Occurrence in Chick Embryo Vitreous Humor of a Type IX Collagen Proteoglycan with an Extraordinarily Large Chondroitin Sulfate Chain and Short α1 Polypeptide*

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We previously isolated from chick embryo cartilage a minor proteoglycan component from the vitreous humor of 13-day-old chick embryos. Using immunoblot analysis coupled with chondroitinase digestion, we demonstrate that the purified preparation is composed predominantly of type IX collagen-like chondroitin sulfate proteoglycan with an α1(IX) chain $M_\text{r} \approx 23,000$ shorter than the known α1 in cartilage type IX. Also different from cartilage type IX is the size of the chondroitin sulfate chain attached to the α2(IX) polypeptide; its $M_\text{r}$ is $\sim 350,000$ indicating that it is $\approx 10$ times larger in vitreous humor than in cartilage. Examination of vitreous bodies at different developmental stages indicates that a transition occurs in the size of α1(IX) in a well defined temporal pattern; at about stage 31, a cartilage-type α1(IX) of $M_\text{r} \approx 84,000$ is the predominant species, whereas at stage 36 and thereafter, a $M_\text{r} \approx 61,000$ species appears with a concomitant disappearance of the $M_\text{r} \approx 84,000$ species.

Immunostaining for type IX collagen followed by electron microscopic observation of 13-day-old chick embryo vitreous humor reveals a regular D-periodic arrangement of vitreous type IX collagen proteoglycan along thin fibrils. It seems possible that the chondroitin sulfate chains of extraordinarily high viscosity and high molecular weight may extend away from the fibrils, thus contributing to structural as well as functional properties of this unique matrix.

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an intact form of type IX collagen proteoglycan with the chain composition α1(IX), α2(IX), α3(IX), of which the α2(IX) chain bears chondroitin sulfate chain(s) (Vaughan et al., 1985; Bruckner et al., 1985; Huber et al., 1986; Konomi et al., 1986; McCormick et al., 1987; Huber et al., 1988).

In vitricle, it has been established that type IX molecules are localized in a periodic manner along cartilage collagen fibrils (Müller-Glauser et al., 1986; Vaughan et al., 1988; Bruckner et al., 1988) and are cross-linked to type II collagen molecules within such fibrils (Dyer et al., 1987; van der Rest and Mayne, 1988). Thus, type IX molecules on the fibrils have been implicated in interacting with each other and with other components of the matrix (Vasios et al., 1988). However, the role played by the glycosaminoglycan chains of type IX collagen proteoglycan is not precisely understood.

Embryonic chick corneas and vitreous humor have been shown to contain immunodetectable protein for type IX collagen (Fitch et al., 1988). Likewise, Ayad and Weiss (1984) have reported evidence for the occurrence in bovine vitreous humor of type IX collagen along with type II collagen. Not only these specific collagens but also some glycosaminoglycans have been demonstrated in avian and mammalian vitreous humor, e.g. chondroitin sulfate in chick embryo vitreous humor (Smith and Newsome, 1978) and hyaluronic acid in bovine vitreous humor (reviewed by Laurent and Fraser, 1986, among others). In an effort to understand the role played by the glycosaminoglycans in cartilage matrix and the vitreous humor, we have investigated the nature, expression, and organization of vitreous humor type IX collagen in the chick embryos at different developmental stages. Here we show that the vitreous humor type IX collagen molecule is unique in containing a short form of α1(IX) polypeptide and an extraordinarily large chondroitin sulfate chain, the results suggesting a contribution of the structural modulation to the functional differences between cartilage matrix and the vitreous humor. We also give immunohistological data as to its longitudinal arrangement along thin fibrils in the vitreous humor. A preliminary account of this work has appeared (Yada et al., 1988).

Recently, a report of electron microscopic studies on the collagen fibrils of adult chicken vitreous humor has been

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‡ The abbreviations used are: PG, proteoglycan; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline (0.1 M, pH 7.2); HMW and LMW, high and low $M_\text{r}$ fragments, respectively, isolated from picpic digestes of chick embryo cartilage type IX collagen.

§ As shown in the present report, the type IX collagens (PG-Lt) of chick embryo cartilage and vitreous humor are different in both polypeptide and polysaccharide structures. Moreover, recent evidence indicates that some of the type IX collagen molecules of cartilage lack a chondroitin sulfate side chain (T. Yada, M. Arai, S. Suzuki, and K. Kimata, unpublished observation). To avoid nomenclatural confusion caused by the structural variation, the chondroitin sulfate-carrying form of chick embryo cartilage type IX collagen is referred to as "cartilage type IX collagen proteoglycan" in the present paper.
presented by Wright and Mayne (1988). The results of these authors clearly show that the collagen fibrils are different from those in cartilage in that they are completely invested by proteoglycans that have a high content of chondroitin sulfate and a high degree of sulfation. These results are consistent with the findings of other groups (see below) that have used biochemical methods to analyze the proteoglycans of cartilage. The proteoglycans of cartilage are known to be highly sulfated and to have a high content of chondroitin sulfate, which is different from PG-Lt, PG-Lb, Yamagata et al., 1986), were prepared as described.

**Materials**—Fertile eggs (White Leghorn) were obtained from a local supplier; DNase I, RNase A, bovine serum albumin, and phenylmethylsulfonyl fluoride were from Sigma; chondroitinase ABC, chondroitinase AC, shark cartilage chondroitin sulfate (average M = 45,000), and bovine tracheal cartilage chondroitin sulfate (average M = 15,000) were from Seikagaku Kogyo Co., Tokyo, Japan; Sepharose CL-4B was from Pharmacia Japan, Tokyo; Bio-Gel A-1.5m was from Bio-Rad Laboratories Japan, Tokyo; complete and incomplete Freund's adjuvant and skin milk were from Difco; horseradish peroxidase-conjugated protein A was from E-V Laboratories, San Mateo, CA; 131I-protein A was from ICN Radiochemicals; x-ray film (Fuji RX) was from Fuji Photo Film Co., Kanagawa, Japan; and ribi adjuvant system (monophosphoryl lipid A and trehalose dimycolate) was from Ribi ImmunoChem Research Inc., Hamilton, MT.

Generous gifts of the following compounds are acknowledged: type II collagen (adult rat skin) from R. Hata, the National Institutes of Health, Bethesda, MD; type IV collagen (a mouse Engelbreth Holm-Swarm sarcoma) from M. Kato, The Rockefeller University, New York, NY; type V collagen (human placenta) from P. Bornstein, The University of Washington, Seattle, WA; and a standard sample of the high molecular weight fragment derived from chick sternal cartilage type IX collagen by pepsin digestion (HMW) from R. Mayne, The University of Alabama, Birmingham. Collagen type I from rat tail tendon (Linsenmayer et al., 1978), types II, IX (HWM), and XI from chick embryo sternal cartilage (Reese and Mayne, 1981), and type X from mass chondrocyte cultures (Schmid and Linsenmayer, 1983), were prepared by the methods described in the indicated literature. Hyaluronic acid solutions of different molecular weights were prepared from chicken combs by extraction with water followed by lyophilization and reconstitution procedures including Pronase digestion and ethanol fractionation. To obtain samples of relatively small molecular weights, some preparations were subjected to either heating in an autoclave or digestion with testicular hyaluronidase before ethanol fractionation. The average molecular weights of the resulting fractions were determined by the measurement of low angle laser light scattering according to Ueno et al. (1988). PG-H (a major PG species of chick embryo cartilage which is much higher in both molecular weight and buoyant density than PG-Lt and PG-Lb, Oike et al., 1980), PG-Lb (a second major species of chick embryo cartilage which is smaller in size than PG-H), and PG-Lt (a minor species) were isolated by sucrose gradient centrifugation in 0.1% SDS (Noro et al., 1983), and PG-Lt or cartilage type IX collagen proteoglycan (Noro et al., 1983) from chick embryo epiphyseal cartilage, and PG-M (a major PG of chick embryo mesenchyme and cultured fibroblast which is different in core protein structure from PG-H, PG-Lt, and PG-Lb, Yamagata et al., 1986), were prepared as described.

**Extraction and Separation of Vitreous Humor Proteoglycan**—Fertile eggs were incubated for the periods indicated in individual experiments. After hatching, the eyes were removed, rinsed in Hank’s solution, and staged according to Hamburger and Hamilton (1951). The vitreous humor was removed from embryos in a P500 homogenizer in a final volume of 8 ml guanidine HCl, 0.1 M Tris-HCl, pH 8.0, 2 mM phenylmethylsulfonyl fluoride, 20 mM N-ethylmaleimide, 20 mM EDTA, and the mixture was stirred at 4°C for 12 h. The suspension was centrifuged at 4°C for 30 min at 17,000 × g. The residue was discarded. CsCl was added to the clarified extract to give an initial density of 1.36 g/ml. A successive gradient was established by centrifugation in a Hitachi RPS-50T2 rotor (145,800 × g, 10°C, 48 h) and then fractionated from the bottom of the tubes. Hexuronate and protein contents in the fractions were determined by the carboxyl method of Bitter and Muir (1962) and by the method of Lowry et al. (1951), respectively.

**Antiseras to Chick Embryo Sternal Cartilage HMW and Affinity-purified Antibodies to the 61-kDa Polypeptide of Vitreous Humor Proteoglycan**—0.5 mg of HMW in 0.5 ml of 0.1 M PBS (pH 7.2), 0.05 mg of monophosphoryl lipid A plus 0.05 mg of trehalose dimycolate (Ribi adjuvant system) emulsified with 0.5 ml of Freund's complete adjuvant was injected into a lymph node of the popliteal region of a rabbit. At three subsequent 2-weeks intervals, 0.2 mg of HMW in 0.5 ml of PBS, 0.05 mg of monophosphoryl lipid A plus 0.05 mg of trehalose dimycolate, 0.5 ml of Freund's incomplete adjuvant was injected to the intradermal site. The antibody titer and specificity were examined by enzyme-linked immunosorbent assay (Rennard et al., 1976) and by immunoblotting with chick vitreous humor (see below). The serum was collected 3 weeks after the third booster. The antisera were shown to react with both HMW and intact cartilage type IX or PG-Lt but not with collagen types I, II, III, IV, V, X, or XI.

From the anti-HMW antisera, antibodies specific for 61-kDa polypeptide of vitreous humor proteoglycan (see below for preparation of this compound) were affinity-purified as follows. A purified sample of the proteoglycan was treated with chondroitinase AC and subjected to SDS-polyacrylamide gel electrophoresis under reduced conditions (see below), followed by electrotransfer to a nitrocellulose membrane. The membrane was treated with 10% (w/v) skin milk in PBS and then with the anti-HMW antisera prepared as above. After washing with PBS, 0.05% (w/v) Tween 20 (five times), the 61-kDa band complexed with antibodies was cut out and eluted with 0.2 ml of 0.2 M glycine HCl, pH 2.4. The eluate was immediately neutralized with 0.02 ml of 1 M Tris-HCl, pH 9.0.

**Analyses of Proteoglycan Core Polypeptides by SDS-Polyacrylamide Gel Electrophoresis**—Proteoglycan core polypeptides were separated by SDS-polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) SDS was carried out in 5% (w/v) running gel (Laemmli, 1970) under nonreducing and reducing conditions. Before electrophoresis, proteoglycan samples were desalted by precipitation with 3 volumes of 95% (v/v) ethanol, 15% (w/v) potassium acetate acetate (Mavne et al., 1984) to remove chondroitin sulfate chains, ethanol precipitates (about 40 μg each as hexuronate) were dissolved in 30 μl of 0.05 M Tris-HCI, pH 7.6, 0.05 M sodium acetate containing 0.1 unit of chondroitinase ABC or chondroitinase AC and incubated at 37°C for 2 h. Incubated mixtures and control samples were heated in a boiling water bath for 3 min. Proteoglycans and/or their core molecules were precipitated with ethanol (see above) and dissolved in the sample buffer by heating at 100°C for 3 min. Reduction was carried out by adding 2-mercaptoethanol to a final concentration of 5% (w/v) in sample buffer. The molecular masses of the proteoglycans were estimated with collagenous peptides as standards. After electrophoresis, proteins and proteoglycans in gels were detected by staining with Coomassie Blue and Alcian Blue, respectively. For immunoassaying, the proteins in a gel were transferred to a nitrocellulose membrane by the method of Towbin et al. (1979). The membrane was then incubated successively in the following solutions: 1) 10% (w/v) skin milk, PBS at 37°C for 1 h, 2) anti-HMW antibodies in PBS, 0.05% (w/v) Tween 20; 3) five changes of PBS, 0.05% (w/v) Tween 20; 4) horseradish peroxidase-conjugated protein A or 131I-protein A in PBS, 0.05% (w/v) Tween 20 at room temperature for 1 h; 5) five changes of PBS, 0.05% (w/v) Tween 20; 6) 0.05% (w/v) 4-chloro-1-naphthol, 0.01% (v/v) H2O2 in PBS for 5 min to develop the color. For autoradiographic detection of bound 131I-protein A, the membranes were dried and exposed to Fuji RX x-ray film at -80°C (Fig. 4).

**Analyses of Glycosaminoglycans**—Glycosaminoglycans were released from proteoglycan samples by treatment with 0.3 M NaOH at room temperature for 24 h. After neutralization with acetic acid, the mixture was digested in 0.02 M Tris-HCl, pH 8.0, with Pronase (one-fifth the total amount of proteins) at 60°C for 1 h. Glycosaminoglycans were collected by ethanol precipitation, dissolved in either 0.4 M ammonium acetate or 4 M guanidine HCl, 0.05 M Tris-HCl, pH 6.5, and chromatographed on a Bio-Gel A-5m column on a Sephacryl CL-4B column equilibrated and eluted with sample buffer. Viscosities of glycosaminoglycans were measured in a Cannon-Manning microviscometer in 0.15 M phosphate buffer, pH 7.0, 0.2 M NaCl at 25°C, according to Ueno et al. (1988).

For determination of disaccharide unit compositions of glycosaminoglycans, chondroitin sulfate digestion was performed as previously described (Saito et al., 1968), and separation of the products was carried out by high-performance liquid chromatography on a Lichrosorb-NH2 column (0.26 × 25 cm) eluted with a linear gradient of NaOH-P04 (Yoshida et al., 1989). The unsaturated disaccharide products were detected and quantified by virtue of their absorbance at 232 nm.

**Electron Microscopy**—Vitreous bodies were dissected from 13-day-old chick embryos and immediately fixed in 0.1 M sodium periodate, 0.07 M lysine, 0.037 M phosphate, pH 6.2, 2% (w/v) paraformaldehyde at 4°C for 24 h. The specimens were dehydrated with a gradual
RESULTS

Isolation of Vitreous Humor Proteoglycan—When the vitreous humor obtained from 13-day-old (stage 39) chick embryos was extracted with 4 M guanidine HCl as outlined under “Experimental Procedures,” about 85% of the total carboxyl reaction-positive material was brought into solution. The insoluble material was not studied further. However, it probably represents so-called pecten, a major component of the vascular network in avian vitreous humor (Romanoff, 1960).

The extract was partitioned by CsCl isopycnic centrifugation under dissociative conditions. As Fig. 1 shows, the carboxyl reaction-positive material yielded three peaks, a, b, and c, of which a and b were located in a lower one-third where little labeled material was found and where peak c was seen. Peak a was digested with Streptomyces hyaluronidase-susceptible material, all of the carboxyl reaction-positive material in peak a, 10–20% of the material in peak b, and approximately one-half of the material in peak c were digested with chondroitinase ABC, as assessed by the corresponding reduction in ethanol-precipitable, carboxyl reaction-positive materials. None of the peak fractions, however, was digestible by Streptomyces hyaluronidase treatment. The resistant material in peak b probably represents DNA, as assessed by its strong adsorption at 260 nm, by its susceptibility to DNase I, and by its failure to react with RNase A (data not shown). The resistant material in peak c, on the other hand, was characterized as heparan sulfate in proteoglycan form; successive treatment of peak c material with alkaline and Pronase yielded a glycosaminoglycan sample which behaved as a mixture (about 1:1 with respect to the color intensity in carboxyl reaction) of chondroitin sulfate and heparan sulfate when examined by electrophoresis on a cellulose acetate membrane before and after treatment with chondroitinase ABC or heparitinase, or both (data not shown).

To test the possible occurrence of a proteoglycan form of type IX collagen, the gradients were partitioned into three fractions, I, II, and III, as in Fig. 1, and aliquots of each pooled fraction were examined by SDS-polyacrylamide gel electrophoresis in the running gel (lane a) and in the gel front (indicated by the open arrowheads) can be attributed to proteins from the chondroitin sulfate and heparan sulfate contents by the measurement of the hexuronate which was released from macromolecules by chondroitinase ABC digestion, 87, 12, and 1% of the total chondroitin sulfate were recovered in fractions I, II, and III, respectively. It is likely therefore that the Alcian Blue-positive material represents a large chondroitin (dermatan) sulfate proteoglycan a core molecule corresponding in M, to the core of type IX collagen proteoglycan (270 kDa) and at least 87% of the total chondroitin sulfate of the vitreous was derived from type IX collagen proteoglycan. Similar enzymatic treatment of fraction II also resulted in the appearance of a 270-kDa protein band (lane d) but its amount was far smaller than in fraction...
I. In fraction III, no chondroitinase-sensitive component was detected (lane f).

To test whether the 270-kDa core protein is related to cartilage type IX collagen proteoglycan, samples were electrophoresed as above and then transferred to nitrocellulose membranes. The membranes were stained using antisera raised against one of the triple-helical portions (HMW) of cartilage type IX collagen proteoglycan. As the immunostaining pattern (Fig. 2, lanes g–l) shows, the 270-kDa bands in lanes h and j (the band in lane j cannot be photographically reproduced) but no other protein bands were immunoreactive to anti-HMW antisera.

The results, taken together, indicate that fraction I of the CsCl gradient (Fig. 1) contains a proteoglycan form of type IX collagen as a sole protein component, although its buoyant density (~1.52 g/ml) is much higher than the value reported for cartilage type IX collagen proteoglycan (~1.34 g/ml, see Noro et al., 1983).

For further characterization of the vitreous humor proteoglycan, about 50 ml of the vitreous humor collected fresh from 150 chick embryos were extracted and partitioned as above and the fractions with buoyant densities higher than 1.52 g/ml (which were free of DNA) were pooled. The yield was about 2 mg/50 ml vitreous humor of 150 embryos; hereafter this preparation is referred to as “vitreous PG.”

Comparison between Vitreous PG and Cartilage Type IX Collagen Proteoglycan—Intact and chondroitinase AC-treated vitreous PG samples were analyzed by SDS-polyacrylamide gel electrophoresis under nonreducing and reducing conditions (Fig. 3). The use of chondroitinase AC in place of cartilage type IX collagen proteoglycan was necessary to avoid an overlapping, under reduced conditions, of protein bands derived from the enzyme preparations with those from proteoglycan samples. In agreement with the results of analyses using chondroitinase ABC (see above), intact vitreous PG did not enter the 5% gel (Fig. 3, lane a) whereas chondroitinase AC-treated vitreous PG gave a single protein band at 270 kDa (Fig. 3, lane b). The bands indicated by the open arrowheads represent proteins from the enzyme preparation. After reducing, both intact (lane c) and chondroitinase AC-treated (lane d) samples yielded a sharp band at 61 kDa, a somewhat broad band at 68 kDa, and a very weak band at 84 kDa. The color intensity of the 68-kDa component was significantly higher in the chondroitinase-treated sample than in the nontreated sample, suggesting that a part of the 68-kDa band represents a polypeptide derived from glycosaminoglycan-bearing α chain. Consistent with this view, an Alcian Blue-positive band was detected at the top of the gel in lane c but not in lane d (data not shown).

When the single protein band at 270 kDa (lane b) was cut out and again subjected to the gel electrophoresis under reduced conditions, three bands at 84, 68, and 61 kDa with the different color intensities were observed (data not shown), similarly to those in Fig. 3, lane d, indicating that protein bands in the lane d were derived from the 270-kDa collagen core protein. It is well established that cartilage type IX collagen proteoglycan is a heterotrimer of three polypeptide chains α1(IX), α2(IX), and α3(IX) with molecular mass 84,000, 68,000, and 61,000, respectively, and that the intact molecules contain chondroitin sulfate or dermatan sulfate covalently linked to the α2(IX) chain. Therefore, the results in Fig. 3 suggest that, although vitreous PG is similar to cartilage type IX collagen proteoglycan, there are tissue-specific variations at the α1(IX) chain leading to the appearance of a 61-kDa chain in place of the 84-kDa chain; hereafter they are referred to as α1(IX)61kDa and α1(IX)64kDa, respectively. The appearance of the weak band at 84 kDa in Fig. 3, lanes c and d, may reflect the fact that the vitreous humor preparation consists of a small amount of the cartilage-type component, α1(IX)64kDa, in addition to the major, vitreous humor-type component, α1(IX)61kDa.

When chondroitinase AC-treated vitreous PG and cartilage type IX collagen proteoglycan were run under reduced con-
dions on an SDS gel and then examined by immunoblotting with anti-HMW antisera (Fig. 4), the 84, 68, and 61-kDa bands from vitreous PG (lane a) as well as the 84- and 68-kDa bands from cartilage type IX collagen proteoglycan (lane b) were all immunoreactive to the antisera. When, however, the anti-HMW antisera were replaced by affinity-purified antibodies to the 61-kDa polypeptide (prepared with a nitrocellulose membrane to which the 61-kDa polypeptide had been electrotransferred), the 68-kDa band corresponding to α2(IX) and α3(IX) was no longer detected in both vitreous humor (lane c) and cartilage (lane d) samples. In contrast, the 84- and 61-kDa bands in the vitreous humor sample (lane c) as well as the α1(IX)64kDa band in the cartilage sample (lane d) showed significant reactivities, indicating that the 61-kDa polypeptide of vitreous PG is immunochemically related to the α1(IX)64kDa chains of cartilage type IX collagen proteoglycan and vitreous PG.

In addition to the difference in the polypeptide chains, a marked difference was observed when the glycosaminoglycan chains released from vitreous PG, cartilage type IX collagen proteoglycan, and other proteoglycans were compared on a Bio-Gel A-1.5m column. As Fig. 5 shows, the glycosaminoglycans from vitreous PG were far larger in hydrodynamic size than the chondroitin sulfate or dermatan sulfate with M_r ranging from 32,000 to 60,000 (Kimata et al., 1978; Yamagata et al., 1986), the samples prepared from chick embryo cartilage proteoglycan (PG-H, PG-Lb, and type IX collagen proteoglycan/PG-Lt) and chick embryo fibroblast proteoglycan (PG-M). This behavior of the vitreous humor glycosaminoglycan chains could not be ascribed to their self-aggregation, as a similar elution pattern was demonstrated by chromatography on a Sepharose CL-4B column regardless of whether or not guanidine HCl (4 M) was present in the elution buffer (data not shown). By measurement using high-performance liquid chromatography on two TSK-Gel G6000PW columns straight forward connected (0.75 × 60 cm), the average molecular weight of the vitreous humor glycosaminoglycan chains was estimated to be about 350,000 relative to hyaluronic acid standards with known molecular weights. A similar value was determined with two different elution patterns (prepared with a nitrocellulose membrane to which the 61-kDa polypeptide had been electrotransferred) in the Bio-Gel A-1.5m system. As shown in lanes a (vitreous PG) and b (cartilage type IX collagen proteoglycan), the 84-kDa band of vitreous PG was much larger than the 61-kDa band of cartilage type IX collagen proteoglycan (61 kDa). X, immunologically nonspecific bands.

![FIG. 4. Transfer blot analyses using anti-HMW antisera and affinity-purified antibodies to the 61-kDa polypeptide. Vitreous PG and cartilage type IX collagen proteoglycan were treated with chondroitinase AC and then electroblotted on a 5% SDS-polyacrylamide gel under reduced conditions. The result of staining nitrocellulose blots with anti-HMW antisera is shown in lanes a (vitreous PG) and b (cartilage type IX collagen proteoglycan) show the result of staining with affinity-purified antibodies to the 61-kDa polypeptide to see whether the 61-kDa polypeptide is related to 84-kDa α1(IX). The closed arrowheads indicate the positions of α1(IX) (84 kDa), α2(IX) and/or α3(IX) (68 kDa), and a short form of α1(IX) (61 kDa). X, immunologically nonspecific bands.](http://www.jbc.org/)

![FIG. 5. Bio-Gel A-1.5m chromatography of the glycosaminoglycans released from vitreous PG. Glycosaminoglycans obtained after alkaline Pronase treatment of vitreous PG were chromatographed on a Bio-Gel A-1.5m column. Positions for the glycosaminoglycans of PG-H (Mr. 32,000), cartilage type IX collagen proteoglycan (LI, M_r 40,000), and PG-Lb (Lb, Mr. 52,000) from chick embryo cartilage, and those of PG-M (M_r 60,000) from chick embryo fibroblasts. V_v, void volume. V_t, total column volume.](http://www.jbc.org/)

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<th>Disaccharide units</th>
<th>Composition</th>
<th>Vitreous humor</th>
<th>Cartilage</th>
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<tr>
<td>IdoA-GalNAc(4-SO_4)</td>
<td>7</td>
<td>12</td>
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<tr>
<td>GlcA-GalNAc(4-SO_4)</td>
<td>17</td>
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<td>GlcA-GalNAc(6-SO_4)</td>
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<td>GlcA-GalNAc</td>
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TABLE I Composition of glycosaminoglycans derived from vitreous PG and cartilage type IX collagen proteoglycan

Shown is the percentage of unsaturated disaccharides released by chondroitinase ABC or AC digestion.

suggested when viscosities of the glycosaminoglycan samples were compared; the reduced viscosity of the vitreous humor sample was 6.5 dl/g compared to a value of 9.2 dl/g for hyaluronic acid with a light-scattering molecular weight 460,000. The molecular weight of the vitreous humor chondroitin sulfate was calculated to be 330,000, based on its reduced viscosity. Far lower values, 0.45 and 1.4 dl/g were obtained with bovine cartilage chondroitin sulfate (molecular weight 15,000) and shark cartilage chondroitin sulfate (molecular weight 43,000), respectively.

As Table I shows, analysis of the glycosaminoglycan samples with chondroitinase ABC and AC indicates that the vitreous humor glycosaminoglycan is rich in chondroitin 6-sulfate units (63%), compared with the sample of cartilage type IX collagen proteoglycan at the same developmental stage which is rich in chondroitin 4-sulfate units.

Transition in α1(IX) Chain Size during the Development of Embryonic Chick Vitreous Humor—The demonstration in the 13-day-old (stage 39) embryonic chick vitreous humor of two different size α1(IX) polypeptides suggests that transitions may occur in the type of vitreous PG synthesized during the development of vitreous body. To test this possibility, the chain compositions of vitreous PG samples prepared from younger (stages 31 and 36) and older (stages 42 and 45) embryos and adult chicks (60 days old) were analyzed accord-
Vitreous Humor Proteoglycan

All the vitreous humor samples gave proteoglycan fractions with buoyant densities higher than 1.52 g/ml, suggesting the occurrence of proteoglycan molecules bearing very large chondroitin sulfate chains. Analyses of the resultant proteoglycan fractions by immunoblotting using anti-HMW antisera (Fig. 6) indicate that stage 31 vitreous humor (lane a) contained a vitreous PG which showed, after chondroitinase AC digestion followed by mercaptoethanol reduction, an immunostaining pattern for $\alpha_1(IX)^{64kDa}$, $\alpha_2(IX)$, and $\alpha_3(IX)$ as predominant core peptides. At later stages, the vitreous humor showed a progressive increase of $\alpha_1(IX)^{61kDa}$ with a concomitant decrease of $\alpha_1(IX)^{64kDa}$ and the specimens from stage 42-45 chick embryos and 60-day-old chicks (lanes b-f) contained predominantly $\alpha_1(IX)^{61kDa}$, $\alpha_2(IX)$, and $\alpha_3(IX)$.

**D-Periodic Distribution of Vitreous PG along Thin Collagen Fibrils**—A combination of immunological and electron microscopic methods was used to analyze the distribution of vitreous PG in 13-day-old embryonic chick vitreous humor. The sections immunolabeled with anti-HMW antisera followed by poststaining with uranyl acetate/lead citrate (Fig. 7a) showed that immunoreactive materials (vitreous PG; arrowheads) are distributed in a regular D-periodic arrangement of 66.9 nm (S.D. 8.7 nm, 193 fibrils) along individual thin fibrils (composed presumably of type II collagen) of a uniform diameter of 10 nm. These observations indicate that the epitopes of our polyclonal antibodies were not evenly distributed along a type IX collagen proteoglycan molecule in the fibril, as described by Bruckner et al. (1988) on human cartilage type IX collagen that had been stained with polyclonal antibodies raised against HMW + LMW. In Fig. 7a, clustered fibrils decorated by immunoreactive materials were frequently present, perhaps representing artifacts derived from individual thin fibrils by a shrinkage of vitreous humor during dehydration procedures (apparent distances of immunoreactive materials on these clustered fibrils are not included in the above data for D-periodicity). Control sections where anti-HMW antisera had been replaced by nonimmune sera revealed only thin and clustered fibrils with no periodically arranged materials (Fig. 7b).

**Fig. 6.** Results of transfer blot analyses using anti-HMW antisera to show a transition in the size of vitreous humor $\alpha_1(IX)$ polypeptide during chick embryo development. Vitreous PG samples prepared from the vitreous humor specimens at developmental stage of 31 (lane a), 36 (lane b), 39 (lane c), 42 (lane d), and 45 (lane e), and at 60 days (adult chick; lane f) were treated with chondroitinase AC and electrophoresed under reduced conditions as in Fig. 4. The result of staining nitrocellulose blots with anti-HMW antisera is shown (the top and bottom parts of the blots are not shown). $\bullet$, positions for $\alpha_1(IX)^{84kDa}$, $\alpha_2(IX)$ and/or $\alpha_3(IX)$ (68 kDa), and a short form of $\alpha_1(IX)^{61kDa}$.

**Fig. 7.** Electron micrograph of immunolabeled fibrils in the vitreous humor of 13-day-old chick embryo. $a$, vitreous PG molecules ($\uparrow$) are visualized by the immunoperoxidase method using anti-HMW antisera; $b$, control using nonimmune serum in place of the HMW antisera; bar, 100 nm.
DISCUSSION

The results presented here indicate that vitreous humor of chick embryos contains a modified form of type IX collagen proteoglycan which is characterized by the occurrence of a short form of $\alpha_1(IX)$ polypeptide and by the attachment of extraordinarily large chondroitin sulfate chain(s).

Using probes specific for $\alpha_1(IX)$ and $\alpha_2(IX)$ mRNAs, Svozil et al. (1988) have demonstrated that, although the size of $\alpha_2(IX)$ mRNA is the same in chick embryo cornea as in cartilage, the $\alpha_1(IX)$ mRNA is significantly smaller in the cornea than in the cartilage because 700 nucleotides are absent at the 5’ end of the mRNA encoding the major portion of the amino-terminal globular domain (NC4). It seems possible that a similar, if not identical, alteration may have occurred in the mRNA for vitreous humor $\alpha_1(IX)$ polypeptide, leading to the synthesis of $\alpha_1(IX)^{\text{short}}$, a smaller form presumably lacking the major portion of the amino-terminal globular domain (see below). It is noteworthy in this respect that, at early developmental stages (some time between stage 34 and 40), an epithelium (the neural retina) is responsible for the synthesis and secretion of collagens and gycosaminoglycans into vitreous humor, whereas at later stages these components are being produced predominantly by a fibroblastic cell population (hyalocytes) within the vitreous matrix itself (Newhouse et al., 1976; Smith and Newhouse, 1978). It is possible that the observed transition of $\alpha_1(IX)$ polypeptide from the long to the short form is closely associated with the temporal and spatial transition in cell type during the development of chick embryo vitreous humor. Whether the fibroblastic cells (hyalocytes) in the vitreous humor represent a population of cells that have emigrated from the neural retina remains to be established.

The most unusual feature of the present investigation is the demonstration in vitreous PG of chondroitin sulfate chains with an average molecular weight around 350,000, about 10 times the average value of ordinary chondroitin sulfate samples. The unusual chondroitin sulfate chain is detected as a covalently bound form in the 68-kDa polypeptide (presumably $\alpha_2(IX)$). In cartilage type IX collagen proteoglycan, the chondroitin sulfate attachment site is the serine residue of the sequence Val-Glu-Gly-Ser-Ala-Asp contained in the nonhelical NC3 domain of the $\alpha_2(IX)$ chain (Irwin et al., 1986; McCormick et al., 1987; Huber et al., 1988). Rotary shadowing of the fibrils prepared from chick embryo cartilage has revealed a D-periodic distribution of 35-40 nm long projections, each capped with a globular domain, corresponding to the amino-terminal globular and collagenous domains, NC4 and COL3, of type IX collagen proteoglycan (Vaughan et al., 1988). These authors suggest that, in cartilage matrix, type IX collagen proteoglycan must be distributed in a regular D-periodic arrangement along the fibrils, with the chondroitin sulfate either extending away from the fibril to potentially interact with other matrix components or interacting with molecules in the fibrils themselves. Our immunoelectron microscopic examination of the vitreous humor of chick embryo also revealed a D-periodic distribution of vitreous PG along the fibril, thus suggesting that the distribution pattern of the chondroitin sulfate chains on collagen fibrils should principally be identical in both vitreous humor and cartilage matrix. An intriguing question then arises as to whether the differences between the vitreous humor and cartilage matrix in the size of $\alpha_1(IX)$ and chondroitin sulfate chain may contribute to the functional difference between these matrices.

The vitreous humor provides a transparent medium for the transmission of light as well as the maintenance of the spherical shape of the eye. In mammals, the vitreous humor is rich in hyaluronic acid (reviewed in Laurent and Fraser, 1986, among others) which has the capacity to trap the flow of water, to exert an osmotic pressure, and to interfere with diffusion of macromolecular solutes (reviewed in Toole, 1976). These characteristics of hyaluronic acid can be related to various features of the vitreous humor, e.g., highly hydrated, swollen structure, low serum protein content, viscosity, osmotic pressure, transparency, and other mechanical properties. Our studies reported here indicate that little or no hyaluronic acid is present in the vitreous humor of 13-day-old chick embryos and further suggest that the periodically arranged chondroitin sulfate chains of high molecular weight may provide a vitreous matrix for physicochemical support to maintain internal eye structure and function. It is noteworthy in this respect that a regular arrangement of keratan sulfate- and dermatan sulfate-proteoglycans along collagen fibrils has been demonstrated in corneal stroma of rabbit, rat, and cow (reviewed in Scott, 1988). The transparency of the cornea depends largely on the regular spacing of the collagen fibrils (Maurice, 1957), and it is thought that this regularity is maintained by the swelling pressure of the proteoglycans, which keeps the fibrils apart.

Another aspect to be considered is the orientation of the amino terminal domain of $\alpha_1(IX)$ in fibrils. Since the globular domain of cartilage $\alpha_1(IX)$ is reported to have a pI value of 9.7 (Vasios et al., 1988), it could participate in ionic interactions with the polyanionic chondroitin sulfate chains attached to $\alpha_1(IX)$ (see Laurent and Scott, 1964; Mathews, 1970, for the examples of chondroitin sulfate/protein interaction), thereby affording a force opposing chondroitin sulfate-induced osmotic swelling, hydration, viscosity, etc. If so, transition of $\alpha_1(IX)$ polypeptide from the long to the short form would eliminate this force, driving the spherical expansion of the vitreous humor during development.

Using rotary shadowing procedures, Wright and Mayne (1988) have recently shown that the collagen fibrils prepared from adult chick vitreous humor are coated by glycosaminoglycan which could be removed by chondroitinase ABC digestion. The result contrasts with the previous observation with the collagen fibrils isolated from embryonic chick sternal cartilage which did not appear to have this glycosaminoglycan coat (Vaughan et al., 1988). The difference between the vitreous humor and cartilage in the morphological feature of collagen fibrils may be interpreted as reflecting a difference between these two tissues in the size of chondroitin sulfate chains associated with the collagen fibrils. That the vitreous humor type IX collagen from adult chick has a very large chondroitin sulfate chain (just like the chain from embryonic chick vitreous humor) has been confirmed by our recent analysis. Another unique feature of the vitreous humor collagen fibrils of adult chick is the absence of the knob of NC4 domain in the type IX collagen-proteoglycan associated with the fibrils (Wright and Mayne, 1988). In cartilage, the NC4 knob was detected in every projection of type IX collagen-proteoglycan from the surface of the collagen fibrils (Vaughan et al., 1986). Apparently, these electron microscopic observations are compatible with the hypothesis that the short form of $\alpha_1(IX)$ found in the vitreous humor of chick embryo (and adult chick, see Fig. 6) may represent an $\alpha_1(IX)$ chain lacking the NC4 domain which was never detected in chick cartilages.

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Occurrence in chick embryo vitreous humor of a type IX collagen proteoglycan with an extraordinarily large chondroitin sulfate chain and short alpha 1 polypeptide.


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