Partial characterization of the C-terminal non-collagenous domain (NC1) of collagen type X

Ruth E. BARBER*‡ and Alvin P. L. KWAN†§

*School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, and †School of Molecular and Medical Biosciences, University of Wales, Cardiff, P.O. Box 911, Museum Avenue, Cardiff CF1 3US, U.K.

Collagen type X is composed of three identical α1(X) chains of 59 kDa, each containing a triple-helical region of 45 kDa flanked by a short N-terminal sequence and a larger non-collagenous C-terminal (NC1) domain of approx. 15 kDa. Collagen type X molecules can associate via their C-termini to form a regular hexagonal lattice *in vitro*, which *in vivo* may provide a modified extracellular matrix for the events of endochondral ossification. The NC1 domain of chick collagen type X was isolated and purified from a highly purified bacterial collagenase digest of hypertrophic chondrocyte medium proteins. The structure and aggregation properties of the NC1 domain of collagen X were investigated, independently of the triple helix. A trimer, a dimer and a monomer of the individual α-chain NC1 polypeptides were identified from a bacterial collagenase digest of cartilage collagens using [^{14}C]tyrosine labelling, *N*-chlorosuccinimide peptide mapping and N-terminal sequencing. The trimer (50 kDa) remained intact in Laemmli sample buffer unless boiled, upon which it dissociated into the dimer (38 kDa) and the monomer (20 kDa). The dimer persisted even after prolonged periods of heating or reduction with β-mercaptoethanol, and in preparations obtained from chondrocyte cultures treated with β-aminoproprionitrile, indicating the presence of non-reducible, non-lysine-derived, covalent cross-links. Hexamers of the individual C-termini were observed in rotary-shadowed preparations of purified NC1 domain, reflecting the ability of collagen type X to self-assemble via its C-termini under appropriate conditions.

INTRODUCTION

Collagen type X is a low-molecular-mass collagen that is expressed exclusively in the hypertrophic zone of the growth plate where mineralization and vascularization of the cartilage matrix occur. Mineralization of the matrix is followed by invasion of osteoprogenitor cells which deposit new bone matrix, a process known as endochondral ossification [1]. Collagen type X has been detected in cell or organ cultures from the hypertrophic or presumptive calcification regions of the growth plate [2–5], and in cultures of chondrocytes that have reached hypertrophy *in vitro* [6,7]. The temporal and spatial distribution of collagen X *in vivo* has been reported at several different sites of endochondral ossification during development of the chick embryo [8–11]. Synthesis of the collagen type X protein or mRNA was found to commence at different ages depending on the site investigated (tibia, vertebra or sternum), but in all cases collagen X expression preceded mineralization and cartilage erosion. Collagen type X has also been reported at sites of endochondral ossification during fracture repair [12] and in disease processes such as osteoarthritis where bone formation occurs at normally cartilaginous sites [13–15]. The evidence from both *in vivo* and *in vitro* studies of collagen type X expression strongly suggests that collagen type X synthesis and chondrocyte hypertrophy are closely related processes. In fact, collagen type X expression is frequently used as a marker for chondrocyte hypertrophy [16]. More recently, several mutations within the human *COL10A1* gene were found to be associated with the skeletal disorder known as metaphyseal dysplasia type Schmid, in which dwarfism and bone malformation are frequently observed [17–20]. These findings strongly suggest a role for collagen type X in normal endochodral bone formation.

Collagen type X is a homotrimer comprising three identical α1(X) chains of molecular mass 59 kDa [21,22]. Each α1(X) chain contains a pepsin-resistant triple-helical domain of 45 kDa flanked by two pepsin-sensitive non-collagenous regions [21,23]. The N-terminal region (NC2) consists of only 52 amino acids, 18 of which form the signal peptide [24], but the C-terminal domain (NC1) is considerably larger (162 amino acids) [25]. Unlike with the fibrillar collagens, the NC1 domain of collagen type X is not removed following secretion into the extracellular matrix, suggesting that it may play a part in the function of the collagen. Furthermore, analysis of the chick, bovine, human and mouse α1(X) gene sequences has revealed a high degree of identity in the NC1 domain (80–90 % at the amino acid level), including 13 conserved tyrosine residues, a single conserved cysteine and a potential site for N-glycosylation [24,26–28].

It has been postulated that the NC1 domain of collagen type X may have a function in the association and alignment of the α1(X) chains during biosynthesis [29], and this hypothesis is supported experimentally by work examining the denaturation/renaturation properties of collagen type X [30]. It has also been observed that, in rotary-shadowed preparations, collagen type X molecules are able to aggregate via their NC1 domains into hexagonal lattice structures consisting of nodules composed of clustered NC1 domains, interconnected by a filamentous network of helices [31]. Such observations have led to speculation that the NC1 domain may play an important role in supramolecular assembly, as has been demonstrated for collagen type IV [32]. Furthermore, the α1(X) chain has been shown to be highly

Abbreviations used: LSB, Laemmli sample buffer; NC1, non-collagenous C-terminal domain; NC2, non-collagenous N-terminal domain; NCS, *N*-chlorosuccinimide.

‡ Present address: Chiroscience Ltd., Cambridge Science Park, Milton Road, Cambridge CB4 4WE, U.K.

§ To whom correspondence should be addressed.

similar to the α chains of collagen type VIII [25,33], a collagen known to associate into hexagonal sheet-like structures via its non-collagenous domains in Descemet's membrane [34]. In studies using immunoelectron microscopy, Schmid and Linsenmayer [35] and Kwan et al. [31] have identified fine filamentous mats which react with antibodies recognizing collagen type X, and which may be related to the hexagonal lattices seen *in vitro*.

There is considerable evidence to suggest that the NC1 domain of collagen type X is important in the overall function of this molecule, and we have attempted to characterize this domain further. Here we report the isolation and partial characterization of the NC1 domain of chick collagen type X, and studies of its structural and aggregational properties independently of the triple helix.

MATERIALS AND METHODS

Embryonic chick chondrocyte culture

Primary cultures of chick embryonic hypertrophic chondrocytes were used as a source of cartilage collagens. These cultures were set up according to the procedures described previously [4]. Briefly, the hypertrophic zone was dissected from the tibial epiphyses of 17-day embryonic chicks, digested for 3 h in 10 ml of filter-sterilized Dulbecco's modified Eagle's medium (Gibco) containing 10 mg/ml crude bacterial collagenase (Sigma, type IA) and 0.03 % (w/v) trypsin (Gibco) at 37 °C in a shaking water-bath. Cells were washed and plated out in Dulbecco's modified Eagle's medium supplemented with 10 % foetal calf serum (Gibco) at a density of 0.5×10^6/ml. Cells were incubated at 37 °C in a moist atmosphere of 5 % CO_2 and 95 % air. The culture medium was changed every 48 h and the used medium was pooled and stored at −20 °C in the presence of protease inhibitors [0.025 M Na_2EDTA, 0.02 % (w/v) sodium azide, 0.1 M Tris/HCl, pH 7.4] (Sigma Chemical Co.). In some experiments, confluent cultures were labelled for 16 h with either 10 μCi/ml L-[5-^{3}H]proline or 2.5 μCi/ml L-[U-^{14}C]tyrosine (Amersham International). In order to determine whether collagen type X is a substrate for tissue transglutaminase, cells were labelled with 4 μCi/ml [1,4-^{14}C]putrescine (Sigma) for 16 h in the presence of ascorbate. Separate cultures were maintained for 16 h in the presence of 0.5 mM of the transglutaminase substrate monodansylcadaverine (Sigma). Control cultures were treated with 0.5 mM of the substrate analogue dansylamidopentanol-5 (supplied by Dr. Daniel Aeschlimann, University of Wisconsin, Madison, WI, U.S.A.) [36] for 16 h. Collagens secreted into the culture medium were precipitated by the addition of solid ammonium sulphate to 30 % saturation at 4 °C. These crude fractions were stored at −20 °C before use. In some experiments, cultures were pretreated with culture medium containing 100 μg/ml β-aminoproprionitrile (Sigma) for 6 h prior to radiolabelling, to inhibit formation of lysine-derived cross-links. Biosynthetic products were then radiolabelled and harvested as described above.

Preparation of NC1 domain of collagen type X

Crude collagen preparations (30 % ammonium sulphate precipitates from hypertrophic chondrocyte culture medium) were resuspended at 1 g wet weight/10 ml in 0.05 M Tris/HCl, pH 7.2, containing 0.01 M calcium acetate and digested for 24 h at 37 °C with 225 units of ultrapure bacterial collagenase (*Clostridium histolyticum* type III; activity 225 units/84 μg of protein; Advanced Biofactures, New York, NY, U.S.A.). The digested product was applied directly to a Sephacryl 300HR (Pharmacia-LKB) gel filtration column for purification.

N-Chlorosuccinimide (NCS) peptide mapping

NCS mapping of the trimer, dimer and monomer was performed based on the method of Lischwe and Ochs [37]. Polypeptides were separated by SDS/PAGE [38] on a 12.5 % gel and stained with Coomassie Blue. The bands to be mapped were excised using a scalpel blade. The gel slices were washed twice for 10 min in 10 ml of ultrapure water (Millipore), then twice for 10 min in urea solution [1 g of urea/ml of 50 % (v/v) acetic acid]. The slices were treated for 30 min at room temperature with a 2 mg/ml solution of NCS in urea solution, washed twice for 10 min in ultrapure water, and then equilibrated in 1 × Laemmli sample buffer (LSB) for 30 min. The bands were loaded on to a second SDS/polyacrylamide gel (15 %) and the products of NCS cleavage were separated by electrophoresis.

N-terminal sequencing

Partial sequencing of NC1 was performed by the method of Matsudaira [39]. Protein samples were separated by SDS/PAGE as described above. After electrophoresis, the polypeptides were transferred on to an Immobilon membrane (Millipore) by electroblotting for 45 min with a constant current of 45 mA using an LKB 2117 Multiphor II blotter. The membrane was stained for 5 min in a 0.1 % (w/v) solution of Coomassie Brilliant Blue R250 in 50 % (v/v) methanol, and then rapidly destained in 90 % (v/v) methanol/7 % (v/v) acetic acid. The bands to be sequenced were cut out of the membrane, and the first 13 N-terminal amino acids were sequenced by automated Edman degradation using an Applied Biosystems 476A Protein Sequencer (University of Manchester and University of South Florida).

Western blotting

Incorporation of dansyl groups into collagen type X was investigated by immunoblotting. Culture medium proteins from chick hypertrophic chondrocytes maintained in the presence of monodansylcadaverine were separated by SDS/PAGE as described above. After electrophoresis, the polypeptides were transferred on to a Gelman N20 nitrocellulose membrane by electroblotting for 30 min with a constant current of 45 mA using Bio-Rad Trans-blot SD semi-dry electrotransfer cell. The membrane was rinsed with PBS and blocked at room temperature for 30 min with PBS containing 0.05 % (v/v) Tween 20 and 0.1 % (w/v) BSA (PBST-BSA). After washing with PBS containng 0.05 % (v/v) Tween 20 (PBST), the membrane was incubated with a diluted (1 : 500 in PBST-BSA) polyclonal rabbit anti-(dansyl moiety) antiserum (a gift from Dr. Daniel Aeschlimann, University of Wisconsin) for 60 min. After extensive washing (3 × 15 min in PBST), the membrane was then incubated with 1 : 1000-diluted peroxidase-conjugated swine anti-rabbit IgG (Dakopatts) at room temperature for 60 min. Bound antibodies were visualized using the Amersham ECL reagent kit.

Rotary shadowing electron microscopy

Rotary shadowing of the purified NC1 domains was performed according to the mica sandwich technique of Mould et al. [40]. Samples were prepared at a concentration of 1 μg/ml in 0.05 M Tris/HCl, pH 7.2, containing 0.01 M sodium acetate, or in 0.5 M acetic acid. A piece of ruby mica (20 mm × 20 mm × 0.125 mm) was removed with forceps, and a 5 μl drop of the sample solution was placed in the centre of the newly formed

surface. The other newly formed surface was gently replaced on top, causing the drop to spread evenly between the two surfaces. The specimen was allowed to adsorb to the mica surface for 5 min, then excess salt was washed away by separating the mica pieces under 0.2 M ammonium acetate. The sandwich was frozen rapidly by plunging it into liquid nitrogen and the two pieces of mica were separated under the liquid nitrogen. The pieces were then immediately freeze-dried. Rotary shadowing was carried out according to previously described procedures [31]. Specimens were examined in a JEOL 1200EX electron microscope at an accelerated voltage of 60 kV.

RESULTS

Isolation and purification of the NC1 domain of collagen type X

The NC1 domain of collagen type X was purified from the culture supernatant collected from chick hypertrophic chondrocyte primary cultures. [^{14}C]Tyrosine-labelled cartilage collagens (including collagen type X) from the culture medium were isolated by ammonium sulphate precipitation, and collagenous domains were removed by digestion with ultrapure bacterial collagenase. Figure 1 shows a fluorogram of the [^{14}C]tyrosine-labelled polypeptides before and after bacterial collagenase digestion. The heavily labelled α1(X) chains (lane 1) were not observed following bacterial collagenase treatment, and a new lower-molecular-mass band was apparent (lane 2). This polypeptide, which was dissolved in LSB but was not boiled prior to electrophoresis, migrated as a single band of approx. 46 kDa. This molecular mass is approximately three times that expected for the C-terminus of a single α1(X) chain, which suggests that this species represents a trimer of α1(X) C-termini, i.e. the intact NC1 region of a single collagen X molecule. When the collagenase-digested collagen sample was boiled prior to electrophoresis, two [^{14}C]tyrosine-labelled bands of approx. 37 kDa and 18 kDa were observed (Figure 1, lane 3), thought to be a dimer and a monomer respectively of the α1(X) C-termini. The molecular masses of the trimer, dimer and monomer of the collagen type X NC1 domain were determined by SDS/PAGE on 12.5% and 15% mini-slab gels in 10 separate experiments

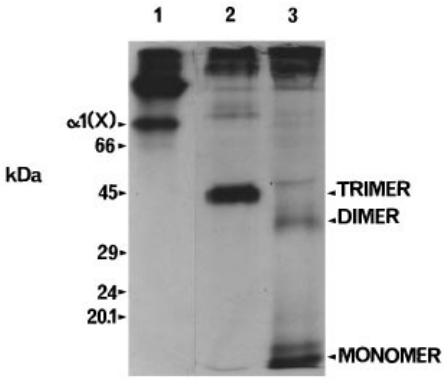


Figure 1 Fluorogram of [^{14}C]tyrosine-labelled collagenous polypeptides isolated from embryonic chick hypertrophic chondrocyte culture medium

Proteins in the culture medium were precipitated by addition of ammonium sulphate to 30% saturation. Lane 1, [^{14}C]tyrosine-labelled medium polypeptides containing the intact α1(X) chain; lane 2, bacterial collagenase digest of [^{14}C]tyrosine-labelled medium proteins (samples not boiled in LSB prior to electrophoresis); lane 3, bacterial collagenase digest of [^{14}C]tyrosine-labelled medium polypeptides (samples boiled before electrophoresis). Molecular masses of globular protein standards are indicated.

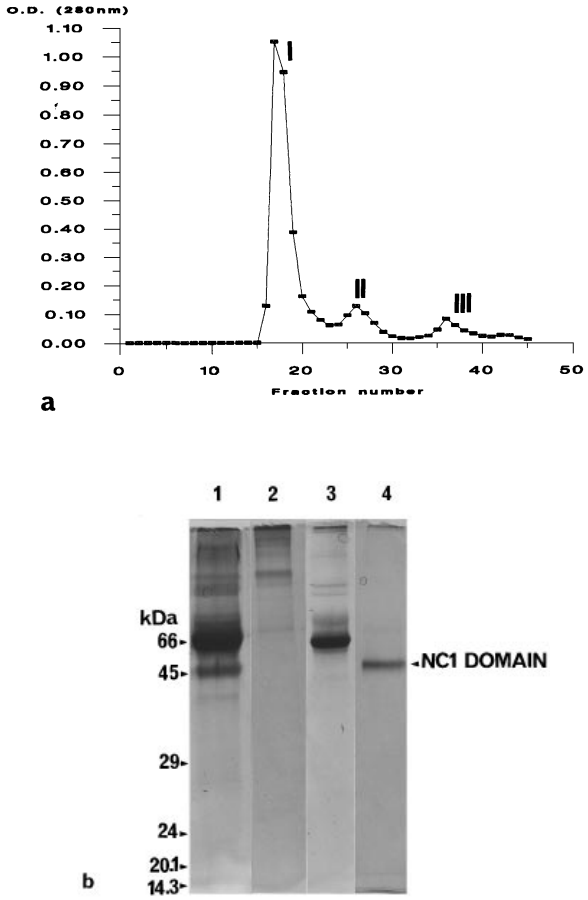
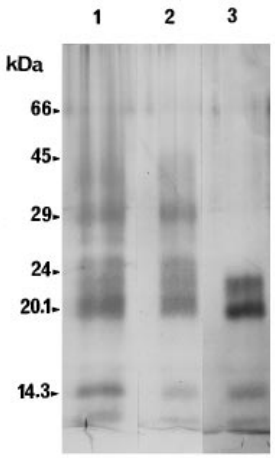


Figure 2 Purification of the NC1 domain of collagen type X

Collagens precipitated from the medium of hypertrophic chondrocyte cultures were digested with ultrapure bacterial collagenase and applied to a Sephacryl 300HR column in 0.01 M sodium acetate/0.05 M Tris, pH 7.2. Fractions of 1.3 ml were eluted in the same buffer and peaks were analysed by SDS/PAGE. (**a**) Elution profile of a typical gel-filtration chromatography of the collagenase digest in 0.01 M sodium acetate/0.05 M Tris, pH 7.2. Absorbance (O.D.) was measured at 280 nm. (**b**) Silver-stained gel of eluted peaks. Lane 1, bacterial collagenase digest applied to column; lane 2, peak I containing type II collagen and high-molecular-mass proteins; lane 3, peak II containing predominantly BSA from the culture medium; lane 4, peak III containing NC1 domains (trimers). Samples were not boiled in LSB prior to electrophoresis. Molecular masses of globular protein standards are indicated.

using a standard curve constructed from the R_F values of six proteins of known mass. The molecular mass of the trimer was found to vary between 44.7 and 52.5 kDa, with a mean ($\pm$S.D.) value of 49.2$\pm$2.6 kDa. The dimer had a molecular mass of between 33.9 and 41.7 kDa (mean value 38.1$\pm$2.6 kDa), and the monomer had a molecular mass of between 18.6 and 21.4 kDa (mean value 20.5$\pm$0.9 kDa). The experimentally determined molecular masses of all three species correspond well to the predicted molecular masses.

Purification of the NC1 domain of collagen type X from large-scale collagenase digests was achieved by size-fractionation chromatography using a Sephacryl 300HR gel-filtration column. Figure 2(a) shows a typical elution profile. SDS/PAGE analysis (Figure 2b) showed that the first peak contained high-molecular-mass proteins, including undigested collagens (lane 2). Peak II contained predominantly a 66 kDa polypeptide which was also present in samples of culture medium only and is therefore likely to be a serum protein such as albumin. Higher-molecular-mass



Published sequence :	**N**	-GPPGQSTIPEGYV-	**C**
Trimer, dimer & Monomer band :	**N**	-GPPGQSTIPEGYV-	**C**

Figure 3 NCS peptide maps (upper panel) and N-terminal sequences (lower panel) of the trimeric, dimeric and monomeric forms of the NC1 domain of collagen X

Upper panel: lane 1, trimer; lane 2, dimer; lane 3, monomer of the NC1 domain of collagen type X. Molecular masses of globular protein standards are indicated. Lower panel: the NC1 bands were separated by SDS/PAGE and transferred to an Immobilon membrane by electroblotting. The bands were stained, cut out of the membrane and the first 13 amino acids from the N-terminus sequenced. The sequence obtained was compared with the published sequence of the collagen type X NC1 domain.

bands were also present, which may correspond to undigested collagen chains (lane 3). The NC1 domain was eluted in peak III (lane 4), very close to the V_t of the column, and appeared as a single polypeptide when examined by SDS/PAGE.

Composition of the NC1 domain

NCS peptide mapping was used to confirm that the three polypeptides of the NC1 domain are related. The NCS peptide maps obtained for the trimer, dimer and monomer (Figure 3, lanes 1–3) suggested that they are indeed derived from the same source. N-terminal sequencing of the first 13 amino acid residues of the trimer, dimer and monomer revealed that their sequences were identical to the published sequence of chick collagen type X [25] (Figure 3, lower panel). Densitometric scanning of a typical SDS/PAGE gel of the [^{14}C]tyrosine-labelled polypeptides as in Figure 1 allowed the quantification of the relative proportions of the trimer, dimer and monomer in a preparation. Comparison of the area under the trimer peak (3.36×10^7 arbitrary units) with the total under the dimer and monomer peaks (3.05×10^7 arbitrary units) shows that the total area of the dimer and monomer peaks together is approximately equal to that of the trimer band. As equal numbers of counts were applied on to both lanes, this provides additional evidence that the dimer and monomer result from dissociation of the trimer after boiling in LSB. Densitometric analyses of Figure 1 also showed that the intensity of the monomer band was approximately twice that of the dimer band. As one dimer is equivalent to two monomers, it should contain twice as many radiolabelled tyrosine residues as a single monomer, i.e. one dimer should have double the intensity of one monomer. Assuming that all three chains were equally labelled with [^{14}C]tyrosine, this would suggest a ratio of four monomers to one dimer, and implies that only one in two trimers contains a cross-linked dimer.

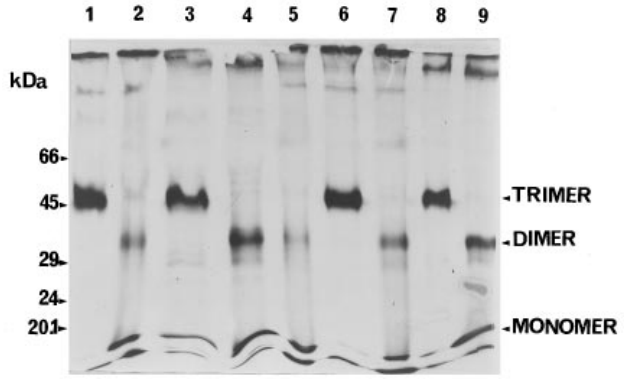


Figure 4 Fluorogram of bacterial collagenase digests of [^{14}C]tyrosine-labelled collagenous polypeptides obtained from chick hypertrophic chondrocyte cultures in the presence and absence of β-aminoproprionitrile

Lanes 1–5, collagenase-digested polypeptides from untreated cultures; lanes 6–9, collagenase-digested polypeptides from β-aminoproprionitrile-treated cultures. Lanes 1, 3, 6 and 8, samples not boiled in LSB prior to electrophoresis; lanes 2, 4, 7 and 9, samples boiled for 2 min in LSB before electrophoresis; lane 5, sample boiled for 10 min. The samples in lanes 3, 4, 8 and 9 were reduced with β-mercaptoenthanol prior to electrophoresis. Molecular masses of globular protein standards are indicated.

Characterization of the NC1 domain

Following boiling in LSB, the trimeric NC1 domain of collagen type X dissociates into a dimer and a monomer. The dimer persists even after boiling for 10 min in LSB (Figure 4, lane 5), indicating that it is held together by covalent interactions. Further evidence that the dimer is covalently cross-linked in at least one place can be seen from the NCS peptide maps (Figure 3, upper panel). It is interesting to note that all three maps contain some bands of higher molecular mass than would be predicted from the published chick collagen type X sequence [25]. NCS cleaves very specifically at tryptophan residues, and therefore the NC1 domain of collagen X should be cleaved in two places, generating three complete cleavage products of 10.3, 4.4 and 3.2 kDa, and two partial cleavage products of 14.7 and 7.6 kDa. Bands of approx. 14.7 and 10.3 kDa can be seen on a 15% gel (Figure 3). The bands of 20.4, 39.8 and 44.7 kDa probably correspond to uncleaved monomer, dimer and trimer respectively. In addition, the peptide map of the monomer contains a band of 24.0 kDa and those of the dimer and trimer contain four bands between 20 and 35 kDa. It is likely that these extra bands are cross-linked cleavage products generated both by intra- and inter-chain cross-links.

Treatment of the collagenase-digested [^{14}C]tyrosine-labelled samples with the reducing agent β-mercaptoethanol prior to fluorographic analysis was found to have no effect on the mobility of the NC1 trimer, dimer or monomer (Figure 4, lanes 3 and 4). It is therefore unlikely that the covalent cross-links are disulphide bonds. The possibility that the covalent cross-links are derived from lysine was also investigated by analysing samples collected from cultures pretreated with β-aminoproprionitrile, which is known to inhibit the formation of such lysine-derived

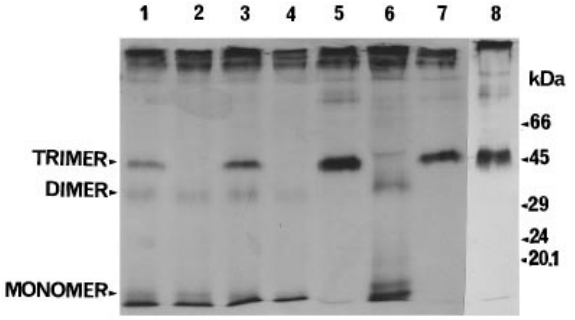


Figure 5 Dissociation/reassociation of the collagen type X NC1 domain

[^{14}C]Tyrosine-labelled collagens were precipitated from chondrocyte culture medium and dialysed into collagenase buffer, 0.5 M acetic acid or 0.1 M acetic acid. Samples in collagenase buffer were digested with bacterial collagenase immediately (lanes 5 and 6). Samples in 0.5 M acetic acid and 0.1 M acetic acid were either freeze-dried, resuspended in collagenase buffer and digested (lanes 1/2 and 3/4 respectively) or dialysed from acid into collagenase buffer, then digested (lanes 7 and 8 respectively). Lanes 1, 3, 5, 7 and 8, samples not boiled in LSB; lanes 2, 4 and 6, samples boiled in LSB prior to electrophoresis.

collagen cross-links. No alterations in band mobility or in the proportions of trimer, dimer and monomer were observed following β-aminoproprionitrile treatment, under either non-reducing (Figure 4, lanes 6 and 7) or reducing (lanes 8 and 9) conditions. These experiments suggest the presence of cross-links other than disulphide or lysine-derived bonds.

In order to ascertain whether collagen type X is a substrate for tissue transglutaminase, hypertrophic chondrocytes were labelled with [^{14}C]putrescine. No radiolabelled collagen type X was recovered from the culture medium. Similarly, no dansyl-labelled collagen type X could be detected, using Western blotting, in proteins isolated from the culture media of cells labelled with monodansylcadaverine (results not shown). These experiments suggest that the covalent cross-link found in the NC1 domain is not a γ-glutaminyl-ϵ-lysine cross-link.

The NC1 region of collagen type X is able to retain its trimeric structure intact even in the presence of SDS (LSB), but it is clear that the assembly can be dissociated by heat. The effect of acetic acid on the NC1 domain was also investigated. [^{14}C]Tyrosine-labelled collagens were precipitated from chondrocyte culture medium and dialysed into collagenase buffer, 0.1 M acetic acid or 0.5 M acetic acid. The samples in collagenase buffer were digested with bacterial collagenase immediately, whereas samples in 0.5 M and 0.1 M acetic acid were either freeze-dried, resuspended in collagenase buffer and digested immediately, or dialysed into collagenase buffer and then digested. All digests were freeze-dried and analysed by fluorography (Figure 5). Samples that had remained in collagenase buffer throughout did not dissociate unless boiled (lanes 5 and 6). Samples treated with 0.5 or 0.1 M acetic acid were partially dissociated whether heated or not prior to electrophoresis (lanes 1–4), unless these samples had been dialysed into collagenase buffer in which case complete reassociation into trimers was observed (lanes 7 and 8). These results indicate that acetic acid dissociates the trimer into its subunits, at least partially, and that under neutral conditions the subunits are able to reassociate fully.

Rotary shadowing electron microscopy

Purified NC1 domain was rotary-shadowed either in 0.01 M sodium acetate/0.05 M Tris/HCl, pH 7.2, or in 0.5 M acetic acid. Under these conditions two distinct populations of

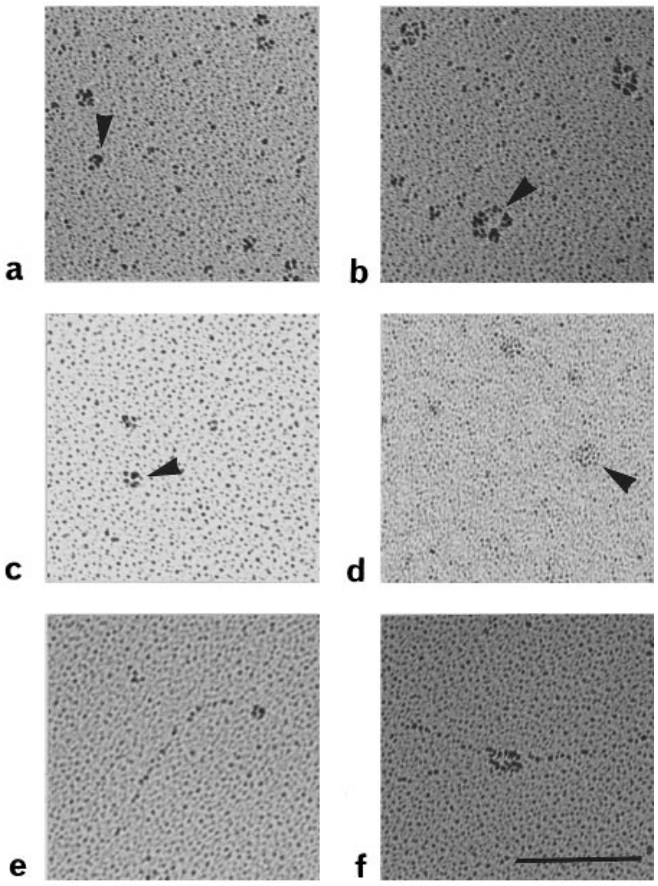


Figure 6 Electron micrographs of rotary-shadowed molecules of NC1 domains of collagen type X (a–d) and of intact collagen type X (e and f)

Samples were prepared in 0.01 M sodium acetate/0.05 M Tris, pH 7.2 (**a**, **b**, **e**, **f**), or in 0.5 M acetic acid (**c**, **d**). Arrowheads indicate molecules typical of the small (**a** and **c**) and large (**b** and **d**) populations of NC1 domains observed. Bar = 50 nm.

molecules appeared (Figures 6a and 6b). The population in Figure 6(a) made up the majority (approx. 79%) of the total number of molecules present. The average diameter of the molecules in each population was calculated from measurements made with a sample size of 224 molecules taken from six micrographs at different magnifications. The diameter of the smaller set of molecules (Figure 6a) was calculated at 7.5 ± 1.6 nm (mean $\pm$ S.D.), half the diameter of the larger polypeptides (14.4 ± 2.4 nm) (Figure 6b). This relationship was found in two separate experiments.

When the NC1 domain was rotary-shadowed in 0.5 M acetic acid (Figures 6c and 6d), there was an increase in the ratio of smaller molecules (88%) to larger ones (12%). The average diameter of the molecules in each population was calculated from measurements made with a sample size of 137 molecules taken from five separate micrographs. The smaller population had a mean diameter of 8.2 ± 1.5 nm, similar to that found in 0.01 M acetate, but the larger population had an average diameter of 12.8 ± 1.1 nm, slightly lower than that found in 0.01 M acetate.

The sizes of the two populations of NC1 domains were compared with those of the NC1 domains of intact collagen type X molecules shadowed at the same time (Figures 6e and 6f). The diameter of the globular domain of a single intact collagen X molecule (Figure 6e) has a similar appearance and size to that of

the smaller population of molecules seen in Figures 6(a) and 6(c). Likewise, the diameter of the globular region joining a pair of intact collagen X molecules together (Figure 6f) is equivalent to that of the larger population of molecules seen in Figure 6(b). It can be tentatively concluded from this evidence that the smaller population of molecules represents trimeric C-terminal domains equivalent to those derived from a single collagen type X molecule, and that the larger population of molecules (in 0.01 M acetate) represents two trimeric C-terminal domains joined together. It is likely that these aggregates were formed under the conditions used during the rotary shadowing process. The NC1 domain would be expected to dissociate into its subunits in 0.5 M acetic acid. However, it seems to be able to reassociate, mainly into trimers, under the conditions used for rotary shadowing.

DISCUSSION

Three related species, believed to be a trimer, a dimer and a monomer of the individual α-chain C-terminal polypeptide of collagen type X, have been identified in a bacterial collagenase digest of cartilage collagens obtained from the culture medium of chick hypertrophic chondrocytes. The three species were detected and shown to be derived from the same molecule by [^{14}C]tyrosine labelling and fluorography, by NCS peptide mapping and by N-terminal sequencing. Using SDS/PAGE, the trimer was found to be a polypeptide of approx. 50 kDa, which remained intact in LSB unless it was boiled, in which case it yielded a dimer and a monomer of approx. 38 and 20 kDa respectively. The dimers of the collagen type X NC1 domain persisted even after boiling in LSB for 10 min. In addition, NCS peptide maps of the trimer and dimer of the NC1 domain contained high-molecular-mass bands which were absent from the peptide map of the monomer, indicating the presence of cross-linked peptides. These results suggest that the NC1 dimers contain at least one covalent cross-link, but the nature of this cross-link is still uncertain. From the data presented, this cross-link is neither a disulphide bond nor a lysine-derived cross-link (e.g. lysyl pyrridinoline). We have also investigated the possibility of the presence of a transglutaminase cross-link in the collagen type X NC1 domain. Although hypertrophic chondrocytes have been shown to produce transglutaminase, we have not demonstrated that collagen type X is a substrate for this enzyme. One possibility is that the dimer contains di- or tri-tyrosine cross-links which are derived from tyrosine residues and are known to exist in some invertebrate collagens [41] as well as in the extracellular protein, elastin [42]. Preliminary experiments using HPLC and IR fluorescence spectrophotometry have indicated that trace amounts of dityrosine cross-link (approx. 0.2 mol/mol of collagen X) are present in these NC1 domain preparations (K. Horiuchi and A. P. L. Kwan, unpublished work). The amount of dityrosine found was extremely low (one cross-link in 200 molecules of collagen type X), and further analyses are required to ascertain the significance of dityrosine in collagen type X NC1 domain cross-links. The identification of the cross-linking amino acids and the significance of such covalent linkage are currently under investigation.

Densitometric scanning of a number of fluorograms indicated that the ratio of dimer to monomer in a [^{14}C]tyrosine-labelled digest was 1:4, implying tentatively that only one in two trimers contains a cross-linked dimer. However, these experiments provided qualitative proof of the existence of a covalently linked dimer within the NC1 domain of collagen type X. It is not known whether this is the case *in vivo* or is a result of non-optimum cell culture conditions leading to inefficient post-translational modification during collagen synthesis. In either case, it is evident that the presumptive intramolecular cross-links within the NC1 domain are not essential for successful assembly and secretion of collagen type X, although they may provide the molecule with additional stability.

It has been proposed that NC1 plays a role in bringing together and aligning the α-chains during synthesis and assembly of the collagen type X molecule [29,30]. It has now been shown, by studies of the genetic skeletal disorder metaphyseal dyschondroplasia type Schmid [17–20] and from studies of mutant recombinant collagen type X molecules [43], that the NC1 domain is essential for triple-helix formation. The present work provides additional evidence that powerful non-covalent interactions play a part in bringing together and maintaining the integrity of the trimeric collagen type X NC1 domain. Although the NC1 domain of collagen type X is able to retain its intact trimeric structure in LSB, it was found to dissociate in 0.1 M and 0.5 M acetic acid. However, full reassociation of the NC1 regions occurred on dialysis back into a neutral buffer. These results support the hypothesis that the NC1 subunits possess the intrinsic ability to self-associate, probably through hydrophobic or electrostatic interactions.

Studies *in vitro* have demonstrated that collagen type X is able to form into hexagonal lattice structures consisting of clusters of NC1 domains interconnected by a filamentous network of helices [31]. The ability of the NC1 domain to aggregate independently of the triple helix was investigated by rotary shadowing of purified NC1 domains in buffer at pH 7.2 and in 0.5 M acetic acid. Under both sets of conditions, two distinct populations of molecules were observed, thought to represent 'trimers' and 'hexamers' of the C-termini of individual α1(X) chains. Dissociation of trimers into monomers and dimers was not observed in acetic acid, which may be a consequence of random reassociation on the surface of the mica used during the rotary-shadowing technique. This hypothesis is supported by the observation that the mean diameters of the hexamers and trimers in 0.5 M acetic acid were slightly different from those found at neutral pH, and that the ratio of hexamers to trimers was lower.

Although hexamers of C-termini were observed during rotary shadowing, they were not detected in digests of [^{14}C]tyrosine-labelled collagens, even after SDS/PAGE on 6% gels (results not shown). It is therefore possible that these species were only formed under the conditions used during rotary shadowing. Larger aggregates were not observed either by rotary shadowing or on fluorograms, suggesting that the formation of the hexagonal lattice structures observed *in vitro* by Kwan et al. [31] is likely to involve the triple-helical as well as the C-terminal domains of collagen type X. However, it should be noted that all the collagen type X used for these studies was purified from the culture medium, whereas supramolecular aggregation would be more likely to take place in the cell layer. Aggregates of both intact collagen type X and NC1 domains observed during rotary shadowing may reflect the ability of these molecules to assemble into supramolecular structures in an appropriate environment. Assembly of the collagen type X network may also be dependent on the polyanionic environment of the cartilage extracellular matrix, the presence of chaperones or interactions with other matrix macromolecules.

The financial support of the Medical Research Council is gratefully acknowledged. Special thanks are due to Ms. Linda Berry (Wellcome Centre for Cell Matrix Research, University of Manchester) and Professor Peter Neame (University of Southern Florida, Tampa, FL, U.S.A.) for the amino acid sequencing of the trimer, dimer and monomer of the NC1 domain. We are grateful to Dr. Daniel Aeschlimann, (University of Wisconsin) for supplying the dansylamidopentanol-5 and the anti-dansyl antiserum. Most of the experimental work described in this paper was conducted at the School of Biological Sciences, University of Manchester.

REFERENCES

1 Horton, W. A. (1992) in Connective Tissue and its Heritable Disorders: Molecular, Genetic and Medical Aspects (Royce, P. M. and Steinmann, B., eds.), pp. 73–84, Wiley-Liss, New York
2 Gibson, G. J. and Flint, M. H. (1985) J. Cell Biol. **101**, 277–284
3 Grant, W. T., Sussman, M. D. and Balian, G. (1985) J. Biol. Chem. **260**, 3798–3803
4 Kielty, C. M., Kwan, A. P. L., Holmes, D. F., Schor, S. F. and Grant, M. E. (1985) Biochem. J. **227**, 545–554
5 Jimenez, S. A., Yankowski, R. and Reginato, A. M. (1986) Biochem. J. **233**, 357–367
6 Capasso, O., Gionti, E., Pontarelli, G., Ambesi-Impiombato, F. S., Nitsch, L., Tajana, G. and Cancedda, R. (1982) Exp. Cell Res. **142**, 197–206
7 Castagnola, P., Moro, G., Descalzi, F. and Cancedda, R. (1986) J. Cell Biol. **102**, 2310–2317
8 Schmid, T. M. and Linsenmayer, T. F. (1985) Dev. Biol. **107**, 373–381
9 Kwan, A. P. L., Freemont, A. J. and Grant, M. E. (1986) Biosci. Rep. **6**, 155–162
10 Iyama, K.-I., Ninomiya, Y., Olsen, B. R., Linsenmayer, T. F., Trelstad, R. L. and Hayashi, M. (1991) Anat. Rec. **229**, 462–472
11 LuValle, P., Daniels, K., Hay, E. D. and Olsen, B. R. (1992) Matrix **12**, 404–413
12 Grant, W. T., Wang, G. and Balian, G. (1987) J. Biol. Chem. **262**, 9844–9849
13 Hoyland, J. A., Thomas, J. T., Donn, R., Marriott, A., Ayad, S., Boot-Handford, R. P., Grant, M. E. and Freemont, A. J. (1991) Bone Miner. **15**, 151–164
14 von der Mark, K., Kirsch, T., Nerlich, A., Kuss, A., Weseloh, G., Gluckert, K. and Stoss, H. (1992) Arthritis Rheum. **35**, 806–811
15 Aigner, T., Reichenberger, E., Bertling, W., Kirsch, T., Stoss, H. and von der Mark, K. (1993) Virch. Archiv. B Cell Pathol. **63**, 205–211
16 Pacifici, M., Golden, E. B., Oshima, O., Shapiro, I. M., Leboy, P. S. and Adams, S. L. (1990) Ann. N. Y. Acad. Sci. **599**, 45–57
17 Dharmavaram, R. M., Elberson, M. A., Peng, M., Kirson, L. A., Kelley, T. E. and Jimenez, S. A. (1994) Hum. Mol. Genet. **3**, 507–509
18 McIntosh, I., Abbott, M. H., Warman, M. L., Olsen, B. R. and Francomano, C. A. (1994) Hum. Mol. Genet. **3**, 303–307
19 Wallis, G. A., Rash, B., Sweetman, W. A., Thomas, J. T., Super, M., Evans, G., Grant, M. E. and Boot-Handford, R. P. (1994) Am. J. Hum. Genet. **54**, 169–178
20 Warman, M. L., Abbott, M. H., Apte, S. S., Hefferon, T., McIntosh, I., Cohn, D. H., Hecht, J. T., Olsen, B. R. and Francomano, C. A. (1993) Nature Genet. **5**, 79–82
21 Schmid, T. M. and Conrad, H. E. (1982) J. Biol. Chem. **257**, 12444–12450
22 Schmid, T. M. and Conrad, H. E. (1982) J. Biol. Chem. **257**, 12451–12457
23 Schmid, T. M. and Linsenmeyer, T. F. (1983) J. Biol. Chem. **258**, 9504–9509
24 LuValle, P., Ninomiya, Y., Rosenblum, N. D. and Olsen, B. R. (1988) J. Biol. Chem. **263**, 18378–18385
25 Yamaguchi, N., Benya, P. D., van der Rest, M. and Ninomiya, Y. (1989) J. Biol. Chem. **264**, 16022–16029
26 Thomas, J. T., Cresswell, C. J., Rash, B., Nicolai, H., Jones, T., Solomon, E., Grant, M. E. and Boot-Handford, R. P. (1991) Biochem. J. **280**, 617–623
27 Thomas, J. T., Kwan, A. P. L., Grant, M. E. and Boot-Handford, R. P. (1991) Biochem. J. **273**, 141–148
28 Apte, S. S., Seldin, M. F., Hayashi, M. and Olsen, B. R. (1992) Eur. J. Biochem. **206**, 217–224
29 Brass, A., Kadler, K. E., Thomas, J. T., Grant, M. E. and Boot-Handford, R. P. (1992) FEBS Lett. **303**, 126–128
30 Schmid, T. M. and Linsenmayer, T. F. (1984) Biochemistry **23**, 553–558
31 Kwan, A. P. L., Cummings, C. E., Chapman, J. A. and Grant, M. E. (1991) J. Cell Biol. **114**, 597–604
32 Timpl, R., Wiedemann, H., van Delden, V., Furthmayr, H. and Kuhn, K. (1981) Eur. J. Biochem. **120**, 203–211
33 Muragaki, Y., Jacenko, O., Apte, S., Mattei, M.-G., Ninomiya, Y. and Olsen, B. R. (1991) J. Biol. Chem. **266**, 7721–7727
34 Sawada, H., Konomi, H. and Hirosawa, K. (1990) J. Cell Biol. **110**, 219–227
35 Schmid, T. M. and Linsenmayer, T. F. (1990) Dev. Biol. **138**, 53–62
36 Aeschlimann, D., Kaupp, O. and Paulsson, M. (1995) J. Cell Biol. **129**, 881–892
37 Lischwe, M. A. and Ochs, D. (1982) Anal. Biochem. **127**, 453–457
38 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
39 Matsudaira, P. (1987) J. Biol. Chem. **262**, 10035–10038
40 Mould, A. P., Holmes, D. F., Kadler, K. E. and Chapman, J. A. (1985) J. Ultrastruct. Res. **91**, 66–76
41 Sakura, S. and Fujimoto, D. (1984) Photochem. Photobiol. **40**, 731–734
42 LaBella, F., Waykole, P. and Queen, G. (1967) Biochem. Biophys. Res. Commun. **26**, 749–753
43 Chan, D., Cole, W. G., Rogers, J. G. and Bateman, J. F. (1995) J. Biol. Chem. **270**, 4558–4562

Received 5 July 1996; accepted 30 July 1996