Bovine cartilage types VI and IX collagens

Characterization of their forms *in vivo*

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1. Collagens were extracted from bovine cartilage by 4 M-guanidinium chloride in the presence of proteinase inhibitors and identified by immunoblotting with specific anti-collagen sera. 2. The collagens retained their native conformations (shown by the resistance of their triple-helical domains to pepsin digestion), and the molecular masses of their component α -chains indicated that the chains were intact. 3. Type VI collagen was extracted as a large-molecular-mass disulphide-bonded aggregate composed of components of molecular mass 140 kDa and 200–240 kDa, and was therefore similar to type VI collagen identified in non-cartilaginous tissues. Immunoblotting established the 200–240 kDa components as intact forms of the $\alpha 3(\text{VI})$ chain. 4. Type IX collagen consisted of three clearly separable components of molecular mass 84 kDa, 72 kDa and 66 kDa, which were assigned to the $\alpha 1(\text{IX})$ -, $\alpha 3(\text{IX})$ - and $\alpha 2(\text{IX})$ -chains respectively, and a large proportion of this collagen had no covalently bound glycosaminoglycan attached to the $\alpha 2(\text{IX})$ -chain. 5. Differences between the type IX collagen extracted from bovine cartilage and that identified in biosynthetic studies on chick cartilage are discussed.

INTRODUCTION

The original concept that cartilage is a homogeneous tissue, comprised of a network of type II collagen fibrils inflated by highly hydrated large-molecular-mass proteoglycans, has been modified in recent years (Grant *et al.*, 1988). The chondrocytes and matrix macromolecules are not randomly disposed but exhibit a high degree of organization and compartmentalization, which is in part related to the type and anatomical site of the cartilage as well as to the species and age of the organism (Kuettnner *et al.*, 1986). Most types of cartilage contain two quantitatively minor collagens, types IX (van der Rest & Mayne, 1987) and XI (Eyre & Wu, 1987), but a third collagen, namely type X collagen, is present specifically in those cartilages that calcify and are ultimately replaced by bone (Schmid & Linsenmayer, 1987). The isolation of all these collagens from cartilaginous tissues has depended largely on the release of their triple-helical domains from the insoluble matrix by digestion with pepsin, and in the case of type IX collagen considerable fragmentation occurs because the triple helix is interspersed by several non-helical domains (van der Rest & Mayne, 1987). Our understanding of the intact forms of the different cartilage collagens *in vivo* is based on either biosynthetic studies with the use of cell or organ culture of avian cartilages (Gibson *et al.*, 1983; Bruckner *et al.*, 1985; Clark & Richards, 1985) or extraction studies on the lathyrctic rat chondrosarcoma (Duance *et al.*, 1984).

Immunofluorescence studies from this laboratory indicated that type VI collagen was also present in cartilage and was localized specifically to the immediate pericellular environment of the chondrocytes (Ayad *et al.*,

1984). This observation was surprising, since type VI collagen had not been detected in pepsin digests of cartilage. However, the effective solubilization of cartilage collagens by digestion with pepsin depends on the prior removal of proteoglycans by extraction with 4 M-guanidinium chloride (Hardingham, 1979), and this solvent is known to extract type VI collagen from non-cartilaginous tissues in an intact form (Ayad *et al.*, 1985; Gibson & Cleary, 1985). It seemed probable, therefore, that cartilage type VI collagen was being preferentially removed together with the proteoglycans. Consequently, we have fractionated the guanidinium chloride extracts of different types of cartilage and have observed that not only is type VI collagen present in these extracts, and therefore constitutes an integral component of the cartilage matrix, but that also the intact forms of all other cartilage collagens, types II, IX, X and XI, can be detected. The characterization of type X collagen in growth-plate cartilage has been reported (Ayad *et al.*, 1987), and attention is focused here on types VI and IX collagens in different bovine cartilages.

EXPERIMENTAL

Materials

Guanidinium chloride (GuCl; grade 1), CsCl (A.R.), pepsin (EC 3.4.23.1) from pig stomach mucosa (1:60000), chondroitin ABC lyase (EC 4.2.2.4), non-collagenous protein standards for polyacrylamide-gel electrophoresis, proteinase inhibitors and reagents used in immunoblotting were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). DE-52 DEAE-cellulose was purchased from Whatman (Hoylake, Cheshire, U.K.).

Abbreviations used: GuCl, guanidinium chloride.

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Sephacrose CL-4B was purchased from Pharmacia Fine Chemicals (Milton Keynes, Bucks., U.K.).

Foetal calves (130–270 days gestation) were obtained from the abattoir within 1 h of maternal death. Epiphyseal cartilage was dissected from the femoral and tibial condyles, excluding the surface layers and the region immediately adjacent to the secondary centre of ossification, which was present in embryos of greater than 180 days gestation. Growth-plate cartilage was dissected from each end of the long bones of the femur and tibia. Cartilage was also dissected free of perichondrium from adult bovine nasal septa.

Preparation of antisera to pepsin-digested collagens

Type II collagen from human nucleus pulposus and types IX and XI collagens from bovine nasal cartilage were purified from pepsin digests of the respective tissues as described previously (Ayad *et al.*, 1981, 1982; Ayad & Weiss, 1984). The type IX collagen preparation consisted of three major triple-helical fragments C-PS1, C-PS2 and C-PS3, corresponding approximately to the COL2, COL1 and COL3 domains respectively (Grant *et al.*, 1988). The COL1 and COL2 domains were separated by differential salt precipitation at neutral pH (Ayad *et al.*, 1982). Type VI collagen was prepared from pepsin digests of bovine uterus (Abedin *et al.*, 1982) and purified further by chromatography on Sepharose CL-4B (Poole *et al.*, 1988). Pure type VI collagen was eluted at the void volume (V_0) and consisted of a mixture of short and (less extensively pepsin-digested) long forms (Timpl & Engel, 1987).

Antisera to the native pepsin-digested collagen types II, VI and XI and the separated COL1 and COL2 domains of type IX collagen were raised in either guinea pigs or rabbits by established techniques (Evans *et al.*, 1983; Poole *et al.*, 1988). Antisera were assayed for cross-reactivity against other native pepsin-digested collagens by the e.i.s.a. technique (Morgan *et al.*, 1987) and against denatured pepsin-digested collagens by immunoblotting (see below).

Extraction of intact forms of types VI and IX collagens

Most experiments were carried out on the epiphyseal cartilage (200 g wet wt.) from a foetus of 240 days gestation. However, an initial wet weight of 10–20 g is sufficient for all the analyses. Cartilage was powdered under liquid N_2 in a stainless-steel mill and the powdered tissue was washed overnight with 50 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.4. The tissue was then extracted twice with 4 M-GuCl/50 mM-Tris/HCl buffer, pH 7.4, followed by 4 M-GuCl/50 mM-Tris/HCl buffer, pH 8.3, containing 100 mM-2-mercaptoethanol. All extractions were performed at 4 °C for 24–48 h in the presence of a mixture of proteinase inhibitors: 0.5 mM-phenylmethanesulphonyl fluoride, 100 mM-6-aminohexanoic acid, 2 mM-EDTA (tetrasodium salt) and 10 mM-N-ethylmaleimide. The extracts were concentrated by ultrafiltration on an Amicon PM10 membrane and stored at 4 °C. Similar extracts were prepared from foetal growth-plate and adult nasal cartilages but were used only for pepsin digestion studies (see below).

CsCl-density-gradient centrifugation (associative conditions)

Collagens and other proteins were separated from proteoglycans by CsCl-density-gradient centrifugation

under associative conditions (Aletras & Tsiganos, 1985). The 4 M-GuCl extracts were first dialysed against 7 vol. of 50 mM-Tris/HCl, pH 7.4, containing proteinase inhibitors (as above), and the resulting 0.5 M-GuCl solutions were clarified by centrifugation at 30 000 g for 1 h. Solid CsCl was then added to a final density of 1.6 g/cm³ and the resulting mixture was centrifuged at 100 000 g for 72 h at 20 °C. The protein gel at the top of the gradient was suspended in, and extensively dialysed against, 0.5 M-acetic acid, and the resulting acid-soluble and acid-insoluble fractions were separated by centrifugation at 30 000 g for 1 h. The acid-soluble fraction was then fractionated by differential salt precipitation at 0.86 M-, 1.2 M- and 3 M-NaCl (Ayad *et al.*, 1987). The acid-insoluble fraction was analysed by chromatography on DEAE-cellulose and Sepharose CL-4B.

DEAE-cellulose chromatography

The acid-insoluble fraction was equilibrated in 7 M-urea/50 mM-Tris/HCl buffer, pH 8.3, containing 0.2% Triton X-100 and the proteinase inhibitors as above (referred to below as 7 M-urea buffer). The solubilized material (approx. 50 mg of protein) was applied to a DEAE-cellulose column (1.6 cm × 14 cm) equilibrated with the 7 M-urea buffer, and the column was eluted with the same buffer at the rate of 50 ml/h until the non-retarded material was eluted, after which a linear gradient of 0–0.5 M-NaCl in the 7 M-urea buffer was applied over a total volume of 300 ml. Appropriate fractions were pooled, dialysed against distilled water and freeze-dried.

Sepharose CL-4B chromatography

The acid-insoluble fraction of the protein gel was equilibrated in 4 M-GuCl/50 mM-Tris/HCl buffer, pH 7.4, containing proteinase inhibitors (referred to below as 4 M-GuCl buffer) and heated at 60 °C for 1 h. The solubilized material was applied to a Sepharose CL-4B column (96 cm × 1.5 cm) equilibrated with the 4 M-GuCl buffer and eluted with this buffer at a rate of 20 ml/h at room temperature. Fractions that were eluted at V_0 and just after V_0 were pooled separately from several runs and rechromatographed on Sepharose CL-4B.

SDS/polyacrylamide-gel electrophoresis and immunoblotting

Samples were analysed in the presence (reduced) and in the absence (non-reduced) of 50 mM-dithiothreitol by the method of Laemmli (1970) and either stained with Coomassie Brilliant Blue R or transferred to nitrocellulose (Towbin *et al.*, 1979) and immunoblotted with the primary anti-collagen serum (1:1000 dilution) followed by the secondary alkaline-phosphatase-conjugated antiserum (1:1000 dilution) by the method of Blake *et al.* (1984). Molecular-mass values quoted for collagens are based on collagenous standards of known molecular mass, except for the intact form of type VI collagen, two-thirds of which is composed of non-collagenous sequences. Molecular-mass values for the latter and for all non-collagenous proteins are based on non-collagenous standards of known molecular mass.

Enzyme digestions

Portions of the 4 M-GuCl extracts from foetal epiphyseal, growth-plate and adult nasal cartilages were equilibrated into 0.5 M-acetic acid, pH 2.5, and digested

with pepsin (0.5 mg of pepsin/ml of extract) for 72 h at 4 °C. The pepsin digests were then fractionated by the method of Trueb *et al.* (1987) as follows. Solid NaCl was added to a final concentration of 2 M and the suspension was stirred for 4 h at 4 °C. Precipitated proteins were recovered by centrifugation (30 000 *g* for 1 h) and re-dissolved in 1 M-NaCl/50 mM-Tris/HCl buffer, pH 7.5, with re-adjustment of the pH to 7.5 with 10 M-NaOH for complete dissolution. The NaCl concentration was increased to 3 M and the resulting precipitate (3 M_{pH 7.5}) was collected by centrifugation. The pH of the supernatant was then adjusted to pH 2.5 with acetic acid and a second 3 M-NaCl precipitate (3 M_{pH 2.5}) was recovered by centrifugation.

Samples of interest were digested with chondroitin ABC lyase by the method of Oike *et al.* (1980).

RESULTS

Characterization of anti-collagen sera

All antisera were active at dilutions between 1:10 000 and 1:50 000 against the immunizing native collagen and showed only low or no cross-reactivity against the other native collagens at dilutions $\geq 1:1000$ in the e.l.i.s.a. (results not shown). The specificities of the antisera for the denatured collagens were assessed by immunoblotting the pepsin-digested forms of the different collagen types. The anti-(type II collagen) serum reacted strongly with the $\alpha 1(\text{II})_p$ - and $\alpha 3(\text{XI})_p$ -chains of types II and XI collagens respectively (Fig. 1, tracks 1 and 2). (Subscript 'p' indicates the pepsin-digested form of the α -chains; no subscript denotes the intact form of the α -chains.) The anti-(type XI collagen) serum reacted with all three chains of type XI collagen [being most active against the $\alpha 3(\text{XI})_p$ -chain] and cross-reacted with the $\alpha 1(\text{II})_p$ -chain (Fig. 1, tracks 3 and 4). The anti-(type VI collagen) serum was highly specific and cross-reacted with no other collagen types. When immunoblotted against a mixture of the short and long forms of type VI collagen it reacted

strongly with the $\alpha 3(\text{VI})_p$ -chain and the less extensively pepsin-digested form of this chain [designated $\alpha 3(\text{VI})_p^*$] and weakly with components migrating in the positions of $\alpha 1(\text{VI})_p$ - and $\alpha 2(\text{VI})_p$ -chains (Fig. 1, track 5). However, since the antiserum did not react with $\alpha 1(\text{VI})_p$ - and $\alpha 2(\text{VI})_p$ - chains in preparations comprising only the short form of type VI collagen (Fig. 1, track 6, and Fig. 5, track 7), the minor immunoreactive components seen in Fig. 1 (track 5) may represent forms of the $\alpha 3(\text{VI})_p$ -chain that are intermediate between the $\alpha 3(\text{VI})_p^*$ - and $\alpha 3(\text{VI})_p$ -chains. The antisera to the COL1 and COL2 domains of type IX collagen [designated anti-[COL1(IX) domain] serum and anti-[COL2(IX) domain] serum respectively] showed no cross-reactivity with the non-immunizing domain (Fig. 1, tracks 7–10). The anti-[COL2(IX) domain] serum, however, did detect traces of the COL2 domain present in the COL1 domain preparation (Fig. 1, track 8). Each anti-(type IX collagen) serum reacted with all three distinct chains within the respective triple-helical domains (van der Rest & Mayne, 1987). Both antisera initially cross-reacted strongly with the $\alpha 1(\text{II})_p$ -chains (results not shown) and were therefore immunoadsorbed against $\alpha 1(\text{II})_p$ -chains (transferred and bound to nitrocellulose) immediately before use.

Immunodetection of intact cartilage collagens

To facilitate the purification of the various collagenous and non-collagenous proteins in successive GuCl extracts, the bulk of the proteoglycans in the first and second GuCl extracts were removed by CsCl-density-gradient centrifugation. The third (reductive GuCl) extract was analysed directly. Fig. 2 shows identical amounts of protein from the three GuCl extracts of epiphyseal cartilage electrophoresed under reducing conditions and immunoblotted with antisera to types II, VI and IX collagens. The anti-(type II collagen) serum reacted with a major component of molecular mass 100 kDa in all three extracts, identifying this as the intact $\alpha 1(\text{II})$ -chain. In addition, two other minor immuno-

Fig. 1. Specificity of antisera for denatured pepsin-digested collagens

Samples of bovine collagens were analysed by SDS/polyacrylamide-gel electrophoresis on either 8% (w/v) gels (tracks 1–6) or 12% (w/v) gels (tracks 7–10) under reducing conditions and immunoblotted with the anti-collagen sera as shown. All collagens (except type VI) were prepared from nasal cartilage. Samples were: tracks 1 and 4, type II collagen; tracks 2 and 3, type XI collagen; tracks 5 and 6, type VI collagen from uterus (mixture of long and short forms) and nuchal ligament (short form only) respectively; tracks 7 and 9, COL2 domain of type IX collagen; tracks 8 and 10, COL1 domain of type IX collagen.

Fig. 2. Immunodetection of intact collagens in successive GuCl extracts of foetal epiphyseal cartilage

Identical amounts of protein from the first (1), second (2) and third (reductive) (3) GuCl extracts of foetal epiphyseal cartilage were analysed by SDS/7%-(w/v)-polyacrylamide-gel electrophoresis under reducing conditions and immunoblotted with the anti-collagen sera as shown. Molecular-mass values at left-hand and right-hand sides are based on collagenous and non-collagenous standards respectively of known molecular mass.

reactive components, migrating more slowly than the $\alpha 1(\text{II})$ -chain, were observed. In view of the cross-reactivity between this antiserum and the $\alpha 3(\text{XI})$ -chain (Fig. 1, track 2), one of these components could represent the intact form of the $\alpha 3(\text{XI})$ -chain. Both the anti-[COL1(IX) domain] and anti-[COL2(IX) domain] sera reacted strongly with three components of molecular mass 84 kDa, 72 kDa and 66 kDa, indicating that the three components contain sequences from the COL1 and COL2 domains and probably represent the intact forms of the three genetically distinct chains of type IX collagen. The anti-(type VI collagen) serum reacted with four well-defined components of molecular mass 200–240 kDa, which in view of the specificity of this antiserum (Fig. 1), are identified as the intact form(s) of the $\alpha 3(\text{VI})$ -chain. Indeed this observation confirms studies on other tissues showing the $\alpha 3(\text{VI})$ -chain to be heterogeneous with molecular mass 200–240 kDa (Timpl & Engel, 1987). Both type IX and type VI collagens were disulphide-bonded (Figs. 3, 4b inset and 4c inset). The anti-(type XI collagen) serum did not detect any components in the GuCl extracts, although pepsin digestion of these extracts indicated that type XI collagen was certainly present (see below).

The immunoreactive components detected by each antiserum did not vary significantly in intensity in the three successive GuCl extracts except in the case of type VI collagen, when the intensity was less pronounced in the first extract compared with the second GuCl extract and the third, reductive, GuCl extract. An additional component, of molecular mass approx. 80 kDa, that reacted strongly with the anti-(type VI collagen) serum was consistently observed in the reductive extract. However, it is not yet known whether this is a specific product released by reductive cleavage or arises from non-specific degradation.

Previous studies (Ayad *et al.*, 1987) have shown that, when the protein gel from the CsCl-density-gradient centrifugation was extensively dialysed against 0.5 M-acetic acid, approx. 5–10% (w/w) of the protein was solubilized. Subsequent analysis of the acid-soluble and acid-insoluble fractions indicated that type II collagen and the three components that we now identify as type IX collagen were present in both fractions. The acid-soluble type IX collagen was precipitated at 1.2 M-NaCl on differential salt precipitation. However, attempts to purify this fraction further by dialysis against phosphate buffers (Ayad *et al.*, 1981, 1982) resulted in the degradation of the component of molecular mass 84 kDa. Further characterization of type IX collagen was therefore carried out only on the acid-insoluble fraction, which also contained all the type VI collagen.

DEAE-cellulose chromatography

Biosynthetic studies have shown that the newly synthesized chick type IX collagen is composed of three genetically distinct chains: an $\alpha 1(\text{IX})$ -chain of molecular mass 84 kDa and $\alpha 2(\text{IX})$ - and $\alpha 3(\text{IX})$ -chains each of molecular mass 68 kDa. Approx. 70–80% of the newly synthesized molecules are proteoglycan in nature and carry variable amounts of glycosaminoglycan (chondroitin sulphate/dermatan sulphate) on the $\alpha 2(\text{IX})$ -chain, which consequently increases in molecular mass from 68 kDa to 115 kDa (for reviews see van der Rest & Mayne, 1987; Grant *et al.*, 1988). It was therefore important (a) to ascertain whether the type IX collagen extracted from bovine cartilage was also a proteoglycan and (b) to assign the three immunoreactive components of molecular mass 84 kDa, 72 kDa and 66 kDa to specific α -chains. The bovine type IX collagen was therefore further investigated by DEAE-cellulose chromatography.

Fig. 3 shows the elution profile of the acid-insoluble fraction of the protein gel from the CsCl-density-gradient centrifugation on DEAE-cellulose and the SDS/polyacrylamide-gel-electrophoretic analyses of the various pooled fractions. When the fractions were electrophoresed under reducing conditions and immunoblotted with the anti-[COL1(IX) domain] serum, immunoreactive peptides were found both in the unbound fraction 1 as well as throughout the gradient. The three immunoreactive components of molecular masses 84 kDa, 72 kDa and 66 kDa were observed in fractions 1 and 3 and were present in approximately 1:1:1 proportions when assessed by Coomassie Blue staining. However, in the more strongly bound fractions 4 and 6 the faster-migrating component of molecular mass 66 kDa was replaced by a diffuse staining whose average molecular mass increased with increasing salt concentration, reaching a maximum of approx. 115 kDa at 0.33 M-NaCl. When the fractions were electrophoresed under non-reducing conditions and immunoblotted with anti-[COL1(IX) domain] serum only one component, of molecular mass approx. 250 kDa, was observed, which increased slightly in molecular mass in the more strongly bound fractions. These results suggested that the bovine type IX collagen also carried a glycosaminoglycan chain and that the component of molecular mass 66 kDa was the $\alpha 2(\text{IX})$ -chain. However, in contrast with chick, at least 95% of the bovine type IX collagen was eluted in the unbound fraction 1. Moreover, chondroitin ABC lyase digestion of both the unfractionated protein gel

Fig. 3. Chromatography of the intact forms of types VI and IX collagens on DEAE-cellulose

(a) The acid-insoluble fraction of the protein gel from the CsCl-density-gradient centrifugation was equilibrated in 7 M-urea/50 mM-Tris/HCl buffer, pH 8.3, containing 0.2% Triton X-100 and proteinase inhibitors. The solubilized material was applied to a DEAE-cellulose column (1.6 cm $\times$ 14 cm) and eluted with the above buffer at the rate of 50 ml/h until the non-retarded material was eluted. A linear NaCl gradient (0–0.5 M) was then applied (arrow) over a total volume of 300 ml. (b) SDS/8%-(w/v)-polyacrylamide-gel electrophoresis of fractions 1–6 pooled as shown in (a) and stained with Coomassie Blue (CB) or immunoblotted with the anti-collagen sera as shown. Samples were analysed in the absence (–) or in the presence (+) of dithiothreitol. Molecular-mass values are based on non-collagenous standards of known molecular mass.

and the acid-insoluble fraction of this gel resulted in no apparent change in the relative proportions of the three components of molecular mass 84 kDa, 72 kDa and 66 kDa, either by Coomassie Blue staining or by immunoblotting with the anti-(type IX collagen) sera (result not shown). Most of the type IX collagen extracted from bovine cartilage by 4 M-GuCl therefore carried no glycosaminoglycan.

When the DEAE-cellulose fractions were immunoblotted with the anti-(type VI collagen) serum, immunoreactive peptides of molecular mass 200–240 kDa were observed in fraction 3, which was eluted at approx. 0.1 M-NaCl (Fig. 3). However, the polypeptides of molecular mass 200–240 kDa and the associated 140 kDa polypeptide (see Fig. 4) characteristic of type VI collagen

Fig. 4. Chromatography of the intact forms of types VI and IX collagens on Sepharose CL-4B

The acid-insoluble fraction of the protein gel from the CsCl-density-gradient centrifugation was equilibrated in 4 M-GuCl/50 mM-Tris/HCl buffer, pH 7.4, containing proteinase inhibitors and heated at 60 °C for 1 h. The solubilized material was applied to a Sepharose CL-4B column (96 cm $\times$ 1.5 cm) and eluted with the 4 M-GuCl buffer at a rate of 20 ml/h at room temperature. Fractions 1 (V_0) and 2 from the initial chromatography (a) were pooled separately from several runs and rechromatographed as shown in (b) and (c) respectively. Insets to (b) and (c) show SDS/8%-(w/v)-polyacrylamide-gel-electrophoretic analyses of appropriate pooled fractions indicated by bars, electrophoresed in the absence (–) or in the presence (+) of dithiothreitol and stained with Coomassie Blue (CB) or immunoblotted (Ib) with an anti-(type VI collagen) serum (b) or anti-[COL2(IX) domain] serum (c).

were barely visible by Coomassie Blue staining, the major components in fraction 3 being link proteins of molecular mass 45–50 kDa (Caterson & Baker, 1979), a protein of molecular mass 60 kDa (Heinegård *et al.*, 1986) and a prominent doublet of molecular mass 35 kDa characteristic of the C-propeptide of type II collagen (Niyibizi *et al.*, 1987). We have found that type VI collagen has a tendency to aggregate on DEAE-cellulose and, if Triton X-100 is omitted from the elution buffer, remains bound to the column. Type VI collagen has therefore been characterized by gel-filtration chromatography.

Sepharose CL-4B chromatography

Fig. 4(a) shows the initial elution profile of the acid-insoluble fraction of the protein gel from the CsCl-

Fig. 5. Pepsin digestion of cartilage collagens extracted under dissociative conditions

The 4 M-GuCl extracts of foetal epiphyseal, foetal growth-plate and adult nasal cartilage were equilibrated in 0.5 M-acetic acid and digested with pepsin by the method of Trueb *et al.* (1987). Fractions obtained by precipitating the digests at pH 7.5 with 3 M-NaCl ($3 M_{pH\ 7.5}$) and by subsequently adjusting the resulting supernatants to pH 2.5 with acetic acid ($3 M_{pH\ 2.5}$) were analysed by SDS/8%-(w/v)-polyacrylamide-gel electrophoresis under non-reducing (tracks 1 and 2) and reducing (tracks 3–14) conditions and stained with Coomassie Blue (CB) or immunoblotted with the anti-collagen sera as shown. Samples were: tracks 1 and 3, $3 M_{pH\ 7.5}$ precipitate from epiphyseal cartilage; tracks 2, 4, 8 and 12, $3 M_{pH\ 2.5}$ precipitate from epiphyseal cartilage; tracks 5 and 7, short form of type VI collagen from bovine uterus; track 6, as Fig. 1 (track 5); track 11, type IX collagen (COL2 domain) standard from epiphyseal cartilage; tracks 9 and 13, $3 M_{pH\ 2.5}$ precipitate from nasal cartilage; tracks 10 and 14, $3 M_{pH\ 2.5}$ precipitate from growth-plate cartilage.

density-gradient centrifugation chromatographed on Sepharose CL-4B under non-reducing but denaturing conditions. Fraction 3 contained mainly $\alpha 1(II)$ -chains and a major component of faster mobility characteristic of the unreduced C-propeptide of type II collagen (result not shown). Fractions 1 (V_0) and 2 were pooled separately from several runs and rechromatographed as shown in Figs. 4(b) and 4(c) respectively. The electrophoretic analysis of the V_0 fraction revealed major polypeptides of molecular mass 140 kDa and 200 kDa and three minor bands of molecular mass 200–240 kDa, all of which were present as large-molecular-mass aggregates before reduction of disulphide bonds. The anti-(type VI collagen) serum reacted strongly with the 200 kDa component as well as the three minor components of larger molecular mass (Fig. 4b inset). Rechromatography of fraction 2 resulted in a small peak at V_0 containing type VI collagen (result not shown) followed by a shallow polydisperse peak. When the fraction eluted in the position of type I collagen γ -chains (molecular mass 285 kDa) was analysed, components of large molecular mass were observed that gave rise to several polypeptides on reduction. However, only the components of molecular mass 84 kDa, 72 kDa and 66 kDa were reactive against the anti-[COL2(IX) domain] serum (Fig. 4c inset). Despite rechromatography, $\alpha 1(II)$ -chains were also found to be eluted in this fraction.

Pepsin digestion of the GuCl extract

The GuCl extracts were digested with pepsin and the digests were fractionated by using the modified method of Trueb *et al.* (1987), in which a high NaCl concentration is maintained throughout. This procedure diminishes the problem of aggregation, which arises when type VI collagen is being handled. Fig. 5 (tracks 1–5) shows the Coomassie Blue-stained SDS/polyacrylamide-gel-electrophoretic analyses of the precipitates obtained by

adjusting the total pepsin digest from epiphyseal cartilage to 3 M-NaCl at pH 7.5 ($3 M_{pH\ 7.5}$ precipitate) and by subsequently adjusting the resulting supernatant to pH 2.5 ($3 M_{pH\ 2.5}$ precipitate). The $3 M_{pH\ 7.5}$ precipitate (Fig. 5, tracks 1 and 3) consisted mainly of types II and XI collagens, whereas the $3 M_{pH\ 2.5}$ precipitate (Fig. 5, track 4) contained type II collagen and several small-molecular-mass peptides. Before reduction (Fig. 5, track 2) the small-molecular-mass peptides were present as large-molecular-mass aggregates at the top of the electrophoretic gel or as a component of molecular mass approx. 180 kDa, characteristics exhibited by the pepsin-digested forms of types VI and IX collagens respectively (Abedin *et al.*, 1982; Ayad *et al.*, 1981, 1982). Three of the small-molecular-mass components migrated in positions identical with those of the $\alpha 1$ -, $\alpha 2$ - and $\alpha 3$ -chains of a pepsin-digested type VI collagen standard (short form) from uterus (Fig. 5, track 5). Similar polypeptide profiles were observed for the $3 M_{pH\ 7.5}$ and $3 M_{pH\ 2.5}$ precipitates from foetal growth-plate and adult bovine nasal cartilage (results not shown). The presence of types VI and IX collagens was confirmed, and their relative proportions in three types of cartilage (foetal epiphyseal, growth-plate and adult nasal) were assessed, by immunoblotting identical amounts of the $3 M_{pH\ 2.5}$ precipitate from each tissue with the anti-(type VI collagen) and anti-[COL2(IX) domain] sera (Fig. 5, tracks 6–14). In the case of type IX collagen (Fig. 5, tracks 11–14), the immunoreactive components were identical with those of pepsin-digested standard. In the case of type VI collagen (Fig. 5, tracks 6–10), the major reactive peptide was the $\alpha 3(VI)_p$ -chain, but other components, probably less extensively pepsin-digested forms of this chain, were also observed (see above). The proportion of type VI collagen was markedly increased relative to type IX collagen in the fraction from adult nasal cartilage compared with fractions from the two types of foetal cartilage.

DISCUSSION

We have recently identified the intact form of type X collagen in 4 M-GuCl extracts of foetal bovine growth-plate cartilage (Ayad *et al.*, 1987). We now provide evidence that all other cartilage collagens previously identified by pepsin digestion, namely types II, IX and XI collagens (Grant *et al.*, 1988) as well as type VI collagen demonstrated only by immunofluorescence (Ayad *et al.*, 1984), can be extracted in an intact form from several types of cartilage. The collagens present in the GuCl extracts have (presumably) not been cross-linked by lysine-/hydroxylysine-derived cross-links, particularly the trifunctional hydroxypyridinium cross-links that predominate in cartilage (for review see Eyre, 1987). It should also be noted that all the collagens extracted by GuCl regained their native triple-helical structure following removal of this chaotropic agent, as shown by the resistance of the helical regions to pepsin digestion (Fig. 5). This observation is in contrast with that of Trueb *et al.* (1987), who found that only types III and VI collagens in non-cartilaginous tissues were stable under such conditions.

The intact forms of all cartilage collagens were identified by the characteristic mobilities of their constituent α -chains on SDS/polyacrylamide-gel electrophoresis and/or by their reactivity with specific antibodies raised against their pepsin-digested forms, except in the case of type XI collagen. Although we have raised several antisera to the pepsin-digested form of type XI collagen, none has detected the intact form of this collagen in GuCl extracts by immunoblotting. This problem was not due to the lack of activity towards the denatured compared with the native form, as the antisera reacted with the denatured pepsin-digested chains in immunoblots (Fig. 1). Rather it must mean that the antisera were raised to epitopes generated on pepsin digestion. Interestingly, an antiserum raised to the pepsin-digested form of type V collagen that shows virtually a 100% cross-reactivity with type XI collagen in the e.l.i.s.a. and binds strongly to the $\alpha 1(XI)_p$ - and $\alpha 2(XI)_p$ -chains in immunoblots also failed to detect the intact forms of these chains in the GuCl extracts (S. Ayad, unpublished work). The anti-(type II collagen) serum cross-reacted with type XI collagen, notably the $\alpha 3(XI)_p$ -chain (Fig. 1). The two minor immunoreactive components migrating more slowly than the $\alpha 1(II)$ -chains (Fig. 2) and in positions similar to those of the matrix forms of type XI collagen α -chains observed in biosynthetic studies on chick chondrocytes (Clark & Richards, 1985; Morris & Bachinger, 1987) could represent the intact chains of bovine type XI collagen. However, the possibility that the minor components are newly synthesized partially processed forms of type II collagen cannot be excluded. Proof that type XI collagen was indeed present in the GuCl extracts was provided by the identification of the $\alpha 1(XI)_p$ -, $\alpha 2(XI)_p$ - and $\alpha 3(XI)_p$ -chains after digestion with pepsin and salt precipitation (Fig. 5, tracks 1 and 3).

There are both similarities and differences between the type IX collagen extracted from bovine cartilage by GuCl and the type IX collagen identified in biosynthetic experiments on chick cartilage (van der Rest & Mayne, 1987). One component of the bovine collagen has the same molecular mass (84 kDa) as the chick $\alpha 1(IX)$ -chain and is probably the bovine analogue of this chain. The two other components, of molecular mass 72 kDa and

66 kDa, are clearly separable and distinct from those of chick, which co-migrate with molecular mass 68 kDa. DEAE-cellulose chromatography (Fig. 3) established that the component of molecular mass 66 kDa carries the glycosaminoglycan side chain and therefore represents the $\alpha 2(IX)$ -chain. The component of molecular mass 72 kDa is therefore assigned to the $\alpha 3(IX)$ -chain. This assignment is consistent with the recent identification of the analogous chains of human type IX collagen (Bruckner *et al.*, 1988). Whether the bovine $\alpha 3(IX)$ -chain is longer than that of the chick or is simply more glycosylated is not known. However, it may be noted that both tissue and species differences have been found in the pepsin-digested COL1 domain [also designated C-PS2 for bovine (Ayad *et al.*, 1982) or LMW for chick (van der Rest & Mayne, 1987)]. In particular, the 16 kDa component of the bovine domain is approx. 5 kDa larger than the corresponding chick component LMW1, shown unequivocally to be part of the $\alpha 3(IX)$ -chain (van der Rest & Mayne, 1987). Since all three chains of the COL1 domain are presumably in register, it is more likely that differences among the molecular masses of individual chains within this domain and among species are due to differences in post-translational modification, e.g. glycosylation.

The major difference between the type IX collagen extracted from bovine cartilage by GuCl and the newly synthesized chick collagen is the virtual absence of glycosaminoglycan chains on the extracted bovine collagen. Preliminary biosynthetic studies suggest that bovine type IX collagen is also synthesized largely in a proteoglycan form, but the proportion of bound glycosaminoglycan decreases when this collagen is deposited in the extracellular matrix (S. Ayad, A. Marriott & M. E. Grant, unpublished work). Further studies are required to determine whether the bound glycosaminoglycan plays any role in the association between type IX collagen and type II collagen fibres (Eyre *et al.*, 1987; van der Rest & Mayne, 1988; Vaughan *et al.*, 1988). However, the interaction between type II and type IX collagens *in vitro* is apparently unaffected by the presence or the absence of glycosaminoglycan on the type IX collagen (Wotton *et al.*, 1988).

The presence of type VI collagen in cartilage was first demonstrated by immunofluorescence studies, which localized this collagen to the pericellular environment of the chondrocyte (Ayad *et al.*, 1984). The results presented here confirm that type VI collagen is an integral component of cartilage. The cartilage type VI collagen has a similar chain composition to that of type VI collagen found in 'soft' tissues and eluted from DEAE-cellulose at the salt concentration characteristic of type VI collagen (Ayad *et al.*, 1985). It is also present in the form of disulphide-bonded aggregates of molecular mass greater than 500 kDa, as shown by its exclusion from Sepharose CL-4B. The anti-(type VI collagen) serum preferentially reacted with the $\alpha 3(VI)_p$ - and $\alpha 3(VI)_p^*$ -chains and hence detected the heterogeneous intact $\alpha 3(VI)$ -chain in the GuCl extracts. The nature of this heterogeneity, which varies considerably with tissue and animal species (Ayad *et al.*, 1985; Gibson & Cleary, 1985; Colombatti & Bonaldo, 1987), is not known: in some tissues the fastest of the four components predominates whereas in others they are of equal intensity, as judged by Coomassie Blue staining. In epiphyseal cartilage, the component of molecular mass 200 kDa predominated over the three com-

ponents of larger molecular mass (Fig. 4), as was also observed in extracts of the nucleus pulposus of the cartilaginous intervertebral disc (Wu *et al.*, 1987). Interestingly, however, the four components of the corresponding annulus fibrosus were more nearly equal in intensity (Wu *et al.*, 1987). Differences in heterogeneity could be due to differences in extracellular processing or other factors, although Colombatti & Bonaldo (1987) have ruled out the possibility of differences in both processing and glycosylation in the case of chick type VI collagen. In this context it may be noted that the mRNA for type VI collagen extracted from human skin fibroblasts is heterogeneous with respect to the $\alpha 3(\text{VI})$ -chain and may reflect alternative splicing (Chu *et al.*, 1987; Weil *et al.*, 1988).

It is apparent that many of the 'unusual banded structures', also called 'zebra' collagen or 'Luse bodies' or (wrongly) described as fibrous-long-spacing (FLS) collagen, that have been found in non-cartilaginous (mainly pathological) tissues as well as cartilage (Silberberg *et al.*, 1963) and the nucleus pulposus of the intervertebral disc (Cornah *et al.*, 1970; Buckwalter *et al.*, 1979) are in fact lateral aggregates of the type VI collagen microfibrils (for review see Timpl & Engel, 1987). Their prominence in the nucleus pulposus explains why this tissue is a rich source of type VI collagen (Wu *et al.*, 1987). Type VI collagen microfibrils have been implicated in the stabilization of the major collagen fibrils (Timpl & Engel, 1987) as well as in providing an adhesion mechanism for cell attachment (Carter, 1984) in the case of soft tissues. In adult articular cartilage type VI collagen is associated mainly with the chondrocytes in the mid and deep zones, forming part of the pericellular capsule, which protects the chondrocyte, and possibly linking the capsule to the extracellular matrix and to the cell surface (Poole *et al.*, 1988). In the superficial zones, however, it is localized both around the cells and throughout the matrix (Poole *et al.*, 1988; Keene *et al.*, 1988).

Type VI collagen, unlike all the other cartilage collagens, does not appear to be cross-linked by lysine/hydroxylysine-derived cross-links (Wu *et al.*, 1987), and therefore is preferentially extracted by GuCl and is not found in subsequent pepsin digests of cartilage (Ayad *et al.*, 1984). It accounts for 1–2% of the total collagen present in foetal epiphyseal cartilage. The remaining collagen types II, IX and XI extracted by GuCl represent a further 8–9% of the total collagen in this cartilage and are present in the respective proportions 17:1:2, which is similar to that observed for the collagens present in the pepsin digest of the GuCl-insoluble residue. There was an increase in the proportion of type VI collagen relative to type IX collagen in the GuCl extract of adult compared with foetal cartilage (Fig. 5). However, further studies will be required to ascertain whether this observation reflects age-related changes in one or both collagen types.

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