A Mapping Technique for Probing the Structure of Proteoglycan Core Molecules*

Yasuteru Oike, Koji Kimata, Tamayuki Shinomura, and Sakaru Suzuki†
From the Department of Chemistry, Faculty of Science, Nagoya University, Nagoya 464, Japan
Noriko Takahashi
From the School of Medicine, Nagoya City University, Nagoya 467, Japan
Kazushi Tanabe
From the Aichi Cancer Research Institute, Nagoya 464, Japan

Our previous work showed that treatment of chick embryo cartilage proteoglycan (PG-H) with chondroitinase-AC II and keratanase yielded a protein-rich core fraction having enzymatically modified linkage oligosaccharides. The core sample has now been analyzed by tryptic peptide mapping, in which the isolated core sample contained in a single Coomassie blue-staining band from a dried slab gel is radioiodinated and treated with trypsin, and the resultant tryptic peptides are displayed two-dimensionally on a silica gel thin layer plate. The map thus obtained exhibited 22 major peptide spots, the resolution and location of which were reproducible.

In order to identify regions of the core polypeptide from which the tryptic peptides are derived, PG-H was cleaved with clostripain under conditions that yield a hyaluronic acid-binding fragment with an apparent Mr = 150,000 and chondroitin sulfate-peptide clusters of smaller molecular sizes. Although the peptide maps of the two size classes of clostripain fragments differed significantly from each other, the patterns of spots, as a whole, were extensively similar to those observed with the intact core molecule.

These results have provided additional evidence that PG-H has a single, nonvariable core protein structure. In addition, the technique used here will provide a versatile method for the identification of genetic types in this increasingly complex family of matrix macromolecules.

A detailed knowledge of the structure of proteoglycans obtained from various tissues is an important prerequisite for any study attempting to elucidate the biological significance of these substances. Various types of proteoglycans have been described which differ in molecular weight, hydrodynamic size, buoyant density, amino acid composition, hyaluronate-binding activity, or glycosaminoglycan composition (for chick embryo cartilage proteoglycans, see Refs. 2-7). However, it is not yet established whether the difference in these properties is due to a true heterogeneity in which distinct genetic types of core polypeptide are present or to a heterodispersity arising from post-translational processing events such as variable attachment of glycosaminoglycan chains or variable partial degradation of core polypeptide chains.

We have previously (8) presented an enzymatic procedure for selective removal of the bulk of the chondroitin sulfate and keratan sulfate of PG-H without cleavage of peptide bonds. Upon zonal sedimentation on a sucrose gradient in 0.5% SDS, the resultant core preparation, PG(-CS,KS), gave a single sharp band with a sedimentation coefficient of 6 S, suggesting that PG-H is a single, albeit polydispersed, population of molecules. In the present work, the possibility has been examined more rigorously that the core molecule from PG-H may contain a single polypeptide. The method described here for probing the structure of the core polypeptide includes two-dimensional peptide mapping of the enzymatically prepared core molecules in amounts as small as 5 μg, a technique which may also find application in establishing the presence of genetic variants of proteoglycan.

EXPERIMENTAL PROCEDURES

Materials

[sulfate-35S]PG-H (specific activity 2.0 × 105 cpm/μmol hexuronate) and unlabeled PG-H were isolated from [15S]sulfate-labeled and unlabeled chick embryo epiphyses, respectively, as previously described (8). [35S]PG(-CS) and [35S]PG(-CS,KS) were prepared from [sulfate-35S]PG as previously described (8). Chondroitinase-AC II (9), keratanase (endo-P-D-galactosidase from Pseudomonas sp.) (10), and hyaluronic acid (M, > 850,000) were products of Seikagaku Kogyo Co., Tokyo, and were kindly donated by Dr. T. Okuyama of the company. The commercial enzyme preparations are available from the above company or from Miles Laboratories, Elkhart, IN. Pepstatin (11) was a gift from Dr. H. Umezawa, Institute of Microbial Chemistry, Tokyo. The reagent is available commercially from Protein Research Foundation, Ina, Minoh, Japan, or from Sigma. Na2[35S]iodide (carrier-free) was purchased from the Radiochemical Centre, Amersham, England; trypsin (33 units/mg) and clostripain were from Boehringer-Mannheim Yamanouchi, Tokyo; phenylmethylsulfonyl fluoride and bovine serum albumin (fraction V) were from Sigma; autoradiography enhancer ENHANCE was from New England Nu.

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† To whom correspondence should be addressed.

‡ The abbreviations used are: PG-H, proteoglycan Type H (the major proteoglycan component of chick embryo cartilages); SDS, sodium dodecyl sulfate; PG(-CS)AC, core molecule obtained from PG-H by selective removal of the chondroitin sulfate chains with chondroitinase-AC II; PG(-CS,KS), core molecule obtained from PG-H by selective removal of the chondroitin sulfate and keratan sulfate chains with chondroitinase-AC II and keratanase, respectively; 22K, 35K, 150K, M, = 22,000, 35,000, 150,000, etc.
clear; x-ray film for \(^{3}S\) fluorography (Fuji RX) was from Fuji Film Co., Tokyo; x-ray film for \(^{3}P\) fluorography (Fuji Orthopho) and Comassie brilliant blue R-250 were from Eastman; Diaflo PM-10 membranes were from Amicon; Sepharose CL-2B, CL-4B, and CL-6B were from Pharmacia Japan, Tokyo; and acrylamide, N,N'-methylene bisacrylamide, and other chemicals for the preparation of the electrophoresis gels were from Nakarai Chemicals, Kyoto. Silica gel-coated thin layer plates (20 x 20 cm, 0.25-mm thickness) were from E. Merck, West Germany. A 1-cm width of silica gel layer was cut from each edge, and the plates were stored in a desiccator until use. Bovine serum albumin and RNA polymerase subunits \(\alpha, \beta, \text{and } \beta',\) obtained from Boehringer-Mannheim Yamanouchi were used as molecular weight markers in SDS-polyacrylamide gel electrophoresis. All of the other chemicals were of the highest purity commercially available.

Methods

Preparation of Core Molecules from PG-H by Digestion with Chondroitinase-AC II and Keratanase—The preparative sequence is shown in Scheme 1. In each series of experiments, 125 \(\mu\)g (as protein) of purified PG-H was treated with 4 units of chondroitinase-AC II in the presence of 10 \(\mu\)mol EDTA, 10 \(\mu\)M N-ethylmaleimide, 5 \(\mu\)M phenylmethanesulfonyl fluoride, and 0.36 \(\mu\)M pepstatin (hereinafter the mixture of the four reagents will be referred to as “protease inhibitor mixture”), under the conditions described in our previous paper (8). The reaction was terminated by adding SDS to a final concentration of 2% (w/v) followed by heating at 70 \(^\circ\)C for 30 min. A 3-ml portion of the digest was applied to a Sepharose CL-4B column (1.5 x 91 cm) eluted with 0.2% (w/v) SDS, 40 \(\mu\)M Tris/HCl, pH 7.4, 5 \(\mu\)M EDTA at 4 \(^\circ\)C. Fractions of 2.3 ml were collected and their absorbance at 280 nm or radioactivity content was determined. The “Results”), was collected by precipitation with ethanol, and treated with keratanase (0.2 unit/120 \(\mu\)g of core protein) in the presence of the protease inhibitor mixture, as previously described (8). The reaction was terminated and the digest chromatographed on a Sepharose CL-4B column, as described above. The core molecule, PG(-CS)\(_{\text{AC}}\), that eluted in the retarded fractions (see “Results”), was collected by precipitation with ethanol. A 100-\(\mu\)g (as protein) portion of the core sample was dissolved in 9 ml of sodium acetate containing the protease inhibitor mixture. The reaction was carried out at 37 \(^\circ\)C for 60 min.

Preparation of Proteoglycan Fragments by Clostripain Digestion—The method which was devised for this purpose is based on the method of Caputo et al. (for Swarn rat chondrosarcoma proteoglycans) (13) with several modifications. The preparative sequence is shown in Scheme 2. All isopycnic centrifugations were at 10 \(^\circ\)C for 50 h at 38,000 rpm (93,000 \(\times \) g,) in a Hitachi RPS-65T rotor. The gradients were each fractionated into 10 fractions and their protein (12) or radioactivity content was determined. The core molecule, PG(-CS,KS), was recovered from the bottom fraction (see “Results”) by precipitation with ethanol.

When PG-H (125 \(\mu\)g as protein) was treated with a mixture of chondroitinase-AC II and keratanase (Scheme 1, Procedure B), the procedures were as above except that 4 ml of an enzyme reagent containing 4 units of chondroitinase-AC II, 0.2 unit of keratanase, 0.1 \(\mu\)M Tris/HCl, pH 7.4, and the protease inhibitor mixture was used and the reaction was carried out at 37 \(^\circ\)C for 60 min.

Scheme 1. Preparative sequence of the different core molecules analyzed by SDS-polyacrylamide gel electrophoresis.
in the presence of 5 mM calcium chloride, 0.6 mM diethiothreitol, 1 mM phenylmethanesulfonyl fluoride, and 0.36 mM pepstatin. The reaction was terminated by adding iodoacetamide to a final concentration of 1 mM. CeCl₃ was added to the reaction mixture to give a density of 1.50 g/ml. This solution was then subjected to isopycnic centrifugation. The gradients were partitioned into three equal fractions, and the bottom one-third (a) and top one-third (b) fractions were treated as follows: (a) this fraction was concentrated to 0.3 volume with an Amicon PM-10 filter and applied to a Sepharose CL-2B column (1.2 x 50 cm) eluted with 0.5 M sodium acetate at 4°C. The eluted fractions were assayed for hexuronate and protein. The chondroitin sulfate-peptide clusters were removed from the gel by washing with 200 ml of acetic acid/formic acid/H₂O (3:1:16, by volume). Other Methods—Hexuronate was determined by the procedure of Bitter and Muir (18) with glucuronolactone as a standard. Protein was determined by the method of Lowry et al. (12) with bovine serum albumin (Fraction V) as a standard. Radioactivity was measured by spotting a sample solution on a paper disc (2.4-cm diameter), the disc was then dried in air at 50°C and counted in an Aloka liquid scintillation spectrometer (Aloka Co., Tokyo) with the solvent system recommended by the manufacturer.

RESULTS

Preparation of Core Molecules from PG-H by Successive Digestion with Chondroitinase-AC II and Keratanase—Core molecules were obtained from PG-H by successive digestion with chondroitinase-AC II and keratanase, essentially as described in our previous paper (8). The preparative sequence is illustrated in Scheme 1 (Procedure A). At each step of the digestion and fractionation procedures, aliquots of the products were examined by SDS-polyacrylamide gel electrophoresis (Fig. 1). Since our previous studies (8) had indicated the presence of [³⁵S]sulfate residues in the PG(-CS,KS) preparation, it was due to the fact that although chondroitinase-AC II removes all the sulfated sugar units on the chondroitin sulfate chain, keratanase leaves about 10% of the sulfated sugar units on the keratan sulfate chain (8).

In the preparation of PG-H, no Coomassie blue-positive band was detected (Track a), although [sulfate-³⁵S]PG-H (internal marker), detected by fluorography (Track b), was located at the origin; probably the core protein is masked by the preponderance of glycosaminoglycan chains. When PG-H was digested with chondroitinase-AC II and an aliquot of the digest was mixed with [³⁵S]PG(-CS)Ac and [³⁵S]PG(-CS,KS), prepared from [³⁵S]sulfate-labeled PG-H, advantage was taken in the present study of the fact that core molecules derived from unlabeled PG-H can be readily distinguished from other proteins using the labeled compounds as internal or external standards. The presence of [³⁵S]sulfate residues in the PG(-CS,KS) preparation is due to the fact that although chondroitinase-AC II removes all the sulfated sugar units on the chondroitin sulfate chain, keratanase leaves about 10% of the sulfated sugar units on the keratan sulfate chain.

In order to remove the contaminating materials which would interfere with subsequent digestion or fractionation procedures, the rest of chondroitinase digest of unlabeled PG-H was chromatographed on a column of Sepharose CL-4B, as described under "Experimental Procedures." For comparison, a chondroitinase digest of standard [sulfate-³⁵S]PG-H was chromatographed under the same conditions. As we have described (8), chondroitinase-AC II released about 94% of the [³⁵S] in [sulfate-³⁵S]PG-H as small sized fragments, leaving residual [³⁵S] on a core fraction, PG(-CS)Ac, that eluted in Fractions 25 to 42 (Fig. 2A). When the unlabeled sample was chromatographed under the same conditions, UV (280 nm)-absorbing fractions were diffuse and were not well separated (Fig. 2B). However, analysis on polyacrylamide gels of the column eluates indicated that the material in Area I is almost entirely composed of PG(-CS)Ac (Fig. 1, Track d) whereas Area II contains no PG(-CS)Ac. The material in Area II appears to be derived from the enzyme preparation, as the electrophoretogram (not shown) was essentially identical with acetic acid/formic acid/H₂O (3:1:16, by volume) using a Pharmacia flat bed apparatus PBE 3000 with cooling at 4°C. Chromatography was carried out in 1-butanol/pyridine/acetic acid/H₂O (10:12:8:2, by volume) for about 5 h until the front reached the top. The plate was dried and peptides were located by radioautography with Kodak X-OMAT R film.
that shown in Fig. 1, Track c. The large peak at the total bed volume (Fractions 63 to 79 in Fig. 2B) was not further investigated; probably it represents phenylmethanesulfonyl fluoride, pepstatin, and N-ethylmaleimide, i.e. the UV-absorbing materials of low molecular weight present in the incubation mixture.

The material in Area I of Fig. 2B was recovered by ethanol precipitation; the yield expressed as protein measured by the method of Lowry et al. (12) was 120 µg from 125 µg of PG-H.

The PG(-CS, KS) sample obtained as above was further treated with keratanase. When examined on polyacrylamide slab gels, the keratanase digest (Fig. 1, Track e) contained a highly diffuse band due to the proteins present in the enzyme preparation (see Track f for the pattern of the enzyme alone). However, a single protein band, identified in Fig. 1, Track e, as PG(-CS, KS), could be detected which was more compact and migrated more rapidly than the band from the parent molecule (Fig. 1, compare Tracks d and e). As the fluorogram (Track o) shows, this component migrated with standard [35S]PG(-CS, KS).

In order to remove the contaminating proteins, the keratanase digest (unlabeled sample) was chromatographed on a column of Sepharose CL-4B. For comparison, a keratanase digest of [35S]PG(-CS), was also run under the same conditions. From the 35S profile of the digest of standard [35S]PG(-CS), (Fig. 3A), it was expected that unlabeled PG(-CS, KS) product eluted in Fractions 30 to 42 (Fig. 3B; Area I). That this was indeed the case was shown by analysis of the column eluates in Area I by polyacrylamide gels (cf. Fig. 1, Track g). The fractions in Area II, in contrast, produced a diffuse pattern without discrete bands (not shown).

When the material in Area I of Fig. 3B was recovered by ethanol precipitation and analyzed by gel electrophoresis, it was apparent that considerable purification of PG(-CS, KS) was realized (Fig. 1, compare Tracks e and g). However, the preparation still contained some of the proteins from the enzyme preparation which gave a diffuse band overlapping the PG(-CS, KS) band on gel electrophoresis.

The material from Area I of Fig. 3B was therefore subjected to CsCl isopycnic centrifugation for further purification (Fig. 4B). For comparison, [35S]PG(-CS, KS) was centrifuged under the same conditions (Fig. 4A). Comparison of the sedimentation profiles in A and B indicated that most of the contaminating proteins are less dense than [35S]PG(-CS, KS). In fact, it was possible to obtain a PG(-CS, KS) preparation from the bottom fraction (Fraction 1) of CsCl isopycnic centrifugation (Fig. 4B) which showed, on subsequent gel electrophoresis, a sharp band of PG(-CS, KS) practically devoid of other protein bands (Fig. 1, Track h). The yield of PG(-CS, KS) expressed as protein measured by the method of Lowry et al. (12) was 65 µg from 120 µg of PG(-CS),. Although analysis on polyacrylamide gels indicated that a significant amount of PG(-CS, KS) was present in Fraction 2 in the CsCl gradient (Fig. 4), the core molecule could not be isolated from this fraction because of the presence of a large amount of other proteins.

Preparation of PG(-CS, KS) by Simultaneous Digestion with Chondroitinase-AC II and Keratanase—The above procedure for preparing PG(-CS, KS) is both exacting and time-consuming. Consequently, the need arose for the development of a simpler method which can more readily be applied to the routine preparation of core molecules from proteoglycan samples. Such a method, based on the use of an appropriate mixture of chondroitinase-AC II and keratanase for the removal of glycosaminoglycan chains, has been devised. A graphic summary of this procedure is presented in Scheme 1 (Procedure B). A separation of the PG(-CS, KS) product was
separately chromatographed on a column of Sepharose CL-4B (1.5 x 91 cm), using 0.2% (w/w) SDS, 0.95-M Tris/HCl pH 7.4, 5 mM EDTA as eluant. [35S]Sulfate-containing products (O---O) and UV (280 nm)-absorbing materials (O---O) are shown. V0, void volume. V1, total bed volume.

Fig. 3 (center). Sepharose CL-4B chromatography of keratanase digest of PG(-CS,KS), [35S]PG(-CS,KS), (A) and unlabeled PG(-CS)Ac (B) were each treated with keratanase and separately chromatographed on a column of Sepharose CL-4B (1.5 x 91 cm), using 0.2% (w/w) SDS, 40 mM Tris/HCl pH 7.4, 5 mM EDTA as eluant. [35S]Sulfate-containing products (O---O) and UV (280 nm)-absorbing materials (O---O) are shown. V0, void volume. V1, total bed volume.

Fig. 4 (right). CsCl isopycnic centrifugation of crude PG(-CS,KS) sample. Sedimentation profiles of (A) a purified sample of [35S]PG(-CS,KS) and (B) a crude PG(-CS,KS) sample from Area I of Fig. 3B are shown. Each tube was punctured at the bottom, 0.95-ml fractions were collected, and their radioactivity (O---O) or protein content (O---O) determined.

Fig. 5. Tryptic peptide mapping of PG(-CS)Ac and PG(-CS,KS). Experiments were performed as described in the text. The core molecule samples were labeled with 35S in the gel slices and tryptic peptides were displayed on silica gel thin layer plates by electrophoresis in the first dimension and ascending chromatography in the second dimension. Radioautographs are shown.

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of the plates. The occurrence of these relatively immobile components can be explained by assuming that they represent peptides of relatively large size or those bearing carbohydrate chains. The interpretation is consistent with the fact that the profiles of mobile peptide products were essentially the same regardless of whether or not a large portion of the keratan sulfate chains had been removed from the core molecule.

Comparison of \[^{125}\text{I}\] Peptide Maps of Fragments Produced by Clostripain Digestion of PG-H—Proteases have been used to fragment proteoglycan molecules selectively to define different functional regions for study. For example, Caputo et al. (13) have shown that, after clostripain digestion of proteoglycan aggregates from Swarm rat chondrosarcoma, the hyaluronic acid-binding region remains bound with the link protein to hyaluronic acid while most of the core protein with covalently bound glycosaminoglycan chains is removed. Subsequent density gradient and molecular sieve techniques using dissociative solvents have been used to isolate the hyaluronic acid-binding region.

In order to identify regions of the core polypeptide from which the tryptic peptides are derived, core protein fragments of limit size were prepared from PG-H aggregates by a modification of the clostripain method, as outlined in Scheme 2 and in the legend to Fig. 7, and were separately subjected to tryptic peptide mapping (Figs. 8 and 9). In the fragmentation procedure with clostripain, the following two modifications are particularly important: 1) the addition of hyaluronic acid to the extract of cartilages to make up the natural deficiency of hyaluronic acid, and 2) the use of phenylmethylene sulfonyl fluoride and pepstatin to prevent nonspecific cleavage of peptide bonds caused by other protease activities present in the clostripain preparation. When the exogenous supply of hyaluronic acid was omitted, the yield of proteoglycan-link protein-hyaluronic acid aggregates was reduced by about 50%. Omission of the protease inhibitors from the incubation with clostripain caused about 90% reduction in the yield of the complex of hyaluronic acid-binding region, link protein, and hyaluronic acid.

Aliquots (40 \(\mu\)g as protein) of the fragment samples thus obtained were further treated with chondroitinase-AC II in the presence of protease inhibitors and applied to 5% polyacrylamide slab gels. The gels were stained with Coomassie blue (Fig. 7). Before chondroitinase-AC II digestion, neither the complex of hyaluronic acid-binding region, link protein, and hyaluronic acid nor the chondroitin sulfate-peptide clusters penetrated the gel (Tracks a and c). In the case of the complex of hyaluronic acid-binding region, link protein, and hyaluronic acid, it was noticed that the hyaluronic acid component forms a jelly-like layer at the origin of the slab gel, thereby interfering with penetration of the protein components. After chondroitinase-AC II digestion, the hyaluronic acid-binding region, link protein, and hyaluronic acid complex produced two discrete bands corresponding in mobility to link protein and its fragment (Track b, as in a but treated with chondroitinase-AC II; Track c, the chondroitin sulfate-peptide clusters (CPC) obtained as in Scheme 2; Track d, as in c but treated with chondroitinase-AC II. X, proteins from the chondroitinase preparation. In order to estimate approximate molecular weights of the fragments, the gel slab was calibrated using bovine serum albumin (\(M_\text{r} = 68,000\)) and RNA polymerase subunits \(a (M_\text{r} = 30,000), \beta (M_\text{r} = 155,000), \) and \(\beta' (M_\text{r} = 165,000)\).

The apparent size of the hyaluronic acid-binding region fragment is almost twice as large as that observed for bovine or rat proteoglycans (13). The reason for this discrepancy is unknown, but it is noteworthy that the 150K fragment could not be an intermediate produced by an incomplete digestion. The time course of clostripain digestion has shown that the electrophoretic profiles of the fragments are very similar at all time points from 4 to 6 h, whereas higher molecular weight intermediate profiles were obtained from a 2- or 3-h digest.

After chondroitinase-AC II digestion, the chondroitin sulfate-peptide clusters produced a diffuse weakly staining pattern (track d; 50-80K) without discrete bands. The feeble Coomassie blue reaction suggests that the 50-80K fragments contain a large number of covalently bound carbohydrate chains such as chondroitin sulfate remnants, intact keratan sulfate chains, and N- and O-linked oligosaccharide chains.

Note that the 35K and 22K samples were not reduced prior to gel electrophoresis. The link protein from rat chondrosarcoma has been shown to migrate either as a protein with an approximate \(M_\text{r} = 35,000\) to 38,000 (before reduction) or \(M_\text{r} = 45,000\) (after reduction) on the same gel (13). The 35K protein, but not the 22K protein, has been obtained directly from the aggregate of proteoglycan, link protein, and hyaluronic acid (Y. Oike, K. Kimata, T. Shinomura, S. Suzuki, N. Takahashi, and K. Tanabe, unpublished observations), suggesting that the 22K protein is derived from the 35K protein by cleavage with clostripain.
Tryptic peptide mapping was performed with the gel slices of the hyaluronic acid-binding fragment (Band 150K, Track b) and chondroitin sulfate-peptide clusters (Band CPC, Track c). The radioautographs and traced figures are shown in Figs. 8 and 9. Because the maps of the 150K and chondroitin sulfate-peptide cluster fragments are quite different (of the 22 major spots, only three are common), it is apparent that there are considerable differences between these fragments in their polypeptide structures. However, comparison between the maps in Fig. 9 and the map of PG(−CS)Ac or PG(−CS,KS) in Fig. 6 indicates that there are many common spots between the intact core molecule and each of the clastriprain fragments. Of the 22 major spots detected in the map of PG(−CS,KS), 13 and 12 spots were produced from the hyaluronic acid-binding fragment and chondroitin sulfate-peptide clusters, respectively.

Fig. 10 shows the result of mapping of a reconstructed core protein sample, in which the 125I-labeled peptides released from the 150K and chondroitin sulfate-peptide cluster gel slices were combined before mapping on the thin-layer plate. It is clear that the major spots (1 to 22) are homologous to those in the maps of the original core molecule (Fig. 5).

**DISCUSSION**

The combined results of the variety of experiments described here and in the previous paper (8) constitute strong evidence that PG-H has a single, nonvariable core protein structure. In the previous study, the core fraction prepared from [14C]serine-labeled PG-H by digestion with both chondroitinase-AC II and keratanase sedimented as a narrow band with a sedimentation coefficient of 6 S in velocity gradients. In the present work, the use of SDS-polyacrylamide gel electrophoresis for resolution of core molecules and the tryptic peptide mapping procedure described by Elder et al. (16) permitted us to obtain further evidence to support the view that the core has a uniform structure. Thus, SDS-polyacrylamide gel electrophoresis of PG(−CS,KS) followed by Coomassie blue staining revealed a single narrow band (Fig. 1). Radioiodination of the core molecule, followed by trypsin digestion, resulted in a highly reproducible peptide map (Fig. 5). Furthermore, clastriprain cleavage of the proteoglycan into two size classes of fragments, followed by tryptic peptide mapping, indicated that, although the patterns of peptide spots of these fragments are different from each other (Fig. 8), the combined peptide patterns (Fig. 10) are very similar to those observed with the intact core molecules (Fig. 5). Should the core protein have been variable, the probability of obtaining such a combination of consistent results by chance would have been exceedingly small. Several recent reports have provided evidence that the newly synthesized core proteins of proteoglycans have a uniform size prior to the addition of glycosaminoglycan chains (19–22). Since the unlabeled samples treated in the present work represent proteoglycan molecules accumulated in the cartilage matrix of 12-day chick embryos, our results suggest that there was no significant processing or degradation of the core protein during the time of accumulation of PG-H molecules in the matrix, although a highly selective cleavage of peptide bonds by specific peptidases could not be ruled out by the present data.

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3 Since successful maps were obtained directly from the chondroitin sulfate-peptide clusters which were not treated with chondroitinase-keratanase and which did not penetrate the gel, it is expected that intact PG-H could be radioiodinated and mapped directly without recourse to either the enzyme digests or the SDS gel. Our preliminary experiments have indeed shown that the peptide map obtained directly from PG-H is very similar to that obtained from PG(−CS)Ac or PG(−CS,KS) (Y. Oike, K. Kimata, T. Shinomura, S. Suzuki, N. Takahashi, and K. Tanabe, unpublished observations).
The similarity of peptide maps of PG(-CS)AC and PG(-CS,KS) (Fig. 5) suggests that the bound keratan sulfate groups do not significantly influence our analysis. This is consistent with the observations of Elder et al. (23) that carbohydrate-carrying peptides do not migrate in the apolar solvent used in the thin layer chromatography. Even after removal of the bulk of the glycosaminoglycan chains, the result of core preparation, PG(-CS,KS), contains the residual linkage oligosaccharide regions of the digested chondroitin sulfate and keratan sulfate (8) and perhaps N- and O-linked oligosaccharides (24). It should be stressed, therefore, that the peptide mapping technique may not detect variations of amino acid residues in carbohydrate-carrying peptide regions, although we do not have information concerning the precise distribution of the carbohydrate groups in the core molecule. Also to be considered is the fact that 125I reacts only with the carbohydrate residues of the core proteins obtained. It is likely that this technique will be useful for characterization of other proteoglycan components (PG-Lt and PG-Lb), isolated from tissues or in cell cultures. 

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