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Partial characterization of type X collagen from bovine growth-plate cartilage

Evidence that type X collagen is processed in vivo

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Sequential extraction of bovine growth-plate cartilage with 4 M guanidinium chloride and pepsin was used to identify the intact and pepsinized forms respectively of type X collagen. This collagen occurs predominantly as the processed $[\alpha_1(X)]_3$ form in vivo, although the procollagen $[pro\alpha_1(X)]_3$ form can also be detected. The bovine $pro\alpha_1(X)$ and $\alpha_1(X)$ chains have M_r values identical to the corresponding chick species (M_r 59 000 and 49 000). However, the pepsinized $\alpha_1(X)_p$ chains (M_r 47 000) are larger than those of the chick (M_r 45 000), and the bovine collagen type X is further distinguished by being disulphide-bonded within the triple-helical domain.

Type X collagen; Growth-plate cartilage

1. INTRODUCTION

The growth of mammalian long bones occurs predominantly by endochondral bone formation at the epiphysial growth plates [1]. The chondrocytes within these regions pass through a continuum of stages: proliferation, maturation, hypertrophy and eventual removal when the cartilage matrix becomes calcified and is replaced by bone. The hypertrophic chondrocytes are not degenerating but are viable [2] and are capable of active synthesis of a unique proteoglycan [3] and a distinct type of collagen, now known as type X [4].

Type X collagen has been extensively characterized in the chick [4–14] and is the first example of a transient, developmentally and topographically regulated collagen [5–8]. It is synthesized as a procollagen comprising three apparently identical $pro\alpha_1(X)$ chains of M_r 59000 [4,9–14]. Rotary shadowing [4,15,16] and amino acid sequence [5] studies have identified 3 domains: a large non-collagenous globular carboxy-terminal propeptide; a short (132 nm) triple helix; and a very short non-collagenous amino-terminal propeptide. The secreted molecules of chick type X procollagen with chains of M_r 59000 are not disulphide-bonded. However, the conceptual translation product of the type X collagen gene [5] and biosynthetic studies using matrix-free chondrocytes [17] suggest that chick type X collagen is synthesized initially as a disulphide-bonded molecule that is slightly larger than the secreted form, the disulphide bonds residing within a transmembrane-like sequence close to the carboxy-terminal end [5]. Moreover, disulphide-bonded type X collagen species accumulate in the medium of long-term embryonic chick chondrocyte cultures [18]. Although several studies have found that type X procollagen is not processed prior to its

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deposition in the extracellular matrix [10,12,17], processing to a smaller $[\alpha_1(X)]_3$ species, with chains of M_r 49000, is a frequent observation in our own laboratory [4,16]. Pepsin digestion of either the $[pro\alpha_1(X)]_3$ or $[\alpha_1(X)]_3$ form results in a pepsin-resistant $[\alpha_1(X)_p]_3$ fragment comprised of chains of M_r 45000 [4,11,12].

Type X collagen has been characterized predominantly by biosynthetic studies and has been extracted only from chick cartilage and only as the pepsinized form [22,23]. Investigations on mammalian type X collagen have been restricted to organ culture studies which have produced conflicting results. Rabbit type X collagen is apparently identical to the chick species [19] but evidence has been presented that the bovine collagen is disulphide-bonded within the triple-helical domain [20,21]. In this report we describe the extraction of both the intact and pepsinized forms of type X collagen from bovine growth-plate cartilage and show that the processed $[\alpha_1(X)]_3$ species is the major form in vivo.

2. MATERIALS AND METHODS

2.1. *Extraction of intact type X collagen*

Growth-plate cartilage was dissected from each end of the long bones of foetal calves (130–230 days gestation) and powdered in a stainless steel mill under liquid N_2. The powdered tissue was extracted ($\times$ 3) with 4 M guanidinium chloride/50 mM Tris-HCl buffer, pH 7.4, containing the proteinase inhibitors phenylmethanesulphonyl fluoride (0.5 mM), 6-aminohexanoic acid (100 mM), EDTA (2 mM) and *N*-ethylmaleimide (10 mM). These extracts were dialysed against 7 $\times$ vol. of 50 mM Tris-HCl, pH 7.4, containing proteinase inhibitors, and the resulting solutions subjected to associative CsCl density gradient centrifugation using a starting CsCl density of 1.6 $g \cdot cm^{-3}$ [24]. The protein gel at the top of the gradient was suspended in, and dialysed against, 0.5 M acetic acid at 4°C for 4 days and the acid-soluble and acid-insoluble fractions separated by centrifugation. The acid-soluble fraction was then fractionated by differential salt precipitation at 0.86, 1.2 and 3 M NaCl [25,26]. Identical procedures were also performed on articular cartilage dissected from the femoral and tibial condyles.

2.2. *Extraction of pepsinized type X collagen*

The residue remaining after 4 M guanidinium chloride extraction was washed extensively with water, suspended in 0.5 M acetic acid and incubated with pepsin (2 mg pepsin per g wet tissue) for 48 h ($\times$ 2). The pepsin digests were fractionated by differential salt precipitation at 0.86, 1.2 and 3 M NaCl. The 3 M NaCl fractions were then dialysed against 0.02 M Na_2HPO_4 [25] and the resulting precipitates radiolabelled with [^{3}H]acetic anhydride [18] in order to detect the polypeptide constituents more readily.

2.3. *SDS-polyacrylamide gel electrophoresis and immunoblotting*

The polypeptide composition of the extracts obtained by guanidinium chloride and pepsin were analysed under reducing and non-reducing conditions by SDS-PAGE [27] using 8% (w/v) gels. The separated components were either stained with Coomassie blue or transferred to nitrocellulose [30] and immunoblotted [31] using a 1:100 dilution of an antiserum raised against a mixture of native procollagen type X (95%) and its processed form (5%) isolated from the medium of long-term chick embryo tibiotarsal chondrocyte cultures [29]. Radiolabelled samples were also electrophoresed under non-reducing conditions, and the components of interest excised from the gel after fluorography and analysed under reducing conditions [28]. M_r values are based on collagenous standards of known M_r.

3. RESULTS AND DISCUSSION

3.1. *Extraction of type X collagen by 4 M guanidinium chloride*

Preliminary electrophoretic and immunochemical investigations on the 4 M guanidinium chloride extracts of articular and growth-plate cartilages demonstrated the presence of several collagenous polypeptides, suggesting that such extracts may be a potential source of the intact forms of all cartilage collagens. The extracts were purified by CsCl density gradient centrifugation under associative conditions which allowed the collagens and other proteins to concentrate as a gel at the top of the CsCl gradient and to be separated from the major proteoglycans. A small proportion (approx. 5%, w/w) of the protein gel was soluble

in 0.5 M acetic acid and electrophoretic analysis of the acid-soluble and acid-insoluble fractions is shown in fig.1. Type II collagen predominated in these acid-soluble fractions from both cartilages but there was a much higher proportion of minor collagen types in the fractions from growth-plate cartilage. The identification of collagen types VI, IX and XI (see fig.1) is based on immunoblotting and chromatographic separations, details of which will be published elsewhere. Another minor collagenous polypeptide of M_r 49000 was detected in the fractions (especially the acid-insoluble fraction) from growth-plate cartilage (fig.1, lanes 5 and 7). Differential salt precipitation of the acid-soluble fraction at acid pH, showed that the 49 kDa component was preferentially precipitated at 3 M NaCl (fig.2A, lanes 1–4). The high solubility of this component, its molecular size of 49 kDa, and its preferential extraction from cartilage undergoing endochondral ossification suggested that it might be related to type X collagen. This possibility was confirmed by immunoblotting using a specific anti-(type X collagen) antiserum (fig.2A, lanes 5–10). Two major immunoreactive species were observed in the unfractionated sample (lane 5): one corresponded to the 49 kDa polypeptide, the second appeared as a doublet of approx. 59 kDa but the corresponding Coomassie blue-stained polypeptide was barely visible. This 59 kDa doublet was the more immunoreactive species in the 0.86 and 1.2 M NaCl precipitates (lanes 6 and 7) whereas the 49 kDa species reacted

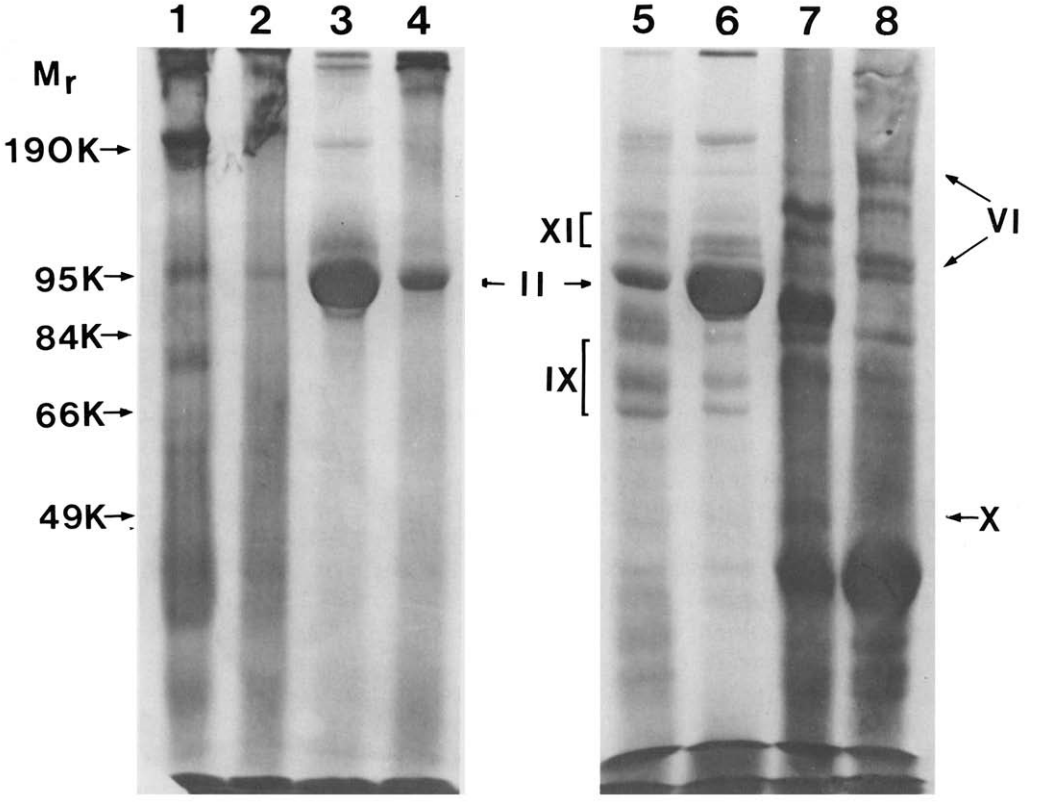

Fig.1. SDS-PAGE of the collagens extracted from articular and growth-plate cartilages by 4 M guanidinium chloride. The 4 M guanidinium chloride extracts were subjected to CsCl density gradient centrifugation and the resulting protein gel partially solubilized in 0.5 M acetic acid. The acid-soluble and acid-insoluble fractions were analysed under non-reducing (lanes 1–4) and reducing (lanes 5–8) conditions. Samples analysed are as follows: acid-insoluble fraction from articular cartilage (lanes 1 and 8) and growth-plate cartilage (lanes 2 and 7); acid-soluble fraction from articular cartilage (lanes 3 and 6) and growth plate cartilage (lanes 4 and 5). Intact chains of types II, VI, XI, X and XI collagens are indicated.

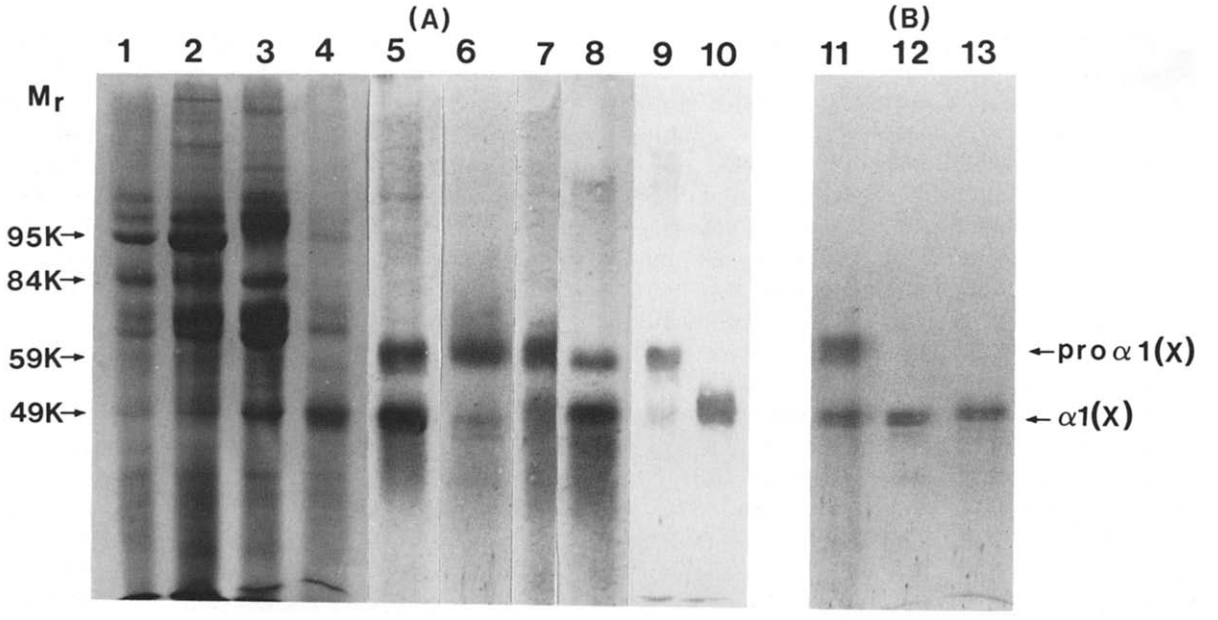

Fig.2. SDS-PAGE of the intact forms of type X collagen from bovine growth-plate cartilage. Samples were analysed under reducing conditions and the separated polypeptides stained with Coomassie blue (lanes 1–4) or immunoblotted with an anti-(type X collagen) antiserum (lanes 5–13). (A) The acid-soluble fraction from the 4 M guanidinium extract (fig.1, lane 5) was fractionated by differential salt precipitation. Unfractionated sample (lanes 1 and 5); 0.86 M (lanes 2 and 6), 1.2 M (lanes 3 and 7), 3 M (tracks 4 and 8) NaCl precipitates; standards of chick proα_1(X) and α_1(X) chains M_r 59000 and 49000 (lanes 9 and 10, respectively). (B) 1st, 2nd and 3rd 4 M guanidinium chloride extracts (lanes 11–13).

more strongly in the 3 M NaCl fraction and only the lower band of the 59 kDa doublet was observed (lane 8). The two species of M_r 49000 and 59000 also had mobilities identical to chick proα_1(X) and α_1(X) chains (lanes 9 and 10). It is therefore concluded that the 59 and 49 kDa components observed in extracts of bovine growth-plate cartilage represent the proα_1(X) and processed α_1(X) chains of type X collagen. The trace amount of type X collagen present in the acid-soluble fraction from articular cartilage (fig.1, lane 6) was probably due to the inclusion of the central region of this cartilage in the original tissue sample. This region ultimately calcifies and gives rise to the secondary ossification centre and contains type X collagen [29].

The occurrence of type X collagen in the acid-insoluble fraction (fig.1, lane 7) was confirmed by immunoblotting the 49 kDa band together with a 59 kDa band (not shown). Attempts to purify the bulk of type X collagen in this fraction by established chromatographic techniques have proved difficult, as was observed for the chick species [12], the problems being due mainly to the extremely hydrophobic nature of this collagen. However, the bovine collagen is disulphide-bonded and also has a tendency to aggregate (see below), these properties resulting in further fractionation anomalies.

The extraction of intact bovine type X collagen predominantly as the processed 49 kDa form agrees with our previous studies on chick cartilage using either 4 M guanidinium chloride extraction of organ cultures or 2 M $MgCl_2$ extraction of cell layers [4,16]. The reason why several laboratories [10,17,19–21] have not detected the processed form could be due to their use of 1 M NaCl rather than the strong dissociative conditions used in our experiments: 1 M NaCl may be able to extract the newly synthesized procollagen X but not the processed type X collagen deposited in the extracellular matrix. In this respect, when three successive guanidinium chloride extracts were immunoblotted with the anti-(type X collagen) antiserum, the proα_1(X) species was observed only in the first extract whereas the successive extracts all contained the processed α_1(X) form (fig.1B).

3.2. *Extraction of type X collagen by pepsin digestion*

The bulk of the cartilage collagens was not extracted by 4 M guanidinium chloride but could be solubilized by subsequent pepsin digestion. Differential salt precipitation of the pepsin-solubilized collagens and direct comparison of the resulting fractions from articular and growth-plate cartilages (fig.3, lanes 1–7) demonstrated the occurrence of a 47 kDa collagenous protein specifically associated with the 3 M NaCl precipitate from growth-plate cartilage (lane 7). The additional components in this precipitate and those copurifying with type XI collagen in the 1.2 M NaCl precipitates (lanes 3 and 4) are pepsinized type IX collagen fragments corresponding to the 'M' and 'N' series respectively described by Yasui et al. [28] (Ayad and Grant, unpublished). The difference between the growth-plate and articular cartilage fractions was further enhanced after dialysing the 3 M NaCl precipitates against 0.02 M Na_2HPO_4 and radiolabelling the resulting phosphate precipitates (fig.3, lanes 10 and 11). The 47 kDa component was precipitated, together with type XI collagen whereas type IX collagen was preferentially soluble in this buffer [25]. The 47 kDa component reacted with the anti-(type X collagen) antiserum (lane 9) and was therefore identified as the bovine $\alpha_1(X)_p$ species. However, it consistently migrated more slowly than the chick $\alpha_1(X)_p$ species (lane 8).

3.3. *Disulphide-bonding of bovine type X collagen*

Both the intact and pepsinized α-chains of bovine type X collagen were detected only after reduction with dithiothreitol (fig.1, lanes 2,4,5 and 7; fig.3, lanes 11 and 13) indicating the presence of

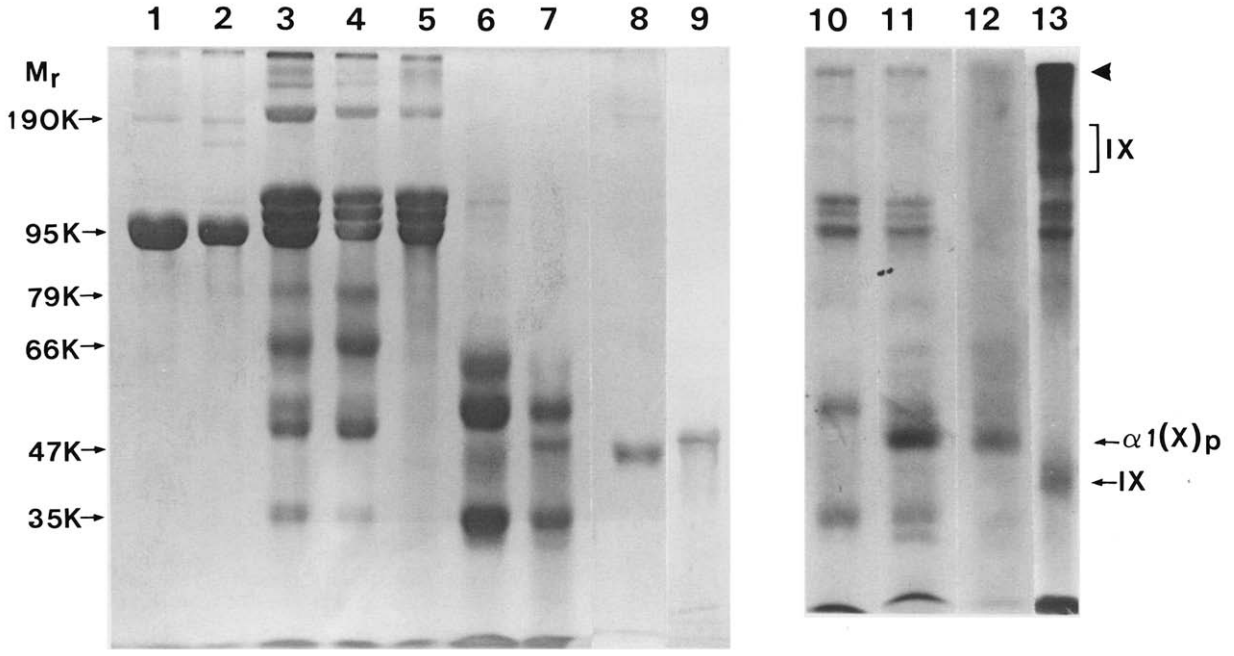

Fig.3. SDS-PAGE of the pepsinized form of bovine type X collagen. Pepsin digests of growth-plate cartilage were fractionated by differential salt precipitation and the precipitates compared directly with those from articular cartilage. Samples were electrophoresed under reducing (lanes 1–12), or non-reducing (lane 13) conditions and the separated polypeptides stained with Coomassie blue (lanes 1–8), immunoblotted with an anti-(type X collagen) antiserum (lane 9) or processed for fluorography (lanes 10–13). Samples analysed are as follows: 0.86 M, 1.2 M and 3 M NaCl precipitates from articular cartilage (lanes 1,3,6); 0.86 M, 1.2 M and 3 M NaCl precipitates from growth-plate cartilage (lanes 2,4,7 and 9); type XI collagen standard (lane 5); chick $\alpha_1(X)_p$ chains M_r 45000 (lane 8); ^{3}H-labelled 0.02 M Na_2HPO_4 precipitates from articular cartilage (lane 10) and growth-plate cartilage (lanes 11 and 13): aggregates excised from lane 13 (arrowed) analysed under reducing conditions (lane 12).

disulphide bonds within the triple helix. Analysis of the phosphate precipitate before reduction showed the presence of type XI collagen, disulphide-bonded fragments of type IX collagen [25,28,33] and also high-molecular-mass aggregates (fig.3, lane 13). When these aggregates (arrow, fig.3, lane 13) were excised and analysed under reducing conditions the 47 kDa $\alpha_1(X)_p$ component was the major species produced (fig.3, lane 12). It is noteworthy that the aggregation of bovine type X collagen has also been observed in organ culture studies [21]. However, the nature of this aggregation is at present unknown.

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