-U, E-CS/DS-L, and E-CS/DS-H were individually digested with chondroitinase B extensively, labeled with the fluorophore 2-aminobenzamide, and then separated by gel filtration on a Superdex Peptide column (1.0 × 30 cm) as described under “Experimental Procedures.” The sizes of the resolved peaks are indicated.
requires specific carbohydrate sequences. E-CS/DS, whereas the higher affinity of PTN for E-CS/DS-H trisulfated units are important factors in the binding of PTN to E-CS/DS.

mixed GlcUA and IdoUA residues are critical for the binding of PTN to E-CS/DS chains in the PTN-bound fractions than in the PTN-unbound fraction and that a long consecutive IdoUA-containing domain in the parent polymers. These results suggest that IdoUA residues are more scattered along the E-CS/DS chains and form the functional neuritogenic domains of the PTN-unbound fraction and that a long consecutive IdoUA-containing sequence is not required, but hybrid sequences composed of mixed GlcUA and IdoUA residues are critical for the binding of PTN to E-CS/DS.

**DISCUSSION**

In this study, endogenous PTN mainly synthesized by glial cells was identified as the predominant ligand for E-CS/DS in the neonatal rat brain and mediated the CS/DS chain-induced outgrowth of dendrite-like neurites toward mouse hippocampal neurons in culture. The results are consistent with the observation that PTN is often located in the glia-rich regions of the developing brain, such as the radial glial processes in the cerebral cortex of embryonic rats (46). The PTN-binding domains largely overlap the 473HD epitopes in the E-CS/DS chains and form the functional neuritogenic domains of the CS/DS chains. Both oversulfated disaccharides and scattered IdoUA-containing disaccharides are critical for the PTN-binding neuritogenic domain.

CS/DS-PGs are mainly located at extracellular matrices and cell surfaces. The E-CS/DS chains, purified from the PBS-soluble PG fractions of embryonic pig brains, mainly represent the CS/DS side chains of secreted CS/DS-PGs, such as neurocan, phosphacan, brevican, and versican, and may also include CS/DS chains of shedded ectodomains of the membrane-associated CS/DS-PGs (NG2 and neuroglycan C, etc.) and hybrid type CS/HS-PGs such as syndecans (1). PTN, a neuritogenic growth factor, binds HS chains of syndecan-3 (51) and also interacts with the core protein and CS chains of phosphacan (44), both of which are involved in the PTN-mediated neurite extension and neuronal migration (44, 52, 53). In this study, PTN specifically bound to E-CS/DS- and CS-B-immobilized gels, but not to CS-A-immobilized gels, suggesting a possible involvement of the IdoUA-containing structures in the E-CS/DS chains in the binding to PTN. This notion was supported by the finding that CS-B strongly inhibited the binding of PTN to immobilized E-CS/DS, whereas CS-C with a comparable degree of sulfation to CS-B exhibited 6.7-fold less inhibitory activity. Oversulfated CS-E and CS-D strongly inhibited the binding of PTN to E-CS/DS, which is consistent with a recent report (8) that PTN bound to CS-E and CS-D with high affinity; a small proportion of D unit (1.3%) in the CS chains of phosphacan mediated the binding of PTN to phosphacan. Thus, both IdoUA-containing disaccharides and oversulfated disaccharides (see below) in the E-CS/DS chains appear to be required for the interaction of PTN with E-CS/DS.

The affinity chromatography on a PTN-coupled column enriched the neurite outgrowth-promoting E-CS/DS chains in the bound subfractions. The endogenous PTN mediated the E-CS/DS-L-induced neurite outgrowth as shown by the inhibition with the anti-PTN antibody but was not required for such activity of E-CS/DS-H, suggesting that the neuritogenic activities of E-CS/DS-L and E-CS/DS-H are expressed via distinct mechanisms. This speculation was supported by the following observations. 1) E-CS/DS-L and E-CS/DS-H induced different cell morphologies. The cells cultured on the substratum coated with E-CS/DS-L exhibited a dendrite-like cell morphology, whereas those cultured on the E-CS/DS-H-coated substratum formed axon-like neurites. It should be noted, however, that identification of dendrite or axon requires longer incubation periods. 2) After the removal of non-neuronal cells from the hippocampal cell culture, the neuritogenic activity of E-CS/DS-L toward purified neurons largely diminished but was restored with the addition of exogenous PTN to the medium. In contrast, the same treatments had little effect on the neuritogenic activity of E-CS/DS-H. 3) The interactions of PTN with E-CS/DS-L and E-CS/DS-H were different. Although PTN bound to E-CS/DS-L and E-CS/DS-H at comparable rates, it was released from the former at a 60-fold higher rate than from the latter, reflecting the tight association with the latter, which suggests that the PTN-binding domains for E-CS/DS-L and -H share a common structure, but either one may contain an additional structural element. Thus, we propose a model for the relation of PTN to the neuritogenic activity of the E-CS/DS chains (Fig. 10). It is likely that E-CS/DS-L efficiently captures and presents PTN produced mainly by adherent glia cells to a high affinity receptor (51, 55) on the neuronal cell surface to promote neurite outgrowth acting as a co-receptor for the PTN signaling. On the other hand, a portion of the E-CS/DS-H chains, which are close to the adherent cells in the culture, would tightly seize the adherent cell-derived PTN and prevent it from being exposed to the neuronal cell surface. At present, the molecular mechanism, through which the E-CS/DS-H chains promote axon-like neurite outgrowth, remains to be investigated. The hippocampal neurons may express a specific receptor for E-CS/DS-H, or a growth factor other than PTN may mediate the neuritogenic activity of E-CS/DS-H.

CS-D and CS-E promote the outgrowth of dendrite- and axon-like neurites, respectively, toward neurons in hippocampal cell cultures (24, 55). In this study, CS-D did not significantly show activity once the non-neuronal cells were removed from the culture, and the addition of exogenous PTN markedly stimulated the activity of CS-D. In contrast, CS-E exhibited neuritogenic activity toward purified hippocampal neurons independently of endogenous or exogenous PTN. Thus, the neuritogenic activities of CS-D and CS-E resemble those of E-CS/DS-L and E-CS/DS-H, respectively. Most likely, the neuritogenic activity of CS-D involves PTN, whereas that of CS-E does not. In fact, the affinity for the binding of CS-E to immobilized PTN is 3.5-fold higher than that for the binding of CS-D to immobilized PTN (8).
mAb 473HD recognizes an epitope embedded in the CS/DS side chains of DSD-1-PG/phosphacan and inhibits the neuritogenic activity of DSD-1-PG/phosphacan (23), suggesting that this epitope is the functional domain of this PG. In the present study, 473HD suppressed the neuritogenic activities of both E-CS/DS-L and E-CS/DS-H, suggesting that the 473HD epitope is shared by both types of CS/DS chains. Most interestingly, the 473HD epitope is also present in CS-C and CS-D (6, 24), and recognizes structures containing an A-D tetrasaccharide sequence [GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S)] (54). Such a sequence may be present in the CS/DS side chains of various PGs including phosphacan in the brain. This study revealed that the 473HD epitope and the PTN-binding domains largely overlap, which may explain the inhibitory effects of 473HD on the neuritogenic activity of CS/DS-L. In addition, 473HD significantly inhibited the neuritogenic activity of E-CS/DS-H for axon-like neurites, suggesting that the functional domain of the neuritogenic activity of the E-CS/DS-H chains also includes the 473HD epitope (Fig. 10).

D/ID and E/EI disaccharides are the structural elements for the neuritogenic activities of various oversulfated CS and DS chains derived from marine organisms (see Refs. 24–27 and 55 and for review see Refs. 3 and 42). Although IdoUA-containing structures are essential for the neuritogenic activity of E-CS/DS (7), the possible contribution of small proportions (2.5–3.6%) of D and E units to E-CS/DS chains to this activity remains to be firmly established. The disaccharides in this study revealed that E-CS/DS-U, E-CS/DS-L, and E-CS/DS-H differed considerably. The enrichment of oversulfated units including D/ID, E/EI, B/Bi, and T/Tt in E-CS/DS-L and E-CS/DS-H indicates that these units are most likely involved in their neuritogenic activities, being consistent with our previous finding that D/ID- or E/EI-containing oversulfated CS/DS chains tend to form multiple neurites or a long neurite, respectively (24, 25, 56). Analysis of the chondroitinase B digests of these three CS/DS preparations showed that critical IdoUA residues in IA, ID, or IE, or iB units were more sparsely distributed along the CS/DS chains in E-CS/DS-L than in E-CS/DS-U, suggesting that a long sequence of consecutive IdoUA-containing disaccharides may not be necessary for the PTN binding.

Further studies are required to elucidate the functional sequences in the E-CS/DS chains responsible for these activities, which would provide valuable information not only for a better understanding of CS/DS-dependent neuritogenesis but also for drug designs for regenerating neurons and treating dementia.

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