THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 48, Issue of December 1, pp. 37407–37413, 2000

Printed in U.S.A.

Neuronal Cell Adhesion, Mediated by the Heparin-binding Neuroregulatory Factor Midkine, Is Specifically Inhibited by Chondroitin Sulfate E

STRUCTURAL AND FUNCTIONAL IMPLICATIONS OF THE OVER-SULFATED CHONDROITIN SULFATE*

Received for publication, March 26, 2000, and in revised form, August 21, 2000
Published, JBC Papers in Press, September 7, 2000, DOI 10.1074/jbc.M002538200

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The heparin-binding neurotrophic factor midkine (MK) has been proposed to mediate neuronal cell adhesion and neurite outgrowth promotion by interacting with cell-surface heparan sulfate. We have observed that over-sulfated chondroitin sulfate (CS) D and CS-E show neurite outgrowth-promoting activity in embryonic day (E) 18 rat hippocampal neurons (Nadanaka, S., Clement, A., Masayama, K., Faissner, A., and Sugahara, K. (1998) *J. Biol. Chem.* 273, 3296–3307). In the present study, various CS isoforms were examined for their ability to inhibit the MK-mediated cell adhesion of cortical neuronal cells in comparison with heparin from porcine intestine and heparan sulfate from bovine kidney. E17–18 rat cortical neuronal cells were cultured on plates coated with recombinant MK in a grid pattern. The cells attached to and extended their neurites along the MK substratum. Cell adhesion was inhibited by squid cartilage over-sulfated CS-E as well as by heparin, but not by heparan sulfate or other CS isoforms. Direct interactions of MK with various glycosaminoglycans were then evaluated using surface plasmon resonance, showing that CS-E bound MK as strongly as heparin, followed by other over-sulfated CS isoforms, CS-H and CS-K. Furthermore, E18 rat brain extracts showed an E disaccharide unit, GlcUAβ1–3GalNAc(4,6-*O*-disulfate). These findings indicate that CS chains containing the E unit as well as heparin-like glycosaminoglycans may be involved in the expression and/or modulation of the multiple neuroregulatory functions of MK such as neuronal adhesion and migration and promotion of neurite outgrowth.

Midkine (MK)[1] was identified as a retinoic acid-inducible differentiation factor in an embryonic carcinoma cell line (1) and constitutes a unique family of heparin (Hep)-binding proteins with the pleiotrophin (PTN)/Hep-binding growth-associated molecule, which was isolated from rat brains as a neurite outgrowth-promoting protein expressed during the developmental stage corresponding to rapid axonal growth (2). MK and PTN have 45% sequence identity at the amino acid level (1, 3, 4) and share many biological activities, including enhancement of plasminogen activator activity (5), and neuroregulatory activities such as promotion of neurite outgrowth (2, 6, 7). MK has a neurotrophic activity for embryonic neurons (6). Their Hep-binding properties possibly indicate that cell-surface heparan sulfate (HS) mediates biological functions of both PTN and MK. Syndecan-3 and syndecan-4, on the cell surface, appear to be high affinity receptors in the PTN-dependent neurite outgrowth of cortical neurons (K_d = 0.6 nM) (8, 9) and in the MK-mediated neural network formation in peripheral nerve tissues (K_d = 0.3 nM) (10), respectively. The cortactin/Src kinase pathway has been implicated in syndecan-3-dependent neurite outgrowth (11). A highly sulfated region of cell-surface HS chains has been suggested to be involved in the MK-mediated adhesion of embryonic rat cortical neurons (12), and the two Hep-binding sites have been localized to the C-terminal half of the molecule (13, 14).

In contrast, PTN binds 6B4-PG/phosphacan (15), which is abundantly expressed in the brain as a chondroitin sulfate proteoglycan (CS-PG) and is the alternatively spliced extracellular domain of the signal-transducing receptor-like protein-tyrosine phosphatase PTPζ/RPTPβ (16–18). Binding to phosphacan is inhibited not only by Hep/HS, but also by shark cartilage chondroitin sulfate (CS) C (15). The PTN-stimulated migration of embryonic rat cortical neurons is also inhibited by CS-C (19). These findings indicate that PTN exhibits its functions through interactions with the CS moiety of PTPζ as well as the HS proteoglycan in the brain.

CS-PGs may play important roles in the regulation of brain development (20, 21). Numerous studies indicate that CS-PGs inhibit neurite outgrowth and serve as inhibitory barriers to migrating neurons or extending axons during brain development (22–27). However, tissues that express CS do not always exclude the axon entry, and CS staining coincides with devel-

* This work was supported in part by the Science Research Promotion Fund from the Japan Private School Promotion Foundation and by Grant-in-aid for Scientific Research 09558082 and Grant-in-aid for Scientific Research on Priority Areas 09470509 from the Ministry of Education, Science, Culture, and Sports of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MK, midkine; Hep, heparin; PTN, pleiotrophin; HS, heparan sulfate; PG, proteoglycan; CS, chondroitin sulfate; CS-PG, chondroitin sulfate proteoglycan; PTPζ, protein-tyrosine phosphatase ζ; DS, dermatan sulfate; GAG, glycosaminoglycan; E, embryonic day(s); GlcUA, glucuronic acid; IdoUA, iduronic acid; $\Delta^{4,5}$-HexUA, 4-deoxy-α-*threo*-hex-4-enepyranosyluronic acid; 2*S*, 3*S*, 4*S*, and 6*S*, 2-, 3-, 4-, and 6-*O*-sulfate, respectively; FGF, fibroblast growth factor; BSA, bovine serum albumin; HPLC, high performance liquid chromatography.

oping axon pathways (reviewed in Ref. 28). Indeed, several studies have suggested that CS-PGs and CS glycosaminoglycans (GAGs) can promote neurite outgrowth *in vitro* (29–32). In particular, the CS-PG designated DSD-1-PG from developing mouse brains promotes neurite outgrowth of embryonic rat mesencephalic (E14) and hippocampal (E18) neurons (32). This PG has recently been identified as the mouse homologue of rat phosphacan (28). Alternatively, it is secreted primarily by immature glial cells and is immunologically detected on the glial cell surface (32), but also on the migrating neuronal surface (15, 33). There are subsets of neurons that express phosphacan as well as PTPζ (34). The neurite outgrowth-stimulating capacity of DSD-1-PG is strongly reduced by monoclonal antibody 473HD, which recognizes a CS epitope. Surprisingly, shark cartilage CS-C inhibits the interactions between the monoclonal antibody and the DSD-1 epitope (32). We have shown that shark cartilage CS-D also exhibits the inhibitory activity and neurite outgrowth-promoting activity for embryonic rat hippocampal neurons as does DSD-1-PG/phosphacan (35, 36). Notably, DSD-1-PG/phosphacan contains the D disaccharide unit characteristic of CS-D (36). During the course of this study, we observed that squid cartilage CS-E also exhibits neurite outgrowth-promoting activity, which, however, is not inhibited by monoclonal antibody 473HD (35, 37), indicating that the mechanism of the neurite-promoting action of CS-E is different from that of CS-D. Thus, rat brain neuronal cells specifically responded to stimulation by cartilage CS chains derived from remote animal species such as shark and squid. Structurally, both shark cartilage CS-D and squid cartilage CS-E are over-sulfated, containing more than one sulfate group/disaccharide unit, whose characteristic positions on each saccharide constituent are different from each other. The D and E units are GlcUA(2*S*)β1–3GalNAc(6*S*) and GlcUAβ1–3GalNAc(4*S*,6*S*), respectively. Note that the A, B, and C units characteristic of CS-A, CS-B, and CS-C are GlcUAβ1–3GalNAc(4*S*), IdoUAα1–3GalNAc(4*S*), and GlcUAβ1–3GalNAc(6*S*).

In view of the neurite outgrowth-promoting activity of CS-D and CS-E and the findings that PTN binds 6B4-PG/phosphacan through interacting with the CS moiety, we explored the possibility that MK, which is highly homologous to PTN, functions in the brain through interacting with over-sulfated CS isoforms. We detected both CS-D and CS-E in E18 rat brains and found that exogenous CS-E, but not CS-D, inhibited MK-mediated adhesion of E18 rat cortical neuronal cells to culture substrates. Preliminary findings have been reported in abstract form (38).

EXPERIMENTAL PROCEDURES

Materials—Recombinant mouse MK expressed in the baculovirus system was prepared as previously reported (7). Recombinant human MK expressed in yeast was from Shinya Ikematsu (Meiji Cell Technology Center, Odawara, Japan) (39). Both recombinant mouse MK and recombinant human MK gave essentially the same findings in cell adhesion assays and GAG binding assays. Recombinant human FGF-1 (acidic FGF) expressed in yeast was purchased from Wako Pure Chemical Industries (Osaka, Japan). Recombinant human FGF-2 (basic FGF) was from Koichi Igarashi (Discovery Research Laboratories II, Takeda Chemical Industries Ltd., Tsukuba, Japan) (40), and FGF-10 expressed in *Escherichia coli* (41) was from Takashi Katsumata (Sumitomo Pharmaceutical Co., Osaka). King crab cartilage CS-K (42) and hagfish notochord CS-H (43) were from Nobuko Seno (Ochanomizu University, Tokyo). Dextran sulfate (5 kDa) was from Atushi Tamada (Kyoto University, Kyoto, Japan). The following materials were purchased from Seikagaku Corp. (Tokyo): unsaturated CS disaccharide standards; CS isoform preparations (super special grade) including CS-A from whale cartilage, CS-A from bovine tracheal cartilage, CS-B or dermatan sulfate (DS) from pig skin, CS-C and CS-D from shark cartilage, and CS-E from squid cartilage (average molecular mass of 70 kDa); porcine intestinal Hep (average molecular mass of 19 kDa) and bovine kidney HS; and chondroitin AC lyase (EC 4.2.2.5) from *Arthrobacter aurescens*, chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris*, and chondro-4-sulfatase (EC 3.1.6.9) and chondro-6-sulfatase (EC 3.1.6.10) from *P. vulgaris*. *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride, *N*-hydroxysuccinimide, and the running buffer for the BIAcore system (10 mM HEPES/NaOH containing 0.15 M NaCl, 3 mM EDTA, and 0.005% Tween 20) were purchased from BIAcore AB (Uppsala). [^{3}H]Acetyl-labeled CS-E (1.875×10^5 cpm/μg) was prepared as described previously (44) by *N*-deacetylation with hydrazine, followed by *N*-reacetylation with [^{3}H]$(CH_3CO)_2O$.

Inhibition Assay of Neurite Outgrowth by GAGs on MK Tracks—Inhibition assays of neurite outgrowth by various GAGs were carried out as described previously (7). Briefly, embryonic rat cerebral cortex (E17–18) was isolated and digested with trypsin and DNase I. After the enzyme treatments, single cells were collected by centrifugation and used for the neurite outgrowth inhibition assays. A 24-well plastic culture plate (Falcon 3057, Becton Dickinson) was coated with recombinant mouse MK at a concentration of 10 μg/ml. After washing with H_2O, the plate was dried, and metal electron microscopy grids (HDL 200, Veco, Amsterdam) were placed in the wells, followed by irradiation with UV light at 315 nm to produce the grid pattern of the substrate. After removing the grids, the wells were incubated with Dulbecco's modified Eagle's medium containing 10 mg/ml bovine serum albumin (BSA) for blocking. After washing with H_2O, the rat brain nerve cells in suspension described above were seeded (0.8×10^6 cells/well) and cultured in medium comprising Dulbecco's modified Eagle's medium, 0.1% fetal bovine serum, insulin/transferrin/sodium selenite, and penicillin/streptomycin in the absence or presence of each test GAG preparation for 24–48 h at 37 °C in an atmosphere of 5% CO_2 and 95% air. Photographs were taken after 20 h.

Filter Binding Assays—Filter binding assays of various GAGs with MK were performed as described previously (44). Various amounts (~0.1–0.4 μg) of recombinant human MK were incubated with [^{3}H]CS-E (80 ng, ~15,000 cpm) in 50 μl of 20 mM HEPES/NaOH, pH 7.3, containing 150 mM NaCl and 0.5 mg/ml BSA at room temperature for 3 h. Incubations for inhibition assays were conducted in the presence of 50 ng of various individual exogenously added GAGs. The growth factor, along with any bound [^{3}H]CS-E, was recovered by quick passage of the samples through nitrocellulose filters (0.45-μ pore size, 25-mm diameter; Sartorius, Göttingen, Germany), which had been placed onto a 12-well vacuum-assisted manifold filtration apparatus. The filters were prewashed with 10 ml of the buffer described above before application of the samples, which was immediately followed by washing five more times with 2 ml of the same buffer. Protein-bound radioactivity was determined after submersion of the filters in 1 ml of 1 M NaCl and 0.05 M diethylamine, pH 11.5, for 30 min; radioactivity in the eluate was determined in a liquid scintillation counter (LSC-700, Aloka Co., Tokyo) using scintillation fluid containing 1.2% (w/v) 2,5-diphenyloxazole and 33% (w/v) Triton X-100.

MK Binding Assays of Various GAGs in the BIAcore™ System—The binding of various GAGs to recombinant human MK was evaluated in the BIAcore™ 2000 system (BIAcore AB), which had been equilibrated with the running buffer (10 mM HEPES-NaOH, pH7.4, containing 0.15 M NaCl, 3 mM EDTA, and 0.005% Tween 20) at a flow rate of 10 μl/min. A CM5 sensor chip (BIAcore AB), which is coated with carboxymethyldextran, was activated by injecting a solution mixture (1:1, v/v) of 200 mM *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride and 100 mM *N*-hydroxysuccinimide at a flow rate of 10 μl/min. A 70-μl aliquot of the recombinant human MK sample (50 μg/ml in 10 mM acetate/NaOH buffer, pH 5.08) was then injected at a flow rate of 10 μg/min for immobilization of MK. The remaining activated *N*-hydroxysuccinimide ester groups were blocked by injecting 1 M ethanolamine hydrochloride/NaOH, pH 8.5, and washed with 10 μl of 1 M NaCl. Binding assays were performed at 25 °C with a constant flow rate of 10 μl/min in both association and dissociation phases. GAGs in a series of concentrations ranging from 0.25 to 4.0 μg/ml in the running buffer were injected into the flow cell, and the changes in resonance units were recorded. After each run, regeneration of the sensor chip surface was accomplished by an injection of 10 μl of 1 M NaCl. The values for rate constants were determined by nonlinear regression analysis using BIAevaluation 2.1 software as described by Karlsson and Stahlberg (45). Association rate constants (k_a) were calculated from the linear portion of sensorgrams during the early association phase. Dissociation rate constants (k_d) were calculated from the early portion of the dissociation phase after the completion of the sample injection during the washout period. The apparent equilibrium dissociation constant (K_d) was calculated as the ratio k_d/k_a. The kinetic constants were determined by three independent experiments.

Analysis of CS Chains of Soluble PGs from Embryonic Rat Brains—A

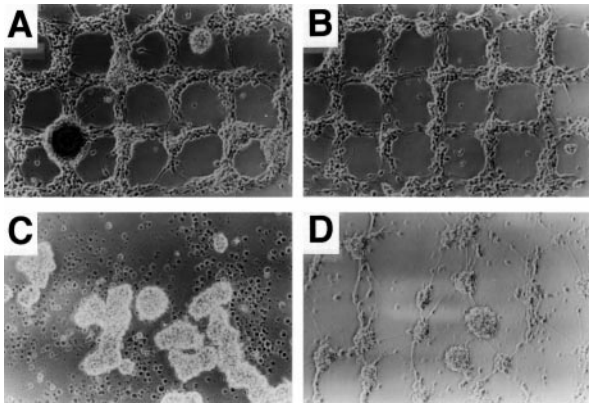

FIG. 1. **Inhibition of the MK-mediated neuronal cell adhesion by various GAGs.** 24-Well plastic culture plates were coated with recombinant mouse MK (10 μg/ml) for 2 h at room temperature. A grid pattern was then made by the UV inactivation technique using electron microscopy grids with a pitch of 125 μm. After blocking with 10 mg/ml BSA, brain cells prepared from rat embryo cerebral cortex (E17–18) were cultured as described under "Experimental Procedures" in the absence (*A*) or presence of 50 μg of CS-A (*B*), 2.5 μg of CS-E (*C*), or 50 μg of CS-H (*D*).

soluble brain CS-PG fraction was prepared according to Oohira *et al.* (46) as described previously (47). Briefly, E18 rat brains were homogenized in 5 volumes (w/v) of ice-cold phosphate-buffered saline containing 20 mM EDTA. The homogenate was centrifuged, and the pellet was subjected to re-homogenization in phosphate-buffered saline. After centrifugation, both supernatant fluids were combined, concentrated, and then dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 50 mM sodium acetate using a Centricon-10 concentrator (Millipore Corp., Bedford, MA). The protein concentration of the PG fractions was determined using the BCA protein assay kit (Pierce) with BSA as a standard. The PG fractions (~100 μg of protein each corresponding to 39.6 mg of brain, wet weight) were first digested using 25 mIU of chondroitin ABC or AC lyase as described (48) and evaporated to dryness. The digests were derivatized with 2-aminobenzamide according to the manufacturer's instructions for the Signal™ labeling kit (Oxford Glycosystems, Abingdon, United Kingdom) as described (49). The labeled disaccharides were analyzed by HPLC on an amine-bound silica PA03 column (4.6 × 250 mm; YMC Co., Kyoto) as described previously (50). HPLC was performed using a linear gradient from 16 to 798 mM NaH_2PO_4 over a 60-min period at a flow rate of 1.0 ml/min at room temperature. The identification and quantification of the resulting disaccharide units were accomplished by comparison with the standard CS-derived unsaturated disaccharides labeled with 2-aminobenzamide as described (49) and by enzymatic digestion using chondro-4-sulfatase and chondro-6-sulfatase as reported (48).

RESULTS

Recently, CS-E derived from squid cartilage was demonstrated to contain novel disulfated and trisulfated disaccharide units, including GlcUA(3*S*)-GalNAc(4*S*), GlcUA(3*S*)-GalNAc-(6*S*), and GlcUA(3*S*)-GalNAc(4*S*,6*S*) (51), in addition to the conventional disulfated disaccharide unit GlcUA-GalNAc-(4*S*,6*S*) (52). In view of such a heavily sulfated structural feature of CS-E, comparable to that of Hep, we examined, using filter binding assays in the preliminary studies, whether CS-E could interact with several Hep-binding growth factors, including MK, FGF-1 (acidic FGF), FGF-2 (basic FGF), and FGF-10 (data not shown). CS-E showed strong binding with only MK, implying that the binding was specific and was not caused merely by electrostatic interactions. Hence, the binding of MK to CS-E was investigated as described below.

Specific Inhibition of MK-mediated Neuronal Cell Adhesion by CS-E—The effects of various CS isoforms on the MK-mediated adhesion of neuronal cells prepared from embryonic rat brain (E17–18) were examined. In the absence of exogenous GAGs, the cells specifically bound to the MK-coated substratum, forming a grid pattern along the MK coating (Fig. 1*A*), as reported previously (7). Most of the tested CS isoforms showed no inhibition even at a high concentration of 50 μg/ml; representative results obtained with CS-A are shown in Fig. 1*B*. In strong contrast, squid cartilage CS-E exhibited potent inhibition at a low concentration of 2.5 μg/ml (Fig. 1*C*), which was comparable to the inhibitory concentration of Hep (Table I). Although Hep showed moderate inhibition even at a lower concentration (0.64 μg/ml), the average molecular mass of CS-E (70 kDa) was 3-fold that of Hep (19 kDa), as has been estimated by gel filtration chromatography (53),[2] suggesting comparable inhibitory activities of CS-E and Hep. It should be emphasized that large cell aggregates were observed even though the cell adhesion was strongly inhibited, supporting the specificity of the inhibition, *i.e.* CS-E inhibited neuronal cell adhesion to the MK-coated substratum, but not intercellular cell adhesion between the neuronal cells. The inhibition observed with CS-E is not attributable to a possible nonspecific binding of CS-E to BSA used for masking the MK-uncoated surface because no binding was observed between CS-E and BSA in the BIAcore system (see below). CS-H, another over-sulfated CS isoform, derived from hagfish notochord, showed appreciable inhibition, although at a high concentration of 50 μg/ml (Fig. 1*D*). The findings from the inhibition studies are summarized in Table I. The specificity of the inhibition of neuronal cell adhesion by CS-E was reinforced by the findings that other over-sulfated CS isoforms such as CS-D from shark cartilage and CS-K from king crab cartilage showed no inhibition even at 50 μg/ml. Thus, the inhibition was dependent not only on the sulfation degree, but also on the sulfation pattern.

Demonstration of Specific Binding of MK to CS-E by Filter Binding Assays—A [^{3}H]acetyl-labeled CS-E preparation was incubated with various amounts of MK, and the binding ability was evaluated using the nitrocellulose filter binding assay, in which MK, along with any bound carbohydrate, was recovered through nitrocellulose filters, which were then examined in a scintillation spectrometer for bound radioactivity. The findings demonstrated direct binding of MK to CS-E in a concentration-dependent manner as shown in Fig. 2, although a saturation curve could not be obtained due to the limited availability of MK.

To characterize the specificity of the binding of CS-E to MK, the effects of various GAGs and dextran sulfate on the binding were examined. [^{3}H]CS-E was incubated with MK in the presence of each tested glycan, and the binding of [^{3}H]CS-E to MK was determined. The findings are shown in Fig. 3. Non-radiolabeled CS-E as well as CS-H and CS-K exhibited marked inhibition of binding. In addition, Hep and dextran sulfate showed even stronger inhibition, whereas bovine kidney HS showed no significant inhibition. These findings indicated that the sulfation degree was an important factor for the binding of CS-E to MK. Interestingly, CS-D, an over-sulfated CS isoform, showed no inhibition, indicating that the binding of CS-E to MK is also dependent on the sulfation profile, at least to some extent. The inhibition by CS-H and CS-K, which are characterized by the GalNAc(4*S*,6*S*) and GlcUA(3*S*) structures, respectively (54, 55), possibly indicates the involvement of such structures in MK binding to CS-E, as will be discussed under "Discussion."

Characterization of the Binding of Various GAGs to MK Using a BIAcore™ System—To further characterize the binding of CS-E to MK, a BIAcore™ biosensor with a surface plasmon resonance detector was used to analyze real-time interactions of GAGs with MK. MK (2.3 ng, 0.18 pmol corresponding to 1902 resonance units) was immobilized on the

[2] K. Takagaki, personal communication.

TABLE I

Inhibition of MK-mediated neuronal cell adhesion by various CS isoforms

Various CS isoforms and Hep as a reference compound were examined for their ability to inhibit neuronal cell adhesion mediated by recombinant mouse MK as described under "Experimental Procedures." Representative photographs are shown in Fig. 1.

GAG	Biological origin	GAG concentration			
		0.64	2.5	10	50
			μg/ml		
CS-A	Whale cartilage	−[a]	−	−	−
CS-A	Bovine tracheal cartilage	−	−	−	−
CS-B	Pig skin	−	−	−	−
CS-C	Shark cartilage	−	−	−	−
CS-D	Shark cartilage	−	−	−	−
CS-E	Squid cartilage	−	++	++	++
CS-H	Hagfish notochord	−	−	−	+
CS-K	King crab cartilage	−	−	−	−
Hep	Porcine intestine	+	++	++	++

[a] −, no inhibition; +, moderate inhibition; ++, strong inhibition.

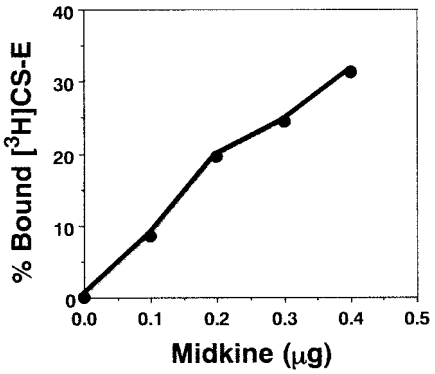

FIG. 2. **Binding of squid cartilage [^{3}H]CS-E to MK.** Squid cartilage [^{3}H]CS-E (80 ng, 1.5 × 10^4 cpm) was incubated with various amounts of recombinant human MK. The radioactivity bound to MK was quantified by the filter binding assay as described under "Experimental Procedures." Values were obtained from the average of two separate experiments and are expressed as percentages of the radioactivity added to the incubation. Estimated errors were within 5%.

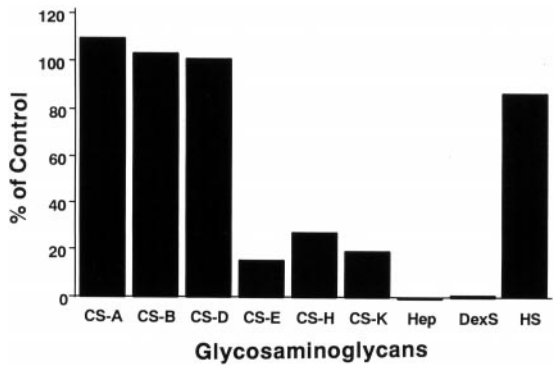

FIG. 3. **Inhibition of the binding of CS-E to MK by various GAGs.** Recombinant human MK (0.2 μg) was incubated with squid cartilage [^{3}H]CS-E (80 ng, 1.5 × 10^4 cpm) in the presence of CS-A, CS-B, CS-D, CS-E, CS-H, CS-K, Hep, dextran sulfate (*DexS*), or HS (50 ng each). The radioactivity bound to MK was quantified by the filter binding assay as described under "Experimental Procedures." Values were obtained from the average of two separate experiments and are expressed as percentages of the control value obtained in the absence of inhibitors. Estimated errors were within 12%.

carboxymethyl-dextran sensor chip surface, and aqueous solutions of various GAGs (100 μg/ml) were individually injected over this surface to examine direct binding. Fig. 4 shows an overlay of sensorgrams obtained with various GAG analytes. Each injection of different GAGs gave a sharp rise when injected at A due to a bulk refractive index change at the sensor chip surface. The gradual increase in response from *arrow A* to *arrow B* was due to binding of GAGs to immobilized MK, where

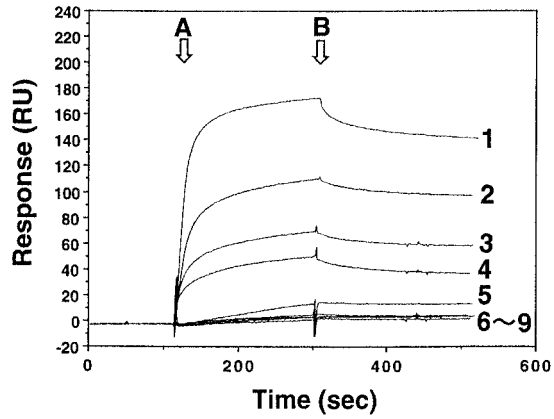

FIG. 4. **Analysis of the binding of Hep and over-sulfated CS isoforms to immobilized MK in the BIAcore™ system.** Recombinant human MK (2.3 ng, 0.18 pmol corresponding to 1902 resonance units) was immobilized on a CM5 sensor chip, and then aqueous solutions (100 μg/ml) of Hep, HS, and various CS isoforms (CS-A, CS-B, CS-C, CS-D, CS-E, CS-H, and CS-K) were individually injected over the immobilized MK surface as described under "Experimental Procedures." *Arrow A* indicates the beginning of the association phase initiated by the injection of each of the various GAG analytes; *arrow B* indicates the end of the sample injection or the beginning of the dissociation phase initiated with the running buffer. Values of the *vertical axis*, expressed in resonance units (*RU*), represent the increments in mass concentration on the MK-immobilized sensor surface due to the binding of each GAG analyte. The increment of 1000 resonance units corresponds to the binding of 1 ng of GAG/mm^2 of surface area. *Trace 1*, Hep; *trace 2*, CS-E; *trace 3*, CS-H; *trace 4*, CS-K; *trace 5*, CS-B; *trace 6*, HS; *trace 7*, CS-A; *trace 8*, CS-C; *trace 9*, CS-D.

arrow A indicates the beginning phase of the association phase, and *arrow B* indicates the beginning of the dissociation phase. The findings indicated strong binding of CS-E, which was comparable to that of Hep. Moderate binding of CS-H and CS-K and weak binding of CS-B (DS) were also observed, whereas HS from bovine kidney and other CS isoforms, including CS-A, CS-C, and CS-D, showed no binding. Neither CS-E nor Hep bound to immobilized BSA, confirming the binding specificity of these GAGs for MK (data not shown).

In the next series of experiments, kinetic analysis of GAG binding to immobilized MK was performed by applying varying concentrations of the GAG samples. As representative examples, overlays of the sensorgrams of the binding of Hep and CS-E, which showed the strongest binding to MK, are shown in Fig. 5 (*upper* and *lower panels*, respectively). They were analyzed using BIAevaluation software to determine the association rate constants (k_a), dissociation rate constants (k_d), and apparent equilibrium dissociation constants (K_d) as described under "Experimental Procedures." Likewise, these parameters were also obtained for other GAGs and are summarized in

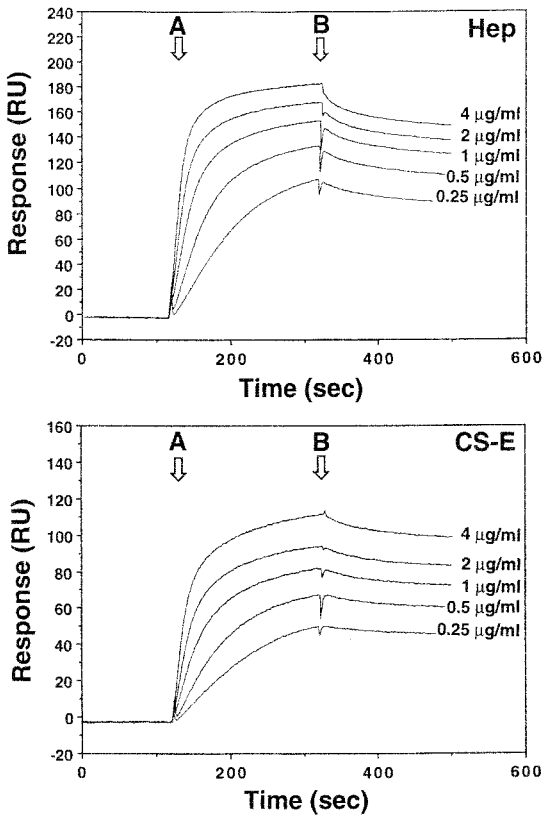

FIG. 5. **Overlaid sensorgrams for the MK-binding kinetics of Hep and CS-E.** The binding assays were carried out as described in the legend to Fig. 4, except that the indicated concentrations of Hep (*upper panel*) and CS-E (*lower panel*) were injected, respectively. The immobilization recombinant human MK level was 1902 resonance units (*RU*).

Table II. These findings indicated that CS-E had a high affinity for MK, which was comparable to that of Hep binding to MK as evidenced by k_a, k_d, and K_d values. Although CS-H and CS-K also exhibited a higher affinity for MK compared with other GAGs, the affinity was lower than those of Hep and CS-E as shown by the K_d value. The parameters indicated that the binding of CS-H and CS-K to MK had lower association rates and comparable dissociation rates compared with the binding of CS-E to MK. The binding of the over-sulfated CS isoforms CS-E, CS-H, and CS-K implies the involvement of the unique structures of GalNAc(4*S*,6*S*) and possibly GlcUA(3*S*) (see "Discussion").

Analysis of CS in Embryonic Rat Brains—To investigate whether an embryonic rat brain contained over-sulfated CS isoforms, the CS chains were analyzed. Most CS-PGs can be extracted from brains using physiological buffers without detergent, and ~70% of total CS-PGs are extracted by phosphate-buffered saline (46). Hence, a soluble CS-PG fraction was prepared from E18 rat brains, and disaccharide composition analysis was carried out by HPLC of the chondroitin AC lyase digest of the PG preparation after derivatization of the resulting disaccharides with the fluorophore 2-aminobenzamide as described previously (47). The findings showed that appreciable proportions of $\Delta^{4,5}$-HexUA(2*S*)α1–3GalNAc(6*S*) (1.7%) and $\Delta^{4,5}$-HexUAα1–3GalNAc(4*S*,6*S*) (1.2%) were present in addition to the major disaccharide components $\Delta^{4,5}$-HexUAα1–3GalNAc(6*S*) (30.3%) and $\Delta^{4,5}$-HexUAα1–3GalNAc(4*S*) (66.8%). Essentially identical findings were obtained with the chondroitin ABC lyase digest. The disaccharide peak of $\Delta^{4,5}$-HexUAα1–3GalNAc(4*S*) was sensitive to chondro-4-sulfatase, whereas $\Delta^{4,5}$-HexUAα1–3GalNAc(6*S*), $\Delta^{4,5}$-HexUA(2*S*)α1–3GalNAc(6*S*), and $\Delta^{4,5}$-HexUAα1–3GalNAc(4*S*,6*S*) were sensitive to chondro-6-sulfatase as determined by HPLC (data not shown), confirming the identity of these components.

DISCUSSION

Faissner *et al.* (32) have demonstrated that DSD-1-PG/phosphacan secreted by immature glial cells promotes neurite outgrowth. They and we (35, 36) have further demonstrated that the neurite outgrowth-stimulating capacity of DSD-1-PG/phosphacan is closely associated with the D disaccharide unit of the CS chains as mimicked by shark cartilage CS-D, which contains the D unit in a high proportion (21.2%) (see Introduction in Ref. 35), although the interacting molecule(s) or the receptor on the neuronal surface has not been identified. However, Maeda *et al.* (15) have isolated the PTN/Hep-binding growth-associated molecule as a 6B4-PG/phosphacan-binding protein in P16 rat brain. PTN binds the CS moiety of phosphacan and neurocan with high affinity (K_d = 0.3–0.8 and 1–8 nM, respectively) (15, 56).

In this study, we demonstrated that MK, in the same gene family as PTN, which utilizes HS proteoglycans as high affinity receptors (K_d = ~1 nM) (8–10), also specifically bound CS-E from squid cartilage and that the MK-mediated adhesion of E18 rat cortical neurons was inhibited by CS-E. The minimum inhibitory concentration (2.5 μg/ml) of CS-E was comparable to that of Hep (Table I). The affinity of CS-E for MK (K_d = 46 nM) was higher than that of Hep (K_d = 159 nM) (Table II), although the number of binding sites on a single CS-E or Hep chain remains to be examined. Recently, Maeda *et al.* (57) also reported the high affinity binding (K_d = 0.58 nM) of MK to PTPζ and the inhibition of the binding by various CS isoforms, including CS-B (DS), CS-C, CS-D, and CS-E, in contrast to the high specificity of MK binding to CS-E demonstrated in this study. The affinity observed for MK with CS-E was lower than that reported for MK with PTPζ, which may be due to a possible conformational change of MK upon immobilization and the usage of the polysaccharides with a heterogeneous population. The low content of the disulfated disaccharide units (Table III) suggests that the putative functional MK-binding domain is present in a small proportion, as in the case of the antithrombin III-binding site in HS from various sources (58) and the Hep cofactor II-binding site in pig skin DS (59). Thus, the apparent lower affinity of CS-E for MK observed in this study is judged to be sufficient to exert molecular interactions under physiological conditions. Likewise, Zou *et al.* (60) recently found that PG-M/versican is a major MK-binding CS-PG in day 13 mouse embryos and that over-sulfated CS is also present in the PG in a small yet significant proportion. Some Hep-binding proteins may not always (but only at certain developmental stages) be physiological ligands for PGs with over-sulfated CS/DS chains.

MK and PTN are strongly expressed in the developing cerebral cortex in the rat, with MK being primarily detected during the embryonic period and not in the postnatal cerebral cortex and with PTN being most intense during the early postnatal period in addition to being present during the embryonic period (61). Thus, they can be differentially involved in cortical development at different stages. The E disaccharide unit, which may constitute part of the binding site for MK, and D unit, which is probably involved in PTN binding, were detected in appreciable proportions of the soluble CS-PG fraction isolated from E18 rat brains (Table III), from which neuronal cells were prepared for the cell adhesion assay. Both D and E units have been demonstrated in appreciable proportions in bovine brains (62) and chick brains, where they exhibit developmentally regulated expression (47). Thus, the over-sulfated D and E motifs appear to be involved in the neuroregulatory activities of PTN and MK, respectively. We recently demonstrated that squid cartilage

TABLE II

Kinetic parameters for the interaction of soluble GAGs with immobilized MK

Apparent association (k_a) and dissociation (k_d) rate constants and equilibrium dissociation constants (K_d) for the interaction of various soluble GAGs with immobilized recombinant human MK were determined by BIAcore analysis as described under "Experimental Procedures."

GAG	k_a	k_d	K_d[a]	
			μg/ml	nM
	$(s^{-1} \times \mu g^{-1} \times ml) \times 10^{-4}$	$s^{-1} \times 10^{-3}$		
Hep	2.57	0.774	3.01	159
HS	ND[b]	ND	ND	ND
CS-A	ND	ND	ND	ND
CS-C	ND	ND	ND	ND
CS-D	ND	ND	ND	ND
CS-E	1.60	0.513	3.21	46
CS-H	0.928	0.458	4.94	—
CS-K	0.574	0.553	9.63	—

[a] K_d values were calculated based on the average molecular masses of 19 and 70 kDa for Hep from porcine intestine and CS-E from squid cartilage, respectively (53).

[b] ND, no specific binding detected; —, not calculated due to the lack of information on molecular mass.

TABLE III

Disaccharide composition of CS-PGs in rat brain

CS-PGs were extracted from embryonic rat brain (E18) and digested exhaustively with chondroitin AC lyase, and the products were identified and quantified by HPLC as described under "Experimental Procedures." Similar findings were obtained using chondroitin ABC lyase instead of chondroitin AC lyase.

Disaccharide[a]	pmol/brain	mol %[b]
ΔDi-4S[c]	1568	66.8
ΔDi-6S	713	30.3
ΔDi-diS_D	40	1.7
ΔDi-diS_E	29	1.2

[a] Although a significant peak (1097 pmol/brain) was observed at the elution position of the non-sulfated disaccharide unit ($\Delta^{4,5}$-HexUAα1–3GalNAc), the peak with a comparable area was also obtained after digestion with hyaluronidase from *Streptomyces hyalurolyticus*, indicating that most of the non-sulfated disaccharide was derived from hyaluronan rather than chondroitin or CS. Hence, it was not included here.

[b] Percent recoveries were calculated based on the peak areas on the HPLC chromatogram and are expressed in molar proportions of the disaccharides.

[c] ΔDi-4S, $\Delta^{4,5}$-HexUAα1–3GalNAc(4*S*); ΔDi-6S, $\Delta^{4,5}$-HexUAα1–3GalNAc(6*S*); ΔDi-diS_D, $\Delta^{4,5}$-HexUA(2*S*)α1–3GalNAc(6*S*); ΔDi-diS_E, $\Delta^{4,5}$-HexUAα1–3GalNAc(4*S*, 6*S*).

CS-E and shark cartilage CS-D promote neurite outgrowth of E18 rat hippocampal neurons, whereas other CS isoforms such as CS-A, CS-B, and CS-C are inefficient (36, 37). The stimulatory effect of CS-D, but not of CS-E, is neutralized by the CS-specific monoclonal antibody 473HD, suggesting two different mechanisms for neurite outgrowth promotion (37). Recently, Maeda *et al.* (57) reported that MK and PTN stimulate the migration of cortical neurons, which is inhibited by CS-B (DS), CS-C, CS-D, and CS-E.

Structural analysis of the CS chains that bind MK is essential for understanding the molecular mechanism of MK signaling. It remains to be investigated, however, which CS-PGs in the brain contain the E motif. Despite the identification of soluble CS-PGs such as brevican (63) and neurocan (64) as well as transmembrane CS-PGs such as NG2 (65) and PTPζ and neuroglycan C (66), the CS chains attached to the core proteins have not been rigorously investigated. Notably, our previous analysis of DSD-1-PG/phosphacan showed the D unit (36). It was proposed that PTPζ is a common receptor of MK and PTN based on the indistinguishable characteristics of PTPζ binding to both proteins, which is inhibited by several CS isoforms, including CS-D and CS-E (57). It is possible that PTPζ and/or phosphacan bears CS chains with distinctive sulfation profiles during different developmental stages. Two populations of phosphacan with different CS structures have been demonstrated immunologically (67). It will be interesting to analyze the CS chains attached to PTPζ and also other CS-PGs in the brain especially in terms of developmental changes.

Another over-sulfated CS isoform (CS-H) exhibited appreciable inhibitory activity in MK-mediated neuronal cell adhesion (Fig. 1*D*), although the affinity for MK was lower than that of CS-E for MK (Table II). CS-H is derived from the hagfish notochord (43), and the corresponding mammalian tissue produces the soluble Sonic Hedgehog protein, implicated in the generation of ventral neurons and the differentiation of motor neurons (68). CS-H is structurally similar to CS-E, containing a high proportion of the E unit (50%) (52), since it also contains the 4,6-*O*-disulfated disaccharide IdoUAα1–3GalNAc(4*S*,6*S*) as the predominant unit (68%) (43, 54). It is different, however, from CS-E because it contains IdoUA instead of GlcUA as a major uronic acid and is regarded as over-sulfated DS. In view of these structural features, the alternative name, DS-E, has been proposed for CS-H (54). DS-E, characterized by the IdoUAα1–3GalNAc(4*S*,6*S*) unit, is copurified with Hep due to its over-sulfated nature and is found in a Hep preparation from porcine intestine (69). Thus, caution should be taken regarding such possible contaminating over-sulfated DS in Hep preparations, which may contribute to the stimulatory or inhibitory activity of Hep for Hep-binding growth factors such as MK and PTN.

Specific oligosaccharide sequences are presumably responsible for the high affinity binding of MK to squid cartilage CS-E and the CS moiety of PTPζ. Several novel discrete tetrasaccharide sequences have been isolated from squid cartilage CS-E, some of which are unique because they contain GlcUA(3*S*) residues linked to GalNAc(4*S*), GalNAc(6*S*), or GalNAc(4*S*,6*S*), forming rare over-sulfated disaccharide units (52). The disaccharide units containing GlcUA(3*S*) are decomposed by chondroitinase ABC digestion and become undetectable upon UV absorbance (52, 55). Since this method is conventionally used for compositional analysis of CS chains, such structures may have been overlooked in previous analyses of CS chains. In this respect, it is interesting that CS-K containing GlcUA(3*S*) (55) inhibited MK binding to CS-E (Fig. 3) and bound to MK (Fig. 4). Precise structural analysis of the putative MK-binding saccharide sequence in squid cartilage CS-E to determine the size and precise positioning of the sulfate groups will give insights into the corresponding binding domain in brain CS chains functioning in neuronal adhesion, survival, and migration and neurite outgrowth promoted by MK. In our ongoing structural studies of squid cartilage CS-E, discrete novel hexasaccharides containing rare sulfated sequences and a glucose-containing unique pentasaccharide have been isolated (70, 71). It is noteworthy that several structurally defined oligosaccharides with D unit-containing characteristic sequences have also been iso-

lated from shark cartilage CS-D (35), which interacts with PTN (57) and exhibits neurite outgrowth-promoting capacity (35).

The molecular interaction between MK and CS-E may reflect that of MK with the CS moiety of the signal-transducing receptor PTPζ at the neuronal surface. Like PTN, MK is primarily associated with the cell surface and is found preferentially in regions where cell migration and neurite outgrowth take place. Both factors are expressed in radial glial processes as well as in the ventricular zone (61). The molecular interaction may also represent binding of MK to soluble CS-PGs such as phosphacan and neurocan, which function as reservoirs for storing MK and/or modulate the interactions between MK and its neuronal surface receptors such as PTPζ and syndecan-4, which is another signal-transducing receptor of an HS proteoglycan type (72). Different neurons may express distinctive PG receptors with HS or CS chain(s) depending on the neuronal lineage and developmental stage, whereas soluble CS-PGs, which possibly bear CS chains with the E unit depending on the developmental stage, would compete with the receptors for the ligand MK or translocate the ligand to the receptors.

Acknowledgment—We thank Megumi Inoda for technical assistance.

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