Characterization of Human Type X Procollagen and Its NC-1 Domain Expressed as Recombinant Proteins in HEK293 Cells

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Type X collagen is a short-chain, network-forming collagen found in hypertrophic cartilage in the growth zones of long bones, vertebrae, and ribs. To obtain information about the structure and assembly of mammalian type X collagen, we generated recombinant human type X collagen by stable expression of full-length human α1(X) cDNA in the human embryonal kidney cell line HEK293 and the fibrosarcoma cell line ITT1080. Stable clones were obtained secreting recombinant human type X collagen (hrColX) in amounts of 50 μg/ml with α1(X)-chains of apparent molecular mass of 75 kDa. Pepsin digestion converted the native protein to a molecule migrating as one band at 65 kDa, while bands of 55 and 43 kDa were generated by trypsin digestion. Polyclonal antibodies prepared against purified hrColX reacted with NC-1 domains generated by collagenase digestion of hrColX at increasing temperatures indicated triple helical molecules with a reduced melting temperature of 31 °C as a result of partial underhydroxylation. Ultrastructural analysis of hrColX by rotary shadowing demonstrated rodlike molecules with a length of 130 nm, assembling into aggregates via the globular noncollagenous (NC)-1 domains as reported for chick type X collagen. NC-1 domains generated by collagenase digestion of hrColX migrated as multimers of apparent mass of 40 kDa on SDS-polyacrylamide gel electrophoresis, even after reduction and heat denaturation, and gave rise to monomers of 18–20 kDa after treatment with trichloroacetic acid. The NC-1 domains prepared by collagenase digestion comigrated with NC-1 domains prepared as recombinant human type X collagen in HEK293 cells, both in the multimeric and monomeric form. These studies demonstrate the potential of the pCMV5s expression system to produce recombinant triple helical type X collagens in amounts sufficient for further studies on its structural and functional domains.

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† The abbreviations used are: NC, noncollagen; hr, human recombinant; ColX, type X collagen; PAGE, polyacrylamide gel electrophoresis; CMV, cytomegalovirus; bp, base pair(s).

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Several lines of evidence indicate that type X collagen may be involved in mineralization of cartilage (3). In the developing growth plate and in chondrocyte cultures, type X collagen expression precedes calcification of hypertrophic cartilage (32–35). Furthermore, type X collagen synthesis is altered in chondrocytes from the rachitic chicken (36, 37), and recently it was shown that purified type X collagen regulates calcium uptake by matrix vesicles isolated from hypertrophic cartilage (38, 39). The putative role of type X collagen in mineralization and endochondral ossification of hypertrophic cartilage is supported by mice with a Col10a1 null mutation showing alterations in the endochondral bone trabecular meshwork in the growth plate, as well as a shift of matrix vesicles from the hypertrophic to the resting zone (40).

A striking feature of type X collagen and their isolated NC-1 domains is the unusual stability of the trimeric molecules which migrate as trimers in SDS-PAGE even after denaturation under reducing conditions (20, 41, 42). However, when pre-α1(X) collagen chains are synthesized with SMDC mutations in the NC-1 domain by cell-free translation and transcription, they are no longer able to form stable trimers in vitro (41, 42). Accordingly, when Col10a1 genes with mutations in the NC-1 domain were transfected into eukaryotic cells, no corresponding proteins were secreted from the cell nor expressed as intracellular protein, indicating failure of the mutated chains to assemble into triple helical molecules, which causes intracellular degradation (42). Mutant collagens lacking the amino-terminal NC-2 domain or containing in-frame deletions in the triple helix, however, were secreted as trimeric molecules (42).

The mechanism of the assembly of α1(X) subunits into trimeric molecules and extracellular meshworks as well as the sequences within the NC-1 and NC-2 domains involved in these events are of central importance for our understanding of the structure-function relationship of type X collagen in hypertrophic cartilage. The question remains, however, what the structural requirements (e.g. degree of prolylhydroxylation, completeness of the globular domains) are for the assembly of α1(X) chains to triple helical molecules in vitro, and which mutations would be tolerated for assembly under less dissociating conditions than those applied prior to SDS-gel electrophoresis (41, 42). Such studies require the production of sufficient amounts of normal and mutated type X collagen molecules or their NC-1 domains, respectively. Here we describe the preparation of human recombinant type X collagen in amounts sufficient to investigate structure and assembly of this collagen. We show that the recombinant protein expressed in HEK293 or HT1080 cells migrates on SDS-PAGE with an apparent molecular mass of 75 kDa, while α1(X) extracted from chondrocyte cultures or from cartilage migrate with an apparent mass of 60 kDa. Although the 75-kDa human recombinant type X collagen (hrColX) is partially underhydroxylated, it is secreted, and triple helical molecules with a melting temperature of 31 °C were obtained that form aggregates in vitro under their globular NC-1 domains. Furthermore, we compare the NC-1 domains released by collagenase digestion of hrColX with NC-1 domains prepared as recombinant proteins in HEK293 cells. Both migrate as aggregates of apparent mass of 40 kDa in SDS-PAGE and are extremely stable and resistant to heat denaturation and reduction in the presence of detergent and urea, but dissociate into monomers of approximately 20 kDa after acid treatment.

MATERIALS AND METHODS

Construction of the Type X Collagen Expression Vectors pCMV-ColX and pCMV-NC-1—A 265-bp HindIII-XhoI fragment corresponding to position +31 to +295 (EMBL no. X68952) of human type X collagen cDNA (43) was cloned into the pBluescript SK+ vector as described previously (44). The XhoI-XhoI insert was combined with a 2080-bp XhoI-SapI fragment (position 295–2383, EMBL no. X68952) from the human COL10A1 gene and cloned into the XbaI-HpaI restricted vector pCMV8 (45, 46), resulting in the expression vector pCMV-ColX. The 2352-bp insert contains the sequence together with 65 bp of a 5′-untranslated region and 249 bp of a 3′-untranslated region.

A cDNA fragment coding for the entire NC-1 domain starting 3′ of the COOH-terminal Gly-Pro-Pro triplet of the α1(X) triple helix (position 1651–2139, according to Reichenberger et al. (44)), was prepared by polymerase chain reaction using the primers NC-1-DW, 5′-CATGCTGTAAGCA/ATGTACGGTGAGGGTTTTTATA-3′; and NC-1-UP, 5′-GTCCGGCGCCCTCACTTGGACCACTATAGGA-3′. The primers introduce an NH2-terminal 5′NheI site, a stop codon, and a COOH-terminal 3′NotI site.

The polymerase chain reaction product (497 bp) was cloned into pUC 18 and sequenced. The insert was excised with NheI and NotI and cloned into the pCMV/AC7, an eukaryotic expression vector containing the signal peptide sequence of BM40 (47). The resulting clone pCMV-NC-1 was verified by sequencing.

Cell Cultures and Transfection—The human kidney epithelial cell line HEK293 and the human fibrosarcoma cell line HT1080 were grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium containing 5% fetal calf serum. One million cells/10-cm culture dish were transfected with the vectors pCMV-ColX or pCMV-NC1, mixed with 1 μg of a selection plasmid pSV2pac containing a puromycin resistance gene according to Niacht et al. (46). Puromycin-resistant HEK293 or HT1080 clones were selected and tested for type X collagen expression by precipitating the culture medium with 10% trichloroacetic acid, analyzing the precipitate by SDS-PAGE, and immunoblotting with rabbit anti-human type X collagen (48). NC-1-expressing clones were screened by Western blotting of the supernatants using the monoclonal antibody X34 specific for the native NC-1 domain (48).

Bovine fetal chondrocytes were prepared from epiphyseal growth plates (49, 50) and cultured in alginate beads as described in Guo et al. (51) and Haushall et al. (52) for 1 week in Ham's F-12 medium containing 10% fetal calf serum and 50 μg/ml ascorbate-phosphate. For type X collagen analysis, the alginate beads were dissolved in 10 mM EDTA, pH 7.5, and the released chondrons were dissolved in sample buffer for SDS-PAGE. Type X collagen production was detected by immunoblotting with a polyclonal antibody prepared against recombinant human type X collagen (see below).

Preparation of Recombinant Human Type X Collagen—Two HEK293 clones (5/16, 6/16) and one HT1080 clone (7/7), which secreted up to 50 μg/ml hrColX were expanded to mass culture. Partial purification of hrColX, dissolved in 50 mM Hepes, 50 mM NaCl, pH 7.4, at 0.25 mg/ml, was achieved by dialysis of the serum-free supernatant against phosphate-buffered saline or against 0.15 mM NaCl, 0.05 mM Tris-HCl, pH 7.4, at 4 °C. Purification to more than 95% homogeneity was achieved by enzyme-linked immunosorbent assay and immunoblotting as described previously (15). The antisera R239 and R244 revealed a titer of 1:60,000 in immunoblotting against hrColX. The titers against type I and II collagen by enzyme-linked immunosorbent assay and immunoblotting as described above. The antisera R239 and R244 revealed a titer of 1:60,000 in immunoblotting against hrColX. The titers against type I and II collagen were 10-fold lower. The specificity of the antibody for type X collagen was verified by the selective staining of the hypertrophic zone of human epiphyseal growth cartilage (see Fig. 4).

Circular Dichroism Analysis—Circular dichroism spectra of purified hrColX, dissolved in 50 mM Hepes, 50 mM NaCl, pH 7.4, at 0.25 mg/ml, were recorded at 20 °C on a Jaeso J-715 dichrograph using 0.01- or 0.05-cm path length thermostated cuvettes. Melting curves were recorded by measuring molar ellipticity at different wavelengths (198, 222, and 295 nm) between 15 and 70 °C, increasing the temperature by 0.5 °C/min. The protein concentration was determined by amino acid analysis in triplicate samples.

Enzyme Digestion—For trypsin/chymotrypsin digestion, serum-free
Recombinant Human Type X Collagen

RESULTS

Preparation of Recombinant Human Type X Collagen—A full-length human type X collagen cDNA was generated from cDNA and genomic clones (32, 33) and cloned into the eukaryotic expression vector pCMVsis. The resulting expression vector pCMV-ColX was stably transfected into human embryonal kidney cells HEK293 and fibrosarcoma cells HT1080. Pymurycin-resistant clones were selected and tested for type X collagen production by SDS-PAGE and immunoblotting of the secreted proteins. Several stable clones were obtained secreting up to 20 μg/ml protein with a molecular mass of 75 kDa, which was not secreted by mock-transfected cells. The band reacted specifically with a rabbit antiserum against human type X collagen (15). The immunoreactive band was purified to homogeneity from serum-free conditioned medium of the HEK293/6/16 clone or from the HT1080-7/7 clone by DEAE-cellulose chromatography, followed by CM-cellulose chromatography, both under native conditions (Fig. 1b). Pepsin digestion of purified hrColX reduced its size to 62 kDa (Fig. 1c).

The hro1(X) was synthesized by pCMV-ColX clones migrated on hrColX containing cell culture supernatants were heated to various temperatures up to 50 °C for 30 min, rapidly cooled to 20 °C, and then digested with 1 mg/ml trypsin (2 × crystallized, Boehringer Mannheim, bovine pancreas, EC 3.4.4.4) and 2.5 mg/ml chymotrypsin (Boehringer, EC 3.4.4.5) for 1 h at 20 °C (53). For pepsin digestion, type X collagen was dialyzed against 0.5 M acetic acid and incubated with 0.2 mg/ml pepsin (swine stomach, 2 × crystallized, Serva, Heidelberg, EC 3.4.4.1) for 18 h at 4 °C. For collagenase digestion, purified type X collagen or serum-free cell culture supernatant was dialyzed against 0.15 M NaCl, 50 mM Tris, pH 7.4, containing 5 mM CaCl2 and 1 mM EDTA (54). The material was heat denatured at 45 °C for 30 min and digested with highly purified collagenase (Clostridium histolyticum, microbial collagenase, EC 3.4.4.19, Advanced Biofacturers, Lynnbrook, NY) for 4 h at 37 °C. After enzyme digestion, the material was either precipitated with 10% trichloroacetic acid, or concentrated by vacuum centrifugation and analyzed by SDS-gel electrophoresis on 12% polyacrylamide-SDS gels.

Analytical Procedures—Amino acid analysis of hrColX after hydrolysis with 6 N HCl was performed on a Biotronic amino acid analyzer (courtesy of Dr. K. H. Mann, Max Planck Institute for Biochemistry, Martinsried, FRG). For amino-terminal sequencing, 20 μg of recombinant type X collagen were subjected to SDS-PAGE in 7% polyacrylamide gels, blotted to a Hybond® nylon membrane, and sequenced on an automatic solid phase amino acid sequencer (Applied Biosystems) (courtesy of Dr. R. Deutzmann, Department of Biochemistry, University of Regensburg, FRG).

Rotary Shadowing—For electron microscopic examination, purified hrColX was dialyzed against 0.2 M ammonium hydrogen carbonate, mixed with an equal volume of glycerol, and sprayed onto mica discs. Rotary shadowing and electron microscopic analysis was kindly performed by H. Wiedemann at the Max Planck Institute for Biochemistry, Martinsried, as described in Kühn et al. (54).

Immunolocalization of Type X Collagen with Antibodies against hrColX—For preparation of antibodies, rabbits were immunized with purified hrColX, and the antisera were tested by enzyme-linked immunosorbent assay and immunoblotting. In the immunoblot, the antiserum reacted specifically with the presumptive amino terminus of a human procollagen A1(X) released after signal peptide cleavage (43, 44) and begins 3 residues carboxyl-terminal from the published amino terminus of chick type X procollagen (19).

Immunoblotting—A rabbit antiserum against hrColX was used to produce a panel of monoclonal antibodies with different epitope specificities, recognizing hrColX in the native, in the denatured, and in the pepsin-digested form, or the native NC-1 domain (48).

Pepsin and Trypsin/Chymotrypsin Digestion of Recombinant Human Type X Collagen—To obtain information on the size and the proteolytic susceptibility of the noncollagenous domains and the protease-resistant, triple helical domain of the

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** hrColX produced by the stably transfected Col10a1 HEK293 cell clone 6/16 (b). The collagen was collected from serum-free culture medium and purified by DEAE- and CM-cellulose chromatography as described under "Materials and Methods." c, hrColX after pepsin digestion at 4 °C. Reducing 10% polyacrylamide-SDS gel, Coomassie Blue-stained. a, molecular mass marker proteins.

**Fig. 2.** Relative migration on SDS-PAGE of type X collagen extracted from bovine hypertrophic chondrocytes cultured for 4 days in alginate beads (a) and recombinant human type X collagen from clone 6/16 (whole cell culture medium) (b). c, supernatant of HEK-293 cells transfected with vector pCis. Samples were separated by SDS-PAGE, blotted, and stained with rabbit anti human type X collagen IgG (15), followed by peroxidase-labeled anti-rabbit IgG.

SDS-PAGE with an apparent mass of 75–80 kDa (Figs. 1 and 2) compared with globular protein standards, while the actual Mr calculated from the cDNA sequence starting at Val20 (see below) is 64149, not considering potential glycosylation. By comparison, the apparent mass of a1(X) extracted from hypertrophic fetal chondrocytes cultured in alginate beads (51, 52) was 60 kDa (Fig. 3), which is in agreement with the mass reported for a1(X) extracted from bovine (14, 50) and human hypertrophic cartilage (15). This suggests that processing of type X collagen may occur in cartilage and in chondrocyte cultures under certain conditions (25–27), but it needs to be confirmed in human cartilage or chondrocyte cultures (see “Discussion”).

The amino-terminal sequence of hrColX was determined by microsequencing. The sequence H2N-V-F-Y-A-E-R-Y- corresponds to the presumptive amino terminus of a human procollagen A1(X) chain, as well as with the pepsin- and trypsin/chymotrypsin-resistant parts of hrColX (Fig. 3). It cross-reacts with murine, bovine, and canine type X collagen (not shown), but not with pepsin-extracted porcine type X collagen. When applied to sections of fetal human epiphyseal cartilage, it stained exclusively the zone of hypertrophic cartilage (Fig. 4). hrColX was also used to produce a panel of monoclonal antibodies with different epitope specificities, recognizing hrColX in the native, in the denatured, and in the pepsin-digested form, or the native NC-1 domain (48).

3 G. Rucklidge, personal communication.
recombinant type X collagen, hrColX was digested for 24 h with pepsin at 4 °C, or with trypsin/chymotrypsin at 20 °C for 1 h. Interestingly, a pepsin-resistant fragment of type X collagen of 62 kDa was obtained (Figs. 1 and 3), while after trypsin/chymotrypsin digestion of hrColX, four fragments in the molecular mass range between 62 and 36 kDa were retained (Fig. 3). By comparison, pepsin digestion of human or bovine type X collagen extracted from hypertrophic cartilage or chondrocyte cultures generates a triple helical fragment of 45 kDa (6, 7, 50). Minor amounts of a fragment of this size were obtained after trypsin digestion of hrColX (Fig. 3).

Circular Dichroism and Thermostability of hrColX—The CD spectrum of purified hrColX at 20 °C revealed a slight peak at 225 nm, as expected for triple helical collagens, and a minimum at 198 nm (Fig. 5a). The melting curve resulting from ellipticity determinations at 198 nm indicated a point of inflection at 31 °C, and further conformational changes at higher temperatures (Fig. 6). This finding is consistent with the results obtained after trypsin/chymotrypsin digestion of hrColX at various temperatures between 4 and 50 °C. Triple helical fragments of 50 and 65 kDa, and smaller, were resistant against enzyme digestion up to 30–31 °C, while minor fractions of the 50- and 65-kDa bands were stable up to 39 °C (Fig. 6).

As the low melting temperature indicated underhydroxylation of hrColX, the hydroxyproline:proline ratio of hrColX was determined by amino acid analysis. A ratio of hydroxyproline:proline of 0.25:1 (Table I) was found, confirming partial underhydroxylation of the recombinant material despite the presence of ascorbic acid in the culture medium (see also “Discussion”). By comparison, in the 59-kDa form of chick type X collagen, the hydroxyproline:proline ratio is 0.7:1 (16).

Ultrastructure of hrColX—Electron microscopic imaging of hrColX by rotary shadowing revealed threadlike molecules with an average length of 130 ± 5 nm, corresponding to that reported for chick type X collagen (20, 22), and a large globular domain at one end, corresponding to the NC-1 domain. In some molecules a small globular domain, probably the NC-2 domain, was detectable at the other end of the molecule. hrColX molecules tended to form aggregates ranging from dimers to multimers by assembling at their NC-1 globules (Fig. 7).

Assembly of hra1(X) Chains—Recombinant hrColX migrated with the approximate mass of a trimer (around 220 kDa) when loaded onto SDS-gels in nondenaturing sample buffer (Fig. 8c). Trichloroacetic acid precipitation resulted in complete dissociation into monomers (Fig. 8e), while partial dissociation to monomers and dimers was achieved by heat denaturation in the presence of 8 M urea and 3% SDS at 20 and 60 °C (Fig. 8). Under these conditions, addition of 5% β-mercaptoethanol caused complete dissociation of dimers into monomers (Fig. 8, a and e). These observations indicate that (i) the dimers and trimers formed by the hra1(X) chains are less stable than the dimers formed by the NC-1 domains (see below) and (ii) interchain disulfide bonds contribute less to the stability of hra1(X) to trimers and dimers than do hydrophobic bonds and other noncovalent bonds susceptible to trichloroacetic acid treatment.

Aggregation of the NC-1 Domain—Digestion of recombinant type X collagen with purified bacterial collagenase gave rise to a noncollagenous domain, which migrated on SDS-gel electrophoresis with an apparent mass of 38.5–40 kDa (Fig. 9). The complex was resistant to heat denaturation up to 100 °C, even
in the presence of 8 M urea, 3% SDS, and 5% mercaptoethanol (Fig. 10), conditions that have been shown to dissociate NC-1 domains prepared from chicken type X collagen (20). Only by precipitation of the material with 10% trichloroacetic acid, in the presence of Triton X-100, did the NC-1 multimer dissociate into a monomer, migrating on SDS-gels with an apparent mass of 18–20 kDa (Figs. 9 and 10).

With an apparent mass of 20 kDa for the monomeric NC-1, the approximately 40 kDa in SDS-PAGE predicts a dimer composition for the molecule in the upper band. However, it is more likely that the 40-kDa band shown in Fig. 8 actually represents a trimeric molecule migrating relatively faster on SDS-PAGE owing to a more compact conformation and smaller Stoke’s radius, in comparison to the trichloroacetic acid-denatured NC-1 monomer. The possibility also existed that the bacterial collagenase unspecifically truncated the NC-1 domain and removed sites required for the assembly of NC-1 into trimers. To investigate this possibility, the molecular weight of NC-1 generated by collagenase digestion was compared with a full-length NC-1 prepared recombinantly in HEK293 cells using the pCMV expression vector.

**DISCUSSION**

Here we report for the first time on the preparation of triple helical recombinant type X collagen in an eukaryotic expression system in amounts sufficient for structural and functional studies. By stable transfection several HEK293 and HT1080 clones were obtained that secreted up to 50 mg/ml intact hrColX as the predominant protein into the culture medium. hrColX was purified to homogeneity under native conditions using DEAE- and CM-cellulose chromatography. The recombinant type X collagen, although underhydroxylated, was secreted into the medium and isolated as a triple helix with a \( T_m \) of 31 °C. With the electron microscope it appeared as threadlike molecules after rotary shadowing with an average length of 130 nm, flanked by a large and a small globular domain at both ends, similar to chick type X molecules described by Schmid and

\[ \text{Asp} \quad 5.4 \quad 4.1 \quad 26 \]
\[ \text{Thr} \quad 3.56 \quad 3.4 \quad 24 \]
\[ \text{Ser} \quad 3.74 \quad 3.8 \quad 25 \]
\[ \text{Glu} \quad 8.73 \quad 6.7 \quad 45 \]
\[ \text{Cys} \quad \text{ND} \quad 0.16 \quad 1 \]
\[ \text{Gly} \quad 23.25 \quad 25.2 \quad 175 \]
\[ \text{Ala} \quad 5.86 \quad 5.5 \quad 37 \]
\[ \text{Val} \quad 4.44 \quad 3.8 \quad 30 \]
\[ \text{Met} \quad 0.436 \quad 1.6 \quad 11 \]
\[ \text{Ile} \quad 3.94 \quad 4.2 \quad 27 \]
\[ \text{Leu} \quad 5.25 \quad 5.0 \quad 35 \]
\[ \text{Tyr} \quad 1.98 \quad 3.4 \quad 21 \]
\[ \text{Phe} \quad 2.46 \quad 2.4 \quad 15 \]
\[ \text{Trp} \quad \text{ND} \quad 0.16 \quad 1 \]
\[ \text{His} \quad 1.57 \quad 1.6 \quad 10 \]
\[ \text{Hyl} \quad 2.61 \quad 5.0 \quad 35 \]
\[ \text{Lys} \quad 4.08 \quad 5.2 \quad 35 \]
\[ \text{Arg} \quad 3.05 \quad 3.0 \quad 19 \]
\[ \text{Hypro} \quad 3.58 \quad 22 \quad 145 \]

\[ ^\text{a} \text{ND, not determined.} \]
Linsenmayer (20), Schmid et al. (55), and Kwan et al. (22). The material was used to produce high titered polyclonal and monoclonal antibodies (48). The affinity-purified antibodies stained specifically hypertrophic cartilage in the growth plate of human fetal epiphyseal cartilage. Monoclonal antibodies prepared against hrColX (38) confirmed our previous finding of enhanced production of type X collagen in osteoarthritic articular cartilage (9, 48, 56).

The recombinant human α1(X) chains consistently migrated on SDS-PAGE with an apparent molecular mass of 75 kDa (compared with globular standards), while in the same gel α1(X) chains extracted from chondrocyte cultures in alginate migrated with an apparent mass of 65 kDa, suggesting processing to shorter forms in the alginate cultures. Retarded migration of hrColX due to overhydroxylation or overglycosylation can be excluded according to the results of the amino acid analysis showing underhydroxylation, and from treatment with glycosidases showing no differences in migration (results not shown). The approximately 75 kDa of the full-length α1(X) chains synthesized in HEK293 and HT1080 cells are in accordance with the size of the α1(X) chains synthesized in cell-free systems in vitro (31, 57). The appearance of shorter forms of α1(X) of 58–65 kDa in cartilage extracts (14, 15, 24, 26) corroborates two previous reports on the processing of a 70-kDa procollagen chain synthesized by chick hypertrophic chondro-
cytes in vitro to a 59-kDa form (24–26). In another study using cartilage organ cultures, however, no processing of a 82 kDa a1(X) chain to smaller forms was observed within a 4-h pulse-chase period (19).

Curiously, after pepsin digestion of hrColX an a1(X) chain of 62 kDa was retained, while pepsin digestion of type X collagen isolated from chondrocyte cultures or from hypertrophic cartilage leaves a fragment of 45 kDa (6, 12–18). Trypsin/chymotrypsin digestion of the native hrColX gave rise to smaller fragments, one of them, 45–50 kDa, the size of the pepsin-resistant fragment of chondrocyte-derived type X collagen.

Whether and how processing of type X collagen occurs after a prolonged time of type X collagen in the extracellular cartilage matrix in vitro, however, needs to be clarified in cartilage cell or organ culture. If there is processing of type X collagen in cartilage, the question remains at which end of the molecule this occurs. Amino-terminal sequencing of chick-type X collagen isolated from chick cartilage cultures revealed an intact amino end, including the complete NC-2 domain (19), even after a 4-h pulse-chase period. However, there are substantial sequence differences between bovine, human, and chick NC-2 domains, so that processing in bovine chondrocytes cultured in 48-h alginate cultures would not necessarily be in conflict with the results described by Summers et al. (19).

However, COOH-terminal processing is also possible. Although Chan et al. (42) have shown that truncation of the Col10A1 gene at the carboxyl terminus by 50 amino acid residues gives rise to a1(X) chains that are unable to assemble to trimers during biosynthesis and thus are degraded intracellularly, extracellular processing of intact type X collagen molecules following secretion may occur, which may not affect sites in the NC-1 domain responsible for intracellular, intermolecular aggregation.

Circular dichroism studies of the purified hrColX revealed a minimum in the molar ellipticity at 198 nm and a slight peak at 225 nm, indicating a triple helical molecule with contributions of large globular domains. Molar ellipticity at 198 nm measured at increasing temperatures indicated a Tm of 31 °C. Amino acid analysis revealed underhydroxylation with a hydroxyproline:proline ratio of 0.25:1, while type X collagen prepared from the culture medium of chick chondrocytes contains hydroxyproline and proline in a ratio of 0.7:1 (16). Nevertheless, the triple helical domain of hrColX produced either in HEK293 or HT1080 cells was resistant to pepsin or trypsin/chymotrypsin digestion up to 31 °C, in agreement with the data obtained by circular dichroism.

The underhydroxylation of the recombinant type X collagen is very likely a result of the high expression levels in the HEK293 or HT1080 cell clones due to the use of the strong CMV promoter. Even in HT1080 cells, the levels of endogenous prolylhydroxylase seem insufficient for complete hydroxylation of the surplus of hro1(X) collagen chains in the endoplasmic reticulum, although HT1080 cells produce endogenous collagen, different from HEK293 cells. In contrast, recombinant type X collagen and type X/II chimeric collagens cloned into the pCDNA3 vector and transfected into HEK293 cells have a Tm of 42 °C, but are expressed at much lower levels (58). Similarly, bovine type X collagen prepared by in vitro transcription and translation of Col10a1 cDNA by using HT1080 microsomal membranes was thermally stable up to 42 °C (57), but expression levels in that system were much lower. In addition, bovine type X collagen contains 2 additional cysteine residues in the triple helical part, thus stabilizing triple helix formation (7, 14, 50). In HT1080 cells, human recombinant type II procollagen also was produced in milligram amounts in fully hydroxylated and glycosylated form (59), while no information is available on the degree of prolylhydroxylation of a recombinant, heterotrimERIC type VI (60) and type IV (61) collagen. Large scale production of a fully hydroxylated type III collagen in the baculovirus system was achieved by cotransfection with the a- and b-units of prolylhydroxylase (62, 63). To achieve full hydroxylation of hrColX in HEK293 cells, a similar experimental attempt is in progress.4

The low melting temperature of hrColX of 31 °C suggests that during biosynthesis only a minor part of the hro1(X) collagen chains are folded into a triple helix within the cell. The level of hydroxylation was, however, sufficient to allow secretion of trimeric hrColX molecules into the medium where they seem to refold into a triple helical structure at room temperature. Previous studies have shown that complete inhibition of prolylhydroxylation by a,α′-dipyridyl prevents secretion, with the unhydroxylated procollagen being retained in the rough endoplasmic reticulum (64, 65). A reduced extent of secretion has been reported for partially underhydroxylated type IV collagen in cultures of lens epithelial cells (66). Satoh et al. (67)
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