Coexpression of α and β subunits of prolyl 4-hydroxylase stabilizes the triple helix of recombinant human type X collagen

Klaus WAGNER*, Ernst PÖSCHL*, Javier TURNAY†, Jeong-mi BAIK*, Taina PIHLAJANIEMI‡, Svenja FRISCHHOLZ* and Klaus VON DER MARK*[1]

*Department of Experimental Medicine I, Nikolaus-Fiebiger Center für Molecular Medicine, University of Erlangen-Nuremberg, Glückstrasse 6, D-91054 Erlangen, Germany, †Departamento de Bioquimica y Biologia Molecular, Facultad de Sciencias Químicas, Universidad Complutense, E-28040 Madrid, Spain, and ‡Collagen Research Unit, Department of Medical Biochemistry, University of Oulu, 90220 Oulu, Finland

We have reported previously on the expression of recombinant human type X collagen (hrColX) in HEK 293 and HT 1080 cells by using the eukaryotic expression vector pCMVsis (in which CMV stands for cytomegalovirus). Several stably transfected clones secreted full-length triple-helical hrColX molecules in large amounts, but the secreted collagen was underhydroxylated, with a hydroxyproline-to-proline ratio of 0.25 and a melting temperature (T_m) of 31 °C. By comparison, native chicken type X procollagen has a T_m of 46 °C. To stabilize the triple helix of hrColX, an hrColX-expressing clone (A6/16) was co-transfected with both α and β subunits of human prolyl 4-hydroxylase. Clones were selected that secreted proα1(X) collagen chains with an apparent molecular mass of 75 kDa and an increased hydroxyproline-to-proline ratio of close to 0.5. As a result of enhanced prolyl hydroxylation, the T_m of the hrColX was increased to 41 °C as measured by CD analysis at various temperatures. The CD spectra indicated a minimum ellipticity at 198 nm and a peak at 225 nm at 20 °C, confirming the presence of a triple helix. The same T_m of 41 °C was measured for the triple-helical core fragments of hrColX of 60–65 kDa that were retained after brief digestion with chymotrypsin/trypsin at increasing temperatures. This shows that the human cell line HEK-293 is suitable for the simultaneous expression of three genes and the stable production of substantial amounts of recombinant, fully hydroxylated type X collagen over several years.

Key words: circular dichroism, hydroxylation, hydroxyproline, thermostability.

INTRODUCTION

Type X collagen is a network-forming collagen with a short triple-helical core 110 nm in length flanked by the non-collagenous C-terminal NC1 and N-terminal NC2 domains (reviewed in [1]). It is expressed almost exclusively by hypertrophic chondrocytes in the growth plate cartilage in long bones, ribs and vertebrae [2–5] and under pathological conditions in osteoarthritic cartilage and some chondrosarcomas [6–9]. Several lines of evidence indicate that type X collagen is involved in calcification and endochondral ossification of hypertrophic cartilage [10–13].

The C-terminal NC1 domain of collagen X, which is structurally related to C1q and tumour necrosis factor α, has been shown to be responsible for the assembly of the nascent proa1(X) chains into trimers and for the formation of the triple helix. Isolated or recombinantly produced $(NC1)_3$ domains reveal unusual thermal stability and are resistant to SDS and heat denaturation up to 90 °C [14–18]. Major progress in our understanding of the role of the NC1 domain in chain assembly and network formation of type X collagen has been made in transcription and translation studies *in vitro* of bovine [19] and human α1(X) cDNA and from mutations inserted into the NC1 domain causing congenital chondrodysplasias (Schmid metaphyseal chondrodysplasia, 'SMCD') in humans [17,20,21].

However, investigations into the structure and assembly of mammalian type X collagen at the protein level have been difficult owing to the restricted amounts available of this collagen, which is expressed only in the narrow, hypertrophic zone of the epiphyseal growth plate [1,22]. In a previous paper [16] we reported on the preparation of a recombinant human type X collagen with the use of the eukaryotic expression vector pCMVsis, in which the expression of the *Col10A1* gene is driven under the control of the cytomegalovirus (CMV) promotor. Stably transfected human HEK 293 or HT 1080 cell clones produced up to 50–100 μg/ml trimeric full-length type X collagen with pro-α1 (X) chains of apparent molecular mass 75 kDa. Although the human recombinant collagen X (hrColX) formed triple-helical molecules that aggregated through their NC1 domains similarly to chondrocyte-derived type X collagen molecules, CD and trypsin digestion studies at various temperatures revealed a melting temperature (T_m) of 31 °C owing to a decreased level of proline hydroxylation. The hydroxyproline-to-proline ratio was 0.25, whereas a ratio of 0.7 was determined for chicken chondrocyte type X collagen [14,23], with a T_m of 46 °C. In a comparable situation, the degree of hydroxylation of recombinant type III collagen produced in the baculovirus system was significantly enhanced to a normal level and the T_m was increased from 32–34 to 40 °C after coexpression with both the α and β subunits of prolyl 4-hydroxylase (P4H) [24]. Furthermore, the formation of trimeric, disulphide-cross-linked type XII minicollagen was considerably increased in a baculovirus expression system after the coexpression of both subunits of P4H [25].

Here we report on the coexpression of both the α and β subunits of human P4H in a clone (A6/16) of HEK 293 cells producing human type X collagen. Clones were selected that

Abbreviations used: CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; hrColX, human recombinant collagen X; NC1, non-collagenous domain 1; P4H, prolyl 4-hydroxylase.

[1] To whom correspondence should be addressed (e-mail kvdmark@molmed.uni-erlangen.de).

overexpressed both the α and β subunits of P4H and secreted recombinant human type X collagen with an increased T_m of 41 °C, as determined by CD spectra at various temperatures. This increase in T_m of 10 °C compared with hrColx produced by the A6/16 clone was consistent with an increase in the hydroxyproline-to-proline ratio from 0.25 to 0.48. The same result was obtained when the thermal stability of the recombinant type X collagen was compared by controlled digestion with trypsin/chymotrypsin at increasing temperatures. Our results indicate that the NC1 domains do not significantly contribute to the thermal stability of the type X collagen triple helix, which substantiates earlier studies on denaturation and renaturation properties of chicken type X collagen [14].

MATERIALS AND METHODS

Antibodies and reagents

Monoclonal antibodies against the β subunit of P4H are described in [26]. The preparation and specificity of the monoclonal antibody X53 specific for type human X collagen is described in [27]. Cell culture medium (DMEM; Dulbecco's modified Eagle's medium), fetal calf serum and antibiotics were from Seromed (Berlin, Germany). Trypsin (EC 3.4.4.4, crystallized twice) and chymotrypsin (EC 3.4.4.5) were from Boehringer/Roche (Mannheim, Germany).

Coexpression of P4H α and β subunits

The HEK 293 clone A6/16 [16] was co-transfected with human P4H α subunit cDNA (S-38) inserted into the multiple cloning sites of pCMVsis [16], and with the human P4H subunit β cDNA (PA-59 [26,28]) cloned into the pcDNA3 expression vector (Invitrogen) containing a neomycin resistance gene. Transcription of both inserts is driven by the CMV promoter. Transfected cells were grown in DMEM/Ham's F12 (1:1) medium containing 10% (v/v) fetal calf serum, pyruvate, penicillin/streptomycin, 50 μg/ml sodium ascorbate and 1 mg/ml G418. Neomycin-resistant clones were selected by testing for the expression of P4H α and β subunits by Northern hybridization and immunoblotting.

Northern hybridization

Total RNA was extracted with guanidinium/thiocyanate [29] from clones producing P4H β subunits, size-fractionated on 1.3% (w/v) agarose/formaldehyde gels and transferred to nylon membranes. The membranes were hybridized with a ^{32}P-labelled P4Hα cDNA probe in a solution consisting of 5 × SSC [1 × SSC = 0.15 M NaCl/0.015 M sodium citrate (pH 7.0)] and 1% N-lauroyl sarcosine at 65 °C and were then washed.

SDS/PAGE and immunoblotting

For identification of type X collagen secretion, serum-free cell culture supernatants of transfected cells were precipitated with 10% (w/v) trichloroacetic acid and analysed by SDS/PAGE [10% (w/v) gel]. Gels were either stained with Coomassie Blue or blotted to nitrocellulose membranes. Membranes were probed for type X collagen synthesis with monoclonal antibody X53 or rabbit antiserum R244 prepared against purified hrColX [16]. For analysis of β subunits of human P4H, lysates from 10^4–10^5 cells were subjected to SDS/PAGE and probed after blotting with a monoclonal antibody specific for the P4H β subunit [26].

hrColX

For CD analysis and enzymic digestion, hrColX produced by clone p52 (hrColX-p52) was purified from serum-free culture medium by affinity chromatography on monoclonal antibody X53 coupled to CNBr-activated Sepharose. Supernatant (100 ml) was applied to 1 ml of X53–Sepharose (2 mg of purified X53/ml of Sepharose); after a wash with 0.1 M NaCl/0.02 M Tris/HCl (pH 7.5), type X collagen was eluted with 0.15 M NaCl/0.05 M $NaHCO_3$ (pH 9.7) and neutralized immediately.

CD analysis

CD spectra of purified hrColX-p52, dissolved in 50 mM Hepes/50 mM NaCl (pH 7.4) at 0.25 mg/ml, were recorded at 20 °C on a JASCO J-715 dicrograph with 0.01 or 0.05 cm pathlength thermostatically controlled cuvettes. Melting curves were recorded by measuring molar ellipticity at different wavelengths (198, 222 and 225 nm) between 15 and 70 °C, increasing the temperature by 30 °C/h [16]. The protein concentration was determined by amino acid analysis.

Enzyme digestion

The resistance of the recombinant collagens to protease in relation to temperature was measured by a brief digestion with chymotrypsin/trypsin at increasing temperatures, followed by SDS/PAGE analysis of the protein fragments. Serum-free supernatants containing hrColX were incubated at temperatures between 4 and 50 °C for 60 min, treated with 10 μg/ml trypsin and 25 μg/ml chymotrypsin at 20 °C for 10 min and rapidly cooled in an ice bath. Samples were then incubated in boiling SDS buffer and applied to SDS/PAGE. Protease-resistant hrColX-fragments were revealed by immunoblotting with the polyclonal antibody R239 [16].

Amino acid analysis

For the analysis of hydroxyproline and proline, purified hrColX was hydrolysed with 6 M HCl and subjected to automatic amino acid analysis with a Biotronic amino acid analyser (performed by Dr K. H. Mann and W. Strashofer, Max-Planck-Institute für Biochemie, Martinsried, Germany).

RESULTS AND DISCUSSION

The role of the collagenous and non-collagenous domains in molecular interactions with annexin V, type II collagen, Ca^{2+} and proteoglycans, and the intermolecular interactions leading to a hexagonal network [30] are still unknown. Previously we have reported on the preparation of recombinant human type X collagen in HEK-293 and HT1080 cells, with the aim of investigating the molecular assembly of this protein [16]. However, owing to the high productivity of the hrColX-producing cells the level of proline hydroxylation was insufficient, so the thermal stability of the recombinantly produced hrColX was significantly lowered.

To enhance the level of proline hydroxylation, the hrColX-producing HEK 293 clone A6/16 [16] was co-transfected with the α and β subunits of human P4H in different expression vectors, under the control of the CMV promoter (pCMVsis and pcDNA3). Neomycin-resistant clones that should express the α subunit of P4H were selected and screened for synthesis of the β subunit by immunoblotting with a monoclonal antibody

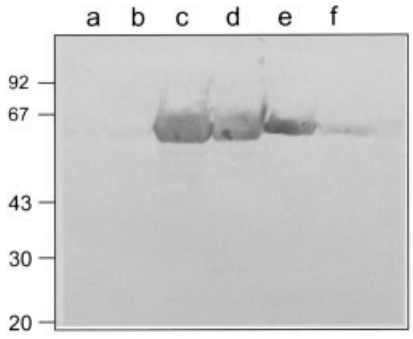

Figure 1 Overexpression of the β subunit of P4H in doubly transfected A6/16 HEK 293 clones

Clone A6/16, which had been stably transfected with human α1(X) [16], was co-transfected with vectors expressing the α and β subunits of P4H cloned into the pcDNA and pCMVsis vectors respectively. Cell lysates of G418-resistant clones were tested for expression of the P4H β subunit by immunoblotting with a P4Hβ-specific monoclonal antibody [26]. Identical amounts of cell lysate from wild-type HEK 293 cells (lane a), from the parental clone A6/16 before transfection with P4H subunits (lane b), and from five different neomycin-resistant clones producing hrColX (lane c, p52; lane d, p36; lane e, p18; lane f, p59) were loaded on SDS/10% polyacrylamide gels and subjected to electrophoresis followed by imunoblotting. The positions of molecular-mass markers are indicated (in kDa) at the left.

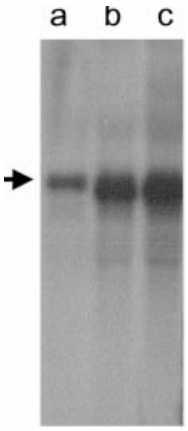

Figure 2 Overexpression of the α subunit of P4H in HEK 293 clones producing type X collagen

Analysis of steady-state levels of P4Hα mRNA by Northern blotting of G418-resistant clones that were positive for P4Hβ production after Western blotting (Figure 1). Lane a, basal levels of P4Hα mRNA (arrowed) in untransfected HEK 293 cells; lanes b and c, P4Hα mRNA in two P4H-transfected clones, p52 and p38 respectively.

specific for P4Hβ [26]. Figure 1 shows a significant increase in the expression of the β subunit in five G418-resistant clones (lanes c–f) in comparison with untransfected HEK-293 cells (lane a) or the parental A6/16 clone (lane b). RNA analysis of the P4Hβ-positive clones by Northern blotting also revealed a severalfold enhancement of P4H α subunits (clones p52 and p38 depicted in Figure 2.) Clone p52 was grown in mass culture and the secreted hrColX was purified from serum-free, ascorbate-enriched culture medium by ion-exchange chromatography [16] or by affinity chromatography on an antibody column with the monoclonal antibody X53 [27]. The purified hrColX/P4H (Figure 3) was subjected to amino acid analysis. The hydroxyproline-to-proline ratio was increased to 0.48 from the ratio of 0.25 for hrColX produced by the parental A6/16 clone.

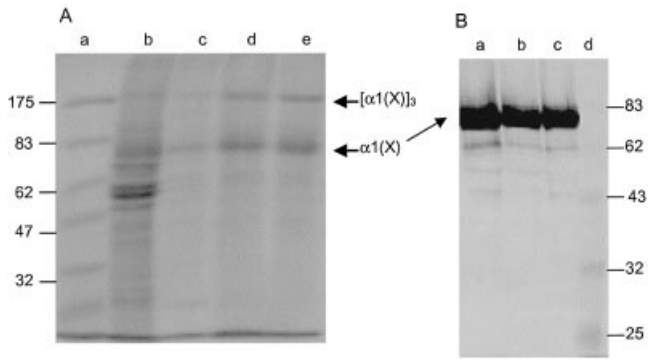

Figure 3 Purification of hrColX by affinity chromatography

The serum-free supernatant of triply transfected HEK 293 clone P52 was subjected to affinity chromatography with monoclonal antibody X53 [27]. (**A**) SDS/PAGE [10% (w/v) gel] under non-reducing conditions; Coomassie Blue staining. Lane a, molecular-mass markers (molecular masses indicated at the left in kDa); lane b, unbound protein; lanes c–e, hrColX/P4H monomers and trimers, eluted in three fractions with $NaHCO_3$. (**B**) Immunoblotting of hrColX/P4H fractions (lanes a–c) with monoclonal antibody X53 (anti-hrColX); lane d, molecular-mass markers (molecular masses indicated at the right in kDa).

CD analysis of native hrColX purified from clone p52 revealed a minimum of ellipticity at 198 nm and a maximum at 225 nm at 20 °C, indicative of the presence of an intact triple helix (Figure 4A). At increasing temperatures the ellipticity at 198 and 225 nm revealed a point of inflexion at 41 °C (Figure 4B), indicating an increase in T_m of 10 °C compared with hrColX produced by the parental cell line A6/16 [16].

This result was confirmed by subjecting hrColX/P4H to brief digestion with trypsin/chymotrypsin at various temperatures between 4 and 45 °C. At 4 or 20 °C trypsin/chymotrypsin converted the 75 kDa hrColX into smaller triple-helical fragments of 60 kDa by removing N- and C-terminal NC2 and NC1 domains [14,16], and smaller fragments of 45 kDa that were probably generated by cleavage at one or more of the eight interruptions of the Gly-Xaa-Yaa triplet structure in the type X collagen helix [31–33]. Interestingly, the 60–65 kDa fragments of hrColX/P4H were also resistant to digestion with trypsin/chymotrypsin up to 41 °C (Figure 5, upper panel), which was consistent with the T_m measured by CD analysis. In contrast, the underhydroxylated hrColX produced by the A6/16 clone was thermally stable only up to 30–35 °C (Figure 5, lower panel).

The enhanced T_m of 41 °C of the triple-helical core of human type X collagen (and even more the T_m of 47 °C reported for native chicken type X collagen) [14] in comparison with fibril-forming collagens (T_m = 39 °C) in the absence of the globular NC1 and NC2 domains is as yet unexplained in view of eight interruptions in the collagen Gly-Xaa-Yaa repeat structure in the collagen X triple helix. However, hydrogen bonds formed by hydroxyproline residues are not the only parameters that contribute to the stability of the triple helix; systematic studies on the role of amino acid side chains in triple helix stability have shown that proline residues in position Xaa and other amino acids such as arginine in position Yaa of the Gly-Xaa-Yaa triplet [34–36] also contribute significantly to stability.

The results presented here indicate that the T_m of the type X collagen triple helix of 41 °C shown here for hrColX is apparently unaffected by the presence of N- and C-terminal globular domains, because both the CD analysis of the intact 75 kDa form of hrColX and the protease resistance analysis of the triple helical core indicated the same T_m of 41 °C. This finding is in

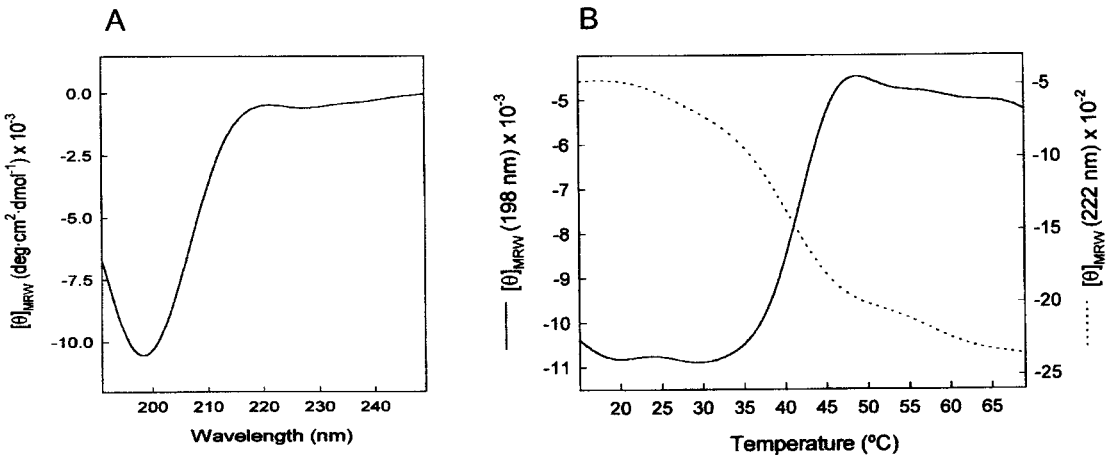

Figure 4 CD spectra of hrColX/P4H produced by clone p52

(**A**) The CD spectrum of hrColX-p52 at 20 °C in the native state shows a minimum at 198 nm and a minor peak at 222 nm. (**B**) The melting curve of hrColX-p52 measured by CD at 198 nm (solid line) and at 222 nm (dotted line) shows a T_m of 41 °C.

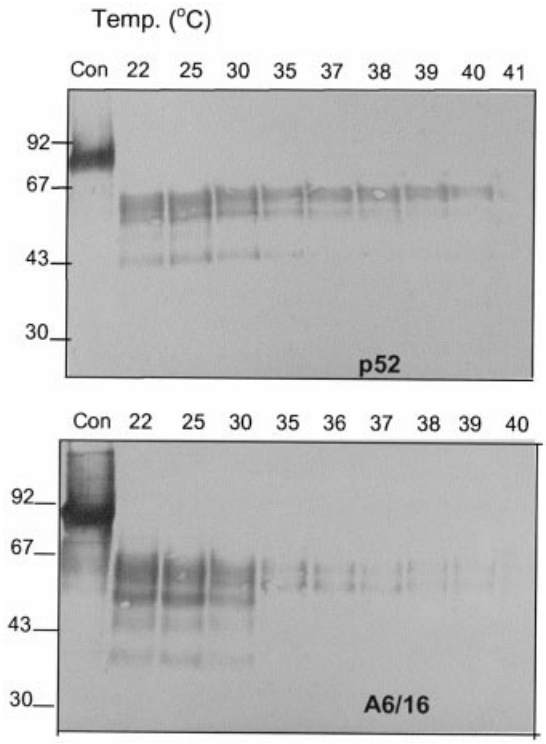

Figure 5 Thermal stability of hrColX/P4H (p52) and the parental clone hrColX-A6/16

hrColX from clone p52 (upper panel) or A6/16 (lower panel) were heated to increasing temperatures (22–42 °C), digested with trypsin/chymotrypsin for 10 min, cooled immediately on ice and applied to SDS/PAGE [8% (w/v) gel]. At 20 °C the 75 kDa undigested form of proα1(X) (Con) is converted into smaller fragments of 60–65 kDa and less, which become further degraded at increasing temperatures. The T_m of the triple-helical core of hrColX/P4H was 41 °C and was therefore identical with that of the non-enzyme-treated hrColX/P4H as measured by CD. For comparison, the melting point of the triple helix of hr(ColX) purified from clone A6/16 [16] was only 31 °C. The positions of molecular-mass markers are indicated (in kDa) at the left.

apparent contrast with the predicted stabilization of the ColX triple helix at the C-terminus by the $(NC1)_3$ domain, which is unusually resistant to dissociation by heat owing to strong hydrophobic interaction sites between the NC1 domains [15,17,20,37]. However, it is consistent with a study on the thermal denaturation and renaturation of native versus pepsin-treated chicken type X collagen [14], showing that the T_m of 47 °C was similar for both forms. However, the renaturation kinetics and the extent of triple helix formation were significantly higher [14] for the native 59 kDa form (corresponding to the 75 kDa form of hrColX reported here). This indicates that the NC1 domains are essential for chain assembly and trimer formation of the pro-α1(X) chain in the rough endoplasmatic reticulum but do not contribute to the thermal stability of the type X collagen molecules once assembled into a triple helical molecule.

In other collagens, such as type XII, a fully hydroxylated triple helix seems more essential for the assembly of a trimeric molecule than the non-collagenous domains [25]: coexpression of a type XII collagen construct, designed as minicollagen containing the C-terminal NC1 domain with P4H, enhanced not only the hydroxylation of the triple helix but also its assembly into a trimeric collagen and the formation of interchain disulphide bonds in the NC1 domain. This might also have been a result of the protein disulphide isomerase activity of P4H. However, the tendency of NC1 domains of collagen XII to form stable trimers seems considerably lower than the trimerization tendency of type X collagen NC1 domains, which is unusually high and has therefore been used to induce the trimerization of non-collagenous proteins by recombinant expression as chimaeric proteins [37]. Thus it is remarkable that the presence of such a tight knot as the ColX-NC1 domain at the C-terminus of the triple helix does not affect its T_m.

In conclusion, we have shown that large amounts of highly hydroxylated and correctly folded recombinant type X collagen can be prepared with a mammalian cell culture expression system by coexpressing both subunits of P4H. Furthermore, our results indicate that the contributions of the N- and C-terminal non-collagenous domains of type X collagen to thermal stability seem neglegible in comparison with the stabilizing role of hydroxyproline residues.

We thank Mrs Eva Bauer for her invaluable help with cell culture and protein purification. This work was financially supported by a grant from Deutsche Forschungsgemeinschaft to K.v.d.M. (Ma 534-10/2).

REFERENCES

1 Schmid, T. M., Cole, A. A., Chen, Q., Bonen, D. K., Luchene, L. and Linsenmayer, T. (1994) Assembly of type X collagen in hypertrophic chondrocytes. In Extracellular Matrix Assembly and Structure (Yurchenco, P., Birk, D. and Mecham, R., eds.), pp. 171–206, Academic Press, San Diego

2 Schmid, T. M. and Linsenmayer, T. F. (1987) Type X collagen. In Structure and Function of Collagen types (Mayne, R. and Burgeson, R. E., eds.), pp. 223–259, Academic Press, Orlando

3 Gibson, G. J., Beaumont, B. W. and Flint, M. H. (1984) Synthesis of a low molecular weight collagen by chondrocytes from the presumptive calcification region of the embryonic chick sterna: the influence of culture with collagen gels. J. Cell Biol. **84**, 208–216

4 Capasso, O., Quarto, N., Descalci-Cancedda, F. and Cancedda, R. (1984) The low molecular weight collagen synthesized by chick tibial chondrocytes is deposited in the matrix both in culture and *in vivo*. EMBO J. **3**, 823–827

5 Remington, M. C., Bashey, R. I., Brighton, C. T. and Jimenez, S. A. (1984) Biosynthesis of a disulphide bounded short-chain collagen by calf growth plate cartilage. Biochem. J. **224**, 227–233

6 von der Mark, K., Kirsch, T., Nerlich, A., Kuss, A., Weseloh, G., Glueckert, K. and Stoess, H. (1992) Type X collagen synthesis in human osteoarthritic cartilage: indication of chondrocyte hypertrophy. Arthritis Rheum. **35**, 806–811

7 Walker, G. D., Fischer, M., Gannon, J., Thompson, R. C. and Oegama, T. R. (1995) Expression of type X collagen in osteoarthritis. J. Orthop. Res. **13**, 4–12

8 Aigner, T., Reichenberger, E., Bertling, W., Kirsch, T., Stoess, H. and von der Mark, K. (1993) Type X collagen expression in osteoarthritic and rheumatoid articular cartilage. Virchows Arch. **63**, 205–211

9 Aigner, T., Frischholz, S., Dertinger, S., Beier, F., Girkontaite, I. and von der Mark, K. (1997) Type X collagen expression and hypertrophic differentiation in chondrogenic neoplasias. Histochem. Cell Biol. **107**, 435–440

10 Reginato, A. M., Shapiro, J. M., Lash, J. W. and Jimenez, S. A. (1988) Type X collagen alterations in rachitic chick epiphyseal growth cartilage. J. Biol. Chem. **263**, 9938–3345

11 Jacenko, O., LuValle, P. A. and Olsen, B. R. (1993) Spondylometaphyseal dysplasia in mice carrying a dominant negative mutation in a matrix protein specific for cartilage to bone transition. Nature (London) **365**, 56–61

12 Kirsch, T. and Wuthier, R. E. (1994) Stimulation of calcification of growth plate cartilage matrix vesicles by binding to type II and X collagen. J. Biol. Chem. **269**, 11462–11469

13 Kwan, K. M., Pang, M. K., Zhou, S., Cowan, S. K., Kong, R. Y., Pfordte, T., Olsen, B. R., Sillence, D. O., Tam, P. P. and Cheah, K. S. (1997) Abnormal compartmentalization of cartilage matrix components in mice lacking collagen X: implications for function. J. Cell Biol. **136**, 459–471

14 Schmid, T. M. and Linsenmayer, T. F. (1984) Denaturation–renaturation properties of two molecular forms of short-chain cartilage collagen. Biochemistry **23**, 553–558

15 Brass, A., Kadler, K. E., Thomas, J. T., Grant, M. E. and Boot-Handford, R. P. (1992) The fibrillar collagens, collagen VIII, collagen X and the C1q complement proteins share a similar domain in their C-terminal non-collagenous regions. FEBS Lett. **303**, 126–128

16 Frischholz, S., Beier, F., Girkontaite, I., Wagner, K., Pöschl, E., Turnay, J., Mayer, U. and von der Mark, K. (1998) Characterization of human type X procollagen and its NC-1 domain expressed as recombinant proteins in HEK293 cells. J. Biol. Chem. **273**, 4547–4555

17 Chan, D., Weng, Y. M., Hocking, A. M., Golub, S., McQuillan, D. J. and Bateman, J. F. (1996) Site-directed mutagenesis of human type X collagen. Expression of α1(X) NC1, NC2, and helical mutations in vitro and in transfected cells. J. Biol. Chem. **271**, 13566–13572

18 Zhang, Y. and Chen, Q. (1999) The noncollagenous domain 1 of type X collagen. A novel motif for trimer and higher order multimer formation without a triple helix. J. Biol. Chem. **274**, 22409–22413

19 Middleton, R. B. and Bulleid, N. J. (1993) Reconstitution of the folding pathway of collagen in a cell-free system: formation of correctly aligned and hydroxylated triple helices. Biochem. J. **296**, 511–517

20 Chan, D., Cole, W. G., Rogers, J. G. and Bateman, J. F. (1995) Type X collagen multimer assembly *in vitro* is prevented by a Gly618 to Val mutation in the α1(X) NC1 domain resulting in Schmid metaphyseal chondrodysplasia. J. Biol. Chem. **270**, 4558–4562

21 Chan, D. and Jacenko, O. (1998) Phenotypic and biochemical consequences of collagen X mutations in mice and humans. Matrix Biol. **17**, 169–184

22 Reichenberger, E., Beier, F., Luvalle, P., Olsen, B. R., von der Mark, K. and Bertling, W. (1992) Genomic organisation and full length cDNA sequence of human collagen X. FEBS Lett. **311**, 305–310

23 Linsenmayer, T. F., Gibney, E. and Schmid, T. M. (1986) Intracellular avian type X collagen *in situ* and determination of its thermal stability using a conformation-dependent monoclonal antibody. Exp. Cell Res. **166**, 15–22

24 Lamberg, A., Helaakoski, T., Myllyharju, J., Peltonen, S., Notbohm, H., Pihlajaniemi, T. and Kivirikko, K. I. (1996) Characterization of human type III collagen expressed in a baculovirus system. Production of a protein with a stable triple helix requires coexpression with the two types of recombinant prolyl 4-hydroxylase subunit. J. Biol. Chem. **271**, 11988–11995

25 Mazzorana, M., Snellman, A., Kivirikko, K. I., van der Rest, M. and Pihlajaniemi, T. (1996) Involvement of prolyl 4-hydroxylase in the assembly of trimeric minicollagen XII. Study in a baculovirus expression system. J. Biol. Chem. **271**, 29003–29008

26 Helaakoski, T., Veijola, J., Vuori, K., Rehn, M., Chow, L. T., Taillon-Miller, P., Kivirikko, K. I. and Pihlajaniemi, T. (1994) Structure and expression of the human gene for the α subunit of prolyl 4-hydroxylase. The two alternatively spliced types of mRNA correspond to two homologous exons the sequences of which are expressed in a variety of tissues. J. Biol. Chem. **269**, 27847–27854

27 Girkontaite, J., Frischholz, S., Lammi, P., Wagner, K., Swoboda, B., Aigner, T. and von der Mark, K. (1996) Immunolocalization of type X collagen in normal fetal and adult osteoarthritic cartilage with monoclonal antibodies. Matrix Biol. **15**, 231–238

28 Vuori, K., Pihlajaniemi, T., Marttila, M. and Kivirikko, K. I. (1992) Characterization of the human prolyl 4-hydroxylase tetramer and its multifunctional protein disulfide-isomerase subunit synthesized in a baculovirus expression system Proc. Natl. Acad. Sci. U.S.A. **89**, 7467–7470

29 Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**, 156–159

30 Kwan, A. P. L., Cummings, C. E., Chapman, J. A. and Grant, M. E. (1991) Macromolecular organization of chicken type X collagen *in vitro*. J. Cell Biol. **114**, 597–605

31 Reichenberger, E., Aigner, T., von der Mark, K., Stoess, H. and Bertling, W. (1991) *In situ* hybridization studies on the expression of type X collagen in fetal human cartilage. Dev. Biol. **148**, 562–572

32 Luvalle, P., Ninomiya, Y. and Rosenblum, N. D. (1988) The type X collagen gene. J. Biol. Chem. **263**, 18378–18385

33 Thomas, J. T., Kwan, A. P. L., Grant, M. E. and Boot-Handford, R. P. (1991) Isolation of cDNAs encoding the complete sequence of bovine type X collagen. Biochem. J. **273**, 141–148

34 Ramshaw, J. A., Shah, N. K. and Brodsky, B. (1998) Gly-X-Y tripeptide frequencies in collagen: a context for host-guest triple-helical peptides. J. Struct. Biol. **122**, 86–91

35 Chan, V. C., Ramshaw, J. A., Kirkpatrick, A., Beck, K. and Brodsky, B. (1997) Positional preferences of ionizable residues in Gly-X-Y triplets of the collagen triple-helix. J. Biol. Chem. **274**, 3632–3641

36 Yang, W., Chan, V. C., Kirkpatrick, A., Ramshaw, J. A. and Brodsky, B. (1997) Gly-Pro-Arg confers stability similar to Gly-Pro-Hyp in the collagen triple-helix of host-guest peptides. J. Biol. Chem. **272**, 28837–28840

37 Marks, D. S., Gregory, C. A., Wallis, G. A., Brass, A., Kadler, K. E. and Boot-Handford, R. P. (1999) Metaphyseal chondrodysplasia type Schmid mutations are predicted to occur in two distinct three-dimensional clusters within type X collagen NC1 domains that retain the ability to trimerize. J. Biol. Chem. **274**, 3632–3641

Received 21 June 2000/24 August 2000; accepted 3 October 2000