Isolation and characterization of a novel chondroitin sulfate from squid liver integument rich in *N*-acetylgalactosamine(4,6-*O*-disulfate) and glucuronate(3-*O*-sulfate) residues

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This paper is dedicated to the 65 birthday of Prof. Johannis P. Kamerling.

Abstract

Novel chondroitin sulfate (CS) chains with an average molecular mass of 79.6 kDa were purified from squid liver integument. A compositional analysis of the CS chains using chondroitinases (CSases) ABC and AC-I revealed a range of variably sulfated disaccharides with GlcAβ1-3GalNAc(6-*O*-sulfate), GlcAβ1-3GalNAc(4-*O*-sulfate), and GlcAβ1-3GalNAc(4,6-*O*-disulfate) as the major ones, significant amounts of rare 3-*O*-sulfated GlcA-containing disaccharides, and a small amount of nonsulfated GlcAβ1-3GalNAc. The CS chains exhibited neurite outgrowth-promoting activity toward embryonic mouse hippocampal neurons, which was abolished completely by digestion with CSase ABC or AC-I. Consequently, whether these CS chains interact with heparin-binding growth factors was tested in a BIAcore system. All of the growth factors exhibited concentration-dependent and specific binding. CS chains from squid liver integment, with their unique composition and strong biological activities, may be a good candidate for therapeutic application.

Keywords: chondroitin sulfate; glycosaminoglycan; growth factor; neurite outgrowth; squid liver; surface plasmon resonance.

Abbreviations: SLI, squid liver integument; GAG, glycosaminoglycan; CS, chondroitin sulfate; HS, heparan sulfate; Hep, heparin; GalNAc, *N*-acetyl-D-galactosamine; GlcA, D-glucuronic acid; CSase, chondroitinase; HPLC, high performance liquid chromatography; 2AB, 2-aminobenzamide; $\Delta^{4,5}$HexA, 4,5-unsaturated hexuronic acid; 2S, 2-*O*-sulfate; 3S, 3-*O*-sulfate; 4S, 4-*O*-sulfate; 6S, 6-*O*-sulfate; P-ORN, poly-DL-ornithine; NOP, neurite outgrowth-promoting; E16, embryonic day 16; rh, recombinant human; MK, midkine; PTN, pleiotrophin; FGF,

fibroblast growth factor; HGF, hepatocyte growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; $VEGF_{165}$, vascular endothelial growth factor-165; DMMB, dimethylmethylene blue; FACE, fluorophore-assisted carbohydrate electrophoresis; AMAC, 2-aminoacridone.

1. Introduction

Glycosaminoglycans (GAGs), typically linked to a protein core forming proteoglycans at the cell surface or in the extracellular matrix, are a family of linear, polydisperse polysaccharides, which participate in a number of physiological phenomena including neuronal development, cell-matrix interactions, and activation of chemokines, enzymes and growth factors.[1,2] The ability of GAGs to regulate these processes is attributed to their complex structure, which arises from extensive modifications of a nonsulfated precursor consisting of hexosamine and either hexuronic acid or galactose residues that are arranged in an alternating linear sequence by modifying enzymes such as sulfotransferases and epimerases.[3]

Heparan sulfate (HS) has attracted much attention because of its involvement in developmental processes and various signaling pathways.[4] Although chondroitin sulfate (CS) has attracted little attention, recent advances in the structural biology of CS chains suggest the importance of these molecules in various biological processes.[5,6] We and others have shown the importance of this class of molecules, from simple chondroitin involved in the cell division of a nematode[7,8] to differentially oversulfated CS-D and CS-E involved in neuroregulatory functions[9–11] and the binding of growth factors in mammalian systems.[12] CS has been isolated from various tissues obtained from a large number of animal species including both vertebrates and invertebrates.[13-19] Even though the structural organization of CS from the tissues of several vertebrates has been studied extensively,[18,20,21] the assessment of CS from the tissues of invertebrate organisms is rather limited. The main purpose of studies of CS chains from invertebrate tissues is to compare the chemical structure of CS chains from invertebrate tissues with the well-known CS chains that occur in vertebrate tissues, and to gain a deeper understanding of their structure with physicochemical and biological properties.

As a part of our continued exploration for sources of CS unique in structure and with therapeutic potential, in this study, we isolated CS chains with novel structural features and multiple biological activities from the integument of squid liver, an industrial waste with a good candidate to be exploited for pharmaceutical purposes.

2. Results

2.1. Preparation of the CS Fraction from Squid Liver Integument (SLI)

GAGs were extracted from SLI by protease digestion, and recovered by ethanol precipitation. This preparation was further fractionated by anion-exchange chromatography using an Accell^{TM} Plus QMA cartridge, which was eluted stepwise with buffers containing 0.15, 0.5, and 2.0 M NaCl. Only trace amounts of CS/dermatan sulfate (DS) were detected in the fractions eluted with a buffer containing 0.15 M NaCl. Twelve and 56% of all GAGs were detected in the fractions eluted with buffers containing 0.5 and 2.0 M NaCl, respectively. The 0.5 M NaCl-eluted fraction lacked E-units (GlcAβ1–3GalNAc(4S,6S)), whereas the 2.0 M NaCl-eluted fraction was rich in E-units (21.6%) and further purified. Since the 2.0 M NaCl-eluted fraction showed trace amounts of HS disaccharides upon high performance liquid chromatography (HPLC) after digestion with heparitinase (data not shown), it was purified further by nitrous acid treatment[22,23] to remove HS. Subsequently, this preparation was subjected to alkali treatment to prepare peptide-free glycan chains,[24] and passed through a C_{18} cartridge to remove peptides (the final yield was 0.12% of the starting material). No significant amount of DS or keratan sulfate was found by anion-exchange HPLC in the digests of the SLI-CS preparations obtained with chondroitinase (CSase) B and keratanase-II, respectively (data not shown).

2.2. Determination of the Molecular Mass of SLI-CS

The molecular size of the purified SLI-CS was determined by gel filtration HPLC (Figure 1), using the calibration curve generated with standard polysaccharides. The elution profile of SLI-CS showed a broad peak with a molecular mass ranging from 37 to 122 kDa, giving an average molecular mass of 79.6 kDa. Notably, the molecular mass of SLI-CS was larger than that of the CS chains derived from squid

skin (40 kDa).[25] In contrast, squid cartilage CS-E was larger than SLI-CS and eluted in the void volume of the Superdex 200™ column (Figure 1), indicating that CS populations from various squid tissues may differ in structure and function.

2.3. Analysis of the Disaccharide Composition of SLI-CS

To determine its disaccharide composition, the SLI-CS preparation was digested with CSase ABC, labeled with a fluorophore 2-aminobenzamide (2AB) for high sensitivity and resolution, and analyzed by anion-exchange HPLC. The analysis of SLI-CS revealed a unique composition consisting of monosaccharides (mono- and di-sulfated GalNAc) and disaccharides with a diverse sulfation pattern as shown in Figure 2 and Table 1. SLI-CS was enriched with disulfated disaccharide ΔE ($\Delta^{4,5}$HexAα1–3GalNAc(4S,6S)) (see "Footnote"), accounting for 21.6% of all its disaccharides, and contained significant proportions of monosulfated disaccharides of ΔC ($\Delta^{4,5}$HexAα1–3GalNAc(6S)) (14.9%) and of ΔA ($\Delta^{4,5}$HexAα1–3GalNAc(4S)) (41.8%). Its sulfate/disaccharide unit (S/unit) ratio was 1.18 (Table 1).

In addition, SLI-CS had small yet appreciable proportions of GalNAc(4S), GalNAc(6S) and GalNAc(4S,6S), indicating the presence of GlcA(3S)-containing disaccharides as revealed in our recent studies, where we identified mono- and disulfated GalNAc as stable end products of the degradation of GlcA(3S)-containing disaccharides by CSase ABC.[26] GalNAc(4S) derived from K-units [GlcA(3S)β1-3GalNAc(4S)] accounted for 5%, while GalNAc(4S,6S) derived from M-units [GlcA(3S)β1-3GalNAc(4S,6S)] accounted for 2.8% of the disaccharides. However, in the case of GalNAc(6S) derived from L-units [GlcA(3S)β1-3GalNAc(6S)], the proportion could not be determined, since it co-eluted with ΔO ($\Delta^{4,5}$HexAα1–3GalNAc) upon anion-exchange HPLC. Hence, a

Fluorophore-assisted Carbohydrate Electrophoresis (FACE) analysis was carried out for the digest of the SLI-CS preparation obtained with CSase ABC. The separation of mono- and disulfated GalNAc residues as well as CS disaccharides can be clearly achieved by FACE.[27] Based on the FACE analysis (Figure 3), the ratio of ΔO to GalNAc(6S) was found to be 1 : 1.2, which corresponded to 6.3% ΔO and 7.6% GalNAc(6S) among the 13.9% disaccharide (Table 1). Since mono- or disulfated GalNAc residues have been reported as non-reducing terminal modifications in CS chains,[28,29] the possibility exists that the released mono- and disulfated GalNAc residues were derived from the non-reducing termini due to the action of CSase ABC. However, it was not the case. Based on the average molecular mass of SLI-CS chains (79.6 kDa), they consist of approximately 170 disaccharide units, and the proportion of the non-reducing termini is calculated to be only 0.6% of the total disaccharides, which was far below the proportion of the monosaccharides (15.4%) generated by digestion with CSase ABC. Therefore, to confirm that these mono- and disulfated GalNAc units were derived from GlcA(3S)-containing disaccharides, SLI-CS was subjected to digestion with CSase AC-II, which cannot act on the *N*-acetylgalactosaminidic linkages to GlcA(3S)[26] but can release the non-reducing terminal monosaccharides.[29] However, the CSase AC-II digest of SLI-CS gave no significant peaks at the positions where mono- and disulfated GalNAc residues were eluted upon anion-exchange HPLC (data not shown). Thus, a unique composition was revealed for SLI-CS with considerable proportions of GlcA(3S)-containing disaccharides in addition to a significant proportion of E-unit [GlcAβ1-3GalNAc(4S,6S)].

2.4. Neurite Outgrowth-promoting (NOP) Activity of the SLI-CS Preparation

To evaluate the biological activity of the purified SLI-CS preparation, NOP activity was tested by using embryonic day 16 (E16) mouse hippocampal neuronal cells. Control cells (cultured on cover slips coated with poly-DL-ornithine (P-ORN) alone) showed no significant promotion of neurite outgrowth (Figure 4A). The SLI-CS preparation exhibited neuritogenic activity that was principally axonic in nature in terms of cell morphology (Figure 4B), and was weaker than that shown by a positive control (CS-E) (Figure 4C). The lower NOP activity level of SLI-CS compared to CS-E may reflect the higher proportion of E-unit [GlcAβ1-3GalNAc(4S,6S)] in CS-E (66.7%) in comparison to SLI-CS (21.6%), since both preparations comprise approximately 10% GlcA(3S)-containing disaccharides. Furthermore, the NOP activity was completely abolished upon digestion with CSase ABC or AC-I (Figure 4D), suggesting that the neuritogenic activity was attributable to the CS polysaccharides not to a contaminant in the preparation.

2.5. Specific Interactions of SLI-CS with Various Heparin (Hep)-binding Growth Factors

Signaling by Hep-binding growth factors is involved in the NOP activity of CS chains.[30,31] Hence, we analyzed the molecular interactions of SLI-CS with various Hep-binding growth factors expressed in the brain during embryonic development using a BIAcore system. The purified SLI-CS preparation was biotinylated and immobilized on the streptavidin-precoated sensor chip. To determine the association and dissociation rate constants (k_a, k_d) as well as the dissociation equilibrium constants (K_d), a kinetic analysis was carried out. Various growth factors (Table 2) at different concentrations were injected individually onto the surface of an SLI-CS immobilized sensor chip. The overlaid sensorgrams, shown in Figure 5, were analyzed

collectively using “the 1:1 Langmuir binding model with mass transfer” of the BIAevaluation 3.1 software package to calculate kinetic parameters, which are summarized in Table 2. The direct binding of pleiotrophin (PTN), midkine (MK), vasucular endothelial growth factor-165 ($VEGF_{165}$), Hep-binding epidermal growth factor-like growth factor (HB-EGF), hepatocyte growth factor/scatter factor (HGF/SF), fibroblast growth factor-2 (FGF-2), and FGF-18 to the immobilized SLI-CS preparation was demonstrated. These bindings were inhibited by addition of the soluble SLI-CS in a concentration-dependent manner but not by the CSase digest of SLI-CS (data not shown), suggesting the specific interaction of the growth factors with the immobilized SLI-CS. The tested growth factors varied in their ability to bind SLI-CS. PTN, MK, HGF/SF, FGF-2, and FGF-18 displayed quick binding and slow dissociation, giving K_d values in the low nanomolar (nM) range, indicating their strong affinity for SLI-CS (Table 2). In contrast, $VEGF_{165}$ and HB-EGF showed weaker affinity for SLI-CS as reflected in the K_d values listed in Table 2. The different characteristics of the kinetics of the binding between SLI-CS chains and various Hep-binding growth factors suggest possible distinct roles for CS chains in the regulation of growth factor signaling.

3. Discussion

In this study, CS purified from SLI was structurally and functionally characterized. Squid tissues have been shown to contain CS chains with considerable variation in molecular mass and degree of sulfation.[15,32-37] Chondroitin and oversulfated CS chains with an average molecular mass (M_r) of 80 and 40 kDa, respectively, were isolated from squid skin.[32,36] Oversulfated CS with a M_r of 72.5 kDa and a small amount of low sulfated CS were isolated from squid cornea,[35] while oversulfated CS-E with a M_r of 300 kDa was isolated from squid cartilage.[33,34] On the other hand, SLI-CS reported here, which has a M_r of 79.6 kDa and S/unit ratio of 1.18, is comparable to squid cartilage CS-E[37] in terms of its disaccharide components including E-units [GlcAβ1-3GalNAc(4S,6S)] together with rare GlcA(3S)-containing disaccharide units, K-units [GlcA(3S)β1-3GalNAc(4S)], L-units [GlcA(3S)β1-3GalNAc(6S)] and M-units [GlcA(3S)β1-3GalNAc(4S,6S)]. In this report, a method developed recently by our group[26] was used to detect rare GlcA(3S)-containing disaccharides, since the disaccharide units containing 3-*O*-sulfated GlcA are decomposed following digestion by CSase ABC.[38]

SLI-CS exhibited marked NOP activity, promoting a few prominent long neurites with relatively small cell soma. This characteristic feature is also displayed by CS-E,[10] although the amount of oversulfated disaccharide differs between CS-E rich in E-units (66%) and SLI-CS, where E-units account only for 21.6% of disaccharides. This implies that NOP activity is dependent on the domain-related structure rather than the content of oversulfated disaccharide and that neuritogenic activity is dependent on not only the charge density but also the sequential arrangement of the disaccharide units.

Although the molecular mechanism behind the actions of CS chains in neuritogenesis is not well understood, the neuroregulatory effects of these chains may be attributable at least in part to their binding with growth factors and the regulation of their signaling.[5,39] Supporting this hypothesis, we demonstrated earlier the involvement of PTN and HGF/SF in the NOP activity of CS/DS hybrid chains isolated from embryonic pig brain and shark liver.[30,31] The growth factors examined in the present study including PTN, MK, FGF-2, FGF-18, HGF/SF, $VEGF_{165}$, and HB-EGF, are broadly expressed in the brain and implicated in its development.[40-44] The high affinity of SLI-CS particularly for PTN, MK, HGF/SF, FGF-2, and FGF-18 suggests that at least one of these growth factors is recruited by SLI-CS to mediate its NOP activities. The K_d values of SLI-CS (Table 2) for PTN, MK, HGF, FGF-2, and FGF-18 are greater than those of CS-E or Hep.[12] GlcA(3S)-containing structures together with E-units in SLI-CS may regulate the biological functions of various Hep-binding growth factors. Although the presence of E-units is demonstrated in brain CS-proteoglycans,[18,45-47] the occurrence of GlcA(3S) residues on CS chains in mammalian systems and their potential involvement in the functions of CS polysaccharides remain to be investigated. Although $VEGF_{165}$ and HB-EGF showed little affinity for SLI-CS, Nandini *et al.*[23] reported strong affinity of $VEGF_{165}$ and HB-EGF for CS-H from hagfish notochord, which contains a significant proportion of dermatan sulfate structures, suggesting that iduronic acid-containing structures may be important for the signaling pathways mediated by $VEGF_{165}$ and HB-EGF.

Most studies on interactions between growth factors and proteoglycans have been concerned with HS-proteoglycans and more specifically with their HS chains. The present findings strengthen the emerging concept that the interactions of CS-proteoglycans with growth/differentiation factors might also play significant roles

in developmental processes of the central nervous system and other systems. SLI-CS with its unique structure, strong biological activities and capacity to bind various growth factors may well serve as a potential candidate for therapeutic agents derived from non-mammalian species. Further studies on the structural motifs of SLI-CS required for the binding of growth factors and neuritogenesis would pave the way for the development of therapeutics for neuronal diseases.

4. Experimental

4.1. Materials

SLI was obtained from Biomatec Japan Inc. (Kushiro, Japan). The following materials and enzymes were purchased from Seikagaku Corp. (Tokyo, Japan): Six unsaturated CS-disaccharide standards, CS-E from squid cartilage, CSase ABC (EC 4.2.2.4) from *Proteus vulgaris*, CSase AC-I (EC 4.2.2.5) from *Flavobacterium heparinum*, CSase AC-II (EC 4.2.2.5) from *Arthrobactor aurescens*, and keratanase-II (EC 3.2.1.103) from *Bacillus sps*. CSase B (EC 4.2.2.19) was obtained from IBEX Technologies (Montreal, Quebec, Canada), GalNAc(4S) sodium salt from Sigma (St. Louis, MO), GalNAc(6S) sodium salt from Dextra laboratories LTD (Berkshire, UK), EZ-Link^{TM} biotin-LC-hydrazide from Pierce (Rockford, IL), and recombinant human (rh) MK from PeproTech EC Ltd. (London, UK). rh-PTN, rh-$VEGF_{165}$, rh-FGF-2, rh-FGF-18, rh-HB-EGF and rh-HGF/SF were purchased from R&D systems (Minneapolis, MN). DMMB was obtained from Aldrich (Milwaukee, WI). Actinase E was from Kaken Pharmaceutical Co. (Tokyo, Japan). All other chemicals and reagents were of the highest quality available.

4.2. Extraction and Purification of CS from SLI

An acetone powder of SLI was used for the extraction of GAGs essentially as described[31] with some modifications. Briefly, 25 g of the powder was treated with actinase E, followed by 5% trichloroacetic acid to precipitate residual proteins and peptides, and with diethylether to extract trichloroacetic acid. A crude GAG fraction was recovered from the extract by precipitation with 80% ethanol containing 1% sodium acetate at 4 °C overnight. The yield of GAGs was 178 mg based on the carbazole reaction. For further purification, the crude GAG fraction (40 mg as GAG)

was loaded on an Accell QMA Plus cartridge (Waters, Milford, MA) pre-equilibrated with 0.05 M phosphate buffer, pH 6.0, containing 0.15 M NaCl. After the column was washed with the equilibration buffer, GAGs were eluted with the same buffer containing 0.5 and 2.0 M NaCl, dialyzed against water, and concentrated. The fraction eluted with 0.5 M NaCl lacked E-units, whereas the 2.0 M NaCl-eluted fraction was rich in E-units and used for the analysis. The 2.0 M NaCl-eluted fraction was subjected to nitrous acid treatment (pH 1.5) to remove Hep/HS as described previously,[22,23] and the resultant HS fragments were removed by MICROCON® YM-10 (Millipore Corp, Bedford, MA). This fraction was then subjected to treatment with 1 M $NaBH_4$/0.05 M NaOH at 4 °C for 15 h to prepare free glycan chains as described,[24] which were desalted using a PD-10 column (GE Healthcare, Tokyo, Japan) followed by a Sep-Pak C_{18} cartridge (Waters) to remove peptides.

4.3. Enzyme Digestion and Fluorophore Labeling

Digestion with CSase ABC was carried out using 1 μg of GAG and 5 mIU of the enzyme in a total volume of 30 μl in a 50 mM Tris-HCl buffer, pH 8.0, containing 60 mM sodium acetate at 37 ^{o}C for 60 min unless otherwise specified. Digestion with CSase AC-I was carried out using 1 μg of GAG and 5 mIU of the enzyme in a total volume of 30 μl in a 50 mM Tris-HCl buffer, pH 7.3, at 37 ^{o}C for 60 min. After incubation, each reaction mixture was boiled at 100 ^{o}C for 1 min, cooled to room temperature, vacuum-dried, and derivatized with 2AB as described.[48] The products were used for the structural analysis.

4.4. HPLC

The analysis of monosaccharides/unsaturated CS disaccharides was carried out by HPLC on an amine-bound silica PA-03 column (YMC Co., Kyoto, Japan) under isocratic conditions with 16 mM of NaH_2PO_4 for the first 20 min followed by a linear gradient from 16 to 798 mM NaH_2PO_4 over 70 min at a flow rate of 1.0 ml/min at room temperature as detailed previously.[26] Elution was monitored using a RF-10A XL fluorometric detector (Shimadzu Co., Kyoto, Japan) with excitation and emission wavelengths of 330 and 420 nm, respectively.

4.5. Determination of Molecular Mass

To determine the molecular mass of SLI-CS, an aliquot (10 μg as GlcA) of SLI-CS or squid cartilage CS-E was chromatographed by gel filtration on a Superdex^{TM} 200 column (10 × 300 mm, GE Healthcare, Uppsala, Sweden), which had been calibrated using a series of size-defined commercial polysaccharides.[49] The sample was eluted with 0.2 M ammonium bicarbonate at a flow rate of 0.3 ml/min for 90 min. Fractions were collected at 3-min intervals, freeze-dried, and dissolved in 50 μl of water. An aliquot was utilized for estimating the total amount of GAGs using DMMB according to the method of Chandrasekhar *et al.*,[50] except that the absorbance was measured at 525 nm.

4.6. Surface Plasmon Resonance Analysis

The interaction of various growth factors with SLI-CS was examined using a BIAcore 2000 system (Biacore life sciences, Tokyo, Japan). The SLI-CS preparation was biotinylated using biotin-LC-hydrazide and 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride as described previously[51] and was immobilized onto a streptavidin-derivatized sensor chip

according to the manufacturer's instructions. For the kinetic analysis, various concentrations of growth factors were injected onto the surface of the sensor chip in the running buffer (HBS-EP, Biacore AB, Uppsala, Sweden), containing 10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% (w/v) Tween-20. The flow rate was kept at a moderate speed (30 μl/min) as recommended by the manufacturer. Each growth factor was allowed to interact with the SLI-CS-immobilized sensor chip for 2 min. Before each injection, baseline stabilization was achieved by injecting 1 M NaCl for 1 min. The kinetic parameters were evaluated with BIAevaluation software 3.1 (Biacore AB) using a 1:1 binding model with mass transfer.

4.7. NOP Assays

Cultures of mouse hippocampal neurons were established from E16 animals as described.[52,53] Briefly, 2 μg/well of the SLI-CS and CS-E preparations were individually coated onto cover slips precoated with P-ORN (Sigma) at 4 °C overnight. To investigate the structural characteristics of SLI-CS responsible for the neuritogenic activity, an aliquot (10 μg as GAG) was digested with 10 mIU of CSase ABC or AC-I, and a 2-μg aliquot of each digest was coated onto the cover slips precoated with P-ORN. Control experiments were carried out using heat-inactivated enzymes. The hippocampal neuronal cells freshly isolated from E16 mouse embryos were suspended in Eagle's minimum essential medium containing supplements described previously.[18,53] Subsequently, the cells were seeded on cover slips at a density of 20,000 cells/cm^2 and allowed to grow in a humidified atmosphere for 24 h at 37 °C, 5% CO_2. Thereafter, the cells were fixed using 4% (w/v) paraformaldehyde for 30 min at room temperature and the neurites were visualized by immunochemical staining using anti-microtubule-associated protein-2 (Lieco technologies Inc, St.

Louis) and anti-neurofilament (Sigma) as described previously.[53] The antibodies were then detected using a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA) with 3, 3'-diaminobenzidine as a chromogen. The stained cells on each cover slip were scanned and digitalized with a x20 objective lens on an optical microscope (BX51, Olympus, Tokyo, Japan) equipped with a digital camera (FX380, FLOVEL Co. Ltd., Tokyo, Japan). One hundred cells were chosen at random to determine the length of the longest neurite using morphological analysis software (FLOVEL Filing System, FLOVEL Co. Ltd., Japan). At least three independent experiments per parameter or condition were carried out.

4.8. FACE

To separate and examine the ratio of GalNAc(6S) and ΔO in the SLI-CS preparation, a FACE analysis[27] was carried out using MONO composition gels and companion buffers (Glyko, San Leandro, CA) as per the manufacturer's instructions. SLI-CS was digested with CSase ABC and the digest was subjected to gel filtration chromatography on a Superdex peptide™ column (10 x 300 mm, GE Healthcare, Uppsala, Sweden) using 0.2 M NH_4HCO_3 as an eluent at a flow rate of 0.4 ml/min. Elution was monitored using a UV detector (Shimadzu Co.) at 232 nm. A fraction containing both mono-sulfated GalNAc and ΔO was collected, repeatedly freeze-dried, and derivatized with a flurophore AMAC (Molecular Probes, Eugene, OR) as described previously.[54] The AMAC-labeled sample was freeze-dried and resuspended in a loading buffer. The electrophoresis was carried out at 30 mA for about 100 min. The gels were washed with distilled water and imaged using a LAS-4000 Image Reader (FUJIFILM, Tokyo, Japan) with excitation and emission wavelengths of 488 and 520 nm, respectively, and the ratio of GalNAc(6S) to ΔO was determined.

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Footnote

Treatment with bacterial lyases including CSases converts the original structures of internal uronic acid residues, β-D-GlcA and α-L-IdoA, in the GAGs into a common product, namely 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid (α-L-ΔHexA).

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Figure legends

Figure 1. Determination of the average molecular mass of SLI-CS by gel filtration.

The CS preparations (10 μg as GlcA) from SLI and squid cartilage (CS-E) were individually loaded onto a Superdex 200^{TM} column calibrated with molecular mass markers as described under "Experimental". Fractions were monitored with the dimethylmethylene blue (DMMB) dye, and the average molecular mass was estimated using the calibration curve (*inset*). The void volume (V_o) and total volume (V_t) were determined using dextran (average mass: 200 kDa) and NaCl, respectively. The *circles* and *squares* indicate the elution profiles of SLI-CS and CS-E, respectively. Note that the CS-E preparation is devoid of HS and keratan sulfate as demonstrated by digestion experiments carried out using either heparinases or keratanase II, respectively.

Figure 2. Anion-exchange HPLC of the digest of the SLI-CS preparation obtained with CSase ABC.

The SLI-CS preparation was digested with CSase ABC. After 2AB-labeling, the digest was analyzed by HPLC on an amine-bound silica PA-03 column using a liner gradient of NaH_2PO_4 as indicated by the *dashed line*. The peaks before 10 min were derived from 2AB-derivatizing reagents. Arrows indicate the elution positions of the mono- and disulfated GalNAc as well as CS disaccharides: a, GalNAc(6S); b, GalNAc(4S); c, GalNAc(4S,6S); 1, $\Delta^{4,5}$HexAα1–3GalNAc; 2, $\Delta^{4,5}$HexAα1–3GalNAc(6S); 3, $\Delta^{4,5}$HexAα1–3GalNAc(4S); 4, $\Delta^{4,5}$HexA(2S)α1–3GalNAc(6S); 5, $\Delta^{4,5}$HexA(2S)α1–3GalNAc(4S); 6, $\Delta^{4,5}$HexAα1–3GalNAc(4S,6S); 7, $\Delta^{4,5}$HexA(2S)α1–3GalNAc(4S,6S).

Figure 3. Comparison of the amount of GalNAc(6S) and ΔO in SLI-CS by FACE.

The SLI-CS preparation was digested with CSase ABC, and the digest was subjected to gel filtration on a column of Superdex peptide. The fraction containing the mono- and disulfated GalNAc and ΔO was derivatized with 2-aminoacridone (AMAC) and analyzed by FACE as shown in the right lane. The AMAC-derivatives of the authentic mono- and disaccharides, ΔO, GalNAc(4S), and GalNAc(6S), were electrophoresed in the left lane. Note that the band with an asterisk might be considered as the AMAC-derivative of GalNAc(4S,6S).

Figure 4. NOP activity of the SLI-CS preparation.

E16 mouse hippocampal cells (20,000 cells/cm^2) were grown for 24 h on substrate precoated with P-ORN (A) and subsequently with SLI-CS (B) or CS-E (C) as a positive control, fixed, and immunostained for microtubule-associated protein 2 and neurofilaments. Note that the neurons cultured on SLI-CS showed a tendency to elongate prominent long neurite(s). Panel D, one hundred randomly selected neurons were used to measure the mean length of the longest neurite. The neurite outgrowth-promoting activity of SLI-CS was almost completely eliminated by digestion either with CSase ABC or with CSase AC-I. The values obtained from two separate experiments are expressed as the mean ± S.E.

Figure 5. Binding of various growth factors to immobilized SLI-CS.

Various concentrations of PTN (A), MK (B), FGF-2 (C), FGF-18 (D), HGF/SF (E), $VEGF_{165}$ (F), or HB-EGF (G) were injected onto the surface of the SLI-CS immobilized sensor chip and the sensorgrams obtained were overlaid using BIAevaluation software (version 3.1). *RU*, resonance units.

Table 1. Disaccharide composition of the SLI-CS preparation

The SLI-CS preparation was digested with CSase ABC. The digest was labeled with a fluorophore 2AB and analyzed by anion-exchange HPLC as described under "Experimental."

Monosaccharides and unsaturated disaccharides	SLI-CS
	CSase ABC
	pmole/μg acetone powder (mol %)
GalNAc(4S)[a]	0.14 (5.0)
GalNAc(4S,6S)[a]	0.08 (2.8)
ΔDi-0S[b] / GalNAc(6S)[a]	0.39 (13.9)
ΔDi-6S	0.42 (14.9)
ΔDi-4S	1.18 (41.8)
ΔDi-diS_D	ND[c]
ΔDi-diS_B	ND
ΔDi-diS_E	0.61 (21.6)
ΔDi-triS	ND
Total	2.82 (100)
Molar ratio of sulfate to disaccharide units	1.18

[a]Note that mono- or disulfated GalNAc was essentially derived from an internal disaccharide unit containing a GlcA(3S) residue, which is degraded by digestion with CSase ABC (24).

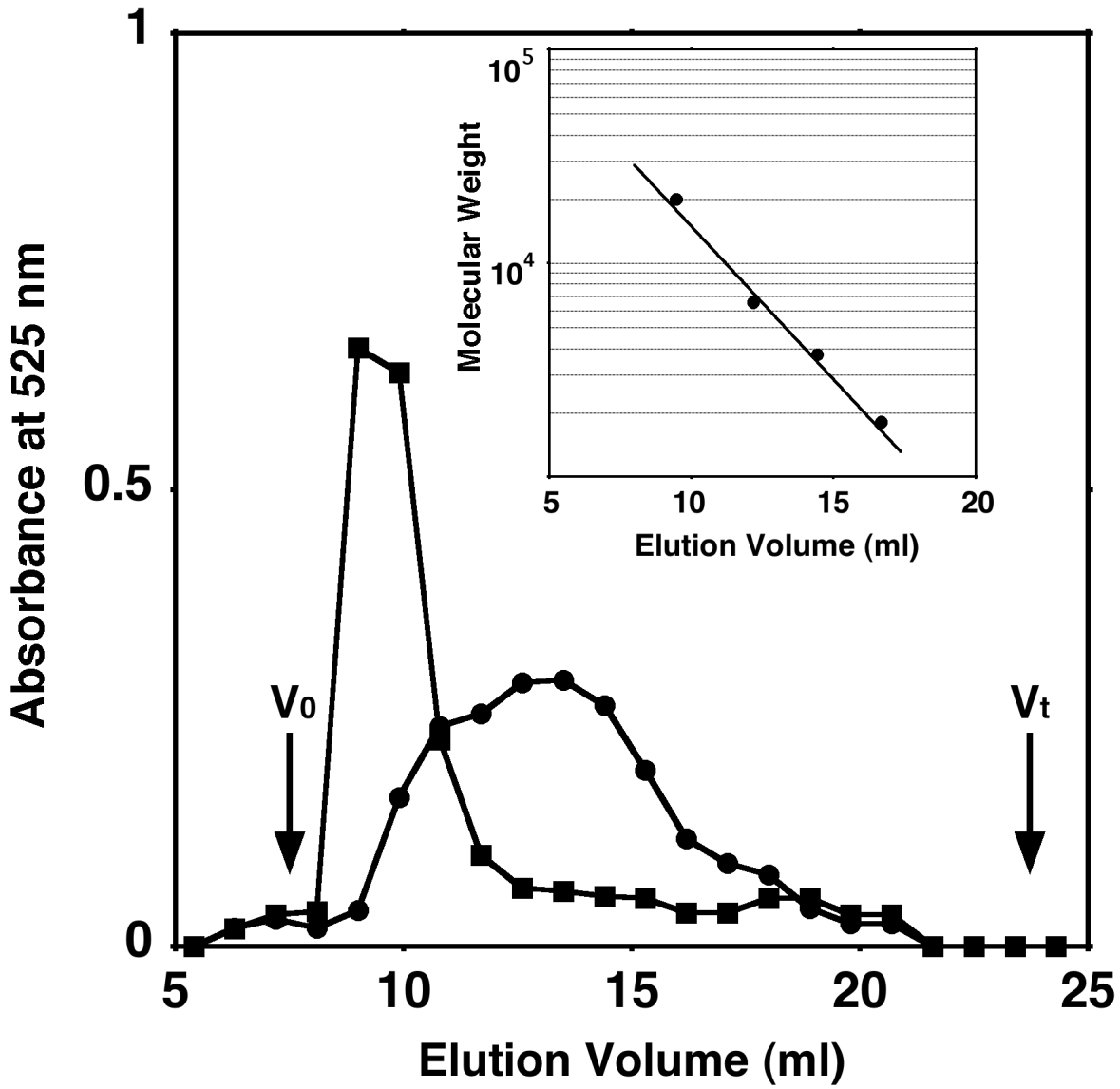
[b]ΔO and GalNAc(6S) could not be separated by HPLC under the conditions used. The ratio of ΔDi-0S to GalNAc(6S) was determined to be 1 : 1.2 by a FACE analysis. Thus, the proportion (mol%) of ΔDi-0S and GalNAc(6S) was calculated to be 6.3 and 7.6%, respectively, among the 13.9%.

[c]ND, Not detected.

Table 2. Kinetic parameters for the interaction of growth factors with immobilized SLI-CS

The k_a, k_d, and K_d values were calculated with BIAevaluation 3.1 using a 1:1 Langmuir binding model with mass transfer as described under "Experimental." The values are expressed as the mean ± S.D.

Growth Factors	k_a	k_d	K_d
	$M^{-1}s^{-1}$	s^{-1}	nM
PTN	$(5.38 \pm 0.28) \times 10^5$	$(3.31 \pm 0.11) \times 10^{-4}$	0.6 ± 0.1
MK	$(8.48 \pm 0.26) \times 10^5$	$(3.38 \pm 0.006) \times 10^{-3}$	4.0 ± 0.01
FGF-2	$(1.49 \pm 0.10) \times 10^5$	$(1.04 \pm 0.31) \times 10^{-3}$	7.0 ± 2.7
FGF-18	$(1.34 \pm 0.17) \times 10^5$	$(7.28 \pm 0.30) \times 10^{-4}$	5.4 ± 1.0
HGF/SF	$(6.25 \pm 0.62) \times 10^5$	$(1.72 \pm 0.14) \times 10^{-3}$	2.8 ± 0.1
$VEGF_{165}$	$(8.79 \pm 3.72) \times 10^4$	$(6.39 \pm 0.21) \times 10^{-3}$	73 ± 57
HB-EGF	$(7.10 \pm 2.97) \times 10^4$	$(2.02 \pm 0.06) \times 10^{-2}$	285 ± 219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Vt
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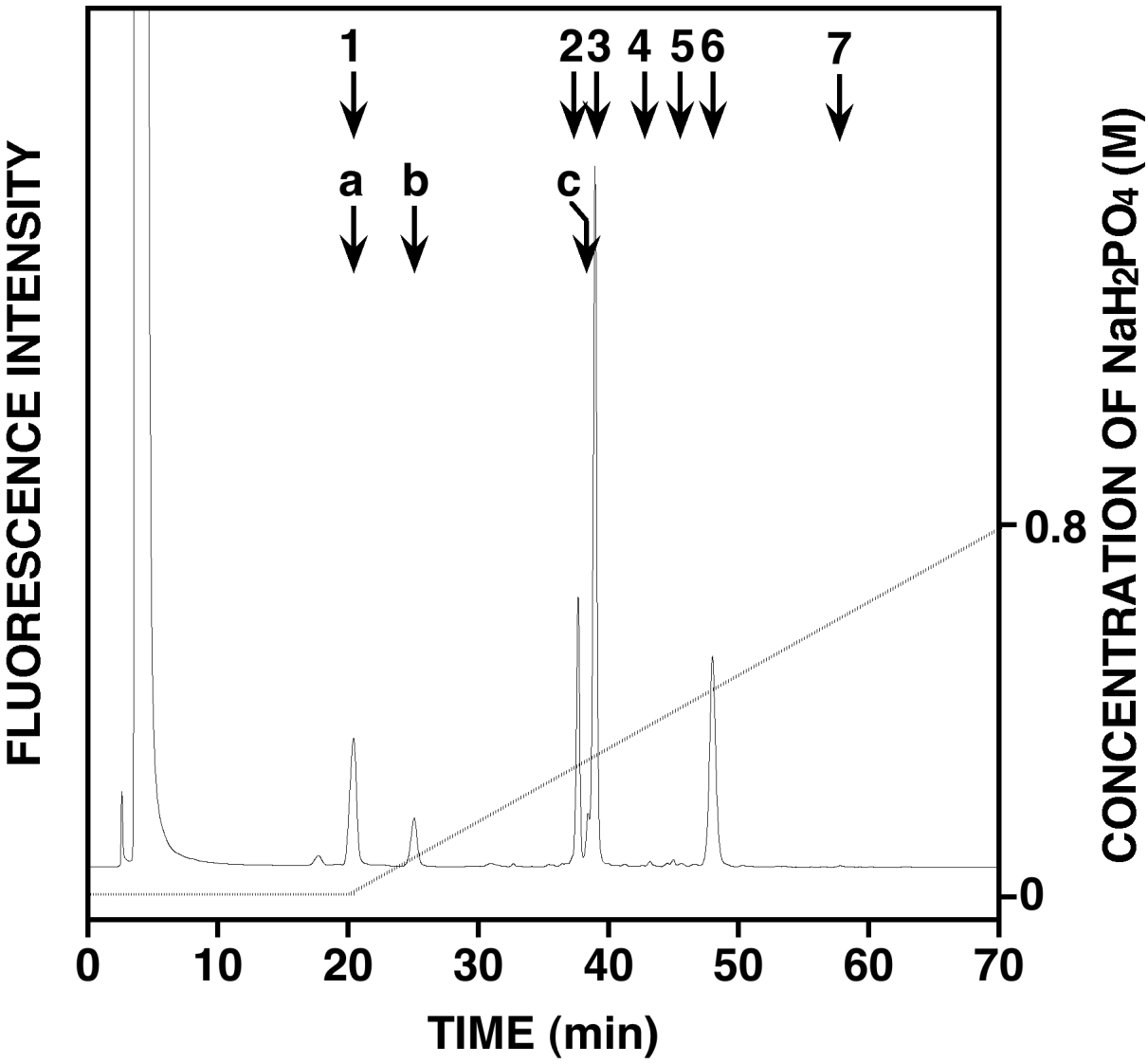
Elution Volume (ml)


1
2
3
4
5
6
7
a
b
c
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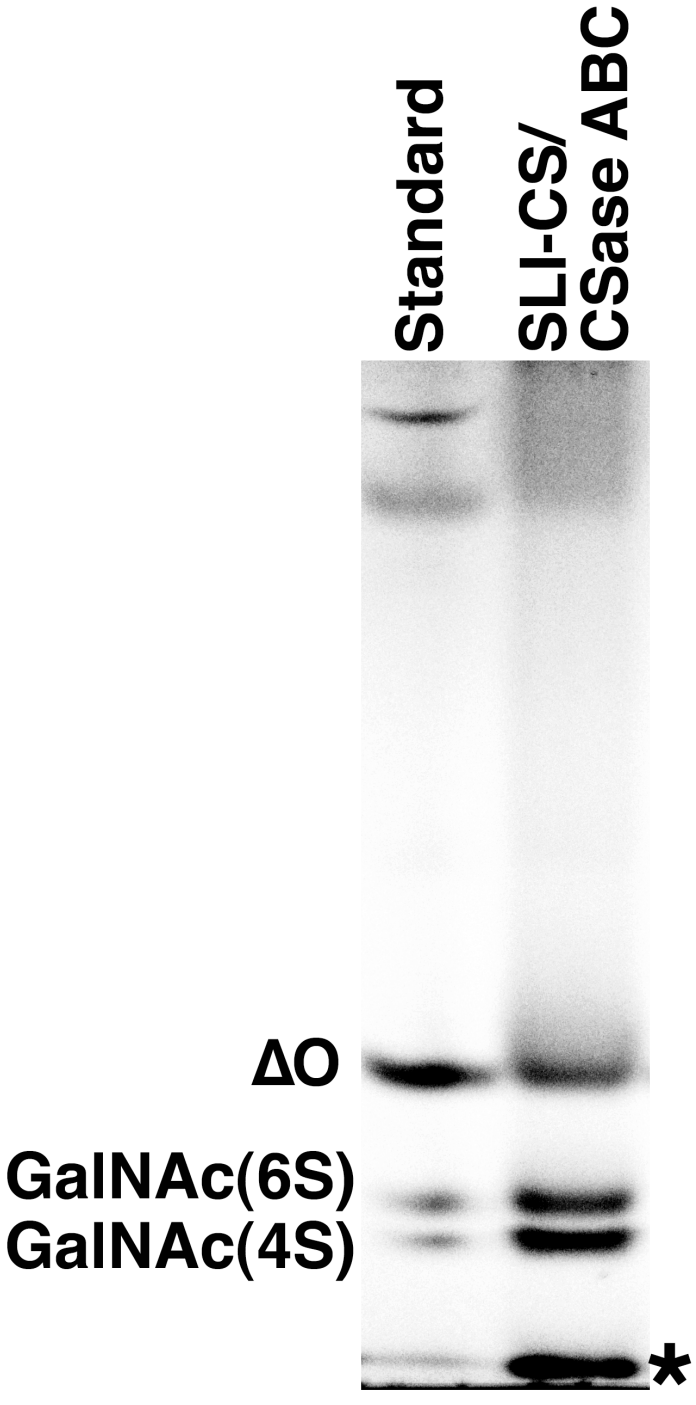
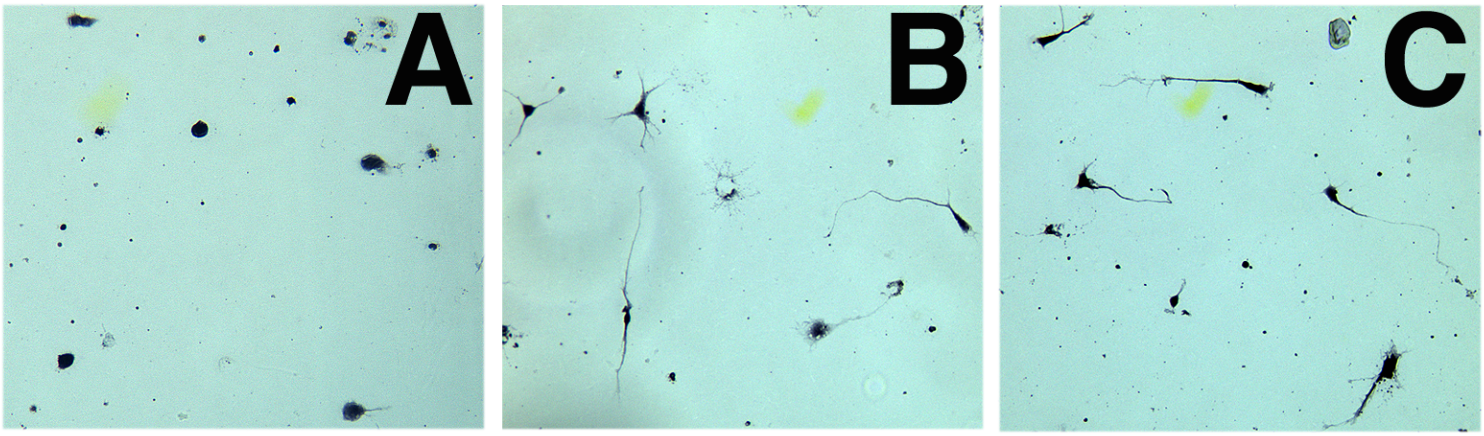
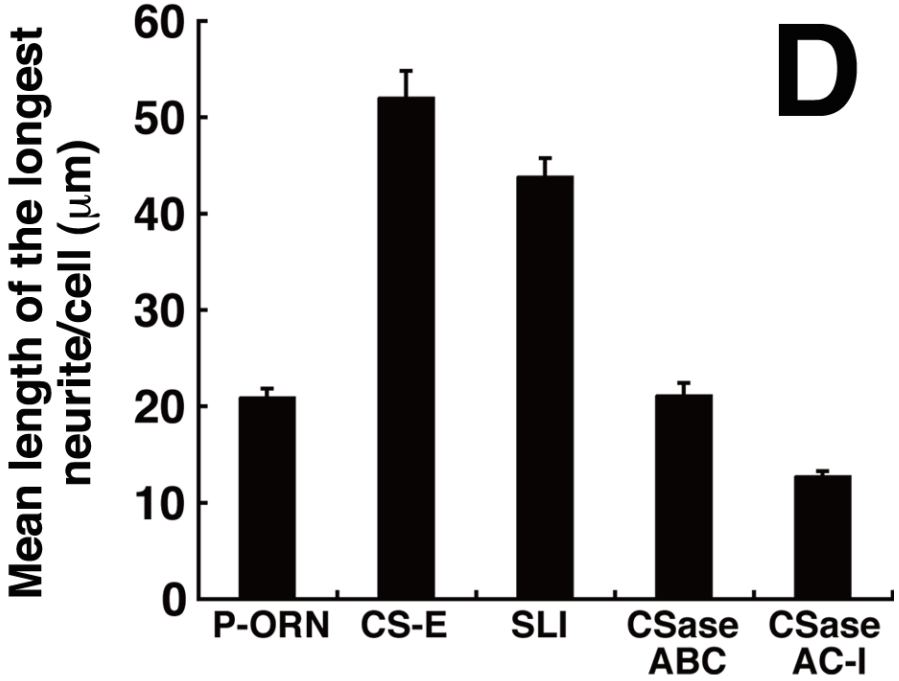
TIME (min)


Standard
SLI-CS/
CSase ABC
ΔO
GalNAc(6S)
GalNAc(4S)
*


A
B
C

100 μm


D
Mean length of the longest neurite/cell (μm)
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40
30
20
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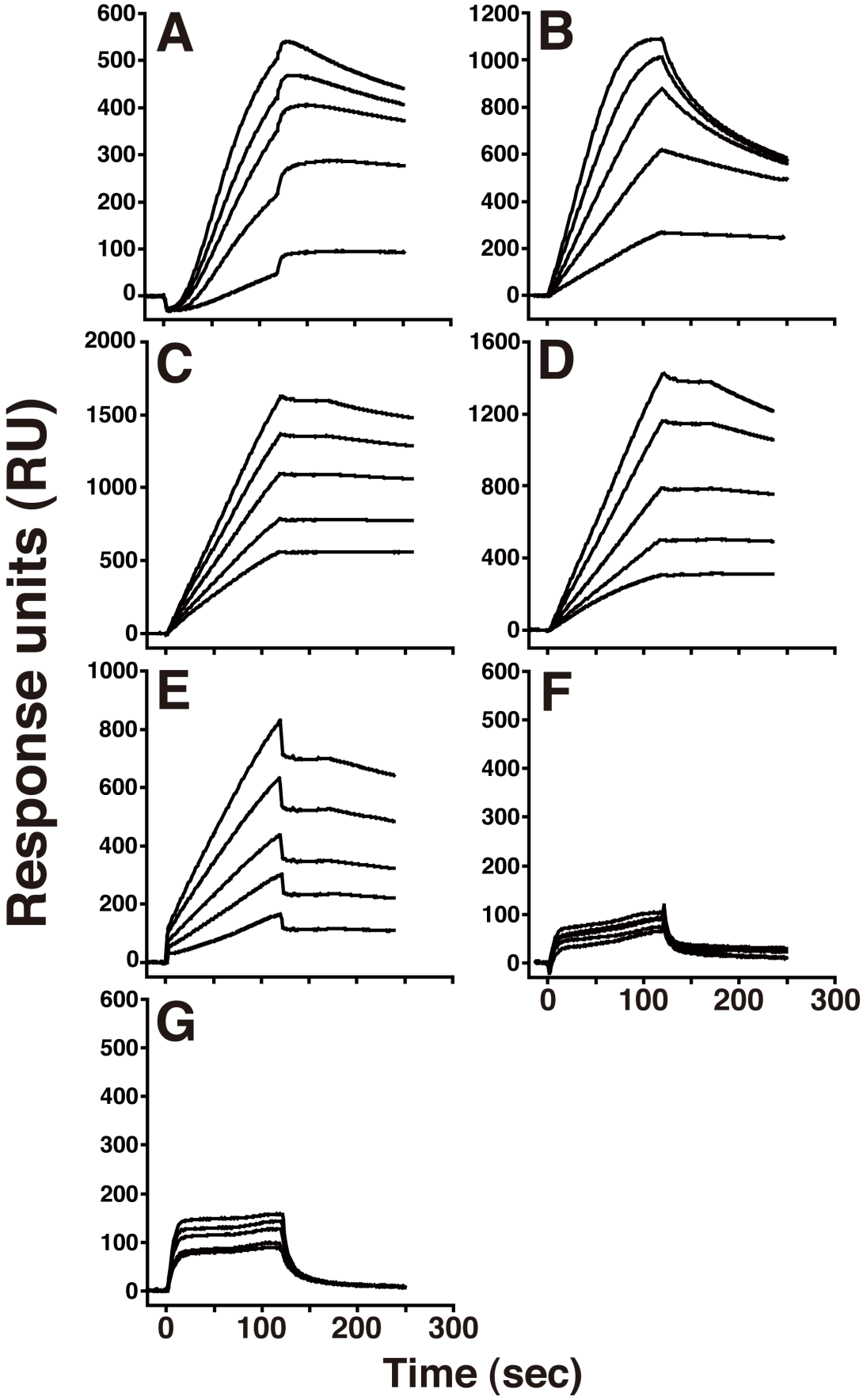
0
P-ORN
CS-E
SLI
CSase ABC
CSase AC-I


A
B
C
D
E
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G
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2000
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