Occurrence of Three Distinct Molecular Species of Chondroitin Sulfate Proteoglycan in the Developing Rat Brain*

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More than 60% of brain chondroitin sulfate proteoglycans were extracted from 10-day-old rat brains by homogenization in ice-cold phosphate-buffered saline containing protease inhibitors. Although the soluble proteoglycan preparation was a mixture of chondroitin sulfate proteoglycans with a different hydrodynamic size as well as a different molecular density, each subfraction of the proteoglycans contained chondroitin sulfate side chains with virtually identical molecular weight (approximately 15,000) and chondroitin sulfate disaccharide composition (high content of 4-sulfate unit). Digestion of the purified proteoglycan preparation with protease-free chondroitinase ABC produced five core proteins with Mr = 250,000 (designated as 250K protein), 220,000 (220K), 160,000 (150K), 130,000 (130K), and 93,000 (93K). All these core proteins were obtained from chondroitin sulfate proteoglycan preparations extracted from various regions of the brain, but their composition varied among different brain regions. Analysis for amino acid composition of these core proteins and two-dimensional mapping of their proteolytic peptides revealed that three major core proteins (250K, 220K, and 150K proteins) were structurally different. These observations indicate that at least three distinct types of chondroitin sulfate proteoglycan occur in the developing rat brain.

Chondroitin sulfate is one of the most ubiquitous pericellular constituents of animals. In tissues, chondroitin sulfate is believed to exist as a protein-bound form or a proteoglycan. It has been shown so far in connective tissues that there are at least two distinct molecular species of chondroitin sulfate proteoglycan with a characteristic core protein moiety. One is the cartilage-type proteoglycan, which has been studied extensively (for a review, see Ref. 1). The other is a chondroitin sulfate proteoglycan synthesized by prechondrogenic mesenchymal cells (2). Prechondrogenic mesenchymal cells in embryonic chick limb buds synthesize a proteoglycan, termed PG-M, which has a core protein (Mr = 550,000) different from that (Mr = 400,000) of the chick cartilage-type proteoglycan, PG-H, biochemically as well as immunochemically (2). These results suggest that there are several distinct molecular species of protein bearing chondroitin sulfate side chains, and that each tissue or organ has a characteristic composition of chondroitin sulfate proteoglycan subtypes as in the case of collagen subtypes.

Chondroitin sulfate is the major sulfated glycosaminoglycan in the mature brain of various species (3–5) and is detected together with heparan sulfate in the brain from early developmental stages to adult of the rat (6). The brain is composed of at least three different cell types: neuronal cells, glial cells, and vascular endothelial cells. Therefore, one can consider that multiple types of chondroitin sulfate proteoglycan occur in the brain and that they are distinct from those isolated from connective tissues. Initial progress in this regard has been made by Margolis and his co-workers (7, 8) who have partially characterized brain chondroitin sulfate proteoglycans both biochemically and immunohistochemically.

In the present study, we isolate five distinct core proteins from the purified brain chondroitin sulfate proteoglycan preparation by digestion with protease-free chondroitinase ABC, indicating occurrence of multiple types of chondroitin sulfate proteoglycan in the rat brain. Additionally, we demonstrate the difference in the composition of these proteoglycan species among various brain regions.

EXPERIMENTAL PROCEDURES

Extraction of Proteoglycans—Brains from 10-day-old Sprague-Dawley rats (Slc:SD strain, Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan) were homogenized with a tight-fitting Potter glass homogenizer in 2.5 ml of ice-cold phosphate-buffered saline (PBS) containing 2 mM EDTA, 10 mM N-ethylmaleimide, and 2 mM phenylmethylsulfonyl fluoride as protease inhibitors. The homogenate was centrifuged at 27,000 × g for 40 min at 4 °C. The pellet was subjected to rehomogenization in PBS. After centrifugation, both supernatants were combined and then lyophilized. In some experiments, rats were injected intracerebrally with 10 μCi/rat of carrier-free Na2[35S]04, or 100 μCi/rat of [35S]methylmethionine (Tran-35S-label’", ICN Radiochemicals, Irvine, CA), dissolved in 10 μl of PBS 24 h prior to decapitation. Labeled proteoglycans were solubilized as described above.

Preparation and Quantitative Determination of Glycosaminoglycans—To an aliquot (10 ml) of PBS extract, 30 ml of 95% ethanol containing 1.3% (w/v) potassium acetate was added to precipitate proteoglycans. The pellet was digested with Pronase-E (1 mg/ml, Kaken Seiyaku, Tokyo) in 2.5 ml of 50 mM Tris-HCl, pH 7.5, containing 3% ethanol at 50 °C for 24 h. PBS-insoluble material (from three brains) was also digested with Pronase-E in 10 ml of the buffer. Glycosaminoglycans were prepared from the Pronase digest by sequential treatments with 0.4 M NaOH, 5% trichloroacetic acid, and precipitation with 75% ethanol containing 1% potassium acetate as described previously (6). Hexuronate in the glycosaminoglycan preparations was determined by the method of Bitter and Muir (9). Glycosaminoglycans were dissolved in distilled water at the hexuronate concentration of 2 nmol/μl. An aliquot (usually 5 μl) of the solution was subjected to two-dimensional electrophoresis on cellulose acetate film (Sepaphore III, Gelman Sciences, Ann Arbor) by the method of Hata and Nagai (10). Quantitative determinations of each glycosaminoglycan thus separated were done after staining glycos-
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aminoglycans with Alcian Blue 8GS (Chroma Gesellschaft Schmid & Co., Stuttgart, FRG) as described previously (6).

** Purification of Soluble Proteoglycans—**Lyophilized material of PBS extract was suspended in 1 ml/brain of 2 M urea, 50 mM Tris-HCl, pH 7.5, containing 15 mM NaCl, 2 mM EDTA, 1 mM N-ethylemaleimide, and 0.2 mM phenylmethylsulfonyl fluoride at a flow rate of 25 ml/h. In some experiments, proteoglycans were eluted from the DEAE-Sephacel column with 0.7 M NaCl in the urea buffer after washing the column with 0.35 M NaCl-containing urea buffer. The sulfated proteoglycan fraction was pooled and concentrated to 5 ml on a Diaflo YM-10 membrane (Amicon Corp.).

The concentrated solution was chromatographed on a column (1.6 × 100 cm) of Sepharose CL-4B (Pharmacia, Uppsala, Sweden) in 1 M guanidine HCl, 50 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 1 mM N-ethylemaleimide, and 0.2 mM phenylmethylsulfonyl fluoride at a flow rate of 15 ml/h. The sulfated proteoglycan fractions were pooled separately and concentrated to 4 ml on a Diaflo YM-10 membrane.

**Ultrafiltration** was carried out in a Centricon 30 cassette at a flow rate of 0.7 M at 4 °C. After centrifugation at 4 °C in 400 ml of the buffer with a linear gradient of NaCl concentration from 15 mM to 0.7 M at a flow rate of 25 ml/h. In some experiments, proteoglycans were eluted from the DEAE-Sephacel column with 0.7 M NaCl in the urea buffer after washing the column with 0.35 M NaCl-containing urea buffer. The sulfated proteoglycan fraction was pooled and concentrated to 5 ml on a Diaflo YM-10 membrane (Amicon Corp.).

**Preparation of Core Proteins of Proteoglycans—**A portion, containing 0.1 ml of hexuronate, of the proteoglycan preparation purified by ultrafiltration was precipitated with ethanol and dissolved in 100 μl of 0.1 M Tris-HCl, pH 7.5, containing 0.02 unit of protease-free chondroitinase ABC (Seikagaku Kogyo, Tokyo), 2 μg of bovine serum albumin (crystallized, Armour Pharmaceutical Co., Kansas, England), 100 μg of protease inhibitors, 0.2 mM phenylmethylsulfonyl fluoride, 0.07 mM mepstatin, and 1 mM sodium azide. The solution was chromatographed at room temperature in the buffer on a column (1.0 × 48 cm) of Sepharose CL-6B (Pharmacia, Uppsala, Sweden) at a flow rate of 5 ml/h. Another aliquot was chromatographed on the same column after subsequent treatments with Pronase-E, 0.4 M NaOH, and ethanol containing potassium acetate to release glycosaminoglycan side chains from the protein core as described previously (11). The glycosaminoglycan fraction obtained by the chromatography was pooled and concentrated as described above.

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**Analysis for Amino Acid Composition—**A Coomassie Brilliant Blue-stained protein band was cut out and washed with distilled water in a test tube. The protein band was extracted from the gel slice with 70% formic acid (10 ml/cm² gel) by shaking gently for 3 h at room temperature. This step was repeated twice to ensure extraction. The extract was evaporated to dryness in a rotary evaporator at 40 °C. The dried material was dissolved in 0.2 ml of 35% formic acid and the solution was chromatographed on a PD-10 column (Pharmacia, Uppsala, Sweden). The poly peptide fraction was hydrolyzed under vacuum in 6 N HCl at 110 °C for 20 h. Analysis for amino acid composition was performed with a Beckman 120-A amino acid analyzer. No corrections were made for the destruction of amino acids during HCl hydrolysis.

**Analysis of Proteolytic Peptides—**Chondroitin sulfate proteoglycans labeled with [35S]methionine were purified as described above and digested with protease-free chondroitinase ABC to obtain core proteoglycan peptides. [35S]Methionine-labeled core proteins isolated by SDS-polyacrylamide slab gel electrophoresis. Coomassie Blue-stained core protein band (usually 3 × 15 × 1 mm) was cut and washed with 25% isopropyl alcohol three times and then with 10% methanol three times in a siliconized test tube at room temperature. The gel slice was dried under vacuum and then treated with 50 μg of trypsin which had been treated with L-tryosylamido-2-phenyl-ethyl chloromethyl ketone to inhibit contaminating endogenous trypsin activity (Worthington, Freehold, NJ), or α-chymotrypsin (Worthington) in 1 ml of 50 mM NH4HCO3, pH 8.4, at 37 °C overnight. The supernatant containing proteolytic peptides was dried under vacuum to remove the salt, and the dried material was dissolved in acetic acid/formic acid/H2O, 9:3:1, by volume. Two-dimensional mapping of the proteolytic peptides was carried out by the method of Tanabe (14). In brief, the peptide solution was spotted on a silica gel-coated thin layer glass plate (20 × 20 cm, Analtech Inc., Newark, NJ) and peptides were resolved using electrophoresis at 800 V in the solution in the first dimension, and ascending chromatography in 1-butanol/pyridine/acetic acid/H2O, 13:10:2:8, by volume, in the second dimension. The plate was dried, and [35S]methionine-labeled peptides were located by fluorography using ENHANCE Spray (Du Pont-New England Nuclear) with Kodak X-Omat AR film.

**RESULTS**

**Extraction of Proteoglycans—**When 10-day-old rat brains were homogenized in ice-cold PBS in the presence of protease inhibitors, about 60% of hexurionate-containing macromolecules were solubilized (Table I). Since three glycosaminoglycans such as chondroitin sulfate, heparan sulfate, and hyaluronate are isolated by Pronase digestion of the brain (6), extractability of each glycosaminoglycan was examined. In the Pronase digest of the PBS extract, chondroitin sulfate and hyaluronate were detected by two-dimensional electrophoresis on cellulose acetate film (data not shown). When the polysaccharides precipitated by Pronase digestion of PBS-insoluble fraction were analyzed electrophoretically, heparan sulfate was detected in addition to chondroitin sulfate and hyaluronate. From the quantitative determination of these glycosaminoglycans, about 60% of chondroitin sulfate proteoglycans was shown to be soluble in PBS, while heparan sulfate proteoglycans were virtually insoluble (Table I). A large part of hyaluronate was also soluble in PBS.

** Purification of Proteoglycans—**[35S]Sulfate-labeled material extracted from brains with PBS was separated by DEAE-Sephacel column chromatography into two peaks; an unabsorbed small peak and a nearly symmetrical large peak eluted at NaCl concentration of 0.40–0.60 M (data not shown). Since the large peak was enriched in chondroitin sulfate, this component was purified further by gel filtration on Sepharose CL-4B (Fig. 1). The labeled material eluted broadly in the area with Kav ranging from 0.1 to 0.6. The eluate was separated into three fractions designated as I, II, and III in the order of

<table>
<thead>
<tr>
<th>Table I</th>
<th>Extraction of proteoglycans with PBS containing protease inhibitors from the brain of 10-day-old rats</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteoglycan</strong> (nmol hexuronate/brain)</td>
<td><strong>Extract</strong></td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>81.4 (63.0)</td>
</tr>
<tr>
<td>Heparan sulfate (μg/brain)</td>
<td>&lt;6.0</td>
</tr>
<tr>
<td>Hyaluronate (μg/brain)</td>
<td>76.1 (76.6)</td>
</tr>
</tbody>
</table>

The values in parentheses represent the percentage of total.

The amounts of glycosaminoglycans were determined by Alcian Blue-binding assay after separation of glycosaminoglycans by two-dimensional electrophoresis on cellulose acetate film as described under "Experimental Procedures."
The radioactive material in fraction I, recovered in the bottom fraction (S1.41 g/ml), but its hexuronate-containing material in fraction I was obtained in the void volume. The radioactive material in fraction III was distributed broadly in the CsCl density gradient, and about 90% of the radioactivity as well as hexuronate was recovered in the bottom half (>1.38 g/ml) of the tube. The radioactive material in fraction 11, purified by ultracentrifugation, before and after Pronase digestion, was eluted from the same column as a more retarded peak with Kav = 0.3, whereas the material digested with Pronase was eluted from the same column as a more retarded peak with Kav = 0.58 (Fig. 2c). The labeled material was also identified to be chondroitin sulfate by its susceptibility to chondroitinases. Based on the elution position of chondroitin sulfates of known molecular weight (16), the molecular weight of the brain chondroitin sulfate was estimated to be 15,000. In the case of fraction III, the amount of peak B (or sulfated glycopeptides) is relatively small (Fig. 2c). The labeled material in fraction II, purified by ultracentrifugation, was eluted from Sepharose CL-6B as a very broad peak (Fig. 2b). This material was also identified to be a proteoglycan bearing chondroitin sulfate with an average molecular weight of 15,000 in the same way described above. Judging from the elution profile shown in Fig. 2b, fraction II seems to be a mixture of fraction I and fraction III.

Table II summarizes the yield of chondroitin sulfate proteoglycans. Each fraction was further purified by ultracentrifugation, before and after Pronase digestion. The radioactive material was eluted as two retarded peaks; the major peak (designated as peak A) with Kav = 0.53 and the minor (designated as peak B) with Kav = 0.85. Peak A was susceptible to digestion either with chondroitinase ABC or with chondroitinase AC-II (Seikagaku Kogyo, Tokyo), indicating that this is chondroitin sulfate. Analysis of the products of the enzymatic digestion by paper chromatography described by Saito et al. (12) revealed that chondroitin 4-sulfate unit and chondroitin 6-sulfate unit accounted for about 80 and 20%, respectively, of total radioactivity (data not shown). On the other hand, peak B was shown to be resistant to digestion not only with chondroitinases but also other glycosaminoglycan lyases such as heparitinase and keratanase. This component may be sulfated glycopeptides derived from core-protein(s) by Pronase digestion (15). These observations indicate that the labeled material in fraction I is a proteoglycan bearing chondroitin sulfate as glycosaminoglycan side chains.

The labeled material in fraction III, obtained in the bottom half of the tube by ultracentrifugation, was eluted from Sepharose CL-6B as a single peak with Kav = 0.3, whereas the material digested with Pronase was eluted from the same column as a more retarded peak with Kav = 0.58 (Fig. 2c). The labeled material was also identified to be chondroitin sulfate by its susceptibility to chondroitinases. Based on the elution position of chondroitin sulfates of known molecular weight (16), the molecular weight of the brain chondroitin sulfate was estimated to be 15,000. In the case of fraction III, the amount of peak B (or sulfated glycopeptides) is relatively small (Fig. 2c). The labeled material in fraction II, purified by ultracentrifugation, was eluted from Sepharose CL-6B as a very broad peak (Fig. 2b). This material was also identified to be a proteoglycan bearing chondroitin sulfate with an average molecular weight of 15,000 in the same way described above. Judging from the elution profile shown in Fig. 2b, fraction II seems to be a mixture of fraction I and fraction III.

To confirm that these sulfate-labeled materials are proteoglycans bearing chondroitin sulfate as glycosaminoglycan side chains, the labeled materials were chromatographed separately on Sepharose CL-6B before and after Pronase digestion. The radioactive material in fraction I, recovered in the bottom by ultracentrifugation, was eluted in the void volume of the column (Fig. 2a). After Pronase digestion, the labeled material was eluted as two retarded peaks; the major peak...
purified proteoglycan preparation. As shown in Table I, chondroitin sulfate proteoglycans and hyaluronic were obtained in the PBS-soluble fraction of young rat brains. Hyaluronic was separated from the proteoglycans by column chromatography on DEAE-Sephacel. Each proteoglycan fraction purified by ultracentrifugation accounts for about 20% of DEAE-Sephacel purified proteoglycan preparation (Table II).

Core Proteins of Brain Chondroitin Sulfate Proteoglycans—
All the results shown above suggest that the brain chondroitin sulfate proteoglycan preparation is a mixture of proteoglycans with a different hydrodynamic size and a molecular density. Because there is no significant difference in the size of chondroitin sulfate side chains among fractions I, II, and III (Fig. 2), the difference in the overall size of proteoglycans may be attributable to the difference in the core protein size and/or the number of chondroitin sulfate side chains bound to core proteins. To clarify this, core proteins were prepared by treatment with protease-free chondroitinase ABC of various proteoglycan fractions purified by ultracentrifugation in a CsCl density gradient.

When proteoglycans in fraction I treated with chondroitinase ABC were analyzed by SDS-polyacrylamide slab gel electrophoresis, two protein bands were detected both under reducing and nonreducing conditions (Fig. 3). The proteoglycans before enzymatic treatment did not penetrate into 6% separating gel. Under the conditions used, enzyme protein(s) of chondroitinase ABC could not be detected, and only bovine serum albumin, which had been added to the enzyme preparation as a stabilizer, could be stained with Coomassie Blue (see fraction E in Fig. 3). Therefore, it is concluded that these two protein bands represent core proteins derived from chondroitin sulfate proteoglycans by the enzymatic treatment. The molecular weight of the major core protein was estimated to be 250,000 (designated as 250K protein) using a molecular weight marker, Electran 56–280 kDa (British Drug House), and the molecular weight of the minor was estimated to be 220,000 (designated as 220K protein).

Proteoglycans in fraction III penetrated slightly into 6% gel (Fig. 3). Chondroitinase treatment of the proteoglycan fraction yielded two major protein bands and one faint band in addition to small amounts of 250K and 220K proteins (Fig. 3). The molecular weights of these core proteins were estimated to be 150,000 (150K protein) and 130,000 (130K protein) for the major ones, and 93,000 (93K protein) for the faint one under reducing conditions. Under nonreducing conditions, 150K band and 93K band migrated more, while the migration position of 130K band remained unchanged (Fig. 3). Digestion of proteoglycans in fraction II with protease-free chondroitinase ABC produced all these five core proteins, and 220K protein was the major (Fig. 3). This observation indicates that fraction II is a mixture of proteoglycan species detected in fractions I and III, as expected from the elution profile on Sepharose CL-4B (see Fig. 1).

These findings show that the chondroitin sulfate proteoglycan preparation, extracted from young rat brains by PBS in the presence of protease inhibitors, is a mixture of proteoglycans with different core protein size. Table III shows the amino acid compositions of 250K, 220K, 150K, and 130K core proteins. These core proteins have similar, but clearly different, amino acid compositions. For example, the largest component, 250K protein, has a higher content of serine and aspartic acid plus asparagine, and a lower content of glycine and alanine, than others. The amino acid composition of 93K core protein was not determined due to the small amount available.

Chondroitin Sulfate Proteoglycans from Different Regions of the Brain—It is of interest to examine whether these five chondroitin sulfate proteoglycans could be isolated from various regions of the brain or whether there would be a difference in the composition of these proteoglycans among different areas of the brain. Chondroitin sulfate proteoglycans were extracted with PBS in the presence of protease inhibitors from the cerebellum, the brainstem, and the cerebrum of 10-day-old rat brain, and purified by stepwise elution from DEAE-Sephacel followed by ultracentrifugation on a CsCl density gradient under dissociative conditions. The yield of chondroitin sulfate proteoglycans during the purification procedure were summarized in Table IV. Extractability of chondroitin sulfate proteoglycans was about 45% in the cerebellum, about 50% in the brainstem, and about 80% in the cerebrum (data not shown, but see Table I for the method). The reason why the extractability is relatively low in the cerebellum and the brainstem is not known.

Core proteins were prepared from the purified chondroitin

![Fig. 3. SDS-polyacrylamide slab gel electrophoresis of proteoglycan fractions (I, II, and III) and core proteins obtained from the proteoglycan fractions by digestion with protease-free chondroitinase ABC. Lane 1, chondroitin sulfate proteoglycans before enzymatic digestion; lane 2, chondroitinase-digested proteoglycans; lane 3, chondroitinase-digested proteoglycans treated with dithiothreitol (DTT). Enzyme reaction mixture without proteoglycan substrate was analyzed under reducing conditions with dithiothreitol (fraction E). Only bovine serum albumin (BSA), which had been added to the chondroitinase preparation as a stabilizer, was stained with Coomassie Blue.](attachment:image)
TABLE IV
Purification of chondroitin sulfate proteoglycans extracted from different regions of 10-day-old rat brain

<table>
<thead>
<tr>
<th>Experimental step</th>
<th>Cerebellum</th>
<th>Brainstem</th>
<th>Cerebrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole tissue</td>
<td>35.3 (100)*</td>
<td>170 (100)</td>
<td>503 (100)</td>
</tr>
<tr>
<td>PBS extractb</td>
<td>19.0 (53.8)</td>
<td>72.3 (42.5)</td>
<td>318 (63.2)</td>
</tr>
<tr>
<td>DEAE-Sephacel (0.35-0.70 M NaCl)</td>
<td>3.80 (10.8)</td>
<td>32.5 (19.0)</td>
<td>154 (30.6)</td>
</tr>
<tr>
<td>Ultracentrifugation (&gt;1.4 Svedberg units)</td>
<td>3.24 (9.2)</td>
<td>27.6 (16.2)</td>
<td>128 (25.4)</td>
</tr>
</tbody>
</table>

* The values in parentheses represent the yield expressed as the percentage.

PBS extract contained both chondroitin sulfate proteoglycans and hyaluronic acid, and hyaluronic acid was largely separated from chondroitin sulfate proteoglycans by stepwise elution (0.35 and 0.70 M NaCl) from DEAE-Sephacel.

The relative amounts of these protein bands among various regions of the brain (Fig. 4). The largest component, 250K protein, was the major one both in the cerebellum (Fig. 4a) and in the brainstem (Fig. 4b), but the amounts of 220K and 150K proteins in the cerebellum were relatively smaller than those in the brainstem. In the cerebrum (Fig. 4c), 220K protein gave the highest peak, and an almost negligible amount of 93K protein was detected. The relative amounts of 150K and 130K proteins in the cerebrum were larger than those in other regions of the brain.

Two-dimensional Mapping of Proteinogenic Peptides from Core Proteins—[35S]Methionine-labeled core proteins from chondroitin sulfate proteoglycans purified from whole brains of 10-day-old rats, to which [35S]methionine was administered intracerebrally on day 9 after birth. Denosimetric scans of the gel stained with Coomassie Blue (a) and its fluorogram (b) are shown. Ordinate, arbitrary unit of optical density; abscissa, mobility expressed as a percentage referred to that of bovine serum albumin (BSA).

Fig. 4. SDS-polyacrylamide slab gel electrophoresis of core proteins prepared from the purified chondroitin sulfate proteoglycan preparation of the cerebellum (a), the brainstem (b), and the cerebrum (c). Proteoglycans were digested with protease-free chondroitinase ABC and then analyzed by gel electrophoresis under reducing conditions. Denosimetric scans of the gel stained with Coomassie Blue are shown; Ordinate, arbitrary unit of absorbance at 550 nm; abscissa, mobility expressed as a percentage referred to that of bovine serum albumin (BSA). Arrowheads indicate a polypeptide component from the chondroitinase ABC preparation.
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FIG. 6. Two-dimensional mapping of tryptic peptides prepared from [35S]methionine-labeled core proteins of brain chondroitin sulfate proteoglycans. Each band of the core protein separated by SDS-polyacrylamide slab gel electrophoresis (Fig. 5) was cut out and digested with trypsin. The resultant proteolytic peptides were mapped two-dimensionally on a silica gel thin-layer plate. a, fluorogram of the peptide map from 250K protein; b, traced figure of the fluorogram. Traced figures of the peptide maps from 220K protein (c) and 150K protein (d) are also shown. Of the peptide spots visualized by fluorography, the darkest five are indicated by closed spots. Arrowhead shown in a indicates the origin.

6d). Since the tryptic peptides of 220K protein were mapped differently from those of 150K protein (Fig. 6, c and d), one can consider that these core proteins have different amino acid sequences. This conclusion was also drawn from the two-dimensional mapping of chymotryptic peptides from [35S]methionine-labeled core proteins (data not shown).

DISCUSSION

We have demonstrated that some chondroitin sulfate proteoglycan species with a different molecular size of the core protein moiety can be isolated from the developing rat brain. Since there was no significant difference either in the molecular size of chondroitin sulfate side chains (Fig. 2) or in the relative amounts of chondroitin sulfate disaccharide units, one may consider that proteoglycans with a lower molecular weight might be artificial proteolytic products of proteoglycans with a higher molecular weight. Alternatively, the difference in the core protein size of proteoglycans would be attributable only to a different content of oligosaccharides bound to core proteins. However, these possibilities can be ruled out at least in the cases of 250K, 220K, and 150K core proteins, because significant differences in the amino acid composition (Table III) as well as in the amino acid sequence (Fig. 6) are found among these core proteins. Therefore, it is concluded that at least three distinct types of chondroitin sulfate proteoglycan occur in the developing rat brain. A chondroitin sulfate proteoglycan fraction prepared from young rat brains by Aquino et al. (8) should correspond to fraction I in our present work, judging from their purification procedure composed of PBS extraction, DEAE-cellulose column chromatography, and gel filtration on Sepharose CL-6B.

Aquino et al. (8, 17) raised an antiserum against their chondroitin sulfate proteoglycan preparation from young rat brains and demonstrated immunohistochemically that the antibody could react with various sites in cerebral tissue at various developmental stages but neither with extracellular space of cartilage nor with pericerebral soft connective tissues. It is well known that cartilage contains a distinct proteoglycan bearing largely chondroitin sulfate chains as polysaccharide side chains (1). The molecular weight of the core protein of the cartilage-unique chondroitin sulfate proteoglycan, PG-H, has been reported to be 340,000-400,000 (2, 18, 19). Soft connective tissues have been shown to contain another type of chondroitin sulfate proteoglycan, PG-M, with a core protein of $M_\text{r} = 500,000-550,000$ (2, 20). Our present data, together with these findings reported by others (2, 8, 17), indicate that some of the brain chondroitin sulfate proteoglycans are different structurally as well as immunologically from the proteoglycan species of connective tissues.

What is the biological significance of the fact that the brain contains at least three distinct species of chondroitin sulfate proteoglycan? At this moment, we do not have the exact
answer to this question. However, there is a possibility that each cell type in the brain synthesizes and accumulates its own chondroitin sulfate proteoglycan molecule structurally different from other molecules synthesized by other cell types. Neural cells and glial cells are the major cell populations in the brain. Additionally, vascular endothelial cells also exist in the brain. These cells and some cell lines with properties similar to those of neural or glial cells can now be maintained separately in culture. Biochemical studies have been done on proteoglycans synthesized by these cells in culture. For example, human normal glial cells in culture have been shown to synthesize and secrete into the culture medium a chondroitin sulfate proteoglycan with a hydrodynamic size almost identical to that of proteoglycans in fraction I (see Fig. 1) of our present work (21). Fraction I contained two different proteoglycans with 250K core protein and 220K core protein (Fig. 3). Because the structure of the core protein of the glial chondroitin sulfate proteoglycan is not known, it is uncertain which is the proteoglycan species synthesized by glial cells.

Recently, a chondroitin sulfate proteoglycan with a core protein of \( M_r = 280,000 \) was isolated from embryonic chick brains (22). This proteoglycan is a neuron-associated ligand for cytotactin, which is known to be involved in neuron-glia adhesion. Because chicken glial cells did not synthesize this proteoglycan molecule, this proteoglycan is supposed to be synthesized by neuronal cells. The largest chondroitin sulfate proteoglycan with a core protein of \( M_r = 250,000 \) in our present study could be the counterpart in the developing rat brain, although there seems to be a slight difference in the molecular weight of the core protein. This difference might be attributable to a different oligosaccharide content in their core proteins.

We have partially characterized sulfated proteoglycans synthesized either by a mouse neuroblastoma-derived cell line, neuro 2a cells, or by a rat pheochromocytoma-derived cell line, PC12 cells (23). Both cell lines synthesized and secreted into the culture medium a chondroitin sulfate proteoglycan with a hydrodynamic size and a molecular density closely similar to those of the brain chondroitin sulfate proteoglycans in fraction III (see Fig. 2). Neuro 2a cells are known to retain many neuronal features such as the presence of neuron-specific proteins and enzymes involved in neurotransmitter metabolism (24) and, therefore, have been used as an \textit{in vitro} experimental model for neuroblasts in the central nervous system. Similarly, PC12 cells are considered to be an \textit{in vitro} experimental model for peripheral neurons (25). From these observations, one may consider that the chondroitin sulfate proteoglycan with 150K core protein and/or the proteoglycan with 130K core protein in fraction III are synthesized by neuronal cells in the brain. Further studies on the structure of these proteoglycan core proteins are underway in this laboratory.

In the brain, there is a network of microvessels composed of vascular endothelial cells. Cultured vascular endothelial cells have been shown to synthesize some sulfated proteoglycan species, including a chondroitin sulfate proteoglycan which is found preferentially in the culture medium (26). This chondroitin sulfate proteoglycan had a hydrodynamic size almost identical to those of proteoglycans species yielded in fraction III in the present work. Although only the identity in the hydrodynamic size is not the sufficient criterion, it is possible to suppose that either of the chondroitin sulfate proteoglycans in fraction III is an extracellular matrix component synthesized by brain capillary endothelial cells.

REFERENCES