Identification of disulphide-bonded type X procollagen polypeptides in embryonic chick chondrocyte cultures

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A high-M_r (M_r 120000), disulphide-bonded collagenous polypeptide was observed to co-purify with the proα_1(X) chain during isolation of cartilage collagens from culture medium of embryonic chick tibiotarsal chondrocytes. This high-M_r polypeptide was subsequently shown by two-dimensional SDS-PAGE and peptide mapping to represent a dimer of the proα_1(X) chain of type X collagen linked by disulphide bonding in the non-collagenous domains.

Collagen *Hypertrophic chondrocyte*

1. INTRODUCTION

The development and growth of long bones occur via differentiation of mesenchymal cells, proliferation, maturation and hypertrophy of chondrocytes, calcification of the cartilage matrix, and replacement of calcified cartilage by bone in the metaphysis. This complicated developmental cascade of bone formation occurs in a continuum in the epiphyseal growth plate [1]. Associated with these cellular transitions is the change in matrix macromolecular components as exemplified by the synthesis of short-chain type X collagen by the hypertrophic chondrocytes [2–16]. The transient appearance of type X collagen during cell hypertrophy and matrix calcification suggests that this collagen may be responsible for mediating one or more of the events of endochondral bone formation.

Several studies have demonstrated that newly synthesised chick type X collagen comprises polypeptides of M_r 59000 made up of three distinct structural domains: a collagenous domain (M_r 45000) and two non-collagenous peptides flanking the helical region, one of which appears in its native state as a globular head attached to one end of the molecule [9–11]. Pulse-chase experiments have revealed a time-dependent processing of the polypeptide (M_r 59000) to species of M_r 49000 analogous to the conversion of type II procollagen to collagen type II [10]. Therefore, the 59 kDa polypeptide is currently referred to as a type X procollagen chain or proα_1(X). The absence of cysteine residues in the α_1(X) chain [8] and proα_1(X) chain together with the observations that their electrophoretic mobilities on SDS-PAGE are not affected by reduction [3–5,10] has led to the general acceptance that type X collagen from chick cartilage contains no disulphide bonds. However, high-M_r disulphide-bonded polypeptides related to type X collagen have been reported in calf growth plate cartilage organ cultures [17,18].

Here, we present evidence for the existence of disulphide-bonded proα_1(X) chains in long-term embryonic chick chondrocyte cultures.

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2. MATERIALS AND METHODS

2.1. *Preparation and purification of type X collagen*

Confluent chick embryo tibiotarsal (zone 3) chondrocytes were maintained in primary cultures for up to 10 weeks [10]. Culture medium was collected from weeks 2 to 5 and pooled (medium A), and further media were collected and pooled from the same cultures from weeks 5 to 10 (medium B). Proteinase inhibitors were added immediately [10] and media A and B were then processed separately as follows. Newly synthesised polypeptides in the culture medium were twice precipitated by $(NH_4)_2SO_4$ at 30% saturation and then salt fractionated at 0.8 and 2 M NaCl [10]. The 2 M NaCl precipitate from medium B was purified by reverse-phase high performance liquid chromatography (HPLC). Lyophilised 2 M NaCl precipitate was dissolved in 0.05% (v/v) trifluoroacetic acid (5 mg/ml) and samples (200 μl) were injected into a C_{18} reverse-phase column (Waters Ultrapore RPSC column) washed with 0.05% (v/v) trifluoroacetic acid at a flow rate of 2 ml/min. Elution of bound proteins was achieved with a step gradient of acetonitrile (HPLC grade, BDH, Poole, Dorset) in 0.05% (v/v) trifluoroacetic acid. Peaks were detected with a Waters 441 UV monitor at 229 nm and fractions (2 ml) were collected, pooled as appropriate and analysed by SDS-PAGE.

2.2. *Polyacrylamide gel electrophoresis*

Salt-fractionated polypeptides from both media A and B, and peaks eluted from the HPLC column were analysed by SDS-PAGE on an 8% slab gel using the Tris-glycine buffer system described by Laemmli [19]. The 2 M NaCl precipitate from medium B was further characterized by two-dimensional SDS-PAGE. A sample was electrophoresed using a 5% disc gel under non-reducing conditions. The disc gel was then incubated in 0.1 M Tris-HCl buffer, pH 6.8, containing 20 mM dithiothreitol at 50°C for 15 min [20]. After reduction, the disc gel was mounted on top of an 8% slab gel with a 3% stacking gel. Electrophoresis was performed at 28 mA and the gel stained with 0.25% (w/v) Coomassie brilliant blue R in 45% (v/v) methanol and 10% (v/v) acetic acid.

2.3. *CNBr peptide mapping*

Lyophilised 2 M NaCl precipitate (medium B) was labelled with 50 μCi [^{3}H]acetic anhydride in 1 ml of 50 mM Tris-HCl buffer, pH 8.9, and the mixture was incubated at room temperature for 10 min. Labelled polypeptides were dialysed extensively against water and separated by SDS-PAGE on a 5% disc gel. After electrophoresis in the first dimension, the gel was immersed in 70% (v/v) formic acid for 15 min, followed by the addition of solid CNBr to approx. 50 mg/ml. Digestion was performed at 25°C for 16 h under N_2 and then terminated by equilibrating the disc gel with 0.1 M Tris-HCl buffer, pH 6.8. Electrophoresis in the second dimension was performed with a 15% separating gel at 28 mA and the gel was then processed for fluorography as described [20].

2.4. S. aureus *V8 protease peptide mapping*

Labelled polypeptides were obtained by incubating cultured hypertrophic chondrocytes (maintained for 6 weeks in culture) with 20 μCi/ml of [^{35}S]methionine for 24 h and then precipitated from the culture medium with $(NH_4)_2SO_4$ at 30% saturation. Collagenous polypeptides were separated on an 8% slab gel. Bands were visualized by immersing the gel in 4 M sodium acetate [21] and were excised from the gel with a scalpel. Gel strips containing bands of interest were incubated at room temperature in 0.125 M Tris-HCl buffer, pH 6.8, containing 0.1% (w/v) SDS, 1 mM EDTA and 30% (v/v) glycerol for 30 min. The gel strips were then loaded on top of a 15% slab gel and overlaid with 20 μl of the same Tris-HCl buffer containing only 20% (v/v) glycerol followed by the addition of 50 μg *S. aureus* V8 protease in Tris-HCl buffer with 10% (v/v) glycerol. Electrophoresis was performed at 20 mA until the dye front entered the stacking gel. The power was switched off for 30 min to allow enzyme digestion to proceed and the resulting peptides were separated by continuing the electrophoretic separation at 28 mA.

3. RESULTS AND DISCUSSION

When proteins synthesised and secreted by chondrocytes in culture for 2–5 weeks (medium A) were partially purified by differential salt precipitation [10] the 0.8 M NaCl fraction contained the α-

chains of collagen types I and II, and the predominant component of the 2 M NaCl fraction was the α_1(X) collagen chain (fig.1). When similar analyses were conducted on the polypeptides present in culture media from aged chondrocytes (5–10 weeks in culture, medium B) marked differences were observed in the composition of the 2 M NaCl fraction. In this fraction two extra bands, labelled HG and HG° (track 4, fig.1), migrating to positions corresponding to approx. M_r 120000 and 69000 respectively, were apparent. Both HG and HG° were collagenous as judged by their susceptibilities to highly purified bacterial collagenase (not shown). After reduction of this 2 M NaCl fraction the only detectable bands were those of the proα_1(X) and α_1(X) chains (track 8, fig.1). The 2 M NaCl fraction from medium B was then subjected to further purification by reverse-phase HPLC and both bound and unbound peaks were analysed by SDS-PAGE (fig.2). Unbound material that eluted off the column (peak 1, fig.2) and material which eluted at low concentration of acetonitrile (peak 2) contained only the α_1(X) chain, whereas at higher concentrations of acetonitrile HG, HG° and proα_1(X) chains co-eluted in peak 3 (fig.2a,b). These observations

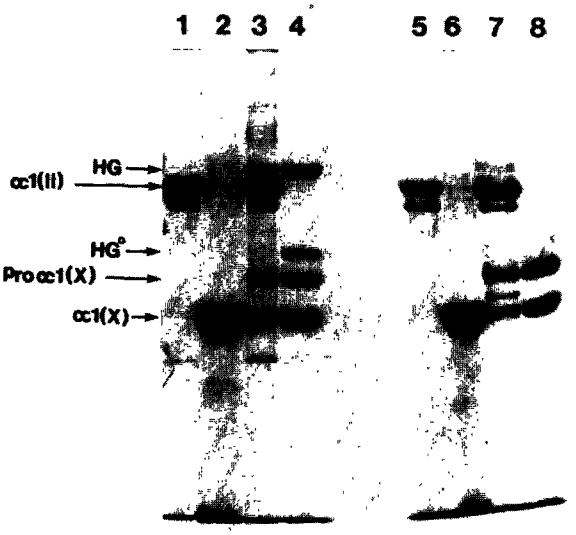

Fig.1. SDS-polyacrylamide gel electrophoresis (8%) of the NaCl-fractionated chick embryo tibiotarsal chondrocyte culture medium proteins. Samples were 0.8 M NaCl precipitates (tracks 1,3,5,7) and 2 M NaCl precipitates (tracks 2,4,6,8). Tracks 1,2,5,6 show collagens isolated from chondrocyte culture medium collected and pooled from week 2 to 5 (medium A). Tracks 3,4,7,8 show collagens isolated from culture medium collected and pooled from week 5 to 10 (medium B). Samples in tracks 5–8 were run after reduction with 2-mercaptoethanol whereas tracks 1–4 contain unreduced samples. Gel was stained with 0.25% (w/v) Coomassie brilliant blue R.

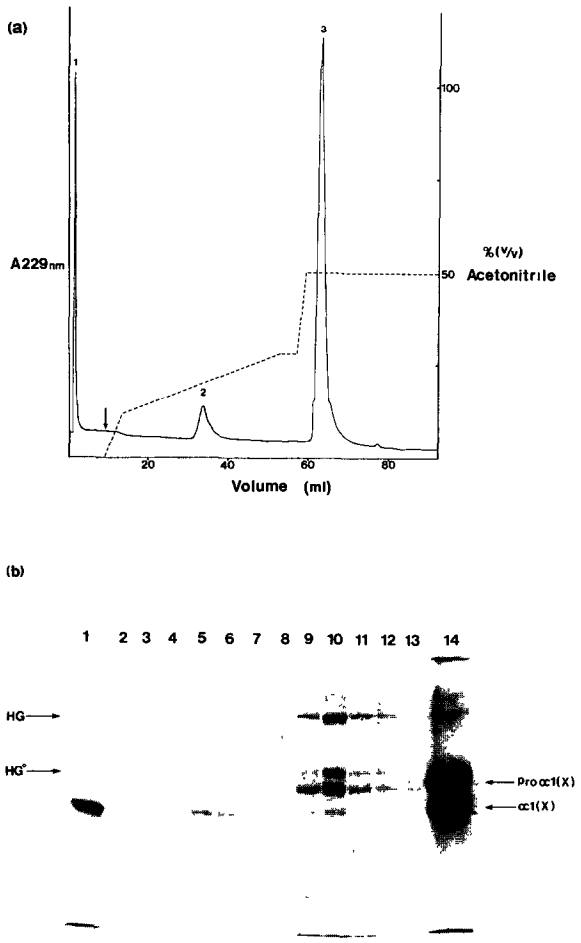

Fig.2. Purification of the 2 M NaCl precipitate (medium B) by reverse-phase HPLC. (a) Redissolved 2 M NaCl precipitate (in 0.05% (v/v) trifluoroacetic acid) was injected into a C_{18} reverse-phase column eluted with an acetonitrile (0–50% (v/v)) step gradient. (b) Fractions from peaks 1–3 were analysed by SDS-PAGE (8%). Tracks: 1, unbound material in peak 1; 2–7, fractions from peak 2; 8–13, fractions from peak 3; 14, standard containing proα_1(X) and α_1(X) chains. Gel was stained with 0.25% (w/v) Coomassie brilliant blue R.

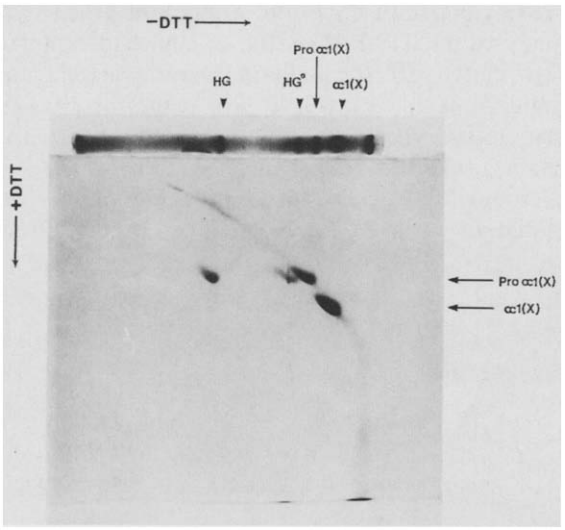

Fig.3. Two-dimensional SDS-PAGE of the 2 M NaCl precipitate (medium B). First dimension electrophoresis was performed on a 5% disc gel without reduction. Second dimension was run on 8% polyacrylamide slab gel after reduction with dithiothreitol (DTT) of the peptides within the disc gel. Gels were stained with 0.25% (w/v) Coomassie brilliant blue R.

were obtained in 10 separate experiments and it can be concluded that the HG, HG° and proα_1(X) chains have similar hydrophobicities.

To investigate the relationship between these disulphide-bonded peptides, the 2 M NaCl precipitate of medium B was analysed by two-dimensional SDS-PAGE. The resulting polypeptides after reduction of HG and HG° migrated to positions identical to that of the proα_1(X) chains

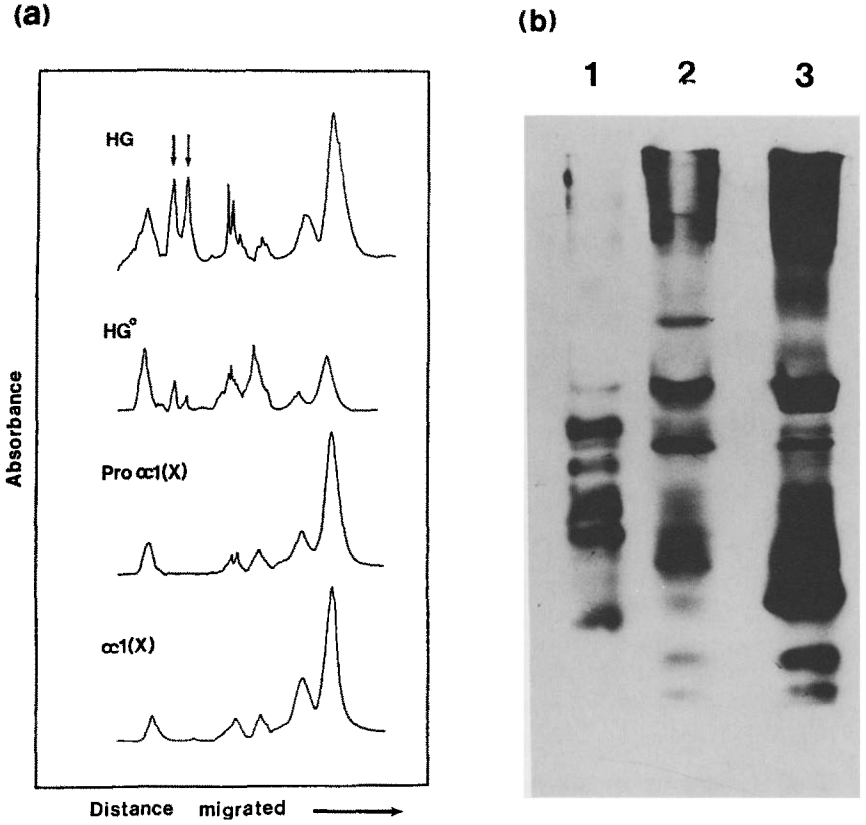

Fig.4. Peptide mapping of type X collagen and related polypeptides. (a) The 2 M NaCl precipitate of medium B was labelled with [^{3}H]acetic anhydride and the polypeptides subjected to CNBr digestion. The resulting peptides were analysed by SDS-PAGE and fluorography and the profiles of CNBr cleavage-products of α_1(X), proα_1(X), HG° and HG polypeptides are presented. The extra peptides of HG and HG° are denoted by arrows. (b) Fluorogram showing *S. aureus* V8 protease digestion products of α_1(II) chains (track 1), HG (track 2) and proα_1(X) chains (track 3). Electrophoresis was performed under non-reducing conditions.

(fig.3). The CNBr peptide patterns of the four polypeptides HG, HG°, proα_1(X) and α_1(X) are identical except two extra high-M_r peptides were prominent in the digests of HG and HG° (fig.4a). Since these peptides were electrophoresed under non-reducing conditions, the two extra peptides may contain regions of disulphide bonding. The *S. aureus* V8 protease peptide maps of HG, proα_1(X) and α_1(II) chains demonstrated the common identity between HG and proα_1(X) chain (fig.4b) which gave a profile distinct from the peptide pattern of the α_1(II) chain. From the above observations it was concluded that HG represents a dimeric form of the proα_1(X) chain linked by disulphide bonds, and HG° represents a partially processed form of this dimer. The disulphide bond(s) are probably located within the globular extension of proα_1(X) for when the unreduced 2 M NaCl (medium B) fraction was subjected to limited pepsin digestion, only the non-disulphide linked helical fragment (M_r 45000) could be recovered (not shown).

These observations provide the first report of the occurrence of disulphide-linked chick type X collagen polypeptides and may explain the recent report [22] that the extractability of type X collagen from chick sterna by pepsin can be increased substantially by the use of dithiothreitol. It is also noteworthy that amino acid sequence data determined from nucleotide sequences of the chick collagen type X gene indicate cysteine residues in the large carboxy-terminal domain [24]. The occurrence of disulphide bonding within mammalian type X collagens has already been documented [17,18] but in contrast to the avian type X collagen, the bovine type X chains exhibit disulphide bonding within their triple-helical domains [17,18].

The failure to detect disulphide-linked forms of proα_1(X) chains in previous studies with chick chondrocytes in this and other laboratories is surprising but the results presented in fig.1 suggest that only after prolonged periods of cell culture (5–10 weeks) do the disulphide linked species become prominent when up to 40% of the proα_1(X) chains occur in disulphide-bonded dimers. At this time type X collagen synthesis represents 50–80% of total protein synthesis by chick tibiotarsal hypertrophic chondrocytes in culture [10] and it is known that the type X collagen in the culture medium is closely associated with matrix vesicles, rich in Ca^{2+} and alkaline phosphatase [23], that appear to play a role in the calcification of cartilage [25]. The hydrophobic nature of type X procollagen polypeptides as reflected by their strong affinities for the reverse-phase HPLC column (fig.2) is consistent with the observations that type X collagen may be associated with membranous structures [23]. However, further studies will be required to define the various processed forms of type X collagen and their function in the extracellular matrix.

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