Isolation and Identification of the Major Heparan Sulfate Proteoglycans in the Developing Bovine Rib Growth Plate*

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Heparan sulfate proteoglycans are thought to mediate the action of growth factors. The heparan sulfate-containing proteoglycans in extracts of the bovine fetal rib growth plate were detected using the monoclonal antibody 3G10, which recognizes a neoepitope generated by heparitinase digestion (David, G., Bai, X. M., Van der Schueren, B., Cassiman, J. J., and Van den Berghe, H. (1992) J. Cell Biol. 119, 961–975). The heparan sulfate proteoglycans that react with this antibody were identified using antisera to known proteoglycans; purified using CsCl density gradient centrifugation, molecular sieve, and ion exchange chromatography; and then characterized. The major heparan sulfate proteoglycans in the growth plate had core proteins of 200 kDa and larger and were identified as perlecan and aggrecan. These two heparan sulfate proteoglycans could be effectively separated from each other by CsCl density gradient centrifugation alone. Perlecan contained 25% heparan sulfate and 75% chondroitin sulfate. The heparan sulfate chains on growth plate perlecan were considerably smaller than the chondroitin sulfate chains, and the heparan sulfate disaccharide content was different than that found for heparan sulfate from either kidney, tumor tissue, or growth plate aggrecan. Aggrecan contained only 0.1% heparan sulfate, which was localized to the CS-1 domain of aggrecan. These results indicate that perlecan and aggrecan would be the principal candidate proteoglycans involved in the action of heparan sulfate-binding proteins in the developing growth plate.

The growth of long bones is determined primarily by the chondrocytes in the growth plate, which undergo a transition from a resting state, to a proliferating state and then to a hypertrophic state (1). The growth that occurs during the transition of the chondrocytes through these developmental stages is due to the proliferation and hypertrophy of the chondrocytes themselves as well as the synthesis, secretion, and deposition of an extensive extracellular matrix by the chondrocytes (1, 2). The major proteoglycan in the extracellular matrix of the growth plate is aggrecan, which is primarily and extensively substituted with chondroitin sulfate (3). Biosynthetic studies using bovine rib growth plates show that the highest level of proteoglycan synthesis occurs in the upper hypertrophic zone (4).

A number of studies have shown the growth plate also contains heparan sulfate proteoglycans (5–7), and several lines of evidence indicate that these proteoglycans play an important function in the growth plate. The EXT-1 gene product is an enzyme directly involved in heparan sulfate synthesis; mice that are homozygous for the EXT-1 null mutation do not undergo gastrulation, but heterozygous embryos survive and develop short limbs, and their cells in culture show a 50% reduction in heparan sulfate synthesis (8). Perlecan is a heparan sulfate proteoglycan that was originally identified in basement membranes (9) and later shown also to be present in the cartilaginous matrix of growth plates (10, 11). Mice that are homozygous for a perlecan null mutation have defective growth plates that result in fetal dwarfism (12, 13), and a similar phenotype is caused by functional null mutations of the perlecan gene in the human Silverman-Handmaker-type dyssegmental dysplasia (14). Mutations in the human perlecan gene have also been shown for patients with Schwartz-Jampel syndrome, a less severe skeletal dysplasia (15). It has been proposed that perlecan may act in the growth plate through binding fibroblast growth factor ligands or modulating the diffusion of Indian hedgehog (12, 16).

Whereas heparan sulfate and the heparan sulfate proteoglycan perlecan are clearly important for normal growth plate function during development, a systematic study to isolate, characterize, and identify the heparan sulfate proteoglycans of the growth plate has not been conducted. Reverse transcription PCR has shown that articular chondrocytes contain mRNA for syndecan 4 (17), and the growth plate may contain this gene product or contain another heparan sulfate proteoglycan that is yet to be identified. In this study, we use a commercially available monoclonal antibody to initially survey extracts of the developing bovine growth plate for heparan sulfate proteoglycans. This antibody, referred to as 3G10, recognizes a neoepitope generated by heparitinase digestion but not chondroitinase digestion and has been previously shown to be specific for heparan sulfate proteoglycans (18). We then identified the 3G10-positive core proteins using antisera to known proteoglycans. The results of this study show that the heparan sulfate proteoglycans in the growth plate have core proteins that are 200 kDa or larger. One of these proteoglycans is, as expected, perlecan, and it is the major heparan sulfate proteoglycan of the growth plate, although it also contained chondroitin sulfate. We also found that heparitinase-digested aggrecan contains the neoepitope recognized by the 3G10 antibody and that the digestion releases heparan sulfate disaccharides from aggrecan. Aggrecan is a multidomain proteoglycan consisting of three globular domains (G1, G2, and G3).
diced into 1 2-mm pieces, and placed in 7.5 volumes (w/v) of ice-cold 0.1 M sodium acetate buffer, pH 6.0, containing 2% CHAPS, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 30 mM benzamidine-HCl. The solution was stirred for 10 min, and then an equal volume of ice-cold 8 M guanidine HCl was added. The tissue was extracted with stirring for 16 h at 4 °C. The insoluble material was removed by centrifugation in a Beckman 50.2 Ti rotor at 33,000 rpm for 1 h at 4 °C. The density of the extract was adjusted to 1.22 g/ml by the addition of solid CsCl and centrifuged in a 50.2 Ti rotor for 72 h at 12 °C. The tubes were fractionated into five equal fractions and immunoassayed (see below) for heparan sulfate proteoglycans. Selected fractions were combined, adjusted to 1.33 g/ml using CsCl, and subjected to a second density gradient centrifugation, fractionated into five fractions, and immunoassayed again as before. The uronic acid content of the fractions was determined using carbazole (22).

### Chondroitin and Heparin Lyases—
Chondroitinase ABC and protease-free chondroitinase ABC (both EC 4.2.2.4), chondroitinase ACII (EC 4.2.2.5), heparitinase, and heparitinase I (both EC 4.2.2.8) and heparitinase II (no EC number) were from Seikagaku America, Inc. Heparitinase I (EC 4.2.2.7) was from Glyko.

### Standards—
The unsaturated CS (Di4S, Di6S) and HS disaccharide standards (DiNHS-NS, DiNHS-6S, DiNHS-S1, DiNHS-S2, and DiNHS-triS) were from Seikagaku America. Purified chondroitin sulfate C (from shark cartilage) and heparan sulfate (from bovine kidney) were from Sigma.

### Proteoglycan Purification—
Perlecan was purified from the top three-fifths of the second density gradient centrifugation. The fractions were combined, exchanged by dialysis into 6 M urea containing 0.05 M sodium

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**Fig. 1.** Immunoanalysis of the five fractions from the initial CsCl density gradient. A, Western blot. Aliquots from each of the fractions were electrophoresed without digestion (−) or after digestion with chondroitinase ABC and heparitinase (+). The presence of heparan sulfate-containing proteoglycan core proteins was detected on the blots using the monoclonal antibody 3G10. Heparan sulfate-containing proteoglycans with 200-kDa core proteins and larger were detected in fractions 1–3. B, dot blot of the fractions using an antiserum to perlecan (○) or an antiserum to aggrecan (●). Perlecan is present primarily in fractions 1–3, whereas aggrecan is exclusively in fraction 1.

**Materials and Methods**

**Growth Plate Isolation—**Ribs were obtained from Pel-Freez (Rogers, AR). Ribs were removed from third trimester bovine fetuses that had been obtained immediately after slaughter of pregnant cows and shipped on ice by overnight courier to Shriners Hospitals for Children, Tampa. The perichondrium surrounding the growth plate in the ribs was removed, and the growth plates were dissected from the ribs as previously described (20) and stored at −80 °C. Histological examination of representative growth plates indicated that the growth plate tissue collected was entirely cartilaginous and extended from the hypertrophic zone to and including the resting zone.

**Proteoglycan Extraction and Isolation—**Proteoglycans were extracted and isolated using methods previously shown to be useful for isolating both cell surface and matrix-associated proteoglycans (21). Thirty grams of frozen growth plate slices were thawed, immediately diced into 1–2-mm pieces, and placed in 7.5 volumes (w/v) of ice-cold 0.1 M sodium acetate buffer, pH 6.0, containing 2% CHAPS, 10 mM EDTA, 600 mM amino hexanoic acid, 2 mM phenylmethylsulfonyl fluoride, 20 mM N-ethylmaleimide, and 30 mM benzamidine-HCl. The solution was stirred for 10 min, and then an equal volume of ice-cold 8 M guanidine HCl was added. The tissue was extracted with stirring for 16–20 h.

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The abbreviations used are: CHAPS, 3-[[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DMMB, dimethylmethylene blue; EHS, Engelbreth-Holm-Swarm.
acetate buffer, pH 6.0, and applied at 5 ml/min to a 2.5 × 110 cm column of Q-Sepharose Fast Flow (Amersham Biosciences) previously equilibrated with the same solution of buffered urea. The column was eluted with a 0–1.5 M NaCl gradient in 6 M urea, 0.05 M sodium acetate buffer, pH 6.0, with 25-ml fractions. The elution position of the perlecan was determined by immunoassay, and the perlecan-positive fractions were combined, concentrated to 5–6 ml by ultrafiltration, and exchanged by dialysis into 4 M guanidine HCl containing 0.05 M sodium acetate, pH 6.0. The elution position of perlecan was again determined using immunoassay, and the positive fractions were combined and used in subsequent analyses.

Aggrecan was purified from the bottom one-fifth of the second density gradient centrifugation. The fraction was exchanged into 0.5 M sodium acetate, pH 6.0, by dialysis, adjusted to 1.56 g/ml with CsCl, and subjected to density gradient centrifugation as described above. The bottom one-fifth of the resulting gradient containing aggrecan was exchanged into 4 M guanidine HCl containing 0.05 M sodium acetate, pH 6.0, and fractionated on a column of Sephacryl S-500 as described above. The elution position of aggrecan was determined by dot blot immunoassays. The aggrecan-positive fractions were combined and used in subsequent analysis.

Chondroitin and Heparan Sulfate Analysis—Aliquots of perlecan and aggrecan, purified from the bovine rib growth plate as well as perlecan previously purified from the EHS tumor (9), were assayed for protein content using NanoOrange (Molecular Probes, Inc., Eugene, OR) with bovine serum albumin as a standard and for glycosaminoglycan content using dimethylmethylene blue (DMMB) (23) with chondroitin sulfate C as a standard. Aliquots containing 50–650 μg of

![FIG. 3. Purification of perlecan by column chromatography.](image)

Column fractions were monitored for protein by absorbance at 280 nm and by dot blot using an antiserum to perlecan. A, chromatography of perlecan-containing fractions from the second CsCl density gradient on Q Sepharose. Fractions 3–5 from Fig. 3 were combined and chromatographed on Q Sepharose using a salt gradient. The fractions containing the major perlecan-positive peak (fractions 22–30) were pooled. B, chromatography of fractions 22–30 from Fig. 3A on Sephacryl S-500. The fractions containing the peak positive for perlecan (fractions 24–31) were pooled as purified perlecan.

![FIG. 4. Analysis of purified perlecan by Western blot using the 3G10 monoclonal antibody (3G10) and antiserum to perlecan (anti-perlecan).](image)

UD, undigested; C, digested with chondroitinase ABC; H, digested with heparitinase; C+H, digested with chondroitinase ABC and heparitinase. The perlecan-positive and 3G10-positive core proteins migrate to identical positions.

![FIG. 5. Chromatography of glycosaminoglycans on Superose 6.](image)

A, chromatography of proteinase K-released glycosaminoglycans from purified perlecan and aggrecan. ●—●, total glycosaminoglycan from perlecan; ○—○, total glycosaminoglycan from aggrecan; △—△, chondroitinase ABC digests of total glycosaminoglycan from perlecan; ▽—▽, chondroitinase ABC and heparitinase digests of total glycosaminoglycans from perlecan; ■—■, total glycosaminoglycan from aggrecan; V_o, void volume; V_t, included volume. B, chromatography of heparan sulfate glycosaminoglycans from different sources on Superose 6. ○—○, kidney heparan sulfate; △—△, heparan sulfate from growth plate perlecan; □, heparan sulfate from EHS perlecan.
protein and 10–500 μg of glycosaminoglycan were digested in 0.5 ml of 25 mM ammonium acetate, pH 7.0, containing 125 μg/ml proteinase K (Roche Molecular Biochemicals) for 18 h at 60 °C. The samples were treated at 100 °C for 10 min to inactivate the protease. Portions of the digest were lyophilized, resuspended in 0.2 ml of 0.5 M sodium acetate, pH 6.0, and applied to a Supersose 6 HR 10/30 column (Amersham Biosciences) eluted at 0.3 ml/min with 0.6 M fractions. Aliquots of the fractions were assayed for glycosaminoglycan content using the DMMB assay. Portions of the proteinase K digest were also digested with chondroitinase ABC with or without additional digestion with heparitinase and/or chondroitinase ACII. The samples were incubated for 18 h at 37 °C. The digests were lyophilized, resuspended in 0.2 ml of 0.5 M sodium acetate, pH 7.0, containing 10 μg/ml each chondroitinase ABC and ACII. The samples were incubated for 18 h at 37 °C. The digest was stopped by the addition of an equal volume of 8 M guanidine-HCl, and the solution was treated at 100 °C for 10 min to inactivate the protease. Portions of the digest were lyophilized, resuspended in 0.2 ml of 0.5 M sodium acetate, pH 6.0, and applied to a Supersose 6 HR 10/30 column (Amersham Biosciences) eluted at 0.3 ml/min with 0.6 M fractions. Aliquots of the fractions were assayed for glycosaminoglycan content using the DMMB assay. Portions of the proteinase K digest were also digested with chondroitinase ABC with or without additional digestion with heparitinase and/or chondroitinase ACII as described below and also chromatographed on Supersose 6 or on a Supersose 12 HR 10/30 column (Amersham Biosciences).

To detect CS and HS disaccharide aliquots of the protease-treated samples were dried and were resuspended in 100 μl of 50 mM ammonium acetate, pH 7.0, containing 1 unit/ml each chondroitinase ABC and ACII. The samples were incubated for 18 h at 37 °C and boiled for 5 min, and aliquots were removed for the determination of CS disaccharide composition by capillary zone electrophoresis. The remainder of each sample was transferred to a Microcon 3 filter unit (3000 molecular weight cut-off; Millipore Corp.) and centrifuged at 10,000 × g at 25 °C to separate the CS disaccharide products from the undigested HS-glycosaminoglycan. HS-glycosaminoglycan (0–25 μg by DMMB) was recovered from each filter as above, dried, and resuspended in 50 μl of digestion buffer consisting of 25 mM ammonium acetate and 5 mM calcium chloride, pH 7.0, containing 10 μg of protease-free bovine serum albumin and 2.5 milliunits each of heparinase, heparitinase I, and heparitinase II. Samples were incubated for 6 h at 37 °C, boiled for 3 min, and stored at −80 °C until analyzed by capillary zone electrophoresis. Quantitation of CS (24) or HS (25) disaccharides by capillary zone electrophoresis was performed essentially as described, using a Dionex Capillary Electrophoresis System I (Dionex Corp.). Commercially prepared unlated CS disaccharide standards (ΔDiOS, ΔDi4S, and ΔDi6S) or unsaturated HS standards (ΔDiHS-OS, ΔDiHS-NS, ΔDiHS-SS, ΔDiHS-triS, ΔDiHS-6S, and ΔDiHS-SS) and chondroitinase ABC- and heparitinase-digested and recentrifuged under the same conditions. The digestion was completed using Quantity One software (Bio-Rad).

Freshly prepared disaccharide standards, chondroitin lyase, and heparin lyase products were processed using an ECL kit according to the manufacturer’s instructions (Novex). The primary antisera to aggrecan G3 domain, and perlecan were used at 1:400 dilutions; the monoclonal antibody 3G10 was used at a 1:1000 dilution; and the secondary antibodies, which were coupled to peroxidase, were used at 1:2000 dilutions. The nitrocellulose membranes were probed with antibody (anti aggrecan) and the secondary antibody (anti peroxidase). The monoclonal antibody and both antisera to aggrecan react with the same high molecular weight core protein. Fractions 1 and 2 were combined for use in subsequent experiments. To detect CS and HS disaccharide aliquots of the protease-treated samples were dried and were resuspended in 100 μl of 50 mM ammonium acetate, pH 7.0, containing 1 unit/ml each chondroitinase ABC and ACII. The samples were incubated for 18 h at 37 °C and boiled for 5 min, and aliquots were removed for the determination of CS disaccharide composition by capillary zone electrophoresis. The remainder of each sample was transferred to a Microcon 3 filter unit (3000 molecular weight cut-off; Millipore Corp.) and centrifuged at 10,000 × g at 25 °C to separate the CS disaccharide products from the undigested HS-glycosaminoglycan. HS-glycosaminoglycan (0–25 μg by DMMB) was recovered from each filter as above, dried, and resuspended in 50 μl of digestion buffer consisting of 25 mM ammonium acetate and 5 mM calcium chloride, pH 7.0, containing 10 μg of protease-free bovine serum albumin and 2.5 milliunits each of heparinase, heparitinase I, and heparitinase II. Samples were incubated for 6 h at 37 °C, boiled for 3 min, and stored at −80 °C until analyzed by capillary zone electrophoresis. Quantitation of CS (24) or HS (25) disaccharides by capillary zone electrophoresis was performed essentially as described, using a Dionex Capillary Electrophoresis System I (Dionex Corp.). Commercially prepared unlated CS disaccharide standards (ΔDiOS, ΔDi4S, and ΔDi6S) or unsaturated HS standards (ΔDiHS-OS, ΔDiHS-NS, ΔDiHS-SS, ΔDiHS-triS, ΔDiHS-6S, and ΔDiHS-SS) and chondroitinase ABC- and heparitinase-digested and recentrifuged under the same conditions. The digestion was completed using Quantity One software (Bio-Rad).

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RESULTS

The 4.0 M guanidine extracts of growth plates were adjusted to 1.22 g/ml by the addition of CsCl and centrifuged for 72 h, and the tubes were fractionated into five equal fractions. Western blot analysis of the fractions using the monoclonal antibody 3G10, which reacts with neoepitope generated by heparitinase digestion (18), revealed the presence of heparan sulfate proteoglycan core proteins in the bottom three fractions after heparitinase digestion (Fig. 1A). The core proteins were ~200 kDa and larger in size. Immunodot blot analysis of these fractions with antisera to aggrecan and perlecan showed aggrecan to be in fraction 1, whereas perlecan was primarily in fraction 2 with lesser amounts in fractions 1 and 3 (Fig. 1B). Fractions 1, 2, and 3 were pooled, adjusted to 1.33 g/ml using CsCl, and again centrifuged under the same conditions and fractionated into five parts. Immunodot blot analysis showed aggrecan present primarily in fraction 1, but perlecan was now mostly in fraction 4 with somewhat lesser amounts in fractions 5 and 3 (Fig. 2A). Heparitinase digestion of these fractions followed by Western blot analysis using the 3G10 antibody showed the heparan sulfate proteoglycan core proteins were now in fractions 1, 4, and 5 with lesser amounts in fraction 3 and only a trace in fraction 2 (Fig. 2B, lanes designated 3). The antiserum to perlecan reacted strongly with the core protein bands in fractions 4 and 5 and reacted less strongly with core proteins in fraction 3 (Fig. 2B, lanes designated P). All of the perlecan-positive proteins coincided with the core proteins recognized by the 3G10 antibody. The core protein bands recognized by the 3G10 antibody in lanes 1 and 2, however, were not reactive with the antiserum to perlecan. Analysis of the fractions using the carbazole assay showed that 94.4% of the total uronic acid in the gradient was in fractions 1 and 2 (data not shown).

Fractions 3–5 were pooled, and perlecan was further purified by ion exchange and molecular sieve chromatography. The combined fractions were exchanged into 6 M urea and applied to a column of Q Sepharose. The column was eluted with a salt gradient, and the fractions were assayed by immunodot blot using the antiserum to perlecan (Fig. 3A). The fractions containing the major perlecan-positive peaks eluting late in the gradient (fractions 22–30) were combined, concentrated, exchanged by dialysis to 4 M guanidine HCl, and applied to a column of Sephacryl S-500 (Fig. 3B). The resulting fractions were again assayed by dot blot using the antiserum to perlecan, and the positive fractions (24–31) were combined as purified perlecan and used for additional characterization.

Western blot analysis of the purified perlecan using the antiserum to perlecan showed the proteoglycan as a high molecular weight band migrating above the 210-kDa marker (Fig. 4, Anti Perlecan, lane UD). Digestion with either chondroitinase ABC (lane C) or heparitinase (lane H) caused perlecan to migrate slightly faster, and digestion with both enzymes together (lanes C and H) even further increased the migration rate. This would suggest that perlecan contains both chondroitin sulfate and heparan sulfate chains. Western blot of the purified perlecan using the 3G10 antibody demonstrated an immunoreactivity with the perlecan band after only heparitinase digestion (Fig. 4, 3G10, lane H) or heparitinase plus chondroitinase digestion (lanes C and H). The purified perlecan was digested with proteinase K, and the size of the glycosaminoglycan chain was determined by chromatography on Superose 6 (Fig. 5A). The perlecan glycosaminoglycans eluted as a single peak with a $K_v$ of 0.54. Digestion with chondroitinase ABC prior to chromatography reduced the amount of glycosaminoglycans substantially and shifted the elution position of the DMMB-positive material to a $K_v$ of 0.68. Digestion with both chondroitinase ABC and heparitinase abolished the glycosaminoglycan peak. These results indicate that perlecan from the growth plate contains both chondroitin sulfate and heparan sulfate chains and that the chondroitin sulfate chains are larger than the heparan sulfate chains. The glycosamin-
The antiserum to aggrecan immunoprecipitated the 3G10-then analyzed by Western blot using the 3G10 antibody (Fig. 7). The immunoprecipitates were combined, and aliquots were immunoprecipitated with antisera to aggrecan or perlecan. The immunoprecipitates were combined, and aliquots were immunoprecipitated with antiserum to recombinant G3 domain of aggrecan (Fig. 6, Anti G3) and with an antiserum to native aggrecan (Fig. 6, Anti Aggrecan).

Fractions 1 and 2 containing the 3G10-positive core protein were combined, and aliquots were immunoprecipitated with antisera to aggrecan or perlecan. The immunoprecipitates were then analyzed by Western blot using the 3G10 antibody (Fig. 7). The antiserum to aggrecan immunoprecipitated the 3G10-positive core protein (Fig. 7, lane 2), but the antiserum to perlecan (Fig. 7, lane 5) did not. Replacing half the aliquot with core protein digested with only chondroitinase ABC reduced the amount of immunoprecipitated 3G10-positive core protein (Fig. 7, lane 3), and replacing all of the aliquot with chondroitinase-only digested core eliminated the 3G10-positive core protein in the immunoprecipitate (Fig. 7, lane 4). These data (Figs. 6 and 7) indicate that the epitope recognized by the 3G10 antibody is on the aggrecan core protein.

Another aliquot of combined fractions 1 and 2 (from Fig. 6) containing the 3G10-positive core protein was digested with trypsin, and the resulting peptides were fractionated on a column of Superose 6 (Fig. 8A). Monitoring the fractions by immunodot blot using the 3G10 antibody showed a major peak at tube 26, which would correspond to a peptide estimated at 13.5 kDa. The minor peak (at tubes 31–32) is at the VI of the column and would correspond to peptides estimated at 0.8–1.2 kDa. Fractions 25–27 were combined and applied to a Mono Q column and eluted with a 0–1.0 M NaCl gradient. Dot blot analysis showed the major 3G10-positive peak eluted at 0.7 M NaCl (Fig. 8B). Fractions 24–28 were combined and sent to the Keck Laboratory (Yale University), where a portion of the contents was hydrolyzed with HCl, and the amino acid composition was determined by ion exchange chromatography. Amino acid composition found (Table II) for the peptide(s) was 15% serine, 15% glutamate, and 17% glycine, and this was consistent with that calculated for chondroitin sulfate domains (CS-1 and CS-2) of bovine aggrecan (27). The globular domains, G1, G2, and G3, have considerably higher levels of aromatic amino acids and lower levels of serine and glycine. The KS-rich domain has a high level of proline and phenylalanine. The amino acid sequence obtained by Edman degradation of the peptide sample was complex, probably due to the sample containing a mixture of peptides, but was consistent with certain repetitive sequences in the CS-1 domain of aggrecan (Table III). This identity included a predicted arginine preceding the sequence and a calculated molecular weight of the tryptic peptides consistent with the elution position seen on Superose 6.

The aggrecan in fraction 1 was further purified by an additional CsCl centrifugation (not shown) and by chromatography on Sephacryl S-500 (not shown). Western blot of the purified...
aggrecan showed reaction of the core protein with the 3G10 antibody only after digestion with heparitinase and chondroitinase (Fig. 9, 3G10). The core protein generated by chondroitinase digestion alone is the same apparent size as the core protein generated by digestion with both chondroitinase and heparitinase (Fig. 9, Anti G3). Heparitinase digestion of perlecan (Fig. 4), which has 25% HS, did produce a shift in the size of the core protein in Western blot. This suggests that there is less heparan sulfate on aggrecan than perlecan. Aggrecan digested with heparitinase alone was not found to react with the 3G10 antibody by Western blot (data not shown). The glycosaminoglycans isolated from the purified aggrecan were digested with chondroitinase ABC and ACII, and the resulting disaccharides were characterized by capillary zone electrophoresis and found to contain approximately equal amounts of Di6S and Di4S with a lesser amount of DiOS (Table I). Subsequent digestion of the remaining glycosaminoglycan with heparitinase released primarily unsulfated DiHHS with lesser amounts of DiHHS-NS. The heparan sulfate content of aggrecan was found to be only 0.1% of the total glycosaminoglycan content of aggrecan.

The heparan sulfate chains on aggrecan were analyzed on Superose 12. Aliquots of the purified aggrecan were digested with either chondroitinase ABC or with chondroitinase ABC and heparitinase. The aggrecan core protein in both samples was isolated by chromatography on Superose 6, and the presence of the 3G10 epitope on the heparitinase-digested sample was confirmed by dot blot (not shown). The core protein preparations were then digested with chondroitinase ACII, to remove the last remaining disaccharide from the linkage region, and with proteinase K, to degrade the core proteins. The digests were chromatographed on Superose 12, and the elution position of the uronic acid-positive material was determined using carbazole (Fig. 10). Both samples had major peaks eluting  of 0.74 and 0.89. These peaks are the linkage region and the residual disaccharide, respectively. The sample not digested with heparitinase also contained two minor peaks with  of 0.32 and 0.47 (tubes 19 and 22) that were not present in the sample digested with heparitinase. These minor peaks would be the heparan sulfate chains on aggrecan. Heparan sulfate chains from perlecan elute at tube 18 on Superose 12 (not shown). This indicates that the heparan sulfate chains on aggrecan that elute at tubes 19 and 22 are similar size to and smaller size than, respectively, those on perlecan.

**DISCUSSION**

The heparan sulfate proteoglycans are thought to play an important role in growth factor-mediated signaling in the growth plate (12, 16). The results of the Western blots in this study using the 3G10 antibody on heparitinase-digested ex-tracts indicate that the predominant heparan sulfate proteoglycans in the developing growth plate have core proteins of 200 kDa or larger. The method used to extract the proteoglycans from growth plate tissue used a brief preincubation with 2% CHAPS before the addition of the denaturing agent, and this is the method of choice for extracting both cell surface- and matrix-associated proteoglycans (21). This suggests that the heparan sulfate cell surface proteoglycans syndecan and glypican, which have core proteins of 100 kDa and smaller, are not major constituents of the growth plate.

The major heparan sulfate proteoglycan in the fetal growth plate is perlecan. Based on carbazole-detected uronic acid, the perlecan in growth plate contains less than 6% of the total glycosaminoglycan found in the growth plate, and it consists of both chondroitin sulfate and heparan sulfate side chains. The chondroitin sulfate chains are considerably larger than the heparan sulfate chains, and they account for 75% of the total glycosaminoglycan content of the proteoglycan. The heparan sulfate chains on growth plate perlecan are smaller than most of the heparan sulfate chains on EHS perlecan but similar to the size of heparan sulfate isolated from kidney. The disaccharide composition of the heparan sulfate on growth plate perlecan is distinct from that of kidney heparan sulfate and EHS heparan sulfate. This difference in disaccharide composition may be important for growth factor binding.

An unexpected finding was the presence of heparan sulfate on aggrecan. Heparitinase, but not chondroitinase ABC digestion, generated an epitope on the aggrecan core protein that is
recognized by the monoclonal antibody 3G10. The epitope recognized by 3G10 was also generated on aggrecan purified from bovine articular cartilage and nasal septum by digestion with heparitinase (data not shown). Previous studies have shown this antibody to be specific for heparan sulfate (18). The amino acid composition of the 3G10 epitope-containing peptides and the sequence obtained by Edman degradation strongly suggests that the epitope was attached to peptides from the CS-1 domain of aggrecan. The amino acid sequence obtained corresponds to sequence in the VNTR polymorphic region of the CS-1 domain (28). This region consists of varying numbers of highly conserved repeats, and allelic variation in the repeat number has been shown to be a risk factor for some forms of osteoarthritis (29).

Heparan sulfate and chondroitin sulfate are attached to serine residues in core proteins via identical linkage regions (30). Previous studies have shown that the presence of acidic residues 7–10 residues N-terminal to the serine attachment site for glycosaminoglycans and repetitive Ser-Gly sequences enhances heparan sulfate synthesis at the site (31–33). The CS-1 region of aggrecan is rich in acidic residues adjacent to potential serine attachment sites and has numerous Ser-Gly-X-Gly sequences. These may influence the synthesis of glycosaminoglycan type on this domain and mediate heparan sulfate substitution on aggrecan. The heparan sulfate on aggrecan contained a different disaccharide composition than the heparan sulfate on perlecian. Removal of heparan sulfate by heparitinase did not produce an appreciable shift of migration of aggrecan core protein on SDS-PAGE, and this is consistent with heparan sulfate only constituting 0.1% of the total glycosaminoglycan on aggrecan. While this may be a very low percentage, it may represent a significant amount because of the high levels of aggregcan present in growth plate and its high glycosaminoglycan content. Based on the uronic acid content of the fractions in the second CsCl density gradient centrifugation, which effectively partitioned aggrecan to the bottom of the gradient and perlecian to the top of the gradient (see Fig. 2), 95% of the total uronic acid in the growth plate was aggrecan, and 5% was in perlecian. Since perlecian was found to contain 25% of its glycosaminoglycan as heparan sulfate, we can estimate that ~1.2% of the total uronic acid in the growth plate is heparan sulfate on perlecian and ~0.1% is heparan sulfate on aggrecan. Thus, ~7% of the total heparan sulfate in the growth plate is on aggrecan.

The 3G10 epitope could only be generated on aggrecan when the heparitinase digestion was accompanied or preceded by chondroitinase ABC digestion. This was not the case with perlecian where heparitinase digestion readily generated the 3G10 epitope. Perlecian has only a limited number of demonstrated glycosaminoglycan attachment sites: three in domain I and two in domain V (33, 34). In contrast, aggrecan has over 100 predicted glycosaminoglycan attachment sites, and its core protein is about half the size of perlecans core protein (19, 27). Consequently, the high density of chondroitin sulfate chains may sterically hinder the action of the heparitinase on the linkage regions bearing heparan sulfate chains. The other possibility is that some of the glycosaminoglycan chains are initiated at the linkage region with a few disaccharides of heparan sulfate before the chondroitin sulfate disaccharides are added, and it is necessary to degrade the chondroitin sulfate region of the chain to allow action of the heparitinase. In any event, the restricted distribution of heparan sulfate to the CS-1 domain of aggrecan argues for functional significance.

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