Variations in the Chondroitin Sulfate-Protein Linkage Region of Aggrecans from Bovine Nasal and Human Articular Cartilages*

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Aggrecan-derived chondroitin sulfate (CS) chains, released by β-elimination, were derivatized with p-aminobenzoic acid or p-aminophenol; radiiodinated; and subjected to graded or complete degradations by chondroitin ABC lyase to generate linkage region fragments of the basic structure ΔGlyUA-GalNAc-GlcUA-Gal-Gal-Xyl-R (where ΔGlyUA represents 4,5-unsaturated glucuronic acid, and R is the adduct), by chondroitin AC lyase to generate the shorter fragment ΔGlyUA-Gal-Gal-Xyl-R, or by chondroitin C lyase to generate the same fragment when it was linked to a 6-O-sulfated or unsulfated GalNAc at the nonreducing end. Fragments were separated by size using gel chromatography, by charge using ion-exchange chromatography, and by size/charge using electrophoresis and then characterized by stepwise degradations from the nonreducing end by using mercuric acetate to remove all terminal ΔGlyUA, by bacterial glycuronidase to remove the same residue when linked to unsulfated or 6-O-sulfated GalNAc/Gal, by mammalian 4-sulfatase to remove sulfate from terminal GalNAc 4-O-sulfate, by chondro-4-sulfatase to remove 4-O-sulfate from other GalNAc/Gal residues, and by β-galactosidase to remove terminal Gal. Results with CS from bovine nasal cartilage aggrecan show that, in nearly all chains, Xyl and probably also the first Gal are unsubstituted, whereas the second Gal is 4-O-sulfated in one CS chain out of five. The first disaccharide repeat is sulfated at C-4 of GalNAc in one chain out of three and unsulfated in the other two. A sulfated first disaccharide is always joined to an unsulfated GlcUA-Gal-Gal sequence. In contrast, CS from human articular cartilage usually has a sulfated first disaccharide repeat. In CS from young human cartilage, sulfate groups are mostly at C-4 of GalNAc in the major part of the chain, but at C-6 in the nonreducing distal portion. In CS from old cartilage, sulfation at C-6 of GalNAc is a major feature from the nonreducing end down to approximately positions 4 and 5 from the linkage region, where GalNAc 4-O-sulfate is common.

Proteoglycans constitute a family of proteins that are characterized by the presence of covalently attached glycosaminoglycan (GAG) side chains and are found at cell surfaces, in pericellular matrices, and especially in the extracellular matrix of connective tissues. Proteoglycans are structurally diverse; core proteins may vary in size from 10 to 400 kDa, and they can contain only a single GAG chain or well over 100. The GAG chains have unique biophysical properties that contribute to the bulk effects of proteoglycans, but they also contain a variety of binding sites for various extracellular cytokines, growth factors, enzymes, and inhibitors (for reviews, see Ref. 1–4). The GAG chains are linear polymers of repeating disaccharides containing hexosamine and hexuronic acid (or, in the case of keratan sulfate, galactose). Hexuronic acid-containing GAGs are bound to serine residues in the core protein via the common carbohydrate sequence -(ΔGlcUAβ1–3Galβ1–3Galβ1–4Xylβ1)n, forming the so-called GAG-protein linkage region, which was shown by Rodén and co-workers over 30 years ago (for review, see Ref. 5). This structure serves as the primer for chain elongation to form either -(ΔGlcUAβ1–4GlcNAcα1)n, the core polymer in heparan sulfate/heparin, or -(ΔGlcUAβ1–3GlcNAcβ1)n, the corresponding one in chondroitin sulfate (CS) and dermatan sulfate (DS). Subsequent modifications of galactosaminoglycan chains by different kinds of O-sulfation and by C-5 epimerization of GlcUA to IdoUA, all in an incomplete and sporadic manner, generate a bewildering complexity. Theoretically, such sequences can encode considerable information, but it is not known if mechanisms for deciphering this information exist.

Sequence analysis of GAGs, as of other oligo- and polysaccharides, is hampered by the fact that the monosaccharides, apart from their ability to assume different ring shapes, can be joined via different types of linkages, varying in anomic con-

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1 The abbreviations used are: GAG, glycosaminoglycan; CS, chondroitin sulfate; DS, dermatan sulfate; GlyUA, glucuronic acid; ΔGlyUA, 4,5-unsaturated GlyUA; IdoUA, iduronic acid; 4S, 4-O-sulfate; 6S, 6-O-sulfate.
The low-charged linkage region tetrasaccharide GlyUA-GalNAc-4S (27, 28). An amount of enzyme preparation corresponding to ∼50 milliliters had no detectable β-galactosidase activity as tested with p-nitrophenyl-β-D-galactoside at pH 7.6 and 37 °C for 18 h. Recombinant mammalian N-acetylgalactosamine 4-sulfatase was prepared as described (29). One µl of a 1.200 dilution hydrolyzed ∼300 pmol of GalNAc-(4S)-GlcUA-2-acetamido-2-deoxy-d-galactopyranoside in 1 h under specified conditions (see below). The column media were Q-Sepharose Fast Flow (Pharmacia Biotech Inc.) and Bio-Gel P-6 and P-2 (Bio-Rad). Other special chemicals, membranes, and films were the same as described earlier (7, 8).

Preparation of Reducing End, 125I-Labeled Chondroitin Sulfate—The procedures have been described in detail previously (7–9). Chondroitin sulfate chains were released by alkaline scission of the Xyl-Ser bond, and the reducing terminal Xyl was coupled by reductive amination to either p-aminobenzoic acid or p-aminophenol. Chains derivatized with p-aminobenzoic acid were radiiodinated under acidic conditions (8), whereas the phenol adduct was iodinated at neutral pH (7).

Degradation Procedures—Digestions with the chondroitin lyses were carried out as follows. Cleavage of GalNAc–GlyUA bonds was achieved using chondroitin ABC lyase in 10 mM EDTA, 0.1 M Tris-HCl, pH 7.3, and cleavage of GalNAc–GlcUA bonds was achieved using chondroitin AC-I lyase in the same buffer or using chondroitin AC-II lyase in 50 mM NaAc, pH 6.0, all at 37 °C. GalNAc–GlcUA bonds involving unsulfated or 6-O-sulfated GalNAc were cleaved with chondroitin C lyase in 0.1 M Tris-HCl, pH 8.0, at 50 °C. In all cases, digestions were monitored by measurement of absorbance at 232 nm as described by Suzuki (27). Nonreducing terminal GlyUA was degraded by treatment with Hg(OAc)₂, as described by Ludwigs et al. (30). Twenty µg of GlyUA-GalNAc-4S disaccharide was added as carrier. Unsulfated, nonreducing terminal GlyUA linked to unsulfated or 6-O-sulfated GalNAc/Gal was removed by treatment with chondro-4-sulfatase acting on GlyUA-GalNAc/Gal-4S was carried out in 50 mM Tris, pH 7.3, containing 0.01% (w/v) bovine serum albumin. 4-O-Sulfate on nonreducing terminal GalNAc was released by treatment with mammalian N-acetylgalactosamine 4-sulfatase in 0.2 M sodium formate, pH 3.5, at 37 °C. Removal of nonreducing terminal Gal was achieved using β-galactosidase in 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.3, at 37 °C.

Separation Methods—Gradient polyacrylamide gel electrophoresis of oligosaccharides, blotting, and autoradiography were carried out as described previously (7). Gel chromatography was carried out on columns (10 × 1000 mm) of Bio-Gel P-2 or P-6 as described (8). Ion-exchange chromatography was performed on columns (10 × 100 mm) of Q-Sepharose Fast Flow connected to a Pharmacia chromatography system and eluted with a linear 160-min gradient of 0–2 M NH₄HCO₃ at a flow rate of 0.5 ml/min. Column effluents were analyzed for 125I using a 1217 Ria-Gamma counter.

RESULTS AND DISCUSSION

Chondroitin Sulfate-Protein Linkage Region of Bovine Nasal Cartilage Aggrecan—CS chains released by β-elimination, derivatized with p-aminobenzoic acid, and radiiodinated were degraded by treatment with chondroitin ABC or AC (type I or II) lyase, followed by gel chromatography on Bio-Gel P-6 (Fig. 1). A nearly complete ABC lyase digest (Fig. 1a) contained saccharides of the general carbohydrate backbone structure GlyUA-(GalNAc-GlcUA)ₐ-Gal-Xyl-R (where n = 1, 2, 3, etc., and R = radiiodinated reducing terminal adduct). Exhaustive digestions with AC lyase generated a shorter fragment, corresponding to n = 0 in the above structure, in accordance with the known specificity of the enzyme. To separate charge variants of the linkage region saccharides, they were subjected to ion-exchange chromatography on Q-Sepharose (Fig. 2). The saccharide pool GlyUA-GalNAc-GlcUA-Gal-Gal-Xyl-R (n = 1) (Fig. 2a) separated into one low-charged (pool A) and one high-charged (pool B) fraction in approximately equal proportions. The saccharide pool GlyUA-Gal-Gal-Xyl-R (n = 0) (Fig. 2b) also yielded two fractions (pools A and B), but in a ratio of ∼4:1.

The low-charged linkage region tetrasaccharide GlyUA-Gal-Gal-Xyl-R (n = 0) (pool A in Fig. 2b) was treated with
glycuronidase to remove the charged, nonreducing terminal residue and then rechromatographed on Q-Sepharose (Fig. 2c).

It is shown that the saccharide was completely degraded to a component of even lower charge density, which should correspond to Gal-Gal-Xyl-R with one remaining negative charge in R. The structure of the tetrasaccharide was confirmed by stepwise removal of monosaccharides from the nonreducing end, followed by gel chromatography on Bio-Gel P-2. As shown in Fig. 3, the size was gradually diminished by successive treatments with glycuronidase (panel b) and then with β-galactosidase (panel c). The final product, the expected Xyl-R, emerged in the same position as phenyl-O-β-D-galactopyranoside. The various fragments were also subjected to electrophoresis, which separates both according to size and charge (Fig. 4). The intact, low-charged linkage region saccharide ΔGlyUA-Gal-Gal-Xyl-R (n = 0) (pool A) is shown in lane 1. After removal of the charged, nonreducing terminal residue ΔGlyUA using glycuronidase, the trisaccharide product Gal-Gal-Xyl-R was obtained (lane 2). Although it is smaller, it migrated more slowly because it had a much lower charge density (−1/3) than the initial tetrasaccharide (−2/4). After further degradation using β-galactosidase (lane 3), the Xyl-R product migrated faster than the previous ones because it both was smaller and had an increased charge density (−1/1).

The less abundant, high-charged forms of the linkage region tetrasaccharide ΔGlyUA-Gal-Gal-Xyl-R (n = 0) (pool B in Fig. 2b) had a high mobility on electrophoresis (Fig. 4, lane 4). Two minor, even faster moving components were also observed. All
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Fig. 3. Stepwise exo-degradation of the low-charged linkage region saccharide ΔGlyUA-Gal-Gal-Xyl-R from bovine nasal cartilage aggrecan. The low-charged variant of saccharide with \( n = 0 \) was eroded stepwise by using first galacturonicidase and then galacturonidase, followed by gel chromatography on Bio-Gel P-2. Material corresponding to 0.4 – 0.6 mg of starting material and containing 1 x 10^6 cpm of \(^{125}\)I was used. a, intact saccharide (pool A in Fig. 2b); b, saccharide treated with galacturonidase (pool A in Fig. 2c); c, saccharide treated with galacturonidase and \( \beta \)-galactosidase (35 units of \( \beta \)-galactosidase in 50 \( \mu \)l of buffer at 37 \( ^\circ \)C overnight). Phe-\( \beta \)-Gal, elution position of phenyl-O-\( \beta \)-galactopyranoside. The nature of the minor component (pool 2) in b is unclear.

high-charged forms of saccharide \( (n = 0) \), except the one with the highest charge, were resistant to digestion with galacturonidase (lane 5), suggesting a preponderance of 4-O-sulfate on the second Gal. This was confirmed by treatment with chondro-4-sulfatase (lane 6). All three components lost one charge, and the major one migrated like unsulfated ΔGlyUA-Gal-Gal-Xyl-R (see also Ref. 31). The charged, nonreducing terminal residue ΔGlyUA of the high-charged saccharides was removed by treatment with Hg(OAc)_2, and products with slightly retarded mobilities were obtained (lane 7). Repeated digestions of the mercury-treated material with excessive amounts of mammalian exo-4-sulfatase (lane 8) generated only trace amounts of Gal-Gal-Xyl-R, suggesting that mammalian exo-4-sulfatase is specific for GalNAc-4S.

The linkage region hexasaccharide ΔGlyUA-GalNAc-GlcUA-Gal-Gal-Xyl-R \( (n = 1) \) obtained after digestion with chondroitin ABC lyase comprised approximately equal proportions of low- and high-charged variants (pools A and B in Fig. 2a). Electrophoretic analysis (Fig. 5) revealed that each pool contained one major, slow-moving (lane 1) or fast-moving (lane 4) component. The slow-moving, low-charged hexasaccharide (lane 1) was sensitive to glycuronidase, and the pentasaccharide product obtained migrated to a more retarded position (lane 2). Apparently, reduction in charge density (from -3/6 to -2/5, i.e. a 20% change) had a greater effect on electrophoretic mobility than reduction in size, which was 16%. The terminal ΔGlyUA-GalNAc disaccharide repeat was removed by treatment with chondroitin AC-II lyase (lane 3), and the tetrasaccharide product obtained migrated to the same position as ΔGlyUA-Gal-Gal-Xyl-R (Fig. 4, lane 1). Removal of one charged and one uncharged residue reduces size (by 33%), but does not alter charge density. The conclusion from these experiments is that -50% of the linkage region in bovine nasal cartilage aggrecan CS consists of an unsulfated hexasaccharide sequence, -GlcUA-GalNAc-GlcUA-Gal-Gal-Xyl- (Structure I in Table I), and was obtained as the low-charged hexasaccharide pool A in Fig. 2a. The other 50% of the linkage region was obtained in the high-charged hexasaccharide pool B in Fig. 2a (lane 4). The charged, nonreducing terminal residue ΔGlyUA of the high-charged variants was removed by treatment with Hg(OAc)_2, and the resulting pentasaccharide products migrated to more retarded positions (lane 5). The major variant appeared to be monosulfated, either at GalNAc or at the second Gal (see structures on the right in Fig. 5). The pentasaccharides were then treated exhaustively with exo-4-sulfatase, but only a partial effect was obtained (lane 6). We therefore conclude that the original high-charged hexasaccharide pool comprised two major isomers, one with sulfate at C-4 of GalNAc and another with sulfate at C-4 of the second Gal; the former was sensitive to exo-4-sulfatase and generated an unsulfated pentasaccharide, GalNAc-GlcUA-Gal-Gal-Xyl-R (see also structures on the right in Fig. 5). Both the high-charged hexasaccharide isomer with GalNAc-4S and the entire pool of unsulfated hexasaccharide (the latter accounted to 50% of total linkage region material) would generate unsulfated tetrasaccharide linkage region fragments after treatment with chondroitin AC lyase (see above).

As the unsulfated tetrasaccharide products amounted to 80% of the linkage region material, the two monosulfated hexasaccharide isomers should represent 30% (the GalNAc 4-O-sulfated isomer) and 20% (the Gal 4-O-sulfated isomer) of the total, respectively (Structures II and III in Table I). Di-, tri-, or tetrasulfated hexasaccharides (Structures IV–VI) are rare. As 50% of the linkage region in bovine nasal cartilage aggrecan CS was unsulfated (Structure I), half of the chains should be sensitive to chondroitin C lyase, which cleaves at both unsulfated and 6-O-sulfated GalNAc (32). However, as shown previously (8), very little linkage region tetrasaccharide (\( n = 0 \)) is released by C lyase. This suggests that C lyase requires a 6-O-sulfate on the second GalNAc in the sequence -GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-Gal-Xyl- in order to cleave between an unsulfated first GalNAc and GlcUA linked to Gal.

To determine if the first Gal and/or Xyl was substituted with charged groups, a different approach was taken. CS chains were coupled to an uncharged iodinatable compound, i.e. p-aminophenol, to avoid repulsion from possible phosphate esters on Xyl. The end-labeled CS was treated with alkaline phosphatase (33) after digestion with chondroitin AC-II lyase. Analysis by electrophoresis (data not shown) indicated that the linkage region saccharides were the same in phosphatase-treated and in untreated samples. The low-charged tetrasaccharide ΔGlyUA-Gal-Gal-Xyl-R, where R’ is now unsulfated, was isolated as in Fig. 2b, treated with galacturonidase, and subjected to electrophoresis, followed by direct autoradiography, omitting...
the transfer-blotting step. The uncharged trisaccharide product migrated only a short distance, and had the same mobility as $^{125}$I-labeled $\beta$-hydroxyphenyl-$\alpha$-D-xylopyranoside (data not shown). Hence, Xyl and the first Gal in bovine nasal aggrecan CS were mostly unsubstituted (see also Table I). It is possible that phosphorylation of Xyl is required for chain biosynthesis and/or intracellular progression and that it is followed by dephosphorylation just before or shortly after secretion.

**Fig. 4.** Structure determination of charge variants of the linkage region saccharide $\Delta$GlyUA-Gal-Gal-Xyl-R from bovine nasal cartilage aggrecan. Linkage region saccharides obtained by chondroitin AC lyase digestion and further enzymatic and/or chemical degradation of reducing end-derivatized and radiiodinated CS chains (see Figs. 1–3) were subjected to gradient polyacrylamide gel electrophoresis. Material corresponding to 250–500 $\mu$g of starting material and containing 0.5–$1 \times 10^6$ cpm of $^{125}$I was used. Lane 1, the low-charged version of the saccharide $\Delta$GlyUA-Gal-Gal-Xyl-R obtained after degradation with chondroitin AC lyase, followed by gel chromatography (see Fig. 1a) and ion-exchange chromatography (pool A in Fig. 2b); lane 2, the same saccharide after treatment with glycuronidase (0.5 milliunits of enzyme in 50 $\mu$l of buffer at 37°C overnight); lane 3, the same saccharide after consecutive degradations with glycuronidase and $\beta$-galactosidase (same material as in Fig. 3c); lane 4, the high-charged version of the saccharide $\Delta$GlyUA-Gal-Gal-Xyl-R (pool B in Fig. 2b); lane 5, the same saccharide after treatment with glycuronidase; lane 6, the same saccharide after treatment with bacterial chondro-4-sulfatase (20 milliunits in 50 $\mu$l of buffer at 37°C overnight); lane 7, the same saccharide treated with Hg(OAc)$_2$ (see “Experimental Procedures”); lane 8, the same saccharide treated consecutively with Hg(OAc)$_2$ and mammalian N-acetylgalactosamine exo-4-sulfatase (1 $\mu$l of undiluted enzyme solution in 24 $\mu$l of buffer at 37°C overnight). BPB, bromophenol blue marker. Structures and migration positions of the various linkage region saccharide fragments as indicated by arrows are shown on the right. The direction of migration is from top to bottom.

**Fig. 5.** Structure determination of charge variants of the linkage region saccharide $\Delta$GlyUA-GalNac-GlcUA-Gal-Gal-Xyl-R from bovine nasal cartilage aggrecan. Linkage region saccharides obtained by chondroitin ABC lyase digestion and further enzymatic and/or chemical degradation of reducing end-derivatized and radiiodinated CS chains (see Figs. 1–3) were subjected to gradient polyacrylamide gel electrophoresis. Material corresponding to 1 mg of starting material and containing 5 × $10^6$ cpm of $^{125}$I was used. Lane 1, the low-charged version of the saccharide $\Delta$GlyUA-GalNac-GlcUA-Gal-Gal-Xyl-R obtained after degradation with chondroitin ABC lyase, followed by gel chromatography (see Fig. 1b) and ion-exchange chromatography (pool A in Fig. 2a); lane 2, the same saccharide after digestion with glycuronidase (0.34 units of enzyme in 0.5 ml of buffer at 37°C overnight); lane 3, the same saccharide after digestion with chondroitin AC-II lyase (6 milliunits of enzyme in 20 ml of buffer at 37°C overnight); lane 4, the high-charged version of the saccharide $\Delta$GlyUA-GalNac-GlcUA-Gal-Gal-Xyl-R (pool B in Fig. 2a); lane 5, the same saccharide treated with glycuronidase; lane 6, the same saccharide after consecutive degradations with glycuronidase and $\beta$-galactosidase (same material as in Fig. 3c); lane 7, the same saccharide treated with glycuronidase; lane 8, the same saccharide after treatment with glycuronidase; lane 9, the same saccharide after treatment with bacterial chondro-4-sulfatase (20 milliunits in 50 $\mu$l of buffer at 37°C overnight); lane 10, the same saccharide treated with bacterial chondro-4-sulfatase. BPB, bromophenol blue marker. Structures and migration positions of the various linkage region saccharide fragments as indicated by arrows are shown on the right. The direction of migration is from top to bottom.
of young or old cartilage were released by β-elimination, derivatized with p-aminobenzoic acid, radioiodinated, and subjected to degradation by chondroitin AC-I or C lyase, followed by gel chromatography on Bio-Gel P-6 (Fig. 6). AC lyase cleaves hexosaminidic bonds to GlcUA when GalNAc is either 4- or 6-O-sulfated, whereas C lyase cleaves only when GalNAc is unsulfated or 6-O-sulfated (32). Exhaustive digestion with AC-I lyase generated the linkage region tetrasaccharide ΔGlyUA-Gal-Xyl-Ser identified by electrophoresis (see the bottom band marked with 1), suggesting sulfation also on the first GlcUA and a high degree of sulfation in the first disaccharide repeat, both of which may decrease the rate of cleavage by AC-I lyase (32). Very little high-mobility hexasaccharide was released by C lyase (see the bottom band marked with 1 in Fig. 7d), indicating that an oversulfated linkage

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**TABLE I**

| Structure | Total (%) |
|-----------|-----------|
| I. ΔGlyUA-GalNAc-GlcUA-Gal-Xyl-R | 50 |
| II. ΔGlyUA-GalNAc-GlcUA-Gal-Xyl-R | 4S 30 |
| III. ΔGlyUA-GalNAc-GlcUA-Gal-Xyl-R | 4S 20 |
| IV. ΔGlyUA-GalNAc-GlcUA-Gal-Xyl-R | 4S 4S Trace |
| V. ΔGlyUA-GalNAc-GlcUA-Gal-Xyl-R | 4S 6S Trace |
| VI. ΔGlyUA-GalNAc-GlcUA-Gal-Xyl-R | 4S 4.6-diS* Trace |

*4,6-diS, 4- and 6-O-sulfate.

**FIG. 6. Isolation of chondroitin sulfate-protein linkage region saccharides from human articular cartilage aggrecan.** Aggrecan preparations from human articular cartilage of a newborn baby and a 63-year-old man were used. CS chains were released by alkaline elimination, derivatized with p-aminobenzoic acid, and radioiodinated as described under "Experimental Procedures." Material corresponding to 0.2 mg and containing 2 × 10⁶ cpm of ¹²⁵I was digested exhaustively with chondroitin lyase and subjected to gel chromatography on Bio-Gel P-6. a, chondroitin AC-I lyase digest of CS from young cartilage (ChACase; 33 milliunits of enzyme in 100 μl of buffer at 37 °C overnight); b, chondroitin C lyase digest of CS from young cartilage (ChCase; 3.3 milliunits of enzyme at 37 °C overnight); c, chondroitin AC-I lyase digest of CS from old cartilage (as in a); d, chondroitin C lyase digest of CS from old cartilage (as in b). The saccharides have the general carbohydrate structure ΔGlyUA-GalNAc-GlcUA-Gal-Xyl-Ser (where n = 0, 1, 2, 3, etc., and R = radioiodinated reducing terminal adduct). Saccharides were pooled as indicated and freeze-dried.
region hexasaccharide sequence can be succeeded by largely 4-O-sulfated disaccharides in position 2. When C lyase cleaves between GalNAc-6S and GlcUA, there is no need for 6-O-sulfate in the disaccharide linked at the nonreducing side (32). Linkage region extended with two consecutive GalNAc 4-O-sulfated disaccharide repeats was analyzed in Fig. 7e, and four to six major components were observed. At least half of them (migrating as the component marked with 2 or faster) must contain oversulfated disaccharides. Analogous results were obtained with saccharides containing three consecutive GlcUA-GalNAc-4S repeats (Fig. 7f). The small amounts of material obtained precluded further detailed analysis.

To further examine the linkage region and the first disaccharide repeat in aggrecan CS from old cartilage, we used a chain preparation that was derivatized with the uncharged adduct p-aminophenol, radiodinated, and then exhaustively degraded with AC-II lyase (to obtain the tetrasaccharide ∆GlyUA-Gal-Gal-Gal-Xyl-R') or with ABC lyase (to obtain the hexasaccharide ∆GlyUA-GalNAc-GlcUA-Gal-Gal-Xyl-R'). The tetrasaccharide was subjected to ion-exchange chromatography on Q-Sepharose (as in Fig. 2; data not shown), and the major charge variant (90% of total material) migrated as a tetrasaccharide with one negative charge, i.e., the carboxylate on ∆GlyUA (data not shown). This indicates that both the Xyl and Gal residues in aggrecan CS from old cartilage were mostly unsubstituted. The hexasaccharide linkage region fragments that included the first disaccharide repeat comprised one major and a few minor components (data not shown). The major component migrated as a monosulfated hexasaccharide. The minor components included unsubstituted, low-mobility hexasaccharide and a couple of presumably oversulfated, high-mobility ones. The nonreducing terminal ∆GlyUA-GalNAc repeat in the major hexasaccharide component was degraded by either Hg(OAc)_2 treatment or glycuronidase digestion. Mercury treatment yielded pentasaccharide that migrated to a more retarded position. Digestion with glycuronidase generated two components; one was resistant to the enzyme, and the other was a pentasaccharide (data not shown). The pentasaccharide generated by glycuronidase should be 6-O-sulfated at GalNAc, whereas the resistant hexasaccharide should contain 4-O-sulfated GalNAc. Hence, the first disaccharide repeat in human articular cartilage aggrecan is most sulfated, either at C-4 or C-6 of GalNAc.

The structures proposed for various linkage region fragments obtained from aggrecan CS of young and old human articular cartilage are summarized in Table II. Structures VII–XI represent segments that, upon degradation by C lyase, would generate linkage region fragments extended with no (VII), one (VIII), two (IX), or three (X) 4-O-sulfated disaccharide repeats. From the results obtained in Figs. 6 and 7, the amounts of saccharides derived from these structures were calculated. In CS from young cartilage, Structures VII–IX accounted for 18, 16, and 14% of the linkage region variants, respectively, whereas the majority of the linkage region was recovered in long 4-O-sulfated segments like Structure X (24%) or higher saccharides (28%). In aggrecan CS from old cartilage, the 6-O-sulfated GalNAc moieties were more frequent and dominated from the nonreducing end down to the vicinity of the linkage region. The majority of the linkage region fragments were recovered as Structures VII–X (81%). Small amounts of Structure XI with, most likely, additional sulfate in one of the Gal moieties and possibly also on GlcUA should also be included.

By using the different charge variants of linkage region saccharides as reference compounds, we examined the electrophoretic banding patterns obtained from whole graded enzymatic digests of articular cartilage aggrecan CS (Fig. 8). CS from young cartilage was treated with AC-II lyase (Fig. 8a), and as expected, bands representing the shortest fragments, i.e., tetrasaccharides (n = 0), increased in intensity with time. Most of these were unsubstituted (see right side of panels a and b in Fig. 8), but minor bands representing tetrasaccharides with, most likely, one, two, or three sulfate groups were also observed. The saccharide with two sulfates appeared as a double band, probably because there were two isomers. A saccharide with three sulfates must be sulfated on both Gal residues and on GlcUA. Saccharides with n = 1 persisted for up to 4 h of digestion and then disappeared; saccharides with n = 2, 3, etc. disappeared gradually at earlier time points. The electrophrogram thus revealed the presence of three overlapping series of saccharides. One started with monosulfated ∆GlyUA-Gal-Gal-Gal-Xyl-R (see right side of panel b in Fig. 8) and included a series of saccharides extended with n = 1, 2, 3, etc. sulfated disaccharide repeats. A second one appeared to be based on monosulfated (or possibly more highly sulfated) ∆GlyUA-Gal-Gal-Gal-Xyl-R extended with sulfated disaccharides (numbers in-
A third one could be an extension of unsulfated \( \text{D GlyUA-Gal-Gal-Xyl-R} \) with likewise unsulfated disaccharide repeats (numbers indicated in parentheses). However, the vast majority of the chains must consist of unsulfated \( \text{D GlyUA-Gal-Gal-Xyl-R} \) extended with sulfated disaccharide repeats. Similar time course analyses were made with chondroitin AC-II lyase digests of CS from young cartilage (ChACase; 33 milliunits of enzyme in 100 \( \mu \)l of buffer at 37 °C overnight); \( b \), chondroitin C lyase (ChCCase; 3.3 milliunits of enzyme at 50 °C overnight). The saccharides have the general carbohydrate structure \( \text{D GlyUA-} (\text{GalNAc-GlcUA})_n \text{-Gal-Gal-Xyl-R} \) (where \( n = 0, 1, 2, 3, \text{etc}, \) and \( R \) = radioiodinated reducing terminal adduct carrying one negative charge). Migration positions of saccharides (\( n = 1-10 \)) are indicated to the left of \( a \) and \( b \). Those with oversulfation are indicated by numbers in brackets, and those with undersulfation are in parentheses. Migration positions of saccharides with \( n = 0 \) and the proposed structures of charge variants of saccharides with \( n = 1 \) and 0 are indicated to the right of \( a \) and \( b \). \( \text{monoS}, \text{diS}, \text{and triS} \), variants with one, two, or three sulfate groups, respectively. The direction of migration is from top to bottom.

\[ \text{Proposed structures of chondroitin sulfate-protein linkage region saccharides from aggrecan of young and old human articular cartilage} \]

| Structures                                                                 | Total | Young | Old |
|---------------------------------------------------------------------------|-------|-------|-----|
| VII. \( \text{GlyUA-GalNAc-GlcUA-GalNAc-GlcUA-Gal-Xyl-R} \)               |       | 18    | 30  |
| S \text{S}6S \text{S}                                                  |       |       |     |
| VIII. \( \text{GlyUA-GalNAc-GlcUA-GalNAc-GlcUA-Gal-Xyl-R} \)             |       | 16    | 25  |
| S                                                   |       | 6S    | 4S  |
| IX. \( \text{GlyUA-GalNAc-GlcUA-GalNAc-GlcUA-Gal-Xyl-R} \)              |       | 14    | 18  |
| S                                                   |       | 4S    | 4S  |
| X. \( \text{GlyUA-GalNAc-GlcUA-GalNAc-GlcUA-Gal-Xyl-R} \)              |       | 24    | 8   |
| S                                                   |       | 2S    | 4S  |
| XI. \( \text{GlyUA-GalNAc-GlcUA-GalNAc-GlcUA-Gal-Xyl-R} \)             |       | ND    | ND  |
| S                                                   |       |       |     |

\( a \) ND, not determined.
of Xyl with phosphate at C-2, of the Gal residues with sulfate at C-4 or C-6, of the first GlcUA with sulfate at C-2 or C-3, as well as un-, mono-, or disulfation of the first repeating GlcUA-GalNAc disaccharide, can be detected. It should be of interest to examine how (and if) microheterogeneity, especially in segments near the core protein, is related to nonrandom variations in the distal part of a chain.

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