Chondroitin sulfate of appican, the proteoglycan form of amyloid precursor protein, produced by C6 glioma cells interacts with heparin-binding neuroregulatory factors

Yuko Umehara[a], Shuhei Yamada[a], Shuji Nishimura[a], Junichi Shioi[b], Nikolaos K. Robakis[b], Kazuyuki Sugahara[a,*]

[a] *Department of Biochemistry, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan*
[b] *Department of Psychiatry and Fishberg Research Center for Neurobiology, Mount Sinai School of Medicine, New York, NY 10029, USA*

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Abstract Appican produced by rat C6 glioma cells, the chondroitin sulfate (CS) proteoglycan form of the amyloid precursor protein, contains an E disaccharide, –GlcUA-GalNAc(4,6-*O*-disulfate)–, in its CS chain. In this study, the appican CS chain from rat C6 glioma cells was shown to specifically bind several growth/differentiation factors including midkine (MK) and pleiotrophin (PTN). In contrast, the appican CS from SH-SY5Y neuroblastoma cells contained no E disaccharide and showed no binding to either MK or PTN. These findings indicate that the E motif is essential in the interaction of the appican CS chain with growth/differentiation factors, and suggest that glial appican may mediate the regulation of neuronal cell adhesion and migration and/or neurite outgrowth.

Key words: Alzheimer's disease; Amyloid precursor protein; Appican; Chondroitin sulfate; Heparin-binding growth factor

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder of the brain, characterized by neuritic plaques and neurofibrillary tangles. The amyloid β (Aβ) peptide, found in the brain amyloid depositions of AD patients, is generated by the proteolytic processing of the amyloid precursor protein (AβPP) [1]. Specific AβPP mutations are associated with familial AD, although it is not clear how AβPP mutations precipitate the AD phenotypes [2]. The AβPP gene consists of 19 exons and alternative splicing of these exons results in the production of several AβPP isoforms [3–6]. An AβPP isoform lacking exon 15 is the protein component of appican, the chondroitin sulfate (CS) proteoglycan (PG) form of AβPP [7]. Although the biological functions of AβPP are not well defined, they have been implicated in cell adhesion and neurite outgrowth promotion [8–10]. Recently we reported that the CS chain of appican produced by rat C6 glioma cells contains the oversulfated E disaccharide motif in significant proportion (14.3%) [11], and may thereby be involved in the biological activities of AβPP.

CS glycosaminoglycans (GAGs) are complex sulfated polysaccharides with a backbone structure composed of a repeating disaccharide building unit of –4GlcUAβ1-3GalNAcβ1–. This simple repeat structure undergoes extensive modification by sulfation at the C2 or C3 position of D-glucuronic acid (GlcUA) residues and/or the C4 or C6 position of *N*-acetyl-D-galactosamine (GalNAc) residues during biosynthesis [12]. Diversity in sulfation results in structural variability of CS chains, which is the basis for their functional diversity. Oversulfated CS variants CS-D and CS-E promote neurite outgrowth [13–15], and are characterized by peculiar disulfated disaccharide motifs including GlcUA(2-*O*-sulfate)β1-4GalNAc(6-*O*-sulfate), or GlcUAβ1-3GalNAc(4,6-*O*-disulfate) (E motif), in CS-D or CS-E, respectively. Recently Deepa et al. [16] demonstrated that squid cartilage CS-E rich in the E motif bound specifically and with high affinity to heparin-binding growth/differentiation factors such as midkine (MK), pleiotrophin (PTN), heparin-binding epidermal growth factor-like growth factor (HB-EGF) and several fibroblast growth factors (FGFs). Zou et al. [17] showed that versican, a CS-PG with a high content of the E disaccharide units, is the MK-binding component in embryonic mouse brains. In view of these findings, we compared the CS chains of appicans produced by rat C6 glioma cells and SH-SY5Y neuroblastoma cells in terms of their structure and growth factor-binding capacities to investigate whether appican with the E unit specifically binds heparin-binding growth/differentiation factors.

2. Materials and methods

2.1. Materials

Appican from rat C6 glioma cells was prepared as described previously [11]. To improve the isolation of appican, we constructed a recombinant cDNA which contains codons for six successive histidines inserted after the signal sequence of the L-AβPP isoform (named after an original finding in leukocytes). This recombinant cDNA was cloned into the pcDNA3 expression vector. His-tagged L-AβPP was expressed in stable transfectants of human neuroblastoma SH-SY5Y cells and rat glioma C6 cells. The His-tagged L-AβPP was processed

*Corresponding author. Fax: (81)-78-441 7571; URL: http://www/kobepharma-u.ac.jp/~biochem/.
E-mail address: k-sugar@kobepharma-u.ac.jp (K. Sugahara).

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β; AβPP, amyloid precursor protein; CS, chondroitin sulfate; GalNAc, *N*-acetyl-D-galactosamine; GlcUA, D-glucuronic acid; GAG, glycosaminoglycan; HPLC, high performance liquid chromatography; HS, heparan sulfate; ΔHexUA, 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid; MK, midkine; PTN, pleiotrophin; bFGF, basic fibroblast growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; PG, proteoglycan

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normally and secreted as soluble L-AβPP and appican. The conditioned medium from the transfectant culture was collected and concentrated by ultrafiltration using a disk membrane of 50 000 molecular weight cut-off. The concentrate was loaded on a nickel column and the column was washed with 20 mM imidazole. The bound His-tagged protein was eluted with 100 mM imidazole and the eluate was dialyzed and concentrated. Silver staining showed more than 95% purity of AβPP consisting of both appican and the non-PG form of L-AβPP. Appican was further separated from L-AβPP using an ion exchange column. More than 100 μg of appican was obtained from 2 l of conditioned medium from SH-SY5Y transfectants cultured with retinoic acid.

Chondroitinase ABC (EC 4.2.2.4), *Flavobacterium* heparinase (EC 4.2.2.7), heparitinase (EC 4.2.2.8), and CS variant preparations (super special grade) were purchased from Seikagaku (Tokyo, Japan). Human recombinant MK and basic FGF (bFGF) were from PeproTech EC (London, UK), and Genzyme-Techne (Minneapolis, MN, USA), respectively. Human recombinant PTN and HB-EGF were obtained from R&D Systems (Minneapolis, MN, USA). NaB^3H_4 (555 GBq/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, USA).

2.2. Preparation of ^{3}H-labeled CS-GAGs of appicans from C6 glioma cells and SH-SY5Y neuroblastoma cells

Purified appican (40 μg) from rat C6 glioma cells was treated with LiOH to liberate *O*-linked saccharides as described previously [18,19]. The sample was then treated with 100 μl of 3.3 mM NaB^3H_4 (555 GBq/mmol)/0.1 M NaOH at room temperature overnight. The appican preparation (30 μg) purified from differentiated neuroblastoma cells was directly treated with 30 μl of 1.3 mM NaB^3H_4 (555 GBq/mmol)/0.15 M NaOH. Non-radiolabeled 1 M $NaBH_4$/0.1 M NaOH was added to each sample and the mixture was incubated at room temperature for 2 h to complete the reaction. The reaction was terminated by adding glacial acetic acid to decompose excess $NaBH_4$. Non-labeled chondroitin (100 μg) was added as a carrier to the mixture, and the ^{3}H-labeled materials were purified by gel filtration [20]. One half of the ^{3}H-labeled polysaccharides purified by Superdex 200 chromatography (1.2×10^5 or 7.4×10^4 dpm derived from C6 glioma or SH-SY5Y neuroblastoma appican, respectively) was exhaustively digested with a mixture of heparinase and heparitinase [20] to remove heparan sulfate (HS) oligosaccharides. An aliquot of each sample (4000 dpm) was digested with chondroitinase ABC [20]. The digests were analyzed by gel filtration chromatography on a column of Superdex 200 using 0.25 M NH_4HCO_3/7% 1-propanol as the eluent at a flow rate of 0.4 ml/min.

2.3. Disaccharide composition analysis of CS chains of SH-SY5Y neuroblastoma appican

The CS disaccharide composition of SH-SY5Y neuroblastoma appican was determined by enzymatic digestion with chondroitinase ABC as reported [21].

2.4. Filter binding assay

Various amounts (0.15–0.5 μg) of growth/differentiation factors were incubated with [^{3}H]CS from appican (4000 dpm) in a total volume of 50 μl of 50 mM Tris–HCl (pH 7.3) containing 130 mM NaCl and 0.5 mg/ml bovine serum albumin (BSA) at room temperature for 3 h. Proteins with any associated [^{3}H]CS were collected on nitrocellulose filters [22], which were monitored by liquid scintillation counting.

2.5. Biotinylation of appican for immobilization

Rat C6 glioma appican was labeled with biotin by reaction of its free amino groups of the core protein. Twenty μg of appican was dissolved in 12 μl of phosphate-buffered saline (PBS) containing 20 μg of EZ-Link™Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) and incubated at room temperature for 30 min. The biotinylated appican was purified using Centricon 30 (Millipore).

2.6. Biosensor analysis of the binding of neuroregulatory factors to appican

Biosensor analysis was performed using an IAsys instrument (Labsystems, Cambridge, UK). Biotinylated appican was immobilized on the sensor chip surface using an IAsys biotin cuvette. The biotin surface of the cuvette was washed with PBS/Tween-20, and streptavidin was added at a concentration of 100 μg/ml. After streptavidin was captured on the surface, BSA was added for blocking. Biotinylated appican (4 μg as appican) was then added to the streptavidin-captured surface. The appican-immobilized cuvette was used in subsequent experiments to examine interactions of appican with PTN and MK. Growth factors were added at the indicated concentrations using PBS/Tween-20, and the association was monitored. In the dissociation phase, proteins were released with PBS/Tween-20, and the dissociation of the binding was monitored. The appican surface was regenerated by the addition of 1 M NaCl. In competition experiments, various CS variants were preincubated with proteins in PBS/Tween-20 at room temperature for 5 min, and then the mixture was added to the cuvette.

3. Results

The biological functions of the CS chain attached to AβPP are mostly unknown. The CS chain of AβPP produced by rat C6 glioma cells contains the oversulfated E disaccharide unit, GlcUAβ1-4GalNAc(4,6-*O*-disulfate), in significant proportion (14%) [11]. Squid cartilage CS, which contains the E unit in high proportion (62%), has neurite outgrowth-promoting activity [13,15] and interacts with various heparin-binding growth factors including neuroregulatory factors such as MK and PTN [16,23]. Hence, the neuroregulatory factors' binding ability of the CS chain of appican produced by rat C6 glioma cells was evaluated to better understand the functions of appican in the brain.

3.1. Preparation of a ^{3}H-labeled CS-GAG fraction from rat C6 glioma appican

A ^{3}H-labeled CS-GAG fraction of appican from rat C6 glioma cells was prepared as follows. Purified appican was subjected to alkaline NaB^3H_4 reduction to liberate *O*-glycans from the core protein and to radiolabel the reducing ends of the liberated saccharides. A CS-containing fraction, recovered by gel filtration on a Superdex 200 column (Fig. 1A), was digested with a mixture of heparinase and heparitinase, and the digest was subjected to gel filtration chromatography to remove the degradation products (Fig. 1B). Approximately 18% of the radiolabeled materials were susceptible to HS lyases, suggesting that this preparation contained HS-GAG chains. It remains unclear whether these HS chains were attached to the appican core protein or derived from co-purified HS-PGs. More than 90% of the radiolabeled materials in the HS lyase-resistant peak was digested by chondroitinase ABC as shown by gel filtration chromatography (Fig. 1C), indicating that the [^{3}H]CS fraction was at least 90% pure.

3.2. Demonstration of a direct interaction of the appican CS purified from C6 glioma cells with heparin-binding growth factors

Purified [^{3}H]CS chain fractions were subjected to the nitrocellulose filter binding assay to examine whether appican CS can interact with several heparin-binding growth factors, MK, PTN, HB-EGF, and bFGF, all of which are expressed in mammalian brains and interact specifically with squid cartilage CS-E [16], despite the fact that they belong to three different gene families. All the heparin-binding growth factors tested bound to the [^{3}H]CS preparation from appican in a concentration-dependent manner (Fig. 2), although saturation of the binding could not be tested due to the limited availability of the commercial growth factors of high costs. The binding of these growth factors to [^{3}H]CS was abolished by chondroitinase ABC digestion (data not shown), confirming that these interactions are dependent on the CS chain.

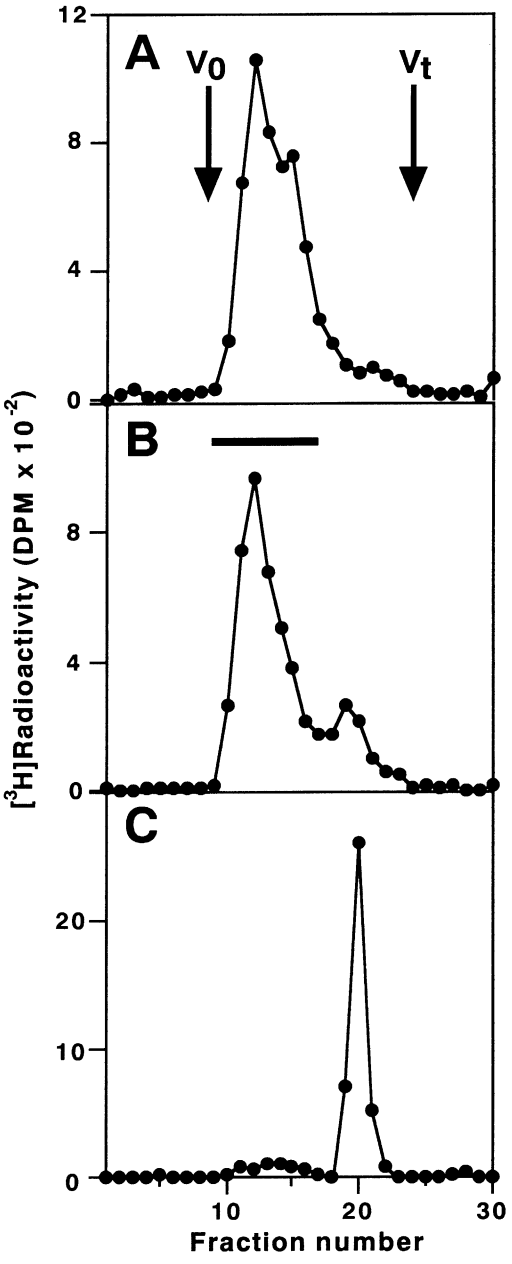

Fig. 1. Preparation of [^{3}H]CS chains from C6 glioma appican. The rat C6 glioma appican preparation was treated with alkaline NaB^{3}H$_4$ and the ^{3}H-labeled CS chains were recovered by gel filtration chromatography as described in Section 2. A: An aliquot (7500 dpm) of the purified fraction was analyzed by gel filtration. B: Another aliquot (1.2×10^5 dpm) was digested with a mixture of heparinase and heparitinase. Approximately one twentieth of each fraction was used for monitoring by liquid scintillation counting. The flow-through fractions, which were resistant to HS lyases and are indicated by the bar (9.8×10^4 dpm), were pooled as [^{3}H]CS chains from appican. C: An aliquot (5000 dpm) of the [^{3}H]CS fraction obtained in B was digested with chondroitinase ABC. At least 90% of the preparation was susceptible to the enzyme.

We also examined the interactions of the growth factors with the appican CS-PG purified from C6 glioma cells using an IAsys resonant mirror optical biosensor. In this binding assay, appican was biotinylated and immobilized on the sensor chip surface coated with streptavidin. MK or PTN was added at a concentration of 1 μg/ml to the immobilized appican, and the time-dependent response (in arc seconds) was recorded. The sensorgrams in an association phase are shown in Fig. 3A,B. Response of 30 and 40 arc seconds were observed for MK and PTN, respectively, demonstrating that both neuroregulatory factors bound to the immobilized appican.

To investigate the specificity of the MK and PTN binding to appican CS-PG, inhibition studies were performed using various kinds of CS variants. When each CS variant was added to the appican surface, no interaction was detected between the appican surface and the CS variant (data not shown). The findings from the inhibition studies are illustrated in Fig. 3C,D. Only the CS-E preparation strongly prevented the binding of both MK and PTN to appican (Fig. 3). Other CS variants exhibited no significant inhibition at 0.5 μg/ml. CS-B showed weak inhibition of the MK binding (44% inhibition) when used at high concentration (1.5 μg/ml) (Fig. 3D). The results suggest that the E disaccharide motif of the CS chain is essential for the binding of MK and PTN to appican CS-PG.

3.3. *Comparison of the disaccharide composition of the CS chain of appican from C6 glioma cells and neuroblastoma cells*

Appican is expressed almost exclusively by glial cells in vivo [24,25], and promotes neuronal cell adhesion and neurite outgrowth [26,27]. To investigate whether the E disaccharide mo-

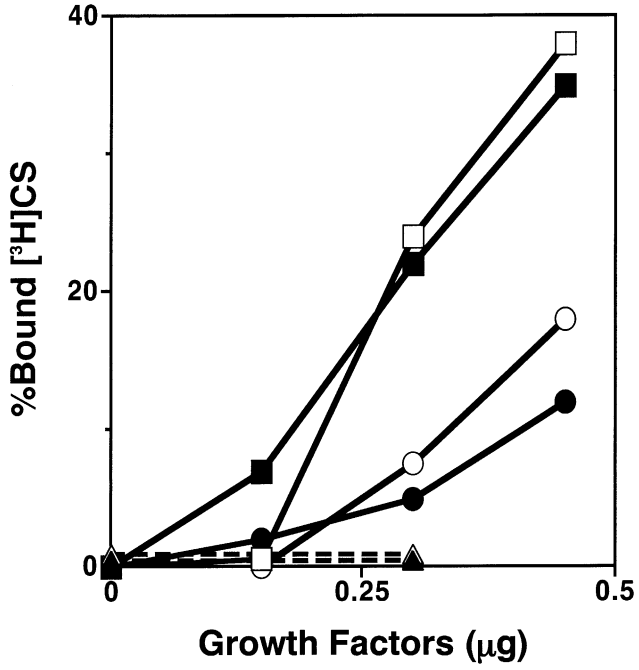

Fig. 2. Demonstration of direct binding of the [^{3}H]CS chains from C6 glioma appican to various heparin-binding growth factors using filter binding assays. The [^{3}H]CS chains from C6 glioma appican (4000 dpm corresponding to 0.82 μg of appican CS-PG) (solid lines) were incubated individually with various amounts of heparin-binding growth factors, MK (open circles), PTN (open squares), HB-EGF (closed circles), or bFGF (closed squares). The [^{3}H]CS chains from SH-SY5Y neuroblastoma appican (4000 dpm corresponding to 0.81 μg of appican CS-PG) (broken lines) were also incubated with 0.3 μg of MK (open triangles) or PTN (closed triangles). The binding to growth factors was quantified by filter binding assays as described in Section 2. Values are expressed as percentages of the radioactivity added to the samples incubated.

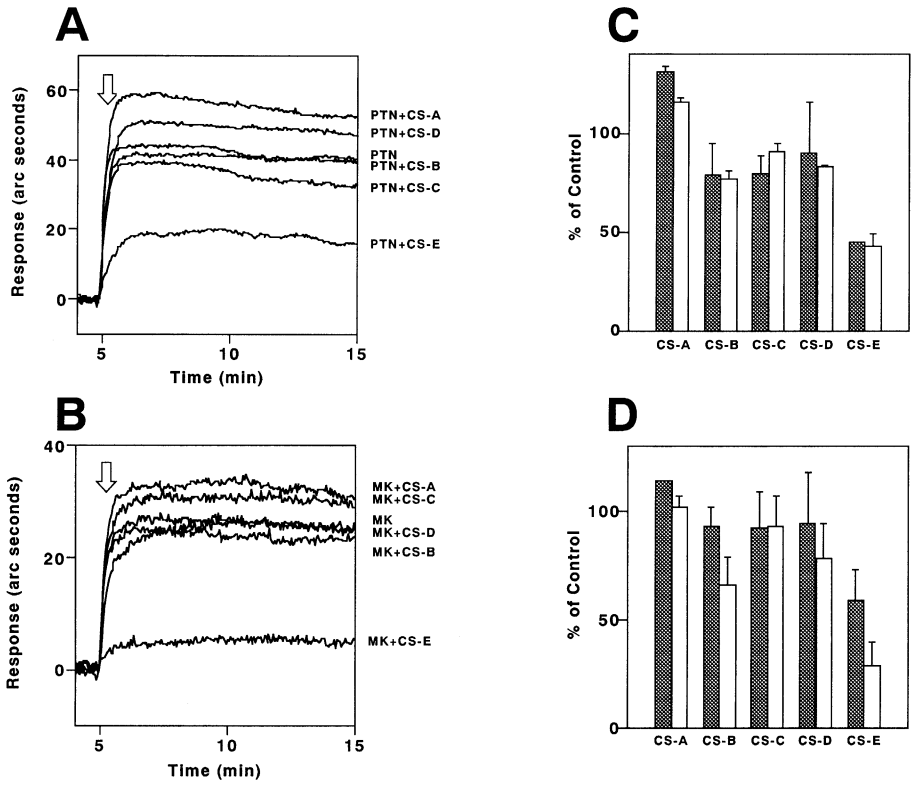

Fig. 3. Specificity analysis of the binding of PTN and MK to the CS-PG appican-immobilized surface in the biosensor IAsys. A,B: Binding of recombinant PTN (1 μg/ml) (A) and MK (1 μg/ml) (B) to the immobilized CS-PG appican purified from C6 glioma was analyzed by IAsys in the presence or absence of various CS variants (0.5 μg/ml), and the overlay sensorgrams are shown. At the position indicated by the open arrow in each panel, the recombinant growth factor was added to the cuvette. C,D: Inhibition of the binding of PTN (C) or MK (D) to the appican-coated surface by various CS variants was analyzed. Recombinant growth factors were preincubated with 0.5 μg/ml (shaded columns) or 1.5 μg/ml (open columns) of various CS variants, and each mixture was then added to the appican-immobilized cuvette. The binding of the growth factors to the sensor surface was quantified based on the response (arc seconds). Values are the averages ± range from two separate experiments and are expressed as percentages of the response without inhibitors.

tif is specific to the appican CS expressed in glioma cells, the disaccharide composition of the CS chain of appican produced by C6 glioma cells and neuroblastoma cells was compared. The disaccharide composition of the former was previously analyzed [11] and that of neuroblastoma appican CS was carried out in this study by digestion with chondroitinase ABC, followed by derivatization with a fluorophore 2-aminobenzamide and anion exchange high performance liquid chromatography (HPLC) (Table 1). The predominant component was ΔHexUAα1-3GalNAc(4-*O*-sulfate) accounting for 77.5% of the total CS. However, in strong contrast to the C6 glioma appican CS, the disaccharide E motif was not detected in the SH-SY5Y neuroblastoma appican CS chain, suggesting that the E disaccharide motif is characteristic for the CS from the C6 glioma cell-derived appican but not for the CS from the SH-SY5Y neuroblastoma cell-derived appican.

To evaluate the importance of the E disaccharide motif for the neuroregulatory factor binding, the CS chain preparation of appican expressed in SH-SY5Y neuroblastoma cells was subjected to the filter binding assay. Neither PTN nor MK exhibited appreciable binding to [^{3}H]CS from SH-SY5Y neuroblastoma appican (Fig. 2). These results altogether indicate that the E disaccharide motif in the appican CS chain is essential in the interaction with MK and PTN.

Table 1
Disaccharide composition of appican CS from C6 glioma and SH-SY5Y neuroblastoma cells

2-Aminobenzamide-labeled disaccharides	Proportion (%)	
	Glioma[a]	Neuroblastoma
ΔHexUAα1-3GalNAc	1.2	ND[b]
ΔHexUAα1-3GalNAc(4-*O*-sulfate)	81.2	77.5
ΔHexUAα1-3GalNAc(6-*O*-sulfate)	3.3	22.5
ΔHexUA(2-*O*-sulfate)α1-3GalNAc(6-*O*-sulfate)	ND	ND
ΔHexUAα1-3GalNAc(4,6-*O*-disulfate)	14.3	ND
ΔHexUA(2-*O*-sulfate)α1-3GalNAc(4,6-*O*-disulfate)	ND	ND

The purified appican from SH-SY5Y neuroblastoma cells was digested with chondroitinase ABC. The digest was derivatized with 2-aminobenzamide [21], and the 2-aminobenzamide-derivatized oligosaccharides were analyzed by HPLC as described in Section 2.
[a]The CS disaccharide composition of C6 glioma appican was taken from the data in [11].
[b]ND, not detected.

4. Discussion

A number of growth/differentiation factors have been postulated to be involved in brain development [28]. In this study, we show that the CS chain of appican, the CS-PG form of AβPP produced by C6 glioma cells, interacts with various heparin-binding growth factors, including MK, PTN, bFGF, and HB-EGF. These data show that CS-GAG chains from central nervous system-derived cells are able to bind growth factors. Recently Zou et al. [17] isolated CS/DS-GAG chains with MK-binding capacity from day 13 embryonic mouse brain using a MK affinity column and found a progressive increase in the E disaccharide unit in the higher affinity fractions, which is consistent with the present findings. Maeda et al. [29] showed that PTN binds to the transmembrane signal-transducing receptor-type protein tyrosine phosphatase 6B4-PG/RPTPζ, a major CS-PG in the brain, and that the CS side chains along with the core protein constitute the binding site. MK and PTN have neuroregulatory activities including stimulation of neuronal cell adhesion [23] and promotion of neurite outgrowth [30]. HB-EGF is found in cerebral neurons and its expression is increased after hypoxic or ischemic injury [31]. bFGF is an important growth factor that influences cell morphology, migration [32] and neural differentiation [33]. We recently reported that CS-E derived from squid cartilage specifically interacts with multiple heparin-binding growth factors including those used in this study [16], suggesting that these proteins interact not only with HS-PG but also with CS-PG on the cell surface. In fact, PTN binds to the cell surface receptor 6B4-PG/RPTPζ through the receptor's CS chains, and the binding is inhibited by CS-B, CS-C, CS-D and CS-E [29].

In contrast to the CS chain of the C6 glioma appican, the CS chain from SH-SY5Y neuroblastoma appican, which lacks the E motif, failed to bind these factors, suggesting that the E motif is necessary for the interaction between appican and the growth factors. It will be interesting to further investigate whether the structural and functional differences in the CS chains of appicans from these two different sources represent glioma cell-specific or cell type-dependent characteristics using multiple glioma and neuroblastoma cell lines.

Based on the data of molecular size and disaccharide composition of the C6 glioma appican CS [11], the number of E units in a single appican CS chain is calculated to be 7–14. These E units could be arranged in clusters forming consecutive sequences such as E-E or E-E-E similar to those reported by Kawashima et al. [34] for the squid cartilage CS-E-derived tetrasaccharide sequence, which interacts directly with L- and P-selectin as well as chemokines. Alternatively, characteristic sequences including scattered E units may serve as functional domain structures recognized by protein ligands.

Salinero et al. [27] purified appican PGs from human control and AD brains and verified their stimulatory effects on the neurite extension of hippocampal neurons. Since the AβPP core protein is much less potent in promoting neurite outgrowth than appican [27], the CS chain of appican is assumed to be mainly responsible for the activity. Compared with appican from control brains, appican from AD brains has higher neurite outgrowth activity [27], indicating that fine sulfation structures of the appican CS chain may differ between control and AD brains. A proportion of the E disaccharide motif would influence the biological activity of appican in pathological situations. An augmentation of appican in the AD brain [27] may represent a positive response aiming at recovering or protecting damaged brains.

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