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Identification of the cartilage α1(XI) chain in type V collagen from bovine bone

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Type V collagen prepared from bovine bone was resolved into three distinct α-chains by high performance liquid chromatography and gel electrophoresis. Peptide mapping established two chains as α1(V) and α2(V) as expected and the third as the cartilage α1(XI) chain (previously thought to be unique to cartilage). In adult bone, the type V collagen fraction was richer in α1(XI) chains than in fetal bone (about 1/3 of the chains in the adult). How these polypeptides are organized into native molecules is not yet clear, though the stoichiometry suggests cross-type heterotrimers between the type V and XI chains.

Collagen; Bone; Cartilage; (Bovine)

1. INTRODUCTION

Type V collagen, originally identified in human fetal membranes [1], is widely co-distributed in small amounts with type I collagen [2]. Variations in its α-chain composition have been noted that seem to depend on tissue source. The most common molecule has the reported composition, $[\alpha 1(V)]_2\alpha 2(V)$ [1,3], but other molecules, $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ [4,5] and $[\alpha 1(V)]_3$ [6], have been described and a fourth chain called α4(V) or α1′(V) was evident in chick tendon fibroblast cultures [7,8]. A related collagen, type XI, has been identified in cartilage. This contains two new chains, 1α or α1(XI) and 2α or α2(XI), that resemble α1(V) and α2(V) in certain properties, though peptide maps establish each as a distinct gene product [9–11].

No specific function for type V or XI collagens is known, but growing evidence by immunolocalization on the chick cornea [12] and from fibril reconstitution experiments in vitro [13] indicates that type V collagen exists in the extracellular matrix copolymerized within collagen type I/V hybrid fibrils. In further exploring the heteropolymeric nature of type V collagen, the chain composition of the protein from bovine bone was determined.

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2. MATERIALS AND METHODS

Samples of mid-shaft cortical bone from the tibiae of adult steers and of mid-shaft cortical (femur) and membrane (skull) bones from fetal calves were dissected from freshly killed animals.

2.1. *Collagen preparation*

Pieces of adult cortical bone were powdered in a heavy duty Waring blender (Eberbach Corporation, Ann Arbor, MI) under liquid N_2. Separate samples of fetal calf skull and femoral bone were cut into small pieces and powdered in a Spex mill (Spex Industries, Inc., Edison, NJ) under liquid N_2. All tissues were exhaustively demineralized (7–14 days) at 4°C in 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.5, containing protease inhibitors (2 mM phenylmethanesulfonyl fluoride, 5 mM *N*-ethyl maleimide and 5 mM benzamidine-HCl), then thoroughly washed with water and suspended in 0.5 M acetic acid at 4°C (5 mg/ml). Pepsin (Boehringer Mannheim) was added (1:50 by weight) and the suspension was stirred at 4°C for 24 h. After removing insoluble material the pepsin-solubilized collagens were resolved by sequentially precipitating at 0.8, 1.2 and 2 M NaCl. Each fraction was collected by centrifugation and its chain composition

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was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2. *High-performance liquid chromatography (HPLC)*

Whole α-chains and derived peptides were resolved by reverse phase HPLC on a C18 column, Vydac 218TP54 (4.6 mm × 25 cm; The Separations Group, Hesperia, CA). For whole α-chains, collagen samples were dissolved in 4 M guanidine-HCl, 1% (v/v) trifluoroacetic acid (TFA), heat denatured and eluted by a linear gradient from 16–30% of acetonitrile/1-propanol (3:1, v/v; solvent B) in 0.1% (v/v) TFA in water (solvent A) over 50 min at 1 ml/min. Tryptic peptides were dissolved in 1% TFA and eluted by a linear gradient from 0–36% solvent B in solvent A over 70 min. Ion exchange HPLC was performed on a Biogel-TSK DEAE-5-PW column (7.5 mm × 7.5 cm; BioRad Laboratories). Samples were heat denatured in 0.02 M Tris-HCl, pH 7.5, containing deionized 6 M urea and eluted with a gradient from 0.01 M to 0.15 M NaCl in 0.02 M Tris-HCl, 5% (v/v) 1-propanol, pH 7.5, over 30 min at 1 ml/min.

2.3. *CNBr-peptide maps*

Samples (100 μg) of each pure α chain were digested with cyanogen bromide in 70% formic acid for 4 h at 30°C. The digests were diluted 10 × with water, freeze dried and then analyzed by SDS–10% polyacrylamide slab electrophoresis according to Laemmli [14].

2.4. *Trypsin digestion*

Samples (0.3–1 mg) of type V collagen α-chains from fetal calf skull bone and of the α1(XI) chain from fetal calf articular cartilage were dissolved in 0.2 M NH_4HCO_3 at 2 mg/ml, heat denatured at 50°C for 10 min and digested with trypsin (1:50, w/w; sequence grade, Boehringer Mannheim) for 6 h at 37°C. A drop of glacial acetic acid was added to each sample before freeze drying. The peptides were analysed by HPLC.

3. RESULTS

Fig.1 shows the elution profile on reverse phase HPLC and subsequent electrophoresis of the type V collagen preparation from adult bovine bone. Material under peak 1 contained α1(V) and α2(V) chains, while peak 2 gave a single protein band that comigrated with the α1(V) chain on electrophoresis. When type XI collagen isolated from cartilage was chromatographed similarly, the α1(XI) chain eluted in the same position as peak 2 (result not shown).

The pool containing the α1(V) and α2(V) chains (fig.1, peak 1) was freeze-dried and its component chains were resolved by anion-exchange HPLC (fig.2). The ratio of α1(V) to α2(V) chains was about 1:1, based on the integrated absorbance units under peaks 3 and 4. In the following discus-

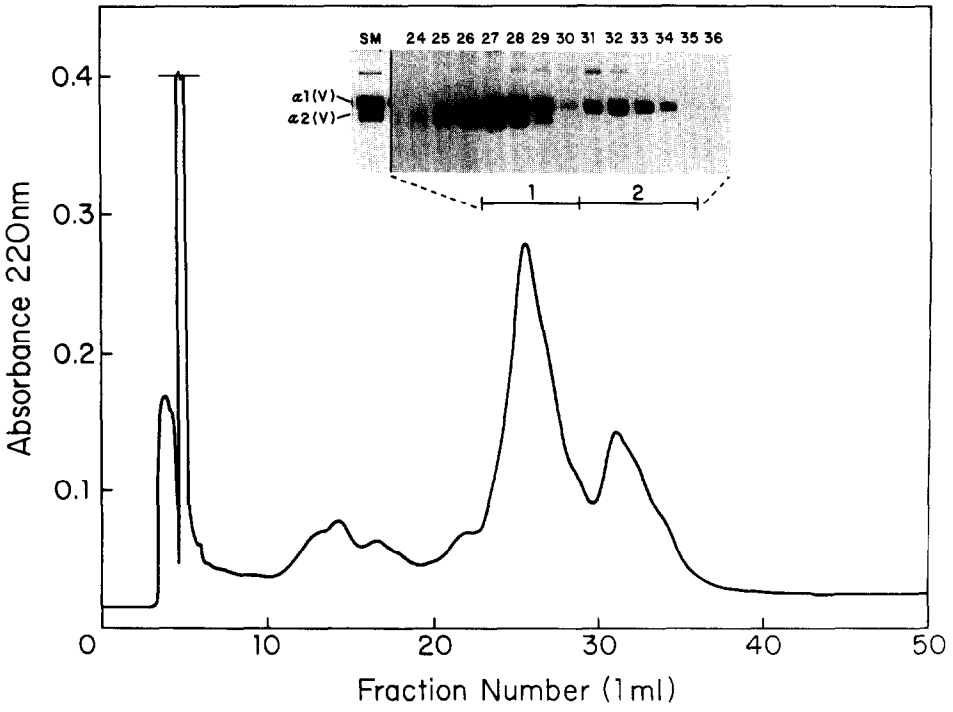

Fig.1. Reverse phase HPLC of the 1.2 M NaCl fraction (type V collagen) of pepsin-solubilized collagen from adult bovine cortical bone. Serial fractions spanning peaks 1 and 2 were analyzed by SDS-7.5% PAGE as shown in the inset (SM = starting material).

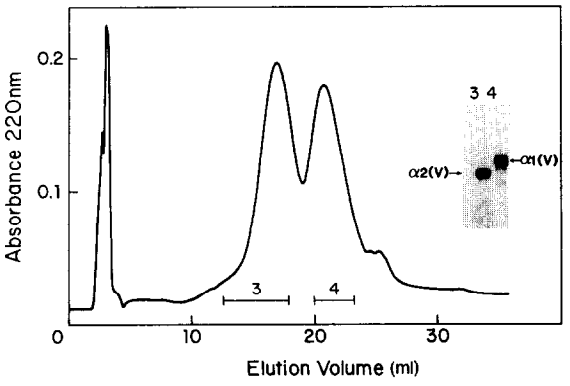

Fig.2. Anion exchange HPLC of material from the pooled fractions in fig.1 (peak 1) on a Biogel-TSK DEAE-5-PW column. The inset shows SDS-7.5%-PAGE of protein in the pooled fractions marked by the bars, identifying peak 3 as α2(V) and peak 4 as α1(V).

sion, the chain eluting in peak 2 (fig.1) will be referred to as the bone α1(XI) chain.

The α1(V), α2(V) and α1(XI) chains from bone each gave a different CNBr-peptide pattern on SDS-PAGE (fig.3), confirming that each had a distinct primary sequence. The CNBr-peptide pattern of the calf bone α1(XI) chain was apparently identical to that of the cartilage α1(XI) chain (fig.3, lanes 2, 3 and 5). Both chains gave two main peptides running as a close doublet of bands at about 45 kDa based on collagen standards (fig.3, lanes 2 and 5). The adult bone α1(XI) chain gave a broad band spanning the position of the 45 kDa doublet (fig.3, lane 3). This band broadening could reflect age-related post-translational differences. The bone α1(V) and α2(V) chains each gave the characteristic CNBr-peptide patterns (fig.3, lanes 4 and 6) of α1(V) and α2(V) prepared from other tissues [15,16]. Longer reaction times with CNBr did not change any of the peptide profiles.

The HPLC elution profiles of tryptic peptides derived from the individual chains are compared in

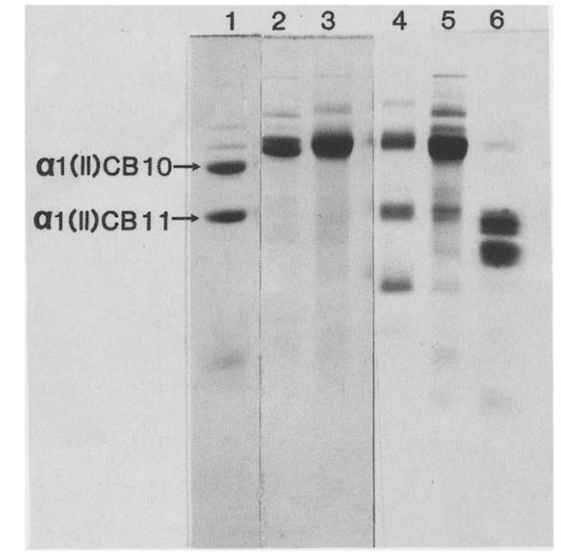

Fig.3. SDS-12.5%-PAGE of CNBr-peptides derived from bone type V collagen α chains compared with collagen chains from cartilage. Lane 1, cartilage α1(II); lane 2, cartilage α1(XI); lane 3, adult bone α1(XI); lane 4, adult bone α1(V); lane 5, calf bone (skull) α1(XI); and lane 6, adult bone α2(V).

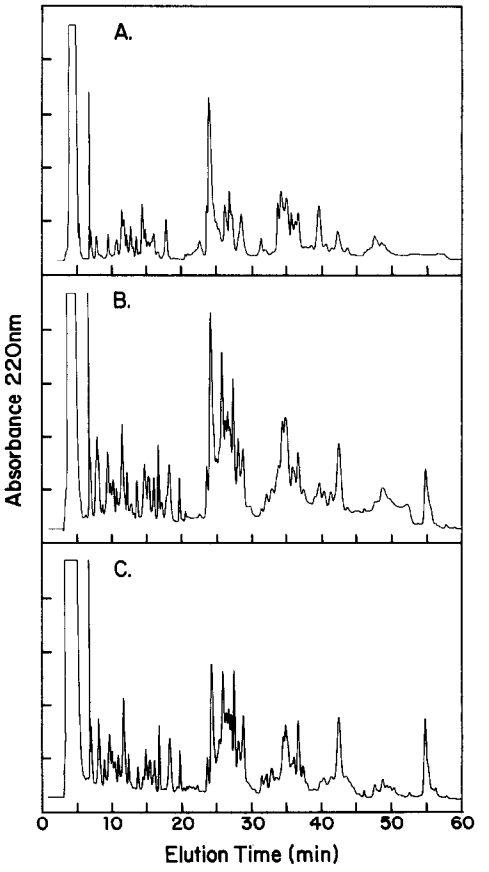

Fig.4. Reverse phase HPLC elution profiles of tryptic peptides from purified α chains of bone type V collagen and cartilage type XI collagen. (A) α1(V) from fetal calf skull bone, (B) α1(XI) from fetal calf skull bone and (C) α1(XI) from fetal calf articular cartilage.

fig.4. Bone and cartilage α1(XI) chains gave essentially identical elution profiles (fig.4B and C) further indicating that they had the same primary sequences. The bone α1(V) chain showed similarities to α1(XI) but nevertheless had a reproducibly distinct pattern (fig.4A) implying a homologous but different primary structure to α1(XI).

Preparations of type V collagen from adult bone were consistently more enriched in the α1(XI) chain than type V from fetal bone. Thus in the fetus the α1(XI) chain accounted for 14% (skull) and 17% (femoral cortex) of the total α-chains, compared with 25–30% in the adult steer, based on the integrated absorbance units of the chromatographic profiles. The ratios between the chains indicated that α1(XI) accumulated at the expense of α1(V) (i.e. the ratio of α1(V) plus α1(XI) to α2(V) was constant at 2/1), suggesting the formation of cross-type hybrid molecules. Neither type II collagen nor the α2(XI) and α3(XI) chains of cartilage type XI collagen were detected in these preparations.

4. DISCUSSION

The results establish that type V collagen prepared from bovine bone contains three distinct α chains, which differ in primary structures. Two of these chains are the well described α1(V) and α2(V) chains seen in other tissues; the third chain appears to be identical to the cartilage α1(XI) chain. It is not clear how these chains are organized into native collagen molecules. The ratio in adult bone of about 1:1:1 for α1(V), α2(V) and α1(XI) is consistent with a single heterotrimer, but a mixture of different heterotrimeric molecules (such as $[\alpha1(V)]_2\alpha2(V)$, $\alpha1(V)\alpha1(XI)\alpha2(V)$, $[\alpha1(XI)]_2\alpha2(V)$) and homotrimers is possible.

The α1(XI) chain from bone is probably distinct from the α3(V) chain identified in human and bovine placenta [4,15,16]. The latter runs between α1(V) and α2(V) as a discrete protein band on SDS-PAGE, and produces one main CNBr-peptide which is much larger than the peptides derived from the α1(XI) chain [15,16]. The $\alpha1'$(V) chain that Fessler et al. [7] described in chick tendon fibroblast cultures may, however, be the α1(XI) chain, since it also could not be resolved from α1(V) once its non-collagenous extension peptides were removed.

Using similar methods, the α1(XI) chain was identified in type V collagen isolated from adult bovine achilles tendon; as in bone it accounted for about 30% of the total α chains (results not shown). A cDNA for α1(XI) was also derived from fibroblast mRNA and shown to hybridize with mRNA from several non-cartilaginous tissues [17]. It seems likely, therefore, that the α1(XI) chain may be a common component of type V collagen preparations. Clearly, types V and XI collagens are closely linked in molecular form, function and expression [11]. It is notable also that the converse situation has been observed in that type XI collagen purified from articular cartilage contains α1(V) chains in addition to α1(XI), α2(XI) and α3(XI), and that the proportion of α1(V) increases with the developmental age of the cartilage [11,18].

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