

Appican, the Proteoglycan Form of the Amyloid Precursor Protein, Contains Chondroitin Sulfate E in the Repeating Disaccharide Region and 4-*O*-Sulfated Galactose in the Linkage Region*

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Chondroitin sulfate (CS)-D and CS-E, which are characterized by oversulfated disaccharide units, have been shown to regulate neuronal adhesion, cell migration, and neurite outgrowth. CS proteoglycans (CSPGs) consist of a core protein to which one or more CS chains are attached via a serine residue. Although several brain CSPGs, including mouse DSD-1-PG/phosphacan, have been found to contain the oversulfated D disaccharide motif, no brain CSPG has been reported to contain the oversulfated E motif. Here we analyzed the CS chain of appican, the CSPG form of the Alzheimer's amyloid precursor protein. Appican is expressed almost exclusively by astrocytes and has been reported to have brain- and astrocyte-specific functions including stimulation of both neural cell adhesion and neurite outgrowth. The present findings show that the CS chain of appican has a molecular mass of 25–50 kDa. This chain contains a significant fraction (14.3%) of the oversulfated E motif GlcUA β 1–3GalNAc(4,6-*O*-disulfate). The rest of the chain consists of GlcUA β 1–3GalNAc(4-*O*-sulfate) (81.2%) and minor fractions of GlcUA β 1–3GalNAc and GlcUA β 1–3GalNAc(6-*O*-sulfate). We also show that the CS chain of appican contains in its linkage region the 4-*O*-sulfated Gal structure. Thus, appican is the first example of a specific brain CSPG that contains the E disaccharide unit in its sugar backbone and the 4-*O*-sulfated Gal residue in its linkage region. The presence of the E unit is consistent with and may explain the neurotrophic activities of appican.

contains Alzheimer's amyloid precursor protein (APP) as a core protein (1–3). The core protein of appican belongs to a group of APP isoforms, termed L-APP, that lacks the peptide sequence corresponding to exon 15 (4). Fusion of exon 14 to exon 16 creates a consensus amino acid sequence containing the specific serine residue for the attachment of the glycosaminoglycan (GAG) chain (4). L-APPs are “part-time PGs,” because they occur in both PG (appican) and non-PG forms. Appican is found in human and rat brains and in primary cultures of rat astrocytes (5). No appican is detected in primary neuronal cultures or in other primary glial cell cultures, although these cultures produce high levels of APP (3). It has been reported that appican promotes adhesion of primary astrocytes and neural cell lines including rat glioblastoma C6, mouse neuroblastoma N2a, and rat pheochromocytoma PC12 cells but fails to promote adhesion of fibroblast cells. The cell adhesion function of appican is mainly due to the CS chain (6). Furthermore, recent evidence suggests that appican promotes neurite outgrowth of primary neuronal cultures (7).

Brain GAGs and PGs have attracted attention in connection with the pathology of Alzheimer's disease (AD). CS, dermatan sulfate (DS), and heparan sulfate (HS) have been found in the pathological lesions of AD including senile plaques and neurofibrillary tangles (8–13) and may accelerate formation of both pathological structures by providing a surface for the initiation of protein aggregation (14, 15). CSPGs participate in the astrocyte-mediated healing processes following brain injury (16, 17), suggesting that appican may function in brain wound healing.

Brain CS modulates neurite outgrowth (18, 19), and several studies indicate that CS GAGs and CSPGs may either inhibit (20–23) or promote neurite outgrowth depending on their structures (24–28). Recent data suggest that the specific neuroregulatory activities of CSPGs are defined by the chemical composition of its CS moiety (reviewed in Ref. 29). For example, the oversulfated CS-D (GlcUA(2S) β 1–3GalNAc(6S)) and CS-E motifs (GlcUA β 1–3GalNAc(4S,6S)) present in shark and squid cartilage, respectively (30, 31), may act as signals for neurite

Appican is a chondroitin sulfate proteoglycan (CSPG)¹ that

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¹ The abbreviations used are: CSPG, chondroitin sulfate proteogly-

can; 2AB, 2-aminobenzamide; AD, Alzheimer's disease; APLP2, amyloid precursor like protein-2; APP, amyloid precursor protein; CS, chondroitin sulfate; DS, dermatan sulfate; GalNAc, *N*-acetyl-D-galactosamine; GlcUA, D-glucuronic acid; GAG, glycosaminoglycan; HPLC, high performance liquid chromatography; HS, heparan sulfate; Δ HexUA, 4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid; Δ Di-OS, Δ HexUA α 1–3GalNAc; Δ Di-6S, Δ HexUA α 1–3GalNAc(6-*O*-sulfate); Δ Di-4S, Δ HexUA α 1–3GalNAc(4-*O*-sulfate); Δ Di-diS_D, Δ HexUA(2-*O*-sulfate) α 1–3GalNAc(6-*O*-sulfate); Δ Di-diS_E, Δ HexUA α 1–3GalNAc(4, 6-*O*-disulfate); Δ Di-triS, Δ HexUA(2-*O*-sulfate) α 1–3GalNAc(4, 6-*O*-disulfate); PG, proteoglycan; 2S, 2-*O*-sulfate; 4S, 4-*O*-sulfate; 6S, 6-*O*-sulfate.

outgrowth in embryonic rat hippocampal neurons (32–34). These oversulfated disaccharides are distinct from the more common monosulfated A (GlcUA β 1–3GalNAc(4S)) and C (GlcUA β 1–3GalNAc(6S)) units, which may either show no neurotrophic activity or may act as inhibitors of neurite outgrowth. Furthermore, oversulfated CS variants specifically interact with and regulate the neurotrophic activity of several growth factors including pleiotrophin (35) and midkine (35, 36). CS chains containing either D or E disaccharide units are rarely detected in peripheral mammalian tissues but are detectable in brain, albeit at low levels (36–38). Despite the large number of CSPGs identified in vertebrate brains, the structure of the CS chains attached to individual core proteins has not been rigorously investigated, and presently no specific examples of brain CSPGs are known that contain the oversulfated E disaccharide units (reviewed in Ref. 39).

Here we report the analysis of the chemical composition of the CS chain of appican. Our data show that appican constitutes the first example of a specific brain CSPG that contains the E disaccharide motif. Furthermore, our findings are consistent with and may explain the neurotrophic activities of appican. Thus, in addition to being a potential precursor of the Alzheimer's amyloid peptide, appican may affect the neuropathology of AD by acting as a neurotrophic factor for the abnormal neurite outgrowth observed in this disorder. Preliminary findings of this work have been reported in abstract form (40, 41).

EXPERIMENTAL PROCEDURES

Materials—Appican was purified essentially as described in our previous report (1). Briefly, rat C6 glioma cells transfected with the L-APP cDNA (6) were grown in Dulbecco's modified Eagle's medium with supplements. Secreted appican, which consists of recombinant and endogenous molecular species, was purified from the conditioned culture medium by column chromatographies using dextran sulfate and Poros HQ/F anion exchange resin (PerSeptive Biosystems, Cambridge, MA). Chondroitinase digest of the final preparation showed a single band in SDS-polyacrylamide gel electrophoresis stained with Gelcode stain (Pierce) and was quantified with a reference of varying amounts of bovine serum albumin within the same gel. Approximately 0.6 mg of purified appican was obtained from a 7-liter culture. Purified APP was also collected in other fractions of the same anion exchange column as mentioned above.

The following authentic tetra- and hexasaccharides were obtained from whale cartilage CS-A as reported (42): Δ HexUA α 1–3Gal β 1–3Gal β 1–4Xyl–2AB, Δ HexUA α 1–3Gal(4S) β 1–3Gal β 1–4Xyl–2AB, Δ HexUA α 1–3GalNAc β 1–4GlcUA β 1–3Gal β 1–3Gal β 1–4Xyl–2AB, Δ HexUA α 1–3GalNAc(6S) β 1–4GlcUA β 1–3Gal β 1–3Gal β 1–4Xyl–2AB, Δ HexUA α 1–3GalNAc(4S) β 1–4GlcUA β 1–3Gal β 1–3Gal β 1–4Xyl–2AB, and Δ HexUA α 1–3GalNAc(4S) β 1–4GlcUA β 1–3Gal(4S) β 1–3Gal β 1–4Xyl–2AB. Other materials were obtained from the following sources: chondroitinase ABC (EC 4.2.2.4), chondroitinase AC-II (EC 4.2.2.5), chondro-4-O-sulfatase (EC 3.1.6.9), and chondro-6-O-sulfatase (EC 3.1.6.10) from Seikagaku Corp. (Tokyo, Japan) and 2-aminobenzamide (2AB) from Nacalai Tesque (Kyoto, Japan).

Derivatization of Oligosaccharides with 2AB—The purified appican (30 μ g containing 20 μ g of the core protein) was treated with 0.5 M LiOH at 4 °C for 16 h to liberate O-linked saccharides including GAG chains from the core protein APP (42, 43). After neutralization with 1 M acetic acid, the sample was applied to a column (300 μ l) of anion exchange resin AG 50W-X2 (H⁺ form; Bio-Rad) equilibrated with water. The flow-through fraction containing the liberated O-linked saccharides was pooled, neutralized with 1 M NH₄HCO₃, and derivatized with 2AB. Derivatization of the saccharides with a fluorophore 2AB was performed as previously reported (44). The 2AB-derivatives were then subjected to gel filtration on a Superdex peptide column (Amersham Pharmacia Biotech) using 20 mM CH₃COONH₄, pH 7.5, as an effluent at a flow rate of 1 ml/min. Eluates were monitored by fluorescence with excitation and emission wavelengths at 330 and 420 nm, respectively. The flow-through fraction containing 2AB-derivatized GAG chains was pooled and lyophilized. Approximately 55 pmol of high molecular weight 2AB derivatives were obtained from 30 μ g of appican.

Enzymatic Digestions—Enzymatic digestion with chondroitinase

ABC was carried out using 5 mIU of the enzyme and 5 pmol of the 2AB-derivatized polysaccharide or 1 μ g of appican in a total volume of 20 μ l of 0.05 M Tris-HCl buffer, pH 8.0, containing 0.05 M sodium acetate at 37 °C for 20 min (45). Chondroitinase AC-II digestion of the linkage hexasaccharides or 1 μ g of appican was conducted using 5 mIU of the enzyme in a total volume of 30 μ l of 0.03 M sodium acetate buffer, pH 6.0, at 37 °C for 10 min (45). Chondro-4-O-sulfatase or chondro-6-O-sulfatase digestion of the linkage hexasaccharides (5 pmol) was carried out using 20 mIU of the enzyme in a total volume of 30 μ l of 0.04 M Tris-HCl buffer, pH 7.5, containing 0.04 M sodium acetate and 100 μ g/ml bovine serum albumin, at 37 °C for 2 h (46). The reactions were terminated by heating at 100 °C for 1 min.

HPLC Analysis—Each digest was treated with a 0.45- μ m C3HV membrane filter (Millipore), and an aliquot was subjected to HPLC on an amine-bound silica PA03 column (4.6 \times 250 mm) (YMC Co., Kyoto, Japan) using a linear gradient of NaH₂PO₄ from 16 to 530 mM over 60 min at a flow rate of 1 ml/min. Eluates were monitored by fluorescence intensity with excitation and emission wavelengths of 330 and 420 nm or by absorbance at 232 nm (44).

RESULTS

Purity of the Appican Preparation—SDS-polyacrylamide gel electrophoresis of a chondroitinase digest of our appican preparation followed by dye staining yielded only one core protein at 110 kDa (data not shown; see also Ref. 1). This protein reacted with anti-APP antibodies that show no reactivity toward amyloid precursor-like protein 2 (APLP2). Because the core protein of APLP2 PG has an SDS-polyacrylamide gel electrophoresis mobility distinct from the appican core protein (47, 48), our preparation had no significant contamination of the APLP2 PG (see also Ref. 47). APP, but not the APLP2, sequence was found in the core protein of our appican preparations from C6 cells following chondroitinase digestion (1).

Determination of the Disaccharide Composition of the CS Chain of Appican—Purified appican was treated with LiOH to liberate GAGs from the core protein. Liberated saccharides were then labeled with a fluorophore 2AB, and most of the labeled sugar chains were recovered in the flow-through fraction on gel filtration using a Superdex peptide column (data not shown). The flow-through fractions containing 2AB-labeled GAG chains were pooled and digested exhaustively with chondroitinase ABC. The resultant disaccharides were labeled again with 2AB and analyzed by HPLC on an amine-bound silica column. Mono- and disulfated disaccharide peaks, designated I and II, were detected at the elution positions of 2AB derivatives of Δ Di-4S and Δ Di-diS_E, respectively (Fig. 1A). Minor peaks eluted at the positions of 2AB-labeled Δ Di-OS and Δ Di-6S were also detected. When the obtained disaccharides were digested further with chondro-6-O-sulfatase, the disulfated disaccharide peak II was shifted to the elution position of 2AB-labeled Δ Di-4S (Fig. 1B). The disulfated disaccharide peak II was resistant to the action of chondro-4-O-sulfatase, whereas the monosulfated peak I was shifted to the elution position of 2AB-labeled Δ Di-OS by the enzyme treatment, confirming that the predominant components in the disaccharide peaks I and II were 2AB-labeled Δ Di-4S and Δ Di-diS_E, respectively (Fig. 1C) (46). The disaccharide composition of the appican CS determined by the analysis is summarized in Table I. The predominant component was Δ Di-4S, accounting for 81.2%, with a considerable proportion (14.3%) of Δ Di-diS_E. Trace amounts of the minor components, Δ Di-OS and Δ Di-6S, were also detected (Table I). Δ Di-diS_E has also been detected in DS chains of various tissues after chondroitinase ABC digestion and has attracted attention (reviewed in Refs. 29 and 49). However, because chondroitinase ABC and AC-II digests of appican showed identical HPLC profiles (data not shown), it is concluded that appican contains CS-E but not DS-E (CS-H) (50, 51).

To examine whether the CS chains of appican with oversulfated units also contain 3-O-sulfated GlcUA residues (49), we

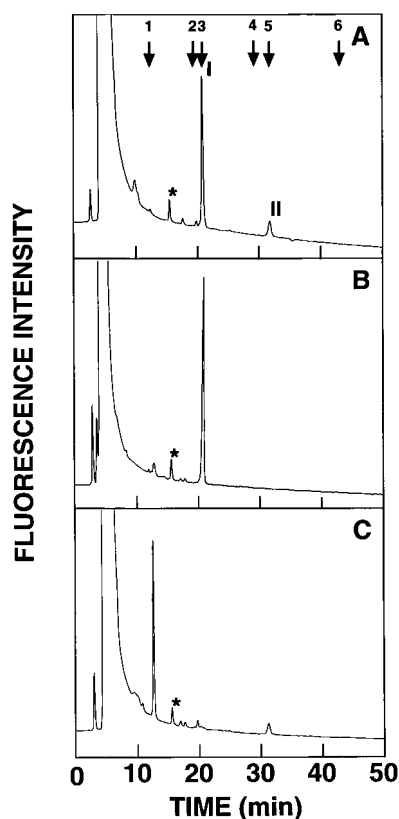


FIG. 1. Identification of the disulfated E disaccharide unit in the CS chains derived from appican. The disaccharide composition of the CS chains obtained from appican was analyzed by HPLC after chondroitinase ABC digestion as described under "Experimental Procedures" (A). To identify the major 2AB-derivatized disaccharide peaks I and II, the appican CS fraction was subjected to successive digestions with chondroitinase ABC and then chondro-6-O-sulfatase (B) or chondro-4-O-sulfatase (C). The digests were analyzed by HPLC using a linear gradient of NaH_2PO_4 as described under "Experimental Procedures." The elution positions of authentic 2AB-derivatized disaccharides are indicated in A by numbered arrows. Arrow 1, $\Delta\text{Di-0S}$; arrow 2, $\Delta\text{Di-6S}$; arrow 3, $\Delta\text{Di-4S}$; arrow 4, $\Delta\text{Di-diS}_D$; arrow 5, $\Delta\text{Di-diS}_E$; arrow 6, $\Delta\text{Di-triS}$. The peak eluted around 15 min and marked by an asterisk is due to an unidentified impurity derived from the 2AB reagent.

TABLE I
Disaccharide composition of the appican CS

Disaccharide composition was determined by enzymatic digestion with chondroitinase ABC followed by HPLC analysis as described under "Experimental Procedures."

Disaccharide	Proportion
	%
$\Delta\text{Di-0S}$	1.2
$\Delta\text{Di-6S}$	3.3
$\Delta\text{Di-4S}$	81.2
$\Delta\text{Di-diS}_D$	ND ^a
$\Delta\text{Di-diS}_E$	14.3
$\Delta\text{Di-triS}$	ND ^a

^a ND, not detected.

took advantage of the differential susceptibility of 3-O-sulfated GlcUA to chondroitinases ABC and AC-II. Oligosaccharide chains containing this sugar residue are decomposed by chondroitinase ABC treatment but are resistant to chondroitinase AC-II (52). However, as described above, we detected no difference in the HPLC profiles of chondroitinase ABC and AC-II digests of appican, indicating that the appican CS chain did not contain a 3-O-sulfated GlcUA residue (data not shown).

CS-E from squid cartilage is known to be occasionally branched with glucose residues (53), and a Glc-containing

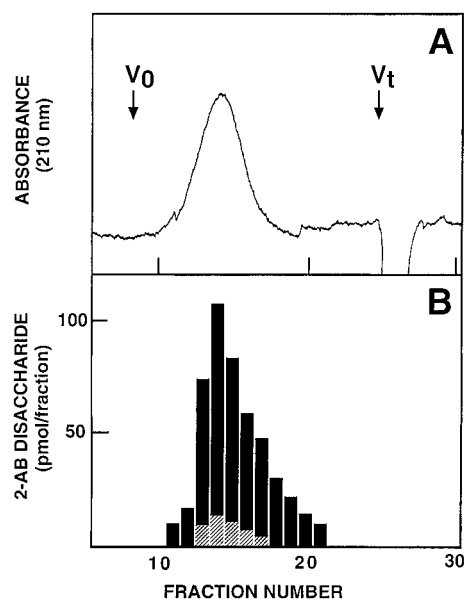


FIG. 2. The molecular size analysis of the CS chain from appican by gel filtration chromatography. A commercial whale cartilage CS-A (A) and the 2AB-GAG fraction (5 pmol) obtained from appican (B) were chromatographed on a column (1.0 \times 30 cm) of Superdex 200 (Amersham Pharmacia Biotech) with 20 mM $\text{CH}_3\text{COONH}_4$, pH 7.5, as the effluent. The fractions shown in A were monitored by measurement of the absorbance at 220 nm. To monitor the CS chain from appican (B), each fraction was evaporated to dryness in a vacuum concentrator, digested with chondroitinase ABC, labeled with 2AB, and then analyzed by HPLC under the conditions used for the experiments shown in Fig. 1. Black bar, $\Delta\text{Di-4S}$; hatched bar, $\Delta\text{Di-diS}_E$.

trisaccharide ($\Delta\text{HexUA}\alpha 1-3(\text{Glc}\beta 1-6)\text{GalNAc}(4\text{S})^2$) and pentasaccharides such as $\Delta\text{HexUA}\alpha 1-3(\text{Glc}\beta 1-6)\text{GalNAc}(4\text{S})\beta 1-4\text{GlcUA}\beta 1-3\text{GalNAc}(4\text{S}, 6\text{S})$ have been isolated after digestion with chondroitinases ABC or AC-II (53, 54). In our HPLC system, the above trisaccharide labeled with 2AB is eluted slightly ahead of and separable from 2AB-labeled $\Delta\text{Di-6S}$.² In this study, no such trisaccharide or the above mentioned pentasaccharide was detected, indicating that the appican CS is not branched with Glc.

Molecular Size Analysis of the Appican CS—The 2AB-GAG fraction obtained from Superdex peptide column chromatography after LiOH treatment of appican was subjected to gel filtration chromatography on a column of Superdex 200. Because of limited amounts of materials, no signal was detected in the eluted fractions by direct fluorescence measurement (data not shown). Therefore, each fraction from the gel filtration chromatography was digested with chondroitinase ABC and labeled with 2AB, and the resultant 2AB-disaccharides were analyzed by HPLC (Fig. 2B). The gel filtration profile of the 2AB-derivatives of disaccharides showed that the molecular mass of the appican CS was similar to that of the commercial whale cartilage CS-A (Fig. 2A), which has been reported to be 25–50 kDa by the manufacturer. Both 2AB-labeled $\Delta\text{Di-4S}$ and $\Delta\text{Di-diS}_E$ gave similar chromatographic profiles (Fig. 2B), suggesting that both disaccharide units were derived from a common CS chain.

Characterization of the CS Protein Linkage Region Oligosaccharides of Appican—To characterize the CS protein linkage hexasaccharide, the appican CS chain was first liberated with LiOH, labeled with 2AB, and then treated with chondroitinase ABC to digest the repeating disaccharide region. The resultant linkage hexasaccharides were analyzed by HPLC on an amine-bound silica column as described under "Experimental Proce-

² A. Kinoshita, T. Nakamura, and K. Sugahara, unpublished results.

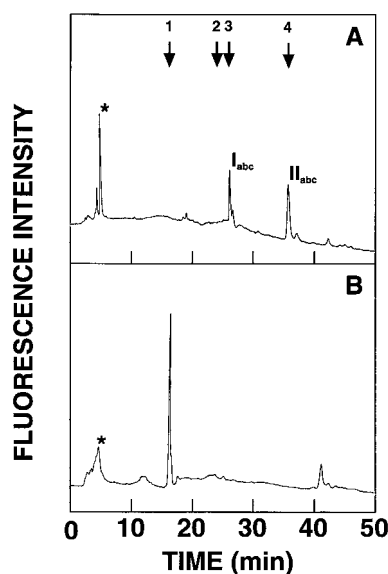


FIG. 3. Characterization of the 2AB-hexasaccharides derived from the CS protein linkage region of the appican CS chain. The 2AB-derivatized GAG fraction obtained from appican was digested with chondroitinase ABC, and the resultant 2AB-derivatized oligosaccharides (5 pmol) were analyzed by HPLC on an amine-bound silica column under the conditions used for the experiments shown in Fig. 1 (A). To identify the two predominant peaks, I_{abc} and II_{abc} , the 2AB-GAG fraction was successively digested with conventional chondroitinase ABC and then chondro-4-*O*-sulfatase (B). The elution positions of the authentic 2AB-hexasaccharides are indicated in A by numbered arrows. Arrow 1, Δ HexUA-GalNAc-GlcUA-Gal-Gal-Xyl-2AB; arrow 2, Δ HexUA-GalNAc-GlcUA-Gal(6S)-Gal-Xyl-2AB; arrow 3, Δ HexUA-GalNAc-GlcUA-Gal(4S)-Gal-Xyl-2AB; arrow 4, Δ HexUA-GalNAc(4S)-GlcUA-Gal(4S)-Gal-Xyl-2AB. The peak marked by an asterisk around 5 min is derived from the 2AB reagent.

dures." Two predominant peaks, designated I_{abc} and II_{abc} , were observed at the elution positions of the authentic 2AB-hexasaccharides, Δ HexUA α 1-3GalNAc(4S) β 1-4GlcUA β 1-3Gal β 1-3Gal β 1-4Xyl-2AB and Δ HexUA α 1-3GalNAc(4S) β 1-4GlcUA β 1-3Gal(4S) β 1-3Gal β 1-4Xyl-2AB, respectively, in a molar ratio of 0.36:0.64 (Fig. 3A). When this sample was co-chromatographed with the standard linkage hexasaccharides, these peaks were co-eluted with the corresponding standards (data not shown). Upon subsequent chondro-4-*O*-sulfatase digestion, both peaks of this sample shifted to the elution position of the authentic nonsulfated 2AB-hexasaccharide, Δ HexUA α 1-3GalNAc β 1-4GlcUA β 1-3Gal β 1-3Gal β 1-4Xyl-2AB (Fig. 3B), indicating that peaks I_{abc} and II_{abc} contained one and two 4-*O*-sulfate groups in the linkage hexasaccharide structure, respectively. The identical sulfated structures were previously detected in the linkage region hexasaccharides isolated from whale cartilage CS chains (55).

To further identify the tetrasaccharide core structures of the linkage region, the 2AB-GAGs were digested first with chondroitinases ABC and then with AC-II. The resultant enzyme digests were analyzed by HPLC on an amine-bound silica column. Two predominant peaks, designated I_{ac} and II_{ac} , were observed at the elution positions of the authentic 2AB-tetrasaccharides, Δ HexUA α 1-3Gal β 1-3Gal β 1-4Xyl-2AB and Δ HexUA α 1-3Gal(4S) β 1-3Gal β 1-4Xyl-2AB, respectively, in a molar ratio of 0.36:0.64 (Fig. 4A). Their molar ratio was in good agreement with that obtained for the 2AB-hexasaccharide peaks I_{abc} and II_{abc} , indicating that peaks I_{ac} and II_{ac} were derived from peaks I_{abc} and II_{abc} , respectively. Furthermore, peaks I_{ac} and II_{ac} were co-chromatographed with the corresponding standards (data not shown), confirming their structural identities. Upon subsequent digestion

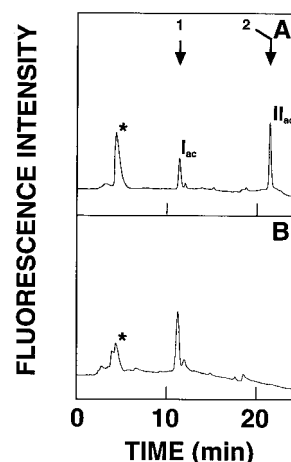


FIG. 4. Characterization of the 2AB-tetrasaccharides derived from the CS protein linkage region of the appican CS. The 2AB-derivatized tetrasaccharides (5 pmol) were analyzed after digestion of the 2AB-GAG fraction with chondroitinase AC-II (A). To identify the two major peaks, I_{ac} and II_{ac} , the 2AB-GAG fraction was successively digested with chondroitinase AC-II and then chondro-4-*O*-sulfatase (B). The elution positions of the authentic 2AB-tetrasaccharides are indicated in A by numbered arrows. Arrow 1, Δ HexUA-Gal-Gal-Xyl-2AB; arrow 2, Δ HexUA-Gal(4S)-Gal-Xyl-2AB. The peak marked by an asterisk around 5 min is derived from the 2AB reagent.

with chondro-4-*O*-sulfatase, peak II_{ac} was shifted to the elution position of Δ HexUA α 1-3Gal β 1-3Gal β 1-4Xyl-2AB as expected (Fig. 4B). Together, these findings indicate that the hexasaccharide linkage region of appican is primarily composed of the structures summarized in Table II. We detected no 2-*O*-phosphorylation of the linkage Xyl residue of appican, although this structure has been detected in the linkage region of other PGs containing CS and heparin/HS (56).

A number of PGs, including L-APP (1, 57), are called part-time PGs because they occur in both PG and non-PG forms (56, 58). However, the biosynthetic molecular mechanism by which a given protein containing the GAG attachment consensus amino acid sequence becomes a PG remains obscure. The non-PG form of thrombomodulin, another part-time PG, contains a truncated linkage tetrasaccharide at the GAG attachment sites, although it lacks the repeating disaccharides (59), suggesting that transfer of the fifth sugar residue, GalNAc, to the linkage tetrasaccharide may be the determining step for the synthesis of the repeating disaccharide region. To examine whether the non-PG form of L-APP contains immature truncated small *O*-linked oligosaccharides, non-PG L-APP was isolated free of appican (see "Experimental Procedures") and treated with 0.5 M LiOH. The released saccharide fraction was derivatized with 2AB, purified by paper chromatography, and then analyzed by anion exchange or gel filtration HPLC (see "Experimental Procedures"). No peak was observed at the expected elution position of the authentic 2AB-tetrasaccharide, GlcUA β 1-3Gal β 1-3Gal β 1-4Xyl-2AB, prepared from α -thrombomodulin (59). Furthermore, none of the obtained peaks was sensitive to the action of β -glucuronidase (59). These results are consistent with our data from the matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis, which showed no signals corresponding to 2AB-derivatives from the linkage penta- or tetrasaccharides (data not shown). Hence, the non-PG form of L-APP contains no appreciable amounts of immature linkage region tetra- or pentasaccharides, suggesting that the addition of a CS chain to the L-APP core protein may be regulated by a mechanism distinct from the mechanism that regulates addition of CS chains on thrombomodulin (see "Discussion").

TABLE II
Yields of the CS-protein linkage hexasaccharides isolated from appican

Structure	Total
Δ HexUA α 1-3GalNAc(4S) β 1-4GlcUA β 1-3Gal β 1-3Gal β 1-4Xy1-2AB	%
Δ HexUA α 1-3GalNAc(4S) β 1-4GlcUA β 1-3Gal(4S) β 1-3Gal β 1-4Xy1-2AB	36
	64

DISCUSSION

In this study, we obtained evidence that, in addition to a predominant A disaccharide unit, the CS chain of appican contains a significant fraction (14.3%) of the uniquely disulfated E disaccharide motif (Table I). Although the E disaccharide unit has been demonstrated in total extracts of bovine, rat, and chick brains (36–38), appican is the first specific example of a brain CSPG that contains the oversulfated E unit. Only a small number of brain CSPGs have been analyzed for their saccharide structures. Among these, chick brain aggrecan is characterized by nonsulfated disaccharide units and by C units (60). Our recent analysis of postnatal mouse brain DSD-1-PG/phosphacan showed that this PG contained the D disaccharide unit in addition to the A, C, and nonsulfated disaccharide units (33). The CS chains of other brain CSPGs, which include versican, neurocan, NG2, glypican, brevican, and neuroglycan C (39), have not been well characterized.

It is known that the E unit is a major disaccharide of the CS chains of serglycin, which is produced by a certain mast cell population (61–63). These mast cells are the only mammalian cells known to produce PGs that contain large amounts of CS-E. Thus, it has been assumed that the small amounts of CS-E found in the CS/DS analysis of various mammalian tissue originates primarily from mast cells. Our study shows that CS-E is expressed in glioma C6 cells. Astrocytes are the only producers of appican among other brain cells (5) and are related to C6 cells. Therefore, we suggest that astrocytes also produce CS-E-containing appican.

Appican promotes adhesion of neural cells (6) and stimulates neurite outgrowth of primary rat hippocampal neuronal cultures (7). The CS chain is mainly responsible for these activities because the core APP protein is much less potent in promoting adhesion and neurite outgrowth than the appican (6, 7). CSPGs may function either as inhibitors or stimulators of neurite outgrowth depending on their chemical composition. The hypothesis that oversulfated disaccharides are mainly responsible for the neurotrophic activities shown by specific CSPGs (33, 34) is based on recent evidence that oversulfated squid CS-E as well as shark CS-D promote neurite outgrowth in cultures of primary rat hippocampal neurons (33, 34). On the contrary, CSPGs lacking oversulfated units either fail to promote neurite outgrowth or show inhibitory activity. Our observation that appican contains the oversulfated E unit supports this hypothesis and suggests that appican may function in the brain to promote neurite outgrowth. Recent evidence suggests that appican from AD brain has higher neurite outgrowth activity than appican from control brains (7). It would be interesting to examine whether appican from AD brain bears a higher proportion of the E unit than appican from control human brain.

The hexasaccharide linkage region of appican contained no E disaccharide unit. The fifth sugar residue, GalNAc, of the appican linkage hexasaccharide was mono-sulfated only at the 4-O-position (Table II). These results are consistent with our previous finding that chondroitinase digests of appican reacted with antibodies specific to 4-O-sulfated stubs but not with antibodies specific to nonsulfated or 6-O-sulfated stubs (1). The second sugar residue (Gal) of the linkage region was not sulfated, whereas 64% of the third residue (Gal) was sulfated at

the 4-O-position. Thus, based on the sulfation of the third residue, the linkage region of appican consists of two distinct subpopulations (Table II). Our findings constitute the first demonstration for the presence of 4-O-sulfated Gal in the linkage region of a brain CSPG. It is noteworthy that the Gal(4S) structure has been found in the linkage region of CSPGs and DSPGs but not of heparin or HSPGs (56). Syndecan-1, a hybrid PG that bears both CS and HS chains, is selectively 4-O-sulfated at the Gal residue of the CS linkage region but not of the HS linkage region (64). Therefore, the 4-O-sulfated Gal residue seems to be specific to the linkage region of CSPGs and DSPGs and may be important for their biological functions and/or the biosynthetic selective assembly of CS/DS chains (see “Discussion” in Ref. 64).

L-APP is expressed as a PG and a non-PG form, suggesting that elimination of exon 15 from the APP mRNA is not a sufficient condition for appican biosynthesis, although it is necessary (1, 4). The purified non-PG form of L-APP contained no tetrasaccharide linkage unit. In contrast, the non-PG form of thrombomodulin, another part-time CSPG, contains a linkage tetrasaccharide at the serine residue used for the attachment of the GAG chain (59). These results indicate that appican biosynthesis has two rate-limiting or determinative steps, first an exon 15 splicing-out and second the synthesis or maturation of the linkage tetrasaccharide on the L-APP serine residue 619 (4). It is possible that synthesis of the linkage region is regulated by the transfer of the first sugar residue, Xyl, to the core protein (65) (see also “Discussion” in Ref. 59). It is noteworthy that APLP2 PG has a similar mechanism of splicing-out a corresponding exon but may not have the second regulation, because the specific splicing isoform of APLP2 is expressed only as a PG form (48). To clarify the biological significance of such diverse regulations in the glycanation of APP core proteins, vigorous examinations are required for detecting possible small biosynthetic intermediate oligosaccharides, which may be generated during the maturation process of the linkage region of the CS chain.

Serglycin CS-E has been implicated in the packaging of tryptases in the secretory granules of the mast cell (66). In this context, it is noteworthy that the secreted form of appican contains a Kunitz type serine protease inhibitor sequence (1, 4). An undefined rat mast cell tryptase (67) has been reported to be a target for the Kunitz type serine protease inhibitor-containing APP. The present finding raises the intriguing possibility that the CS-E in appican may play important roles in the regulation of tryptases in the brain and may be involved in the pathology of AD.

APP, including the core protein of appican, is an integral membrane protein containing a large extracellular domain, a transmembrane sequence, and a small cytoplasmic domain. The proteolytic processing of APP by the actions of β - and γ -secretases gives rise to the amyloid β protein found in neuritic plaques, a pathological hallmark of AD. It was recently reported that sulfated GAGs accelerate amyloid β fibril formation (14, 15). In this context, it would be interesting to examine the effects of appican and/or other CSPGs containing the E units on the formation of amyloid fibrils and neurofibrillar tangles. Characterization of brain CS from AD patients has not been reported, although the distribution and characteristics of

GAGs, especially HS and keratan sulfate, in the lesions of AD have been demonstrated. The HS samples derived from afflicted brains differed minimally from control subjects in quantity and structure (68). In contrast, keratan sulfate GAG is markedly decreased in the cerebral cortex of AD patients (69). A better understanding of the structures and biological activities of CSGAGs in the brain of normal subjects and AD patients may lead to the development of medical strategies designed to control or arrest the progression of AD.

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REFERENCES

- Shioi, J., Anderson, J. P., Ripellino, J. A., and Robakis, N. K. (1992) *J. Biol. Chem.* **267**, 13819–13822
- Pangalos, M. N., Shioi, J., Efthimiopoulos, S., Wu, A., and Robakis, N. K. (1996) *Neurodegeneration* **5**, 445–451
- Shioi, J., Pangalos, M. N., Wu, A., and Robakis, N. K. (1996) *Trend. Glycosci. Glycotechnol.* **8**, 252–263
- Pangalos, M. N., Efthimiopoulos, S., Shioi, J., and Robakis, N. K. (1995) *J. Biol. Chem.* **270**, 10388–10391
- Shioi, J., Pangalos, M. N., Ripellino, J. A., Vassilacopoulou, D., Mytilineou, K., Margolis, R. U., and Robakis, N. K. (1995) *J. Biol. Chem.* **270**, 11839–11844
- Wu, A., Pangalos, M. N., Efthimiopoulos, S., Shioi, J., and Robakis, N. K. (1997) *J. Neurosci.* **17**, 4987–4993
- Salinero, O., Moreno-Flores, M. T., and Wandosell, F. (2000) *J. Neurosci. Res.* **60**, 87–97
- Snow, A. D., Mar, H., Nochlin, D., Kimata, K., Kato, M., Suzuki, S., Hassell, J., and Wright, T. N. (1988) *Am. J. Pathol.* **133**, 456–463
- Perry, G., Siedlak, S. L., Richey, P., Kawai, M., Cras, P., Kalaria, R. N., Galloway, P. G., Scardina, J. M., Cordwell, B., Greenberg, B. D., Ledbetter, S. R., and Gambetti, P. (1991) *J. Neurosci.* **11**, 3679–3683
- Su, J. H., Cummings, B. J., and Cotman, C. W. (1992) *Neuroscience* **51**, 801–813
- DeWitt, D. A., Silver, J., Canning, D. R., and Perry, G. (1993) *Exp. Neurol.* **121**, 149–152
- Snow, A. D., Sekiguchi, R. T., Nochlin, D., Kalaria, R. N., and Kimata, K. (1994) *Am. J. Pathol.* **144**, 337–347
- Goedert, M., Jakes, R., Spillantini, M. G., Hasegawa, M., Smith, M. J., and Crowther, R. A. (1996) *Nature* **383**, 550–553
- Castillo, G. M., Lukito, W., Wight, T. N., and Snow, A. D. (1999) *J. Neurochem.* **72**, 1681–1687
- McLaurin, J., Franklin, T., Zhang, X., Deng, J., and Fraser, P. E. (1999) *Eur. J. Biochem.* **266**, 1101–1110
- Aschner, M., and Lopachin, J. R. M. (1993) *J. Toxic. Envir. Health* **38**, 329–342
- McKeon, R. J., Schreiber, R. C., Rudge, J. S., and Silver, J. (1991) *J. Neurosci.* **11**, 3398–3411
- Herndon, M. E., and Lander, A. D. (1990) *Neuron* **4**, 949–961
- Lander, A. D. (1993) *Curr. Opin. Neurobiol.* **3**, 716–723
- Snow, D. M., Steindler, D. A., and Silver, J. (1990) *Dev. Biol.* **138**, 359–376
- Snow, D. M., Watanabe, M., Letourneau, P. C., and Silver, J. (1991) *Development* **113**, 1473–1485
- Brittis, P. A., Canning, D. R., and Silver, J. (1992) *Science* **255**, 733–736
- Katoh-Semba, R., Matsuda, M., Kato, K., and Oohira, A. (1995) *Eur. J. Neurosci.* **7**, 613–621
- Lafont, F., Rouget, M., Triller, A., Prochiantz, A., and Rousset, A. (1992) *Development* **114**, 17–29
- Streit, A., Nolte, C., Rasony, T., and Schachner, M. (1993) *J. Cell Biol.* **120**, 799–814
- Fernaund-Espinosa, I., Nieto-Sampedro, M., and Bovolenta, P. (1994) *J. Cell Sci.* **107**, 1437–1448
- Faissner, A., Clement, A., Lochter, A., Streit, A., Mandl, C., and Schachner, M. (1994) *J. Cell Biol.* **126**, 783–799
- Garwood, J., Schnädelbach, O., Clement, A., Schütte, K., Bach, A., and Faissner, A. (1999) *J. Neurosci.* **19**, 3888–3899
- Sugahara, K., and Yamada, S. (2000) *Trends Glycosci. Glycotechnol.* **12**, 321–349
- Suzuki, S. (1960) *J. Biol. Chem.* **235**, 3580–3588
- Suzuki, S., Saito, H., Yamagata, T., Anno, K., Seno, N., Kawai, Y., and Furuhashi, T. (1968) *J. Biol. Chem.* **243**, 1543–1550
- Nadanaka, S., Clement, A., Masayama, K., Faissner, A., and Sugahara, K. (1998) *J. Biol. Chem.* **273**, 3296–3307
- Clement, A. M., Nadanaka, S., Masayama, K., Mandl, C., Sugahara, K., and Faissner, A. (1998) *J. Biol. Chem.* **273**, 28444–28453
- Clement, A. M., Sugahara, K., and Faissner, A. (1999) *Neurosci. Lett.* **269**, 125–128
- Maeda, N., Ichihara-Tanaka, K., Kimura, T., Kadomatsu, K., Muramatsu, T., and Noda, M. (1999) *J. Biol. Chem.* **274**, 12474–12479
- Ueoka, C., Kaneda, N., Okazaki, I., Nadanaka, S., Muramatsu, T., and Sugahara, K. (2000) *J. Biol. Chem.* **275**, 37407–37413
- Saigo, K., and Egami, F. (1970) *J. Neurochem.* **17**, 633–647
- Kitagawa, H., Tsutsumi, K., Tone, Y., and Sugahara, K. (1997) *J. Biol. Chem.* **272**, 31377–31381
- Oohira, A., Matsui, F., Tokita, Y., Yamauchi, S., and Aono, S. (2000) *Arch. Biochem. Biophys.* **374**, 24–34
- Tsuchida, K., Takahashi, Y., Yamada, S., Sugahara, K., Shioi, J., Boghosian, G., Wu, A., Gai, H., and Robakis, N. K. (2000) *XXIst Japanese Carbohydrate Symposium, Nagoya, Japan, July 27–29, 2000*, (Muranatsu, T., ed), pp. 102, Abstract No. PI-47, Gakushin Publishing Co., Suita, Japan
- Shioi, J., Tsuchida, K., Yamada, S., Sugahara, K., Boghosian, G., Wu, A., Cai, H., and Robakis, N. K. (2000) *Neurobiol. Aging* **21**, S183
- Sakaguchi, H., Watanabe, M., Ueoka, C., Sugiyama, E., Taketomi, T., Yamada, S., and Sugahara, K. (2001) *J. Biochem. (Tokyo)* **129**, 107–118
- Heinegård, D. (1972) *Biochim. Biophys. Acta* **285**, 193–207
- Kinoshita, A., and Sugahara, K. (1999) *Anal. Biochem.* **269**, 367–378
- Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1523–1535
- Sugahara, K., Shigeno, K., Masuda, M., Fujii, N., Kurosaka, A., and Takeda, K. (1994) *Carbohydr. Res.* **255**, 145–163
- Pangalos, M. N., Shioi, J., and Robakis, N. K. (1995) *J. Neurochem.* **65**, 762–769
- Thinakaran, G., and Sisodia, S. S. (1994) *J. Biol. Chem.* **269**, 22099–22104
- Kinoshita, A., Yamada, S., Haslam, S. M., Morris, H. R., Dell, A., and Sugahara, K. (1997) *J. Biol. Chem.* **272**, 19656–19665
- Anno, K., Seno, N., Mathews, M. B., Yamagata, T., and Suzuki, S. (1971) *Biochim. Biophys. Acta* **237**, 173–177
- Ueoka, C., Nadanaka, S., Seno, N., Khoo, K.-H., and Sugahara, K. (1999) *Glycoconjugate J.* **16**, 291–305
- Sugahara, K., Tanaka, Y., Yamada, S., Seno, N., Kitagawa, H., Haslam, S. M., Morris, H. R., and Dell, A. (1996) *J. Biol. Chem.* **271**, 26745–26754
- Habuchi, O., Sugiura, K., Kawai, N., and Suzuki, S. (1977) *J. Biol. Chem.* **252**, 4570–4576
- Kinoshita, A., Nakamura, T., Haslam, S. M., Morris, H. R., Dell, A., and Sugahara, K. (1998) *XIXth International Carbohydrate Symposium, San Diego, CA, August 9–14, 1998*, abstract AP077, International Carbohydrate Organization
- Sugahara, K., Masuda, M., Harada, T., and Yamashina, I. (1991) *Eur. J. Biochem.* **202**, 805–811
- Sugahara, K., and Kitagawa, H. (2000) *Curr. Opin. Struct. Biol.* **10**, 518–527
- Shioi, J., Refolo, L. M., Efthimiopoulos, S., and Robakis, N. K. (1993) *Neurosci. Lett.* **154**, 121–124
- Fransson, L. Å. (1987) *Trends Biochem. Sci.* **12**, 406–411
- Nadanaka, S., Kitagawa, H., and Sugahara, K. (1998) *J. Biol. Chem.* **273**, 33728–33734
- Li, H., Domowicz, M., Hennig, A., and Schwartz, N. B. (1996) *Mol. Brain Res.* **36**, 309–321
- Razin, E., Stevens, R. L., Akiyama, F., Schmid, K., and Austen, K. F. (1982) *J. Biol. Chem.* **257**, 7229–7236
- Stevens, R. L., Fox, C. C., Lichtenstein, L. M., and Austen, K. F. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 2284–2287
- Thompson, H. L., Schulman, E. S., and Metcalfe, D. D. (1988) *J. Immunol.* **140**, 2708–2713
- Ueno, M., Yamada, S., Zako, M., Bernfield, M., and Sugahara, K. (2001) *J. Biol. Chem.* **276**, 29134–29140
- Vertel, B. M., Walters, L. M., Flay, N., Kearns, A. E., and Schwartz, N. B. (1993) *J. Biol. Chem.* **268**, 11105–11112
- Humphries, D. E., Wong, G. W., Friend, D. S., Gurish, M. F., Qiu, W. T., Huang, C., Sharpe, A. H., and Stevens, R. L. (1999) *Nature* **400**, 769–772
- Kido, H., Fukutomi, A., Schilling, J., Wang, Y., Cordell, B., and Katunuma, N. (1990) *Biochem. Biophys. Res. Commun.* **167**, 716–721
- Lindahl, B., Eriksson, L., and Lindahl, U. (1996) *Biochem. J.* **306**, 177–184
- Lindahl, B., Eriksson, L., Spillmann, D., Caterson, B., and Lindahl, U. (1996) *J. Biol. Chem.* **271**, 16991–16994