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EXTRACTION AND CHARACTERISATION OF THE INTACT FORM OF BOVINE VITREOUS TYPE IX COLLAGEN

Paul Bishop*+, David McLeod+ and Shirley Ayad*

*Departments of *Biochemistry & Molecular Biology and +Ophthalmology, University of Manchester, M13 9PT, U.K.*

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We provide the first biochemical characterisation of intact type IX collagen extracted from bovine vitreous. It possesses a shortened α1(IX) chain (M_r 64K) compared to its cartilage counterpart (M_r 84K). All the vitreous type IX collagen is in a proteoglycan form, its glycosaminoglycan constituent being a chondroitin/dermatan sulphate component of M_r 15-60K attached to the α2(IX) chain. This contrasts with previous findings in chick vitreous where a very long glycosaminoglycan chain of M_r ~350K was demonstrated.

Vitreous and cartilage contain collagen types II and IX (1,2). In both of these tissues type II collagen is the major fibril-forming collagen and type IX collagen molecules are regularly aligned along the surface of these fibrils, in a D-periodic distribution (3,4). Type IX collagen is a disulphide-bonded heterotrimer [α1(IX)α2(IX)α3(IX)] with a multi-domain structure composed of three collagenous domains (COL1 to 3) interspersed with four non-collagenous domains (NC1 to 4). The cartilage form has a highly cationic (pI 9.7) non-collagenous amino-terminal NC4 domain (5) and the α2(IX) chain may have a short covalently-linked chondroitin/dermatan sulphate glycosaminoglycan chain (6,7). In contrast, the chicken vitreous form of type IX collagen has a shortened α1(IX) chain, resulting in most of the NC4 domain being absent, and an extremely long chondroitin sulphate chain (M_r ~350K) attached to the α2(IX) chain which may comprise the major glycosaminoglycan component of chicken vitreous gel (8). As the major glycosaminoglycan component of bovine vitreous is hyaluronan, with chondroitin sulphate being a minor component (9), the intact form of bovine vitreous type IX collagen was extracted and its structure determined.

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MATERIALS AND METHODS

Three to five litres of pooled central and posterior adult bovine vitreous were centrifuged for two hours at 30,000g. The fibrous residue was extracted twice with 1M sodium chloride in 50mM Tris-HCl, pH 7.4, containing 2mM phenylmethylsulphonyl fluoride, 2mM EDTA, 5mM benzamidine and 10mM N-ethylmaleimide for 2 days at 4°C. The salt concentration of the pooled 1M sodium chloride extracts was increased to 4.5M and the resulting precipitate redissolved in 1M sodium chloride, 50mM Tris-HCl, pH 7.4 (with proteinase inhibitors as above). Samples were incubated with and without chondroitin ABC lyase by the method of Oike *et al* (10) for 16 hours at 30°C.

For DEAE-cellulose chromatography 1M sodium chloride extracts were dialysed against 6M urea, 50mM Tris-HCl, pH 8.3 and then applied to a DE52 cellulose column (7.5cm x 1.25cm) equilibrated in this buffer. The unbound fraction was eluted using the same buffer and the bound fraction eluted with 1M sodium chloride in 6M urea, 50mM Tris-HCl, pH 8.3. Absorbance was monitored at 230nm.

The buoyant density of adult bovine vitreous type IX collagen was determined by a modification of the method of Yada *et al* (8). Vitreous was homogenised with an equal volume of 0.1M sodium acetate, 0.3M sodium chloride, 4mM EDTA, 2mM phenylmethylsulphonyl fluoride, 10mM benzamidine and 20mM N-ethylmaleimide. 2000 units of leech hyaluronan lyase (Biopharm Ltd., UK; hyaluronan specific) was added and the mixture stirred at 30°C for 48 hours. Guanidine hydrochloride was then added to a final concentration of 4M and the mixture stirred for a further 48 hours at 4°C. The suspension was centrifuged at 30,000g for 2 hours, and the residue discarded. The supernatant was concentrated by a factor of 10 using Amicon ultrafiltration (membrane cut-off M_r. 30,000), at 4°C. Caesium chloride was added to give an initial density of 1.36 g/ml and a dissociative gradient established by centrifugation at 148,000g (average), 10°C for 60 hours prior to fractionating into five aliquots of equal volume.

Discontinuous SDS-polyacrylamide gel electrophoresis was carried out under nonreducing and reducing conditions (11). Transfer to nitrocellulose (12) was in modified buffer containing 0.048M Tris, 0.039M glycine, 20% (v/v) methanol and 0.0375% (w/v) SDS. Following transfer the nitrocellulose sheets were immunoblotted (13) with antisera (1:1000 in PBS-Tween) to the COL1(IX) and COL2(IX) domains of type IX collagen. M_r values for vitreous type IX collagen were assigned by reference to a collagen II/IX standard prepared from foetal bovine cartilage (14, 15).

RESULTS

The salt extraction procedure employed resulted in the partial purification of small quantities of intact type IX collagen, which were immunoblotted by two antisera that have been fully characterised in previous studies (14, 15). An antiserum raised against the COL2 triple-helical fragment (all three α-chains within the COL2 domain) of pepsinised bovine cartilage type IX collagen (designated anti-[COL2(IX)]), was highly specific for type IX collagen, but immunoblotted the α2(IX) chain weakly, especially when in the glycanated (with attached glycosaminoglycan) form. A similar antiserum raised against the COL1 domain (anti-[COL1(IX)]) immunoblotted the α2(IX) band more strongly, but showed weak cross-reactivity with type II collagen. Both antisera preferentially bound to the α1(IX) chain, allowing distinction of this component by immunoblotting.

Figure 1 shows the results of the partially purified 1M sodium chloride extracts analysed on SDS-8%(w/v)-polyacrylamide gels and immunoblotted with

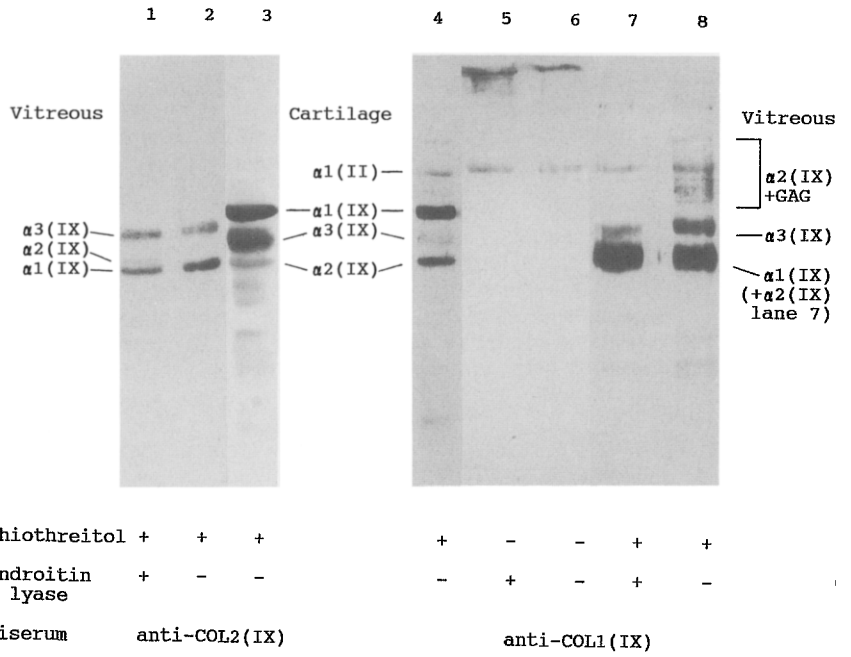

Figure 1. Immunoblots of 1M sodium chloride extracts analysed on SDS-8%(w/v)-polyacrylamide gels, with or without digestion by chondroitin ABC lyase. Lanes 1-3 were immunoblotted with anti-[COL2(IX)] and lanes 4-8 with anti-[COL1(IX)]. Lanes 3-4 are foetal bovine cartilage standards.

the two antisera. Lanes 1-3 were run under reducing conditions and immunoblotted with anti-[COL2(IX)]. The sample that had been digested with chondroitin ABC lyase (lane 1) showed all three α-chains of type IX collagen, with approximate M_r values of α1(IX) 64K, non-glycanated α2(IX) 67K and α3(IX) 78K. In comparison, the foetal bovine cartilage type IX collagen standard (lane 3) exhibited M_r values of α1(IX) 84K, non-glycanated α2(IX) 66K and α3(IX) 72K. Without chondroitin ABC lyase digestion (lane 2) the glycanated α2(IX) component was weak and polydisperse. Following immunoblotting with anti-[COL1(IX)] (lanes 4-8), non-reduced type IX collagen migrated as a high M_r component that just entered the 8%-polyacrylamide gel (lane 6), but after chondroitin ABC lyase digestion showed a slightly increased mobility (lanes 5). After reduction, the α1(IX) and non-glycanated α2(IX) components (lane 7) were too close together to be distinguished. However, the glycanated α2(IX) component (lane 8) was clearly visible as a diffuse band of M_r 80-125K, suggesting that the glycosaminoglycan component is polydisperse with M_r values of approximately 15-60K.

To determine what proportion of the extracted intact type IX collagen possessed a glycosaminoglycan chain, 1M sodium chloride extracts were subjected to DEAE-cellulose chromatography. The unbound and bound fractions were analysed by SDS-5%(w/v)-polyacrylamide gel electrophoresis under

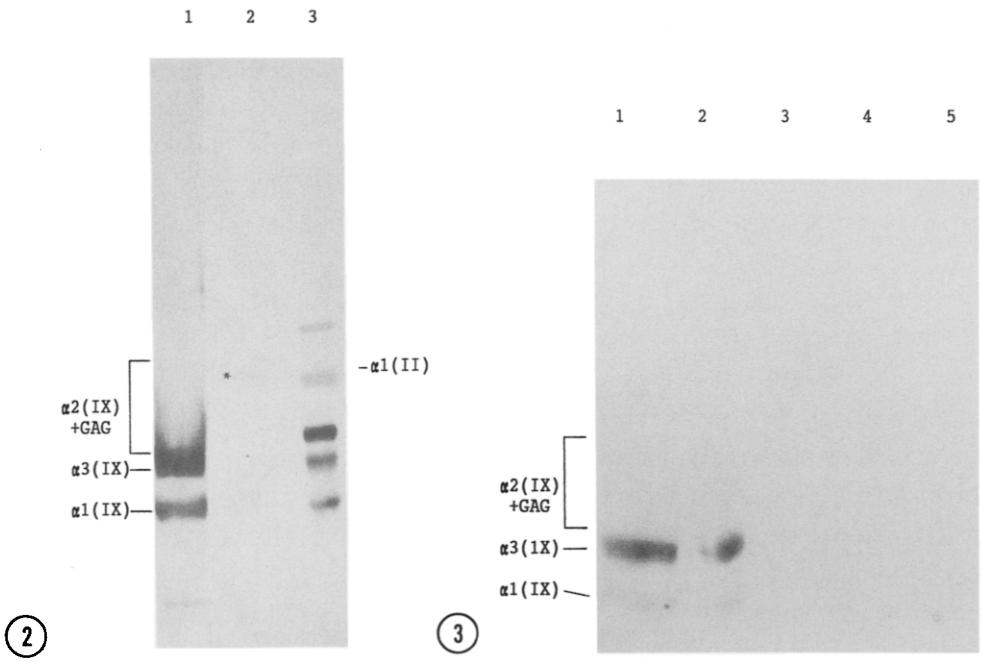

Figure 2. Immunoblot of a 1M sodium chloride extract subjected to DEAE-cellulose chromatography. Bound (lane 1) and unbound (lane 2) fractions were analysed by SDS-5%(w/v)-polyacrylamide gel electrophoresis under reducing conditions and immunoblotted with anti-[COL1(IX)]. Lane 3 is a foetal bovine cartilage standard.

Figure 3. Isopycnic centrifugation of 4M guanidinium chloride-extracted vitreous. Gradient fractions were analysed on SDS-5%(w/v)-polyacrylamide gels under reducing conditions and immunoblotted with anti-[COL1(IX)]. The buoyant densities of each fraction were; lane 1 - 1.22 g/ml, lane 2 - 1.29g/ml, lane 3 - 1.34g/ml, lane 4 - 1.39g/ml, lane 5 - 1.50g/ml.

reducing conditions and immunoblotting with anti-[COL1(IX)] (Figure 2). The bound fraction (Figure 2, lane 1) showed the three α-chains of type IX collagen, with M_r values as indicated above. In contrast, no type IX collagen was immunoblotted in the unbound fraction (lane 2), but faint cross-reactivity with type II collagen was just visible (*). These results indicate that all the extracted type IX collagen was in a proteoglycan form.

Caesium chloride isopycnic centrifugation under dissociative conditions resulted in a density gradient of between 1.22-1.50 g/ml, which was separated into five aliquots of equal volume. Immunoblotting of these fractions (Figure 3) demonstrated that type IX collagen was present only in the two fractions of lowest buoyant density (less than 1.34 g/ml).

DISCUSSION

These data provide evidence that adult bovine vitreous type IX collagen is similar to the chicken form (8) in that it has a shortened α1(IX) chain

and hence an NC4 domain which is largely absent. This conclusion is supported by rotary shadowing experiments which have demonstrated an absence of the amino-terminal NC4 domain in bovine vitreous type IX collagen (4). Foetal chick cornea type IX collagen has similarly been shown to possess a shortened α1(IX) chain and the two different forms have been attributed to the alternative use of two transcription start sites and splice patterns (16).

The α2(IX) chain has a single glycosaminoglycan attachment site at the NC3 domain (17) and consequently our data suggests that all the salt-extractable adult bovine vitreous type IX collagen possesses a single short covalently-linked chondroitin/dermatan sulphate chain of M_r 15-60K. In contrast, Yada *et al.* (8) demonstrated an extremely long, high M_r (~350K), chondroitin sulphate chain attached to chick vitreous type IX collagen. We could find no evidence for a high M_r glycosaminoglycan chain in addition to the short chain of bovine vitreous type IX collagen: 1) there was no evidence of a high M_r component on SDS-5%-polyacrylamide gel electrophoresis under reducing conditions (Figure 2), 2) only a low buoyant density form of bovine vitreous type IX collagen was found (the chick form is of high buoyant density). These findings are further supported by rotary shadowing experiments which have shown that long glycosaminoglycan chains decorating the major type II collagen fibrils of chicken vitreous (18) are not present in bovine vitreous (4; P. Bishop and S. Ayad, unpublished data).

This species difference in type IX collagen glycosaminoglycan chain size may account for quantitative variations in the total vitreous glycosaminoglycan composition. The predominant glycosaminoglycan of the chicken vitreous gel is chondroitin sulphate (8, 19), which is derived from the long chondroitin sulphate glycosaminoglycan chains of type IX collagen. In contrast, the major glycosaminoglycan component of bovine vitreous is hyaluronan (9) and therefore the short chondroitin sulphate/dermatan sulphate chains of type IX collagen contribute to the minor sulphated glycosaminoglycan component observed in this species.

Biosynthetic studies (14) have demonstrated that both proteoglycan and non-proteoglycan forms of type IX collagen are synthesized by bovine cartilage, the proteoglycan form possessing a glycosaminoglycan chain of M_r ~50K (ie. of similar M_r to the bovine vitreous type IX collagen glycosaminoglycan chain). However, when cartilage type IX collagen is extracted using techniques similar to those used in this study, virtually all the extracted type IX collagen is in the non-proteoglycan form (15, 20). In contrast this study demonstrates that all the extracted vitreous type IX collagen is in a proteoglycan form. Clearly there are different forms of type IX collagen which are both tissue and species specific, and these

differences are likely to be of importance in determining the biochemical and morphological characteristics of their respective tissues.

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