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Demonstration of the hepatocyte growth factor signaling pathway in the *in vitro* neuritogenic activity of chondroitin sulfate from ray fish cartilage

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Abstract

Chondroitin sulfate (CS) was isolated from ray fish cartilage, an industrial waste, after protease digestion, and its structure and neurite outgrowth-promoting (NOP) activity were analyzed to investigate a potential application to nerve regeneration. A disaccharide analysis using chondroitinase ABC revealed that the major unit in the CS preparation was GlcUA-GalNAc(6-*O*-sulfate) (63%), where GlcUA and GalNAc represent D-glucuronic acid and *N*-acetyl-D-galactosamine, respectively. Small proportions of other disaccharide units, GlcUA-GalNAc(4-*O*-sulfate) (25%), GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) (7%), and GlcUA-GalNAc (5%), were also detected. The average molecular mass of CS was estimated to be 142 kDa by gel-filtration chromatography. The prepration showed NOP activity *in vitro*, which was eliminated by digestion with chondroitinase ABC, suggesting that a polymeric structure is required for the activity. Antibodies against hepatocyte growth factor (HGF) and its receptor c-Met suppressed the NOP activity, suggesting the involvement of the HGF signaling pathway in the *in vitro* NOP activity of the CS preparation. Since the specific binding of HGF to the CS preparation was also demonstrated by surface plasmon

resonance spectroscopy, the CS chains were fractionated using an HGF-immobilized column into unbound and bound fractions accounting for 44 and 56% of the total yield, respectively. The latter contained a higher proportion of the GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) unit, and showed greater NOP activity than the former, indicating that the HGF-binding domain contains GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) and is involved in the NOP activity. CS from ray cartilage may have potential pharmaceutical applications.

Keywords Chondroitin sulfate, Cartilage, Neurite outgrowth-promoting activity, Hepatocyte growth factor, c-Met receptor

Abbreviations

GAG, glycosaminoglycan; CS, chondroitin sulfate; GlcUA, D-glucuronic acid; IdoUA, L-Iduronic acid; GalNAc, *N*-acetyl-D-galactosamine; PTN, pleiotrophin; MK, midkine; HGF, hepatocyte growth factor; GDNF, glial cell line-derived neurotrophic factor; P-ORN, poly-DL-ornithine; HPLC, high-performance liquid chromatography; CSase,

chondroitinase; NOP, neurite outgrowth-promoting; 2AB, 2-aminobenzamide; ΔHexUA, 4-deoxy-L-*threo*-hex-4-enepyranosyluronic acid; IU, international unit.

1. Introduction

Recent studies using rodents have demonstrated that thousands of fresh neurons arise even in the adult brain every day particularly in the hippocampus, a structure involved in learning and memory [1]. However, little is known about the molecular basis of neuritogenesis. Chondroitin sulfate (CS) is a ubiquitous component of the cell surface and extracellular matrix and participates in diverse biological processes such as growth factor signaling and the nervous system's development [2-4]. Its sugar backbone consists of repeating disaccharide units: D-glucuronic acid (GlcUA)β1-3*N*-acetyl-D-galactosamine (GalNAc). During the chain elongation step of CS biosynthesis, the disaccharide units are modified by specific sulfotransferases at C-2 of GlcUA and/or C-4 and/or C-6 of GalNAc in various combinations, and display enormous structural diversity producing characteristic sulfation patterns critical for binding to a variety of functional proteins [4]. This structural variability of CS is the basis for its wide range of biological activities.

The involvement of CS in the development of the central nervous system has attracted attention from a therapeutic perspective for potential applications to nerve

regeneration. Several studies have shown that the sulfation pattern of CS in the brain alters during development, characterized by an increase in 4-*O*-sulfation and a decrease in 6-*O*-sulfation [5, 6]. The proportion of oversulfated disaccharide units, GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) and GlcUA-GalNAc(4,6-*O*-disulfate), has been demonstrated to be crucial to the neurite outgrowth-promoting (NOP) activity of CS [7-13]. The NOP activity toward embryonic mouse hippocampal neurons of mouse brain CS/DS and shark cartilage CS/DS, which contains a high proportion of the GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) unit [13], has been verified to be executed by capturing endogenous growth/neurotrophic factors produced by non-neuronal cells (glial cells), and then presenting them to neuronal cells [4, 14, 15].

Endogenous hepatocyte growth factor (HGF) in mouse hippocampal neuronal cell cultures is recruited by the oversulfated CS from shark liver and mediates the NOP activity of the CS preparation [15]. HGF is a pleiotropic factor as well as an axonal chemoattractant and a neurothrophic factor for motor neurons [16, 17], and binds to and activates the tyrosine kinase receptor c-Met. HGF cooperates with a nerve growth factor to enhance sensory neuron survival, and outgrowth of sensory and sympathetic axons

[16]. CS from shark liver recruits a minute amount of endogenous HGF to stimulate the outgrowth of neurites in hippocampal neurons [15]. Hence, CS may function as the co-receptor for the HGF-c-Met signaling on the neuronal cell surface.

In this study, CS was isolated from ray fish cartilage, an industrial waste, and its structure and NOP activity were analyzed. NOP activity was demonstrated *in vitro* and involved in the HGF signaling pathway, suggesting that CS from ray cartilage has potential pharmaceutical applications.

2. Materials and methods

2. 1. Materials

The following sugars and enzyme were purchased from Seikagaku Corp. (Tokyo, Japan): CS-C from shark cartilage, CS-E from squid cartilage, standard unsaturated disaccharides derived from CS/dermatan sulfate (DS), chondroitinase (CSase) ABC (EC 4.2.2.20) from *Proteus vulgaris*. Poly-DL-ornithine (P-ORN), anti-neurofilament and polyclonal anti-HGF antibodies, and immunoglobulins from goat serum were from Sigma (St. Louis, MO, USA). Pregnant ddY mice were purchased from SLC Inc. (Shizuoka, Japan). Recombinant human (rh)-pleiotrophin (PTN) and rh-glial cell line-derived neurotrophic factor (GDNF) were from Wako Corp. (Osaka, Japan). Polyclonal anti-PTN and anti-midkine (MK) antibodies, rh-MK, and rh-HGF, were purchased from R&D Systems (Minneapolis, MN). Actinase E, anti-microtubule-associated protein 2 antibodies, and Accell Plus QMA anion-exchange cartridges were obtained from Kaken Pharmaceutical Co. (Tokyo, Japan), Leico Technologies Inc. (St. Louis, MO), and Waters Corp. (Milford, MA), respectively.

2. 2. Extraction and purification of CS from ray cartilage

Ray fish cartilage was obtained by splashing away poultry from the body part after cutting off the fins. Dried and powdered ray cartilage (1 g) was treated in 7 ml of boiling water for 10 min to inactivate proteases and glycosidases, and digested with actinase E in a total volume of 10 ml of 0.1 M borate-sodium buffer, pH 8.0, containing 10 mM calcium acetate as described previously [18] Following incubation, the sample was treated with 5% trichloroacetic acid to precipitate proteins and peptides, and the supernatant was extracted with diethylether to remove trichloroacetic acid. The aqueous phase was treated with 80% ethanol containing 1% sodium acetate at 4C overnight to precipitate polysaccharides. The precipitate was treated with 1.0 M $NaBH_4$/0.05 M NaOH at 4 ˚C overnight to liberate glycosaminoglycan (GAG) chains as described [15]. The sample was passed through a Sep-PakC_{18} (Waters) cartridge to remove peptide fragments and subjected to anion-exchange chromatography on an Accell QMA Plus cartridge, which was eluted stepwise with 0.3 M phosphate buffer, pH 6.0, containing 0.15 and 2.0 M NaCl. Uronic acid in each fraction was quantified by the carbazole method [19]. The 2.0 M NaCl-eluted fraction, which contained the majority of CS, was

used for the structural and functional studies after being desalted using a PD-10 column (GE healthcare).

2. 3. Disaccharide composition analysis

The CS preparation from ray cartilage was digested with CSase ABC [11], and the digest was derivatized with a fluorophore 2-aminobenzamide (2AB), and then analyzed by anion-exchange HPLC on an amine-bound silica PA-03 column (YMC Co., Kyoto, Japan) [20] to determine the disaccharide composition of the CS preparation.

2. 4. Gel-filtration chromatography

The CS preparation from ray cartilage and commercial CS-C (10 μg each as GlcUA) were subjected to gel-filtration chromatography on a column (10 X 300 mm) of Superdex^{TM} 200 10/300GL (Amersham Biosciences, Uppsala, Sweden) using 0.2 M ammonium acetate as an effluent at a flow rate of 0.3 ml/min. Fractions were collected at 3.0-min intervals, as monitered using the dimethymethylene blue dye method as described previously [15, 21], and lyophilized.

2. 5. Assays for NOP activity

NOP activity toward hippocampal cells from E16 mice was assayed as described [9, 13]. Briefly, 2 μg/well of the CS preparation was coated onto coverslips precoated with P-ORN at 4˚C overnight. The hippocampal cells were freshly isolated from E16 mouse embryos, suspended in Eagle's minimum essential medium containing N2 supplements, seeded on coverslips at a density of 20,000 cells/cm^2, and allowed to grow in a humidified atmosphere for 24 h at 37˚C with 5% CO_2. After incubation overnight, the cells were fixed using 4% (w/v) paraformaldehyde for 1 h at room temperature, and the neurites were visualized by immunochemical staining using antibodies against anti-microtubule associated protein 2 and anti-neurofilament. These antibodies were then detected using a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA) with 3,3'-diaminobenzidine as a chromogen. NOP activity was evaluated by determining the length of the longest neurite and the number of the primary neurites of 100 randomly selected neurons using the software FLVFS-LS (Olympus, Tokyo, Japan). For the inhibition assay, polyclonal antibodies including

anti-PTN, anti-MK, anti-c-Met, and anti-HGF (2 or 10 μg/ml) were added to the medium 2 h after seeding of the hippocampal cells.

2. 6. Interaction analysis using surface plasmon resonance

The interaction of various growth/neurotrophic factors with the CS preparation was examined as reported [10], using a BIAcore X system (BIAcore AB, Uppsala, Sweden) at the Open Facility, Hokkaido University Sousei Hall. For the kinetic analysis, various concentrations of each growth/neurotrophic factor were injected onto the surface of a sensor chip, on which the CS chain was immobilized, in the HBS-EP buffer (BIAcore AB) at a flow rate of 30 μl/min. The corresponding reference cell was immobilized with the digest of CS from ray cartilage with CSase ABC. Before each injection, stabilization of the base-line was achieved by injecting 1 M NaCl for 2 min. The kinetic parameters, namely the association rate constant (k_a), the dissociation rate constant (k_d), and the equilibrium dissociation constant (K_d), were evaluated with BIA evalution software 4.1 (BIAcore AB) using a 1:1 binding model with mass transfer.

2. 7. Subfractionation of the CS preparation from ray cartilage on an HGF-immobilized column

HGF (0.6 mg) was coupled to a HiTrap *N*-hydroxysuccinimide-activated column (1 ml) (Amersham Biosciences) according to the manufacturer's protocol. To protect the GAG-binding sites of HGF from possible inactivation by the coupling, 1.2 mg of DS from shark skin [11] was incubated with HGF at room temperature for 30 min prior to the coupling. Before application of the CS preparation from ray cartilage, the HGF-immobilized column was washed with 5 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 2.0 M NaCl, and then equilibrated with 10 ml of the buffer containing 0.15 M NaCl. The CS preparation (10 μg as GAG) was dissolved in 300 μl of 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and applied to the column. Fractions were collected by eluting stepwise with 3 ml of 10 mM Tris-HCl (pH 7.4) containing 0.15 and 0.5 M NaCl. The 0.15 M and 0.5 M NaCl-eluted fractions were designated as the unbound and bound fractions, respectively. After being desalted using Amicon Y-10 (Millipore), each fraction was digested with CSase ABC, and then analyzed by

anion-exchange HPLC on an amine-bound silica PA-03 column [20] to quantify CS disaccharides in the unbound and bound fractions.

3. Results

3. 1. Structural analysis of the CS preparation from ray fish cartilage

The disaccharide composition of the CS preparation from ray cartilage was determined after digestion with CSase ABC followed by 2AB-derivatization and anion-exchange HPLC. A representative chromatogram is shown in Fig. 1. Each peak was identified by comparison with the elution profile of the standard 2AB-disaccharides. The yield of each disaccharide was calculated based on fluorescent intensity and is summarized in Table 1. The major disaccharide unit in the CS preparation was ΔHexUA-GalNAc(6-*O*-sulfate) (63%), with small proportions of other units, ΔHexUA-GalNAc(4-*O*-sulfate) (25%), ΔHexUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) (7%), and ΔHexUA-GalNAc (5%), also detected. Since the disaccharide composition of the CS chains was similar to that of shark cartilage CS-C (Table 1), CS-C was used for comparison with CS from ray cartilage.

Fig. 1 and Table 1

The molecular size of CS from ray cartilage was analyzed by gel filtration

HPLC on a Superdex 200 column. The elution profile was monitored using a dimethymethylene blue dye (Fig. 2), and the molecular size of the peak was calculated using a calibration plot generated with standard polysaccharides (Fig. 2, *inset*). The average molecular size of the CS chains was estimated to be 142 kDa and larger than that of the commercial CS-C (123 kDa) (Fig. 2).

Fig. 2

3. 2. NOP activity of the CS preparation from ray cartilage

To evaluate the biological activity of the CS preparation, its NOP activity was tested. Mouse E16 hippocampal cells were cultured on plastic coverslips coated with P-ORN and then with the CS chains from ray cartilage, CS-C, or CS-E as a control. Representative microscopic fields for P-ORN alone (Fig. 3A) and the CS chains from ray cartilage (Fig. 3B) are shown. The NOP activity of CS from ray cartilage was significantly higher than that of CS-C (Fig. 3C), and eliminated by digestion with CSase ABC (Fig. 3C), suggesting that the polymeric structure of CS chains in the CS preparation is indeed required for the expression of the NOP activity.

Fig. 3

3. 3. Kinetic analysis of the binding of growth/neurotrophic factors to the CS preparation from ray cartilage

Since CS has been considered to act as a co-receptor for various growth factors [22], its interactions with growth/neurotrophic factors may be involved in the promotion of the NOP activity of the CS preparation from ray cartilage as well. To examine this possibility, a kinetic analysis of the binding of growth/neurotrophic factors to the immobilized CS from ray cartilage was carried out using the BIAcore system. The CS preparation was biotinylated and immobilized on the streptavidin-precoated sensor chip. The growth/neurotrophic factors (PTN, MK, HGF, and GDNF) were individually injected at different concentrations onto the surface of the sensor chip. Overlaid sensorgrams are shown in Fig. 4. These sensorgrams were analyzed with the software BIAevaluation 4.1 using the "1:1 binding model with mass tranfer" to calculate the kinetic parameters (Table 2). PTN, MK, and GDNF bound to both the ray cartilage CS and commercial CS-C preparations with a low nM range of K_d values (Fig. 4),

suggesting high affinity and biological significance of the interactions. Although the K_d values for the interaction with PTN, MK, or GDNF (Table 2) as well as the disaccharide composition of the CS chains from ray cartilage and CS-C (Table 1) were similar to each other, HGF had much higher affinity for ray cartilage CS than shark cartilage CS-C (Table 2). Therefore, the CS chains from ray cartilage seem to contain a specific HGF-high affinity domain, and may mediate a function of HGF.

Fig. 4 and Table 2

3. 4. Demonstration of the HGF signaling pathway in the expression of NOP activity by CS from ray cartilage

Since the CS chains from ray cartilage interacted with PTN, MK, and HGF, these growth/neurotrophic factors may be involved in the mechanism of the expression of the *in vitro* NOP activity by CS from ray cartilage. To verify this possibility, the NOP activity of ray cartilage CS toward hippocampal neurons was examined in the presence of an antibody against PTN, MK, or HGF. The addition of anti-HGF antibodies markedly suppressed the NOP activity of the CS preparation in a

concentration-dependent manner, whereas the anti-PTN or anti-MK antibody showed no significant inhibition (Fig. 5). These results suggest that the *in vitro* NOP activity of the CS chains from ray cartilage is most likely mediated by the signaling pathway of HGF, whereas CS from ray cartilage did not use PTN or MK for its neuritogenic activity toward hippocampal neuronal cells under the conditions used. To confirm the involvement of HGF in the NOP activity of CS from ray cartilage, the effect of an antibody against the HGF receptor (c-Met) was also investigated. The anti-c-Met antibody also showed strong inhibition of the NOP activity (Fig. 5).

Fig. 5

Since the NOP activity of the CS chains from ray cartilage seemed to be involved in the HGF signaling, we considered that HGF-bound and -unbound fractions of CS from ray cartilage might exhibit distinct characteristics in terms of neuritogenic activity. Therefore, the CS preparation from ray cartilage was subfractionated by an HGF-immobilized column into unbound and bound fractions, which were eluted with the equilibrating buffer and the buffer containing 0.5 M NaCl, respectively. The

unbound and bound fractions of CS from ray cartilage accounted for 44 and 56% of the total yield, respectively, and their disaccharide composition is summarized in Table 3. The bound fraction of CS from ray cartilage contained a higher proportion of the highly sulfated disaccharide unit, ΔHexUA(2 -*O*-sulfate)-GalNAc(6-*O*-sulfate), than the unbound fraction. The NOP activity of the unbound and bound fractions was assessed. The activity of the bound fraction was significantly higher than that of the unbound fraction (Fig. 6), indicating that the HGF-binding domain enriched in the bound fraction is involved in the expression of the NOP activity of the CS chains from ray cartilage.

Fig. 6 and Table 3

4. Discussion

In this study, CS was isolated from ray fish cartilage, an industrial waste, and its structure and NOP activity were analyzed. The disaccharide composition of ray cartilage CS was very similar to that of shark cartilage CS-C. The major disaccharide unit was GlcUA-GalNAc(6-*O*-sulfate) (63%) and the preparation contained a significant amount of the highly sulfated disaccharide unit, GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) (7%). The average molecular size of ray cartilage CS and shark cartilage CS-C was determined to be 142 and 123 kDa, respectively, larger than that of the CS chains from other sources including embryonic pig brain (40 kDa) [10], shark skin (70 kDa) [11], shark liver (76 kDa) [15], and hagfish notochord (18 kDa) [12]. Although the two had similar structural features, the ray cartilage CS displayed higher NOP activity than shark cartilage CS-C, suggesting that the CS chains from ray cartilage contain a specific saccharide sequence required for interaction with growth/neurotrophic factors.

The NOP activity of the CS chains from ray cartilage was suppressed by the antibodies against HGF and its receptor (c-Met). Although ray cartilage CS showed

high affinity for HGF, PTN, and MK displaying K_d values of 181, 168, and 58 nM, respectively, the NOP activity was not inhibited by the antibodies against PTN or MK (Fig. 5), suggesting that neither PTN nor MK is significantly involved in the NOP activity toward hippocampal neuronal cells. Several studies have demonstrated that endogenous growth/neurotrophic factors such as PTN, MK, and HGF are recruited by the CS chains and then presented to neuronal cells to promote neuritogenesis [14, 15, 23]. Therefore, the endogenous HGF derived from hippocampal glial cells was presumed to be recruited by the CS from ray cartilage and presented to the hippocampal neurons to induce the neurite outgrowth.

HGF has mitogenic activity in various cells such as hepatocytes, epithelial cells, and endothelial cells [24], and has also been proposed to have additional roles in the development and function of the nervous system [16]. Both HGF and c-Met are expressed during the brain's development, and their expression persists into adulthood. HGF promotes neurite outgrowth from neocortical explants [25] and dendritic maturation during hippocampal neuron differentiation [26]. HGF also enhances the neurotrophic activity of nerve growth factor *in vitro* [27]. Although HGF alone had no

outgrowth-promoting effect on cultured dorsal root ganglion explants, it dramatically enhanced nerve growth factor-promoted neurite outgrowth. GAGs have been demonstrated to be involved in the activation of c-Met [28], since HGF, heparin or DS, and c-Met interact to form an active ternary complex [29]. Catlow *et al*. have investigated the interactions of HGF with GAGs and found that the preferred HGF-binding site in CS/DS chains is composed of disulfated, IdoUA(2-*O*-sulfate)-containing sequences [30]. Deakin *et al*. have also characterized the HGF-binding properties of GAG oligosaccharides and revealed that IdoUA-containing sequences varying in their sulfation pattern and/or density from different GAGs interact with a single binding site in HGF [31]. Although they did not examine CS-D, which contains GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) units in a significant proportion, GlcUA(2-*O*-sulfate)-containing motifs might also be vital for interaction with HGF. Since little, if any, IdoUA is present in CS from ray cartilage, the HGF-binding site in this CS preparation is most likely composed of a GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate)-containing motif. In addition, it is worth noting that heparin and heparin oligosaccharides stimulate the production of HGF at the

posttranslational level [32, 33], suggesting an additional possibility, that the CS chains from ray cartilage may also possess such an activity.

CS from ray cartilage was fractionated using an HGF-immobilized column into HGF-bound and -unbound fractions. The HGF-bound fraction contained a higher proportion of the highly sulfated disaccharide unit, GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate), and showed greater NOP activity than the unbound fraction (Fig. 6). The presence of the GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) disaccharide unit in the mouse brain has been reported, especially in the cerebellar region, where neural development dramatically proceeds during the postnatal period [6], and its proportion increased by ~2-fold from postnatal day 7 to 7 weeks of age [6]. CS-D from shark cartilage rich in GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) has been demonstrated to have extensive activity to promote the outgrowth of neurites toward mouse hippocampal neurons and bind PTN more strongly than other CS variants [8, 13, 34]. Oligosaccharides that specifically bind PTN have been isolated from CS/DS co-polymers of embryonic pig brain and characterized to contain the GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate)

disaccharide [35]. Although the saccharide sequence in the CS chains from ray cartilage required for the specific binding to HGF remains to be established, the HGF-binding site is assumed to contain the GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) unit. This is because the proportion of the disulfated disaccharide was greater in the HGF-bound fraction than unfractionated, or intact CS preparation from ray cartilage. More studies are required to elucidate the structure of the HGF-binding site in the CS chains.

In this study, the structure of CS from ray cartilage was characterized, and its NOP activity demonstrated. The activity appears to depend on a specific saccharide sequence containing the GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) unit embedded in the CS chains. Since the CS preparation promoted neurite outgrowth of hippocampal neurons probably through the HGF signaling pathway, the CS chains from ray cartilage appear to be useful for studying the cooperative function of CS and HGF.

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Figure legends

Fig. 1. Anion-exchange HPLC of the CSase ABC digest of the CS preparation from ray cartilage.

The digest of the CS preparation was labeled with 2AB and analyzed by anion-exchange HPLC on an amine-bound silica PA-03 column using a linear gradient of NaH_2PO_4 (indicated by the *dashed line*). The elution positions of authentic 2AB-labeled unsaturated diasaccharides are indicated by *arrows*. 1, ΔHexUA-GalNAc; 2, ΔHexUA-GalNAc(6-*O*-sulfate); 3, ΔHexUA-GalNAc(4-*O*-sulfate); 4, ΔHexUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate); 5, ΔHexUA(2-*O*-sulfate)-GalNAc(4-*O*-sulfate); 6, ΔHexUA-GalNAc(4,6-*O*-disulfate); 7, ΔHexUA(2-*O*-sulfate)-GalNAc(4,6-*O*-disulfate).

Fig. 2. Determination of the molecular mass of the CS preparation from ray cartilage and CS-C from shark cartilage by gel-filtration chromatography.

The ray cartilage CS and shark cartilage CS-C preparations (10 μg each as GlcUA) were subjected to gel filtration chromatography on a column of Superdex 200 using 0.2

M ammonium acetate as an effluent with a flow rate of 0.3 ml/min, and fractions were collected at 3-min intervals and monitored with the dimethylmethylene blue dye method. The *circles* and *squares* indicate the elution profiles of CS from ray cartilage and CS-C from shark cartilage, respectively. The *inset* shows the calibration curve giving a linear relationship between the logMr and elution volume, which was generated using size-defined commercial polysaccharides; dextran (average Mr: 200,000, 66,000, 38,000, and 18,000). V_0 and V_t represent void and total volume, respectively.

Fig. 3. NOP activity of the CS preparation from ray cartilage.

E16 mouse hippocampal cells (20,000 cells/cm^2) were cultured for 24 h on plastic coverslips coated with P-ORN and then with the CS-E, CS-C, CS preparation from ray cartilage (2 μg of each as GAG), or the digest of CS from ray cartilage obtained with CSase ABC. The cells were fixed and stained with anti-microtubule-associated protein 2 and anti-neurofilament antibodies. In the control experiments, cells were cultured on coverslips coated with P-ORN alone. Representative microscopic fields for P-ORN alone (A) or CS from ray cartilage (B) are shown. The mean length of the longest

neurite per cell of 100 randomly selected neurons cultured on various substrates was measured (C).

Fig. 4. Binding of various growth factors to immobilized CS from ray cartilage.

Various concentrations of PTN (A, B), MK (C, D), HGF (E, F), or GDNF (G, H) were injected onto the surface of a ray cartilage CS (A, C, E, and G) or CS-C (B, D, F, and H)-immobilized sensor chip. Sensograms were overlaid using BIA evaluation software (version 4. 1). Concentrations of each growth/neurotrophic factors are shown on the right. Arrowheads and arrows indicate the beginning of the association and dissociation phases, respectively.

Fig. 5. Effects of antibodies against c-Met, HGF, PTN, and MK on the NOP activity of the CS preparation from ray cartilage.

E16 mouse hippocampal cells (20,000 cells/cm^2) were cultured on plastic coverslips coated with P-ORN and then with the CS preparation from ray cartilage (2 μg as GAG). After 2 h, anti-c-Met, anti-HGF, anti-PTN, or anti-MK antibody was added to the

culture, and the cells were incubated for 24 h. IgG from goat serum was used as a negative control. The mean length of the longest neurite was evaluated as described in the legend to Fig. 3.

Fig. 6. The NOP activity of the affinity-fractionated CS subfractions

The CS preparation from ray cartilage was fractionated on a HGF-immobilized column into unbound and bound fractions, which were eluted from the column with 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 and 2.0 M NaCl, respectively. E16 mouse hippocampal cells were cultured for 24 h on the plastic coverslips coated with the unbound or bound CS from ray cartilage (2 μg each as GAG). The mean length of the longest neurite was evaluated as described in the legend to Fig. 3.

Table 1

Disaccharide composition of CS from ray cartilage and CS-C from shark cartilage

	CS from ray cartilage	CS-C
	% Composition	
ΔHexUA-GalNAc	5	ND[a]
ΔHexUA-GalNAc(6S)	63	77
ΔHexUA-GalNAc(4S)	25	15
ΔHexUA(2S)-GalNAc(6S)	7	8

[a]ND, not detected.

Table 2

Kinetic parameters for the interaction of growth/neurotrophic factors with immobilized CS from ray cartilage or CS-C[a]

Growth and neurotrophic factors	CS from ray cartilage			CS-C		
	k_a	k_d	K_d	k_a	k_d	K_d
	$M^{-1}s^{-1}$	s^{-1}	nM	$M^{-1}s^{-1}$	s^{-1}	nM
PTN	9.56×10^{4}	1.61×10^{-2}	168	4.18×10^{4}	1.47×10^{-2}	352
MK	1.66×10^{5}	9.65×10^{-3}	58	1.16×10^{5}	9.20×10^{-3}	79
HGF	1.68×10^{5}	3.05×10^{-2}	181	3.94×10^{4}	6.21×10^{-2}	1580
GDNF	1.44×10^{5}	4.08×10^{-2}	284	7.11×10^{5}	1.11×10^{-1}	157

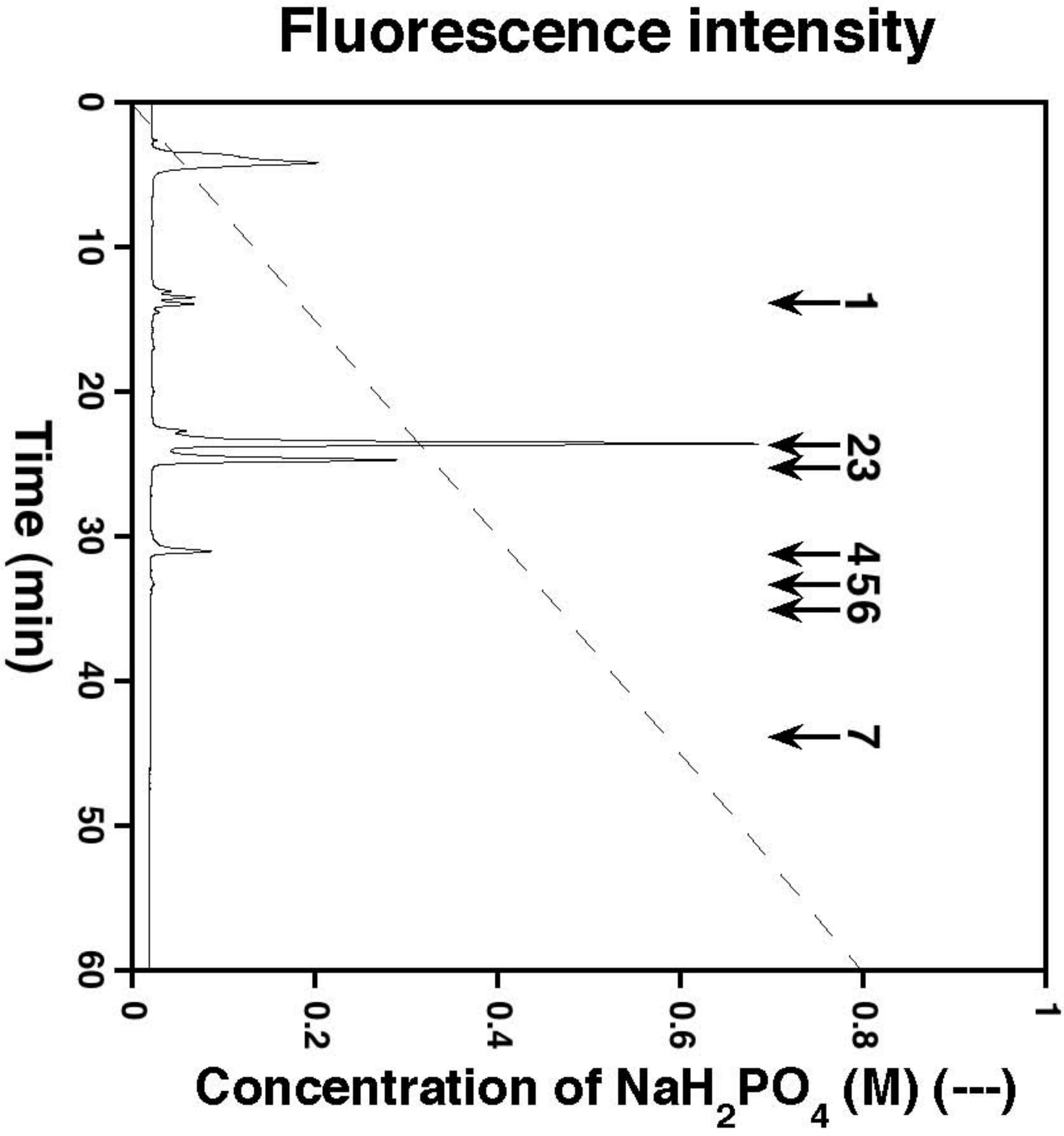
[a]The k_a, k_d, and K_d values were determined using a 1:1 binding model with mass transfer as described under "Materials and methods".

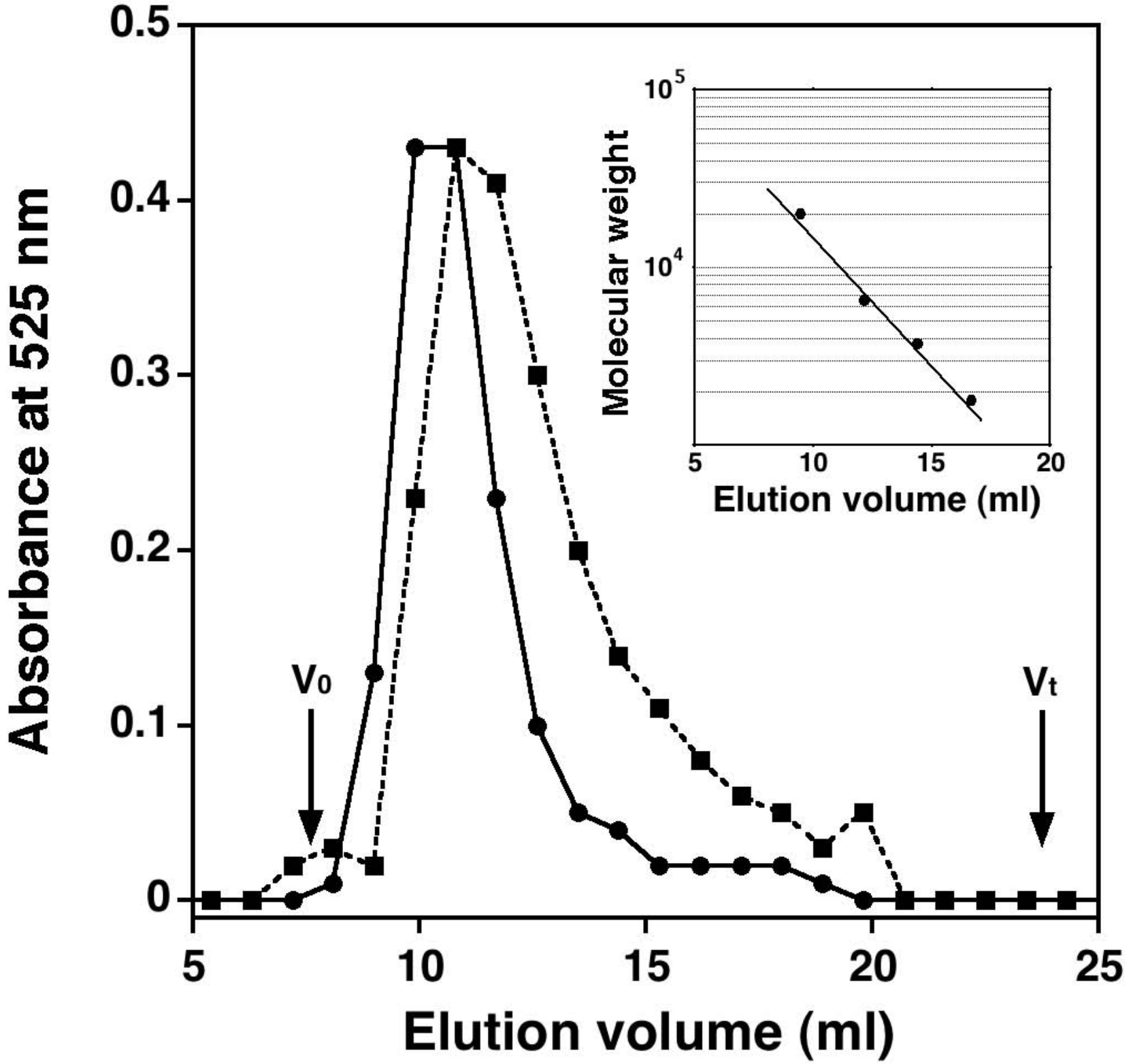
Table 3

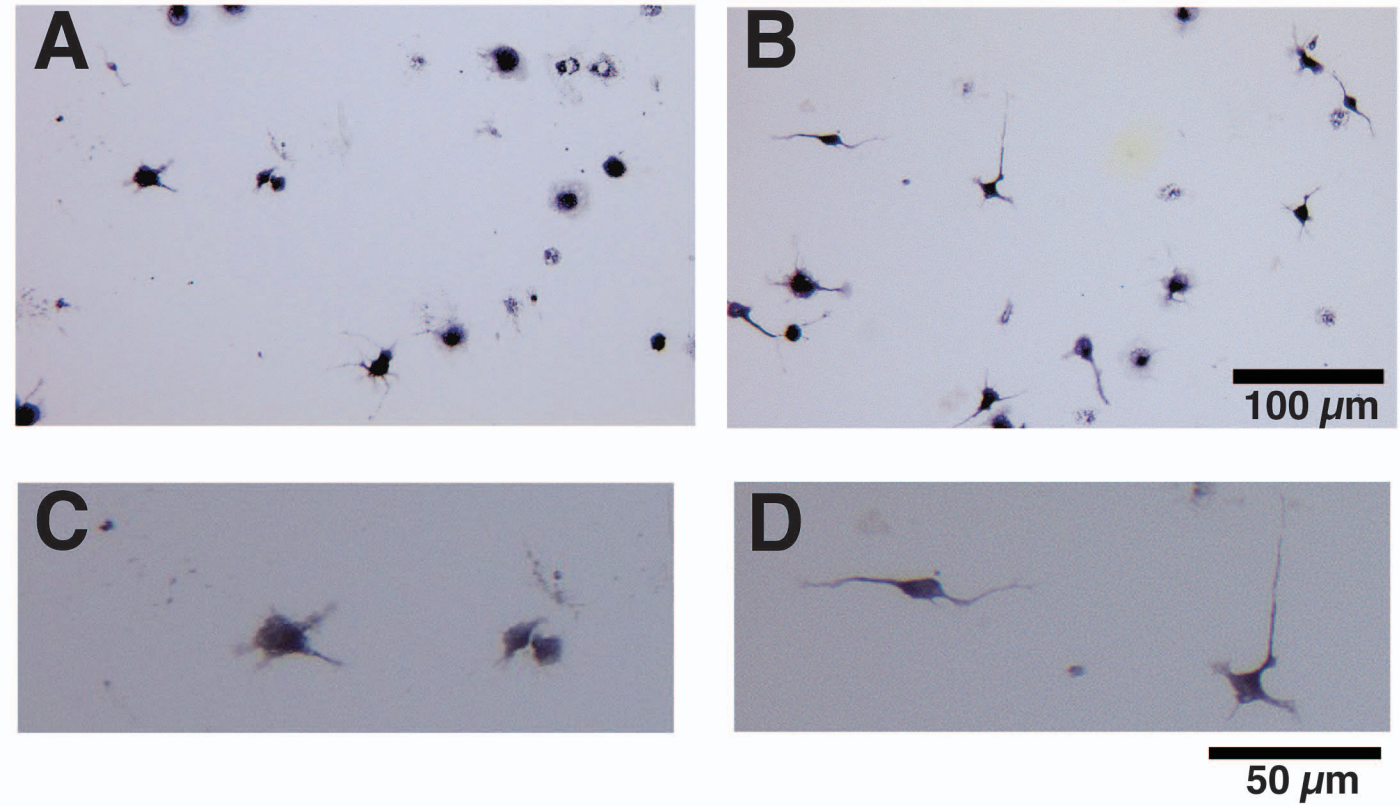
Disaccharide composition of HGF-unbound and -bound fractions of CS from ray cartilage

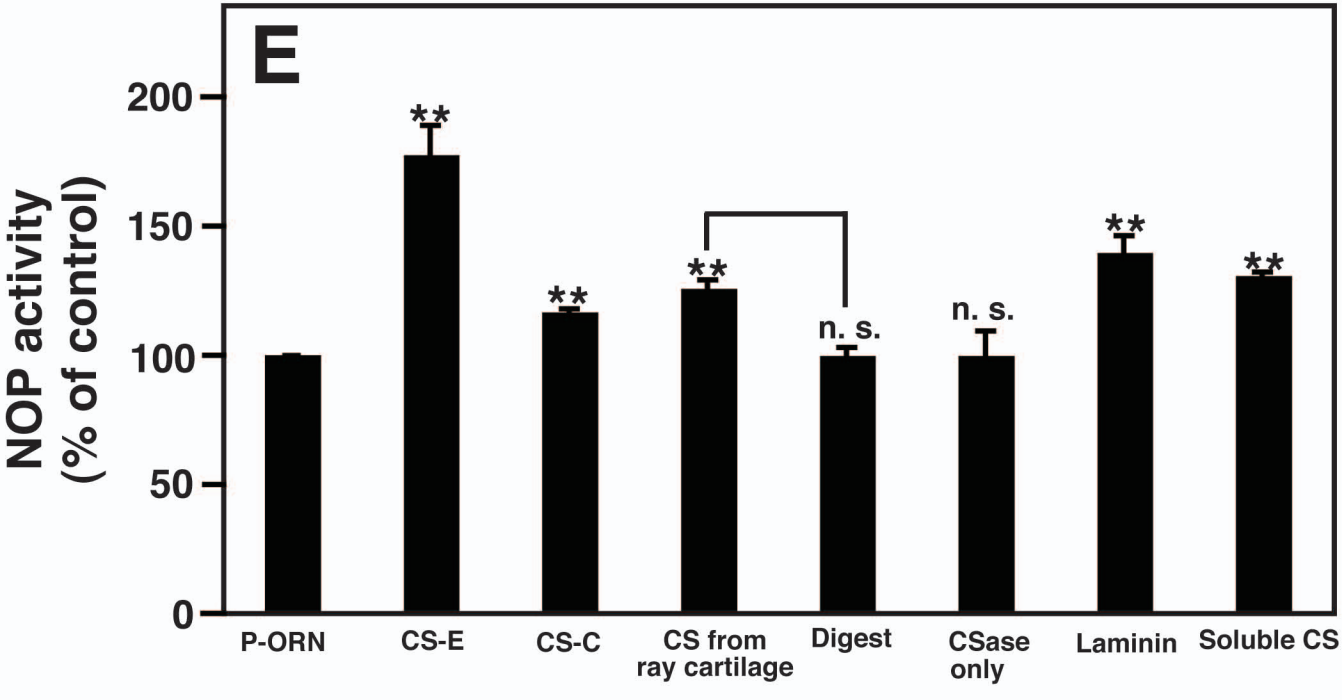
	HGF-unbound	HGF-bound
	% Composition	
ΔHexUA-GalNAc	ND[a]	ND
ΔHexUA-GalNAc(6S)	81	76
ΔHexUA-GalNAc(4S)	13	16
ΔHexUA(2S)-GalNAc(6S)	6	8

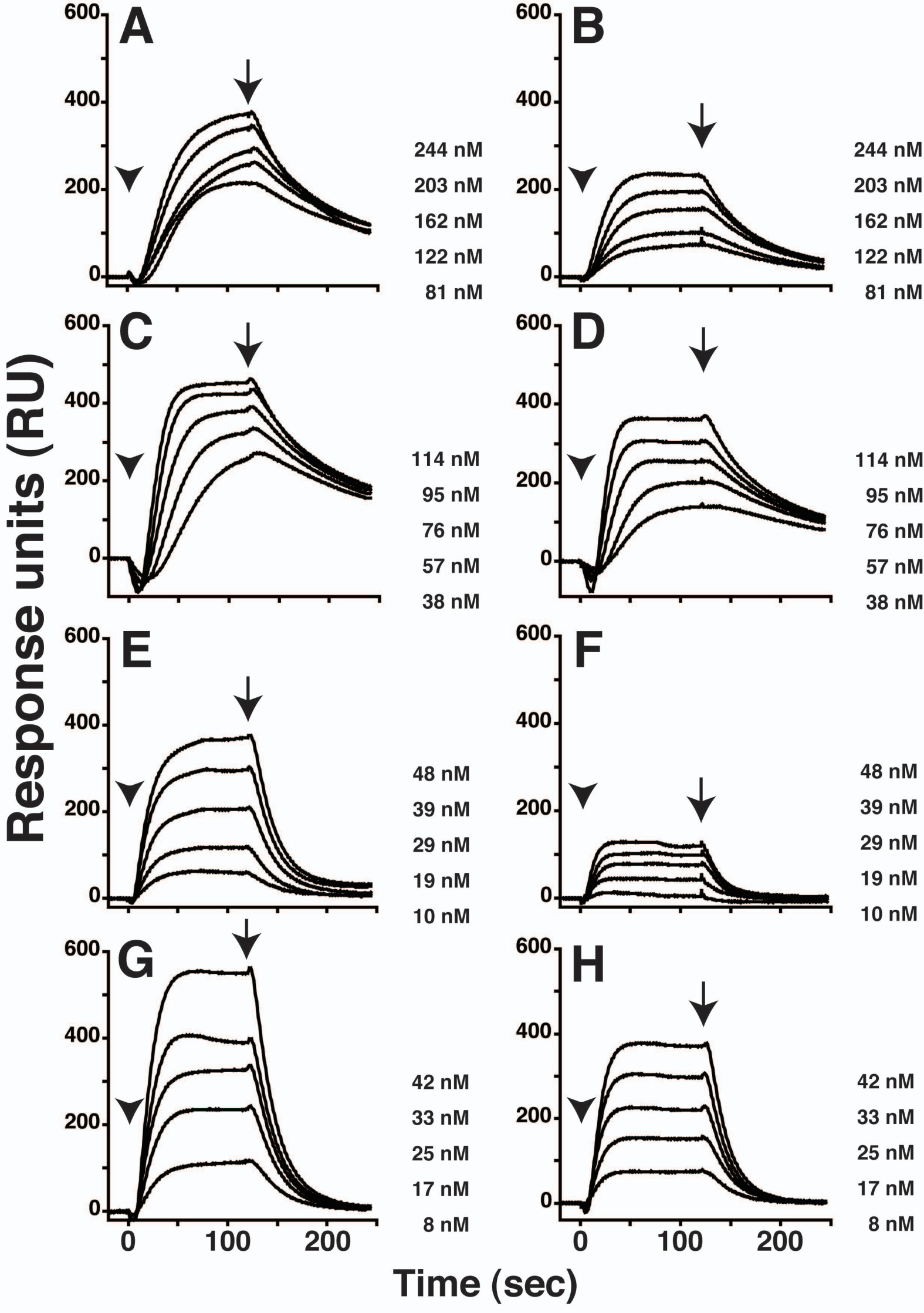
[a]ND, not detected.

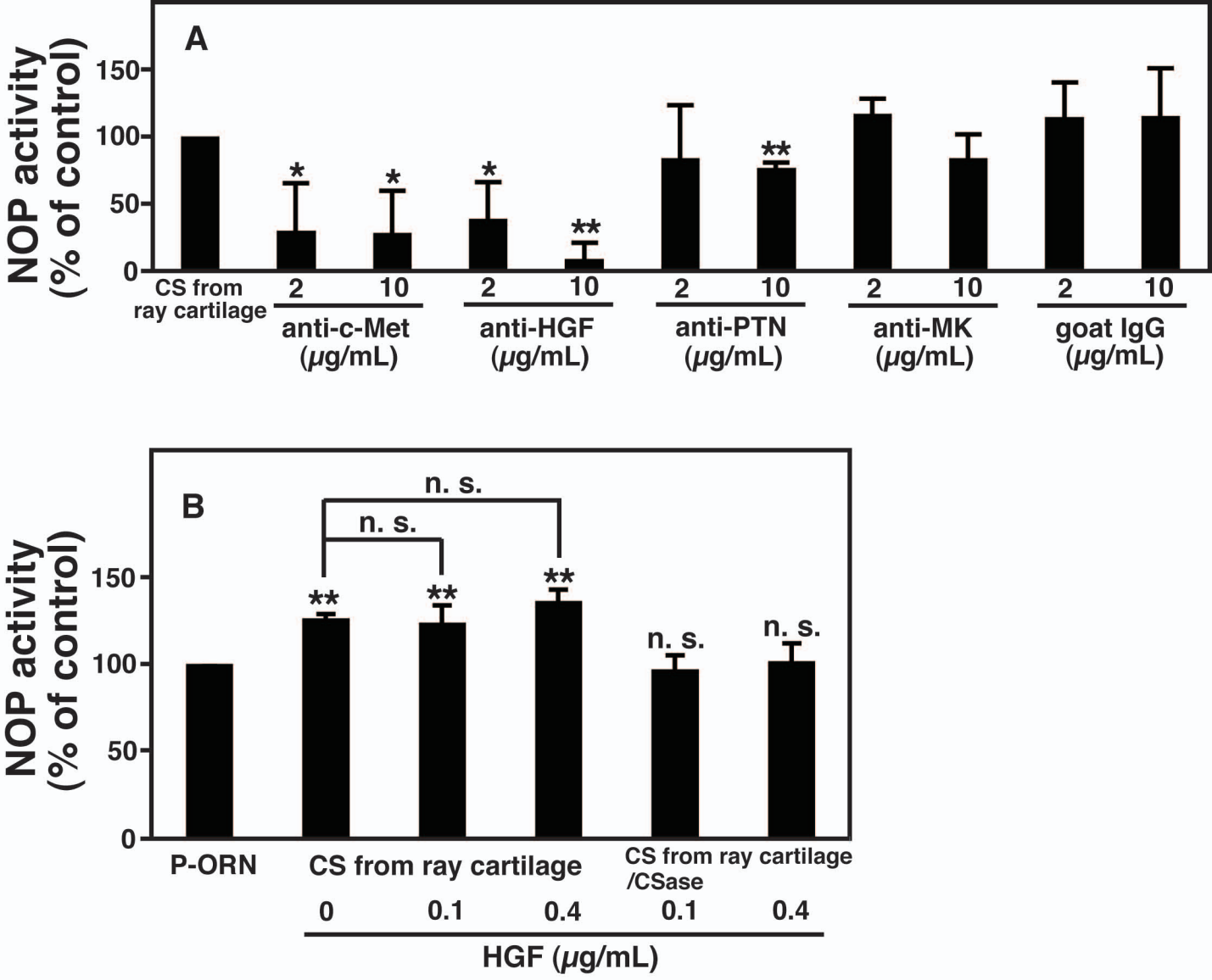

Fluorescence intensity
0
10
20
30
40
50
60
Time (min)
1
23
456
7
0
0.2
0.4
0.6
0.8
1
Concentration of NaH2PO4 (M) (---)

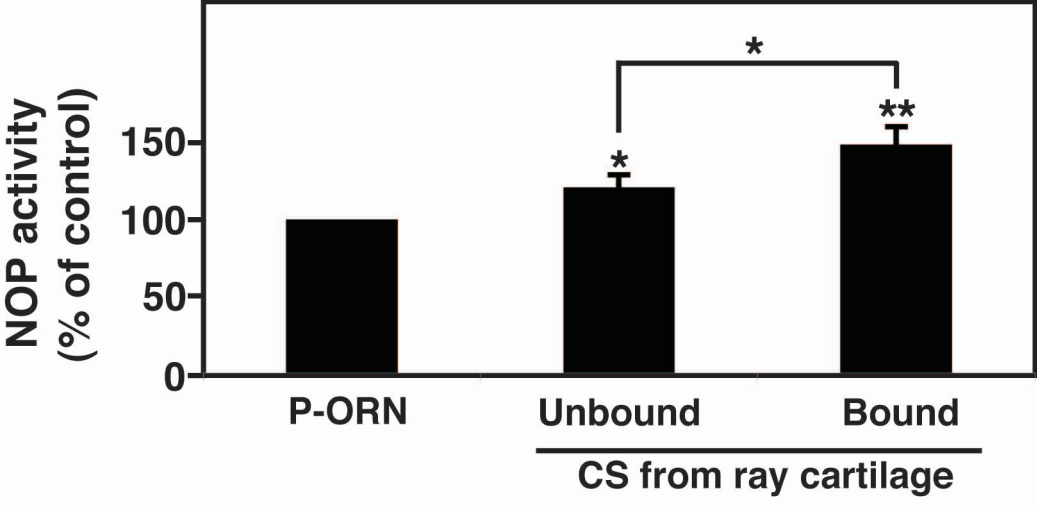

Absorbance at 525 nm
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
Elution volume (ml)
V0
Vt
Molecular weight
10^5
10^4
5
10
15
20
Elution volume (ml)


A
B
100 μm
C
D
50 μm


E
NOP activity
(% of control)
200
150
100
50
0
**
**
**
n. s.
n. s.
**
**
P-ORN
CS-E
CS-C
CS from ray cartilage
Digest
CSase only
Laminin
Soluble CS


A
B
C
D
E
F
G
H
600
400
200
0
244 nM
203 nM
162 nM
122 nM
81 nM
114 nM
95 nM
76 nM
57 nM
38 nM
48 nM
39 nM
29 nM
19 nM
10 nM
42 nM
33 nM
25 nM
17 nM
8 nM
0
100
200
Response units (RU)
Time (sec)


A
NOP activity (% of control)
150
100
50
0
*
*
*
**
**
CS from ray cartilage
2
10
anti-c-Met (μg/mL)
anti-HGF (μg/mL)
anti-PTN (μg/mL)
anti-MK (μg/mL)
goat IgG (μg/mL)
B
n. s.
n. s.
**
**
**
n. s.
n. s.
P-ORN
CS from ray cartilage
CS from ray cartilage /CSase
0
0.1
0.4
HGF (μg/mL)


NOP activity
(% of control)
150
100
50
0
*
**
*
P-ORN
Unbound
Bound
CS from ray cartilage