Proteoglycan Lt from Chicken Embryo Sternum Identified as Type IX Collagen*

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Proteoglycan Lt (PG-Lt), isolated from 17-day-old chicken embryo sterna, appeared to differ from its counterpart from tibia and femur (Noro, A., Kimata, K., Oike, Y., Shinomura, T., Maeda, N., Yano, S., Takahashi, N., and Suzuki, S. (1983) J. Biol. Chem. 258, 9323-9331). The intact disulfide-bonded molecule of approximately 300 kDa was separable into three chains of 115, 84, and 68 kDa on reduction, the molecular masses being relative to those of collagen standards on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This is in contrast to tibial cartilage PG-Lt, from which there was no observed release of a 68-kDa chain (100 kDa relative to globulin protein standards) after reduction. The 115-kDa chain of sternal PG-Lt consists of a core 68-kDa polypeptide to which the chondroitin sulfate chains are attached. The ratio of 4-sulfated to 6-sulfated disaccharides released after either chondroitinase ABC or AC digestion is 3:1. Identity of PG-Lt with type IX collagen was indicated by their similar elution profiles on DEAE-Trisacryl and by the presence in both proteins of co-migrating 84- and 68-kDa bands on SDS-PAGE. This identity was confirmed by immunoblotting PG-Lt after SDS-PAGE, with affinity-purified polyclonal antibodies specific for a triple helical domain (bimolecular) of type IX collagen. The nonreduced high molecular mass material and all three bands of the reduced PG-Lt were immunoreactive, giving immunostaining patterns similar to autoradiographs from the [14C]glycine-labeled protein.

The structural characterization of cartilage has largely focused on its two major macromolecular components, type II collagen and the large aggregating cartilage proteoglycan (reviewed in Refs. 1 and 2). In recent years, the discovery of a new collagen, type IX (M), has been defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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EXPERIMENTAL PROCEDURES

Materials

[U-14C]Glycine and sodium [35S]sulfate were obtained from The Radiochemical Centre (Amersham, United Kingdom) and D-[2-3H]mannose from New England Nuclear (Dreieich, Federal Republic of Germany). Chondroitinase ABC (Proteus vulgaris), chondroitinase AC (Arthrobacter aurescens), and chondroitin sulfates A, B, and C were from Sigma. Unsaturated disaccharide standards were prepared as previously described (33). Bacterial collagenase (grade CLSPA) was purchased from Worthington. Nitrocellulose filters were provided by Schleicher (Feldbach, Switzerland). The horseradish peroxidase-labeled goat anti-rabbit antibody was from Nordic Immunological Laboratories (Tilburg, Netherlands) and goat anti-rabbit immunoglobulin G was from Gibco (New York). DEAE-Trisacryl was from LKB (Bromma, Sweden).

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PG, proteoglycan.
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Methods

**Incubation, Extraction, and Separation of the Proteoglycans**—The sternal cartilages from 17-day-old chicken embryos were dissected free of periostium. They were rinsed into 30 ml of Dulbecco's modified essential medium supplemented with 0.25 mM 3-aminopropionitrile fumarate and 0.25 mM ascorbic acid. Sternas from 12 embryos were used in each experiment. Incubation at 37 °C was commenced after addition of either [14C]glycine (2 μCi/ml), Na[35S]sulfate (10 μCi/ml), or [2-3H]mannose (30 μCi/ml) and continued for 20-24 h with gentle shaking. The medium was then decanted off and the cartilages immersed in cold (0 °C) extraction solution of 4 mM guanidinium HCl buffered with 50 mM Tris-HCl to pH 8.0 and containing a cocktail of proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, 100 μg/ml N-ethylmaleimide, and 10 mM EDTA). The sterna were disrupted by a brief homogenization and then extracted with slow stirring at 0 °C for 16-24 h. The resultant suspension was clarified by centrifugation at 27,000 × g for 30 min at 0 °C. The macromolecular components were precipitated with 3 volumes of cold ethanol containing 1.3% (w/v) potassium acetate. They were redissolved in a minimal volume of extraction solution prior to site zonal centrifugation under dissociative conditions.

**Ultracentrifugation**—Rate zonal centrifugation under dissociative conditions in 10-35% (w/v) glycerol gradients, followed by CsCl isopycnic density centrifugation was performed as previously described (31).

**Ion-Exchange Chromatography**—PG-Lt was purified by DEAE-Trisacryl column chromatography following the procedures of Nero et al. (32). Briefly, 0.25% (w/v) Triton X-100 was added to the top fraction from the CsCl isopycnic gradient, which was then dialyzed against the starting buffer of 7 M urea, 50 mM Tris-HCl, pH 7.4, and 0.2% (w/v) Triton X-100. After dialysis the sample was applied to a 1.5 × 12-cm column of DEAE-Trisacryl. The column was first washed with 50 ml of the starting buffer, material bound to the resin was eluted with a linear gradient of 0-0.6 M NaCl in the same buffer, and 3-ml fractions collected. All procedures were carried out at 4 °C.

**Chain Separation**—Purified PG-Lt labeled with either [14C]glycine or [35S]sulfate was precipitated with 3 volumes of cold ethanol containing 1.3% (w/v) potassium acetate in 1.5-mi plastic centrifuge tubes. After centrifugation at 4 °C, the supernatant was discarded and the precipitate resuspended in 100 μl of 50 mM Tris-HCl, pH 7.0, 8 M urea, 2% (w/v) Triton X-100, and 0.2% (w/v) 2-mercaptoethanol. The samples were reduced at 60 °C for 1 h, with intermittent mixing. The samples were then diluted with an equal volume of buffer A (50 mM citrate HCl buffer, pH 2.5 containing 8 M urea, 0.2% Triton X-100 (w/v), and 1% (w/v) 2-mercaptoethanol) and applied to a 4.5 × 50-mm column of DEAE-Trisacryl equilibrated with the same buffer. Nonbound material was eluted with 3 ml of buffer A, and 300-μl fractions were collected. A linear gradient of 0-0.6 M NaCl in buffer A described by the manufacturer of the bound sample. Increasing the NaCl concentration to 0.6 M failed to elute the unlabeled material. **SDS-Polyacrylamide Gel Electrophoresis**—Gel electrophoresis of 5-20% (w/v) acrylamide overlaid by 3% (w/v) acrylamide stacking gels were prepared by standard methods (34). Fluorography of the gels was performed as previously described (35).

**Immunoblotting**—Proteins were electrotransferred from SDS-PAGE slab gels to nitrocellulose filters (36). The transfer was achieved with 100 mA for 16-24 h. To prevent nonspecific binding of antibodies, the filters were incubated for 1 h with a blocking buffer of 10 mM Tris-HCl, pH 7.4, containing 5% (v/v) fetal calf serum, 1% (w/v) bovine serum albumin, 100 mM NaCl, and 0.5% (w/v) Tween 20 (37). Subsequently the filters were immersed in a minimal volume of this buffer containing the antibodies against type IX collagen at an appropriate dilution and incubated with gentle shaking overnight. The filters were then washed three times for 10 min each with blocking buffer. A peroxidase-labeled secondary antibody (diluted 1:10000 in the blocking buffer and incubated with the filter for 3 h at room temperature. The excess second antibody was removed by three washes of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl. The substrate for the peroxidase was 3-chloronaphthol.

**Enzyme Digestion**—Affinity-purified antibodies to pepsin-released HMW domain of type IX collagen from chicken sternum (4) were prepared and characterized as described previously (17).

**Enzyme Digestion**—Incubation of PG-Lt with chondroitinase ABC or AC was performed in the presence of proteinase inhibitors (100 μg/ml of aprotinin, 10 μg/ml of leupeptin, or 10 μg/ml of soybean trypsin inhibitor) and 100 μM CaCl2 for 16-24 h. To prevent nonspecific binding of antibodies, the filters were incubated for 1 h with a blocking buffer of 10 mM Tris-HCl, pH 7.4, containing 1% (v/v) fetal calf serum, 1% (w/v) bovine serum albumin. After reduction of these samples one diffuse band migrating with an apparent molecular mass of approximately 300 kDa (Fig. 3, b-d). Upon reduction the [14C]glycine-labeled material yielded three bands (Fig. 3e). One ran as a diffuse band centered at 115 kDa and two as sharper bands at 84 and 68 kDa, relative to collagen standards. In contrast, [35S]sulfate labeled only the diffuse band at 115 kDa (Fig. 3f). Both the 84- and the 68-kDa bands could be labeled with [2-3H]mannose, but labeling of the 115-kDa band was inconclusive (Fig. 3g). Occasionally a high molecular mass doublet could be observed in nonreduced samples. After reduction of these samples one diffuse and one sharp high molecular mass band of 210 and 168 kDa, respectively, could be discerned. These presumably represented dimers joined through lysine-derived cross-links. The appearance of these bands was minimized by the inclusion of 3-aminopropionitrile fumarate in the organ culture medium.

**Treatment of the [14C]glycine or [3H]mannose-labeled material with either chondroitinase ABC or AC sharpened the high molecular mass nonreduced band of PG-Lt on SDS-PAGE and decreased its apparent molecular mass by approximately 50 kDa (Fig. 4, a and e). Upon reduction of digested material containing either of the above labels, the diffuse 115-kDa band was no longer visible, but a corresponding increase in the intensity of the 68-kDa band was noted (Fig. 4, g and i). 4C- or 3H-Labeled material could not be detected in the supernatant after ethanol precipitation of the digest nor was there material running as a low molecular mass band(s) on SDS-PAGE (5-20% acrylamide gels). These results indicated that the change in apparent mass of the high molecular mass band was not due to proteolytic degradation. Conversely, more than 98% of the radioactivity could be recovered in the ethanol supernatant after chondroitinase ABC or AC treatment of
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Fig. 1. Separation of the sternum extract into the proteoglycan fractions PG-H, PG-Lb, and PG-Lt by ultracentrifugation. Fractions of 20 drops each are numbered from the bottom of the centrifuge tube. Aliquots of 50 μl were taken for analysis. Absorbance at 280 nm is shown by ---, [14C]glycine by O——O, and [35S]sulfate by □——□. a, rate zonal centrifugation under dissociating conditions to separate the large aggregating proteoglycan population, PG-H, from the smaller PG-L proteoglycans. The latter was pooled as shown (---). b, CsCl isopycnic density gradient centrifugation to fractionate PG-L into PG-Lb and PG-Lt. The lowest density fractions corresponding to PG-Lt were pooled as shown (---).

Fig. 2. Chromatography on a 1.5 X 12-cm column of DEAE-Trisacryl of the pooled lowest density fractions from CsCl isopycnic density centrifugation. Aliquots of 50 μl were taken from each 3.0-ml fraction for analysis. Fractions were pooled as shown (---). a, [14C]glycine-labeled PG-Lt; b, [35S]sulfate-labeled PG-Lt; and c, [2-3H]mannose-labeled PG-Lt. Radioactivity is denoted by --- and the NaCl gradient by ---.

[35S]sulfate-labeled material, with a corresponding disappearance of bands on SDS-PAGE (Fig. 4, d and h).

To more rigorously address the question of whether the 115-kDa diffuse band converts to a 68-kDa band upon chondroitinase ABC treatment, it was first necessary to separate the polypeptide chains. This was achieved by reduction of the protein under denaturing conditions, followed by ion-exchange chromatography at pH 2.5 on DEAE-Trisacryl. Absorbed material was eluted in denaturing conditions with a linear gradient of NaCl. Submitted to this procedure, [14C]glycine-labeled PG-Lt chromatographed as two peaks (Fig. 5a). The first peak eluted with the wash buffer and comprised 38% of the label. The second peak was desorbed with 0.3–0.4 M NaCl and contained 53% of the applied radioactivity. When PG-Lt labeled with [35S]sulfate was treated similarly, the 35S label eluted exclusively in one peak with 0.35 M NaCl (Fig. 5b). Electrophoresis of [14C]glycine-labeled material from peak 1 (Fig. 5a) showed that it consisted almost entirely of the 68-kDa chain (Fig. 6d), with minimal contamination by the 84-kDa chain. In contrast, peak 2 contained mostly the heterogeneous 115-kDa chain (Fig. 6e), which co-migrated with material from the corresponding peak of the [35S]sulfate-labeled chromatogram (Fig. 6c).

Aliquots from the various peaks were digested with chondroitinase ABC. All the [35S]sulfate label was released, causing the diffuse 115-kDa band to disappear on SDS-PAGE (not shown). Similar treatment caused the [14C]glycine label to reappear in a band of 68-kDa mobility (Fig. 6f). The relative proportion and mobility of the contaminating 84-kDa band (lanes e and f) was unchanged by chondroitinase ABC treatment. These results provided further evidence that the polypeptide carrying the chondroitin sulfate chain(s) has an apparent molecular mass of 68 kDa and migrates with the same electrophoretic mobility as the nonsulfated 68-kDa chain (Fig. 6, d and f).

PG-Lt labeled with [35S]sulfate was treated with chondroit-
to 4- to 6-sulfated chondroitin sulfate disaccharides released by either enzyme was identified. Chondroitinase AC effected complete digestion to disaccharides, with none of the oligosaccharide fragments observed which would otherwise be indicative of dermatan sulfate. This shows that the PG-Lt isolated from chicken embryo sternum does not contain dermatan sulfate as was found in PG-Lt isolated from tibia and femur (32).

Treatment of the PG-Lt preparation with bacterial collagenase resulted in the disappearance of the high molecular mass band in the unreduced and all three bands (115, 84-, and 68-kDa) in reduced samples run on SDS-PAGE following digestion with bacterial collagenase. Finally, the unique collagenous nature of the intact proteoglycan as well as that of the individual polypeptide chains is evident from both preparations with the disappearance of all bands on 5–20% SDS-PAGE following digestion with bacterial collagenase. The PG-Lt from tibia was classified as a proteodermatan sulfate (32). We were unable to find any evidence of dermatan sulfate in our preparations of PG-Lt from sternum, which possibly reflects differences between the two tissue sources. Certainly intratissue variation can be expected as the ratio of type IX collagen to type I collagen varies from tissue to tissue. This resulted in a concomitant increase in antibody staining over the 68-kDa band (Fig. 7d).

**DISCUSSION**

Despite the differences between our preparation of PG-Lt and that of Noro et al. (32), the bulk of the evidence favors them being the same molecule. The centrifugation profiles of proteoglycans extracted from sternum (Fig. 1, this paper) and those extracted from tibia and femur (32) are similar. This enables the comparable PG-H, PG-Lb, and PG-Lt fractions to be isolated. In both cases PG-Lt is the major [35S]sulfate-labeled macromolecule with a buoyant density of less than 1.34 g/ml on CsCl density gradient centrifugation. This is evident from the DEAE-column chromatography of this lowest buoyant density fraction, where all but a small proportion of the incorporated [35S]sulfate label elutes in the PG-Lt peak.

On SDS-PAGE a disulfide-bonded molecule of approximately 300 kDa, reducible to a diffuse [35S]sulfate-labeled chain of 115 kDa is observed. This is accompanied by a narrow nonsulfated protein band at 84 kDa, a pattern similar to that previously described by Noro et al. (32) for tibial PG-Lt. The molecular masses of 420 kDa for the intact molecule, 150 kDa for the [35S]sulfate-labeled band, and 120 kDa for a nonsulfated [3H]serine-containing band were calculated by these authors relative to globular protein standards. In our hands, calibration against globular protein standards gave comparable molecular masses of 380, 160, and 110 kDa, respectively. In the interests of simplicity and because of the collagenous nature of PG-Lt, these chains will be subsequently referred to in terms of the values estimated here relative to collagen standards.

A discrepancy between our results and those of Noro et al. (32) is that no 68-kDa band (100 kDa relative to globular protein standards) can be seen in fluorographs published by, nor is it noted by, these authors. This may be due to the labeling intensity, which is very light in the figure shown. It may also be that the content of serine is high in the 84-kDa chain relative to the 68-kDa chain such that the latter is not readily detected when [3H]serine is used as tracer. However in both cases the mobility of the 115-kDa band changes to a band of 68 kDa after chondroitinase ABC digestion. Finally, the unique collagenous nature of the intact proteoglycan as well as that of the individual polypeptide chains is evident from both preparations with the disappearance of all bands on 5–20% SDS-PAGE following digestion with bacterial collagenase.

**Fig. 3.** SDS-PAGE in 5–20% acrylamide-gradient gels of PG-Lt preparation. Samples a–d were nonreduced and samples e–g reduced. a, type I collagen standard; b and e, [14C]glycine-labeled PG-Lt; c and f, [3H]sulfate-labeled PG-Lt; and d and g, [2-3H]mannose-labeled PG-Lt.

**Fig. 4.** Chondroitinase ABC-treated PG-Lt run on 5–20% SDS-PAGE. Nonreduced samples a–c and reduced samples f–i. a, type I collagen standard; b and f, control [14C]glycine-labeled PG-Lt. Chondroitinase ABC-treated samples are: c and g, [14C]glycine-labeled PG-Lt; d and h, [3H]sulfate-labeled PG-Lt; e and i, [2-3H]mannose-labeled PG-Lt.
Fig. 5. Chain separation of reduced PG-Lt on DEAE-Trisacryl under denaturing conditions at pH 2.5. a, [14C]glycine-labeled PG-Lt; b, [35S]sulfate-labeled PG-Lt. Fractions were pooled as shown (——) and analyzed by SDS-PAGE (Fig. 6). Aliquots of 50 μl were analyzed for radioactivity (●). The NaCl gradient is indicated by (—-—). The NaCl gradient is indicated by (—-—).

Fig. 6. Isolated chains of PG-Lt run on 5–20% SDS-PAGE. Lanes a and g are type I collagen standards; b, reduced [14C]glycine-labeled PG-Lt shown as control; c, [35S]sulfate-labeled material which eluted from the DEAE-Trisacryl column (b) with 0.4 M NaCl; lanes d–f, [14C]glycine-labeled chains: d, breakthrough peak off DEAE-Trisacryl column (a); e, peak eluted with 0.4 M NaCl (a); f, chondroitinase ABC digestion of material applied to lane e.

| Disaccharide units | Chondroitinase ABC released | Chondroitinase AC released |
|--------------------|-----------------------------|-----------------------------|
| GlcUA-GalNAc(4-SO₄) | 76%                         | 75%                         |
| GlcUA-GalNAc(6-SO₄) | 24%                         | 25%                         |

Tibia from 12-day chicken embryos (40). Intertissue differences have also been observed. Dermatan sulfate, which is abundant in fibrous cartilage (41), was undetectable in hyaline cartilage (42). Interestingly, the ratio of 4- to 6-sulfated disaccharides of 3:1 is the same for PG-Lt from either source and differs from the ratio of 1:3:1 found for the major aggregating proteoglycan (40).

The incorporation of [2-3H]mannose into PG-Lt, presumably as a constituent of asparagine-linked oligosaccharide chains, was noted previously (32). Here we confirm those observations and show that the 68-kDa chains incorporate the majority of the label. The structure of these oligosaccharides, their number, and sites of attachment must await further studies.

Interestingly, type IX collagen had also been found to migrate as a disulfide-bonded molecule with an apparent molecular mass of 300 kDa (17, 19). In addition, polypeptide chains of 84 and 69 kDa could be released upon reduction. It had an unusually strong anionic nature for a collagen, as evidenced by its relatively high affinity for DEAE-cellulose (19). Furthermore, on closer examination of the published SDS-PAGE patterns of type IX collagen (17, 19) a diffuse
band of about 115 kDa could be discerned in preparations of purified material, in addition to the 84- and 63-kDa bands. The presence of this diffuse band was not addressed in these publications.

The first direct evidence that these two molecules are identical is provided by the immunological identity between PG-Lt and type IX collagen demonstrated here. This conclusion is based not only on the immunoreactivity of the disulfide-linked high molecular mass band of PG-Lt but also on all three of its constituent polypeptide chains. This latter result also demonstrates that all three of these chains, including the glycosaminoglycan-containing 115-kDa chain, must participate in the triple helical HMW domain of type IX collagen.

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