COLLAGENOUS SEQUENCE GOVERNS THE TRIMERIC ASSEMBLY OF COLLAGEN XII*

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Summary

A minicollagen containing the COL1 and NC1 domains of chicken collagen XII has been produced in insect cells. Significant amounts of trimers contain a triple-helical domain in which the cysteines are not involved in inter- but in intrachain bonds. In reducing conditions, providing that the triple-helix is maintained, disulfide exchange between intra- and interchain bonding is observed, suggesting that the triple-helix forms first, and that in favourable redox conditions interchain bonding occurs to stabilize the molecule. This hypothesis is verified by \textit{in vitro} reassociation studies performed in the presence of reducing agents, demonstrating that the formation of interchain disulfide bonds is not a prerequisite to the trimeric association and triple-helical folding of the collagen XII molecule. Shortening the COL1 domain of minicollagen XII to its five C-terminal GXY triplets results in an absence of trimers. This can be explained by the presence of a too short collagenous domain to form a stable triple-helix. In contrast, the presence of five additional C-terminal triplets in COL1 allows the formation of triple-helical disulfide bonded trimers suggesting that the presence of a triple-helix is essential for the assembly of collagen XII.
INTRODUCTION

Collagens are the most abundant proteins of the extracellular matrix. They consist of collagenous triple-helical domains, flanked by non-collagenous domains, that differ in the size and number from one collagen to another. Collagens can be homotrimers or heterotrimers, and their structure is stabilized by interchain disulfide bonds (1, 2).

Collagens IX, XII, XIV, XVI and XIX constitute the FACITs family that are grouped on the basis of similarities in their short C-terminal domain COL1 which is thought to be important for the association with fibrils (3, 4). The association of collagen type IX, XII and XIV molecules with quarter staggered fibrils has been shown by immunofluorescence and electron microscopy (5-9). The biological functions of the FACITs are not yet fully understood. Rapid and reversible production of collagen XII by fibroblasts submitted to mechanical stress has been observed (10, 11). Collagens XII and XIV potentially facilitate the contraction of collagen gels by fibroblasts (12), and they are known to interact with other components of the extracellular matrix (13-15). This suggests that they contribute to the cohesion and the modulation of the mechanical properties of the extracellular matrix. Collagen XIV is also a potential reservoir for procollagen N-proteinase (16) and promotes the adhesion of different cells (17, 18).

The assembly of fibril forming collagens (collagens type I, II, III, V and XI) is a complex process in which three chains have to fold into a triple-helix. This results from chain association, nucleation and propagation of the folding occurring from the C- to N-terminus in a zipper like fashion (19, 20). The C-terminal non-triple-helical domains, called the C-propeptides, play an important role in the initial steps of chain association (21-24). However, in the case of FACITs, the C-propeptides are replaced by significantly shorter non-triple-helical domains, NC1. These small domains consist of just about 75 amino acids for collagen XII and less than 30 amino acids for collagen IX, whereas the C-propeptides of the fibrillar collagens contain some 260 amino acids. The NC1 domains of the FACITs do not share common sequences. In addition, structural variations in the NC1 domains of rat and mouse collagen XII, generated by tissue specific
alternative splicing, have been shown (25). In contrast, FACITs display remarkable similarities in their COL1 domains with respect to their similar size and to two corresponding imperfections within the triple-helix. At the junction of the COL1 and NC1 domains, two cysteines separated by four amino acids are responsible for interchain disulfide bonding. These data suggest that the COL1 domain and the junction may serve a common function in all FACITs.

The critical role of the COL1 domain in the assembly of the collagen XII molecule has first been suggested by the necessity to stabilize this domain by the hydroxylation of prolyl residues to obtain disulfide-bonded trimers. Indeed, reduction or inhibition of prolyl 4-hydroxylase (EC1.14.11.2) prevented the formation of disulfide-bonded trimers in HeLa and insect cells which produced a chicken minicollagen XII consisting of the C-terminal domains COL1 and NC1 (26, 27). In addition, a deletion covering most of the NC1 domain of chicken collagen XII except for seven residues of the COL1/NC1 junction does not prevent the formation of disulfide bonded trimers correctly folded into a triple-helix (28). In this report, we analyze the respective roles of the COL1 domain and of the junction in the formation of the homotrimeric collagen XII molecule. We show that the formation of the COL1 triple-helix is the key step of the trimeric association of the α chains of minicollagen XII which is then stabilized by interchain disulfide bonds.

EXPERIMENTAL PROCEDURES

Materials - HiTrap Q, HiTrap SP, Superose 6 and 12 (HR 10/30) columns were from Amersham Pharmacia Biotech. Chymotrypsin type I-S, reduced glutathione, Triton X-100 (peroxide free) and trypsin type XIII were from Sigma. NADPH and yeast glutathione reductase (EC 1.6.4.2) were from Roche Molecular Biochemicals. Express Five medium, pluronic acid, Dulbecco's Modified Eagle's medium (DMEM), fetal calf serum, penicillin and streptomycin were from Gibco/BRL, Life Technologies.
Construction of the vectors and generation of recombinant viruses - Generation of baculoviruses recombinant for the minigene coding for minicollagen XII (COL XII 23) has been described (27). Recombinant baculovirus transfer vector constructs coding for chicken minicollagen XII with shortened COL1 domains were generated by PCR overlap extension using pRc/CMV COL XII 23 as a template (28). PVL-XII-PCR1 was generated using a 5′ oligonucleotide primer H1 (5′ GCAGTCCTCGAGGGCTCCACAGGATCACGAGG 3′) complementary to the sequence downstream of the second imperfection of the COL1 domain (a Xho I site has been introduced in this oligonucleotide to facilitate subsequent subcloning) and a 3′ oligonucleotide primer H2 (5′ AGGCACAGTCGAGGC 3′) complementary to the sequence of the pRc/CMV vector (Invitrogen Corporation) located 50 nucleotides downstream the Xba I site of the polylinker. PCR yielded a 415 bp fragment (PCR1) which was cut by Xho I and Xba I and subcloned in BKS ENS 23 (28) digested by the same enzymes. The resulting plasmid BKS XII-PCR1 contains also the signal peptide of the α1 chain of human collagen I and two tagging sequences each consisting of a short fragment of human c-myc protein. BKS XII-PCR1 is digested by Hind III and Xba I, and the resulting fragment is then cloned in the mammalian expression vector pRc/CMV, giving rise to pRc/CMV XII-PCR1. Concurrently, BKS XII-PCR1 and the baculovirus transfer vector pVL 1392 (PharMingen) are linearized by Hind III and Not I, respectively, and filled with Klenow. Both are then digested with Xba I. The Hind III filled/Xba I fragment of BKS XII-PCR1 is then ligated to pVL 1392 containing a Not I filled extremity and a cohesive Xba I extremity. pVL XII-PCR2 has been prepared in the same way using a 5′ oligonucleotide primer I (5′ GCAGTCCTCGAGGGAAATGCGGGTATTCG 3′) complementary to the sequence coding for the last fifteen amino acids of COL1 and H2 as 3′ oligonucleotide primer. The PCR2 fragment was 363 bp in size. In the final baculovirus transfer vector pVL1392, the minigenes of interest are inserted downstream of the polyhedrin promoter. In view of homologous recombination, each of the two recombinant pVL constructs has been co-
transfected into *Spodoptera frugiperda* Sf9 cells together with wild type purified DNA of *Autographa californica* polyhedrosis virus. The resultant viral pool was collected, amplified and plaque-purified to screen for recombinant viruses (29). After several rounds of purification, the ensuing recombinant viruses, called XII-PCR1 and XII-PCR2 were checked by a PCR-based method (30), amplified and stored at 4°C in the dark until use.

*Infection of insect cells with recombinant baculoviruses - Trichoplusia ni Tn-SB1-4* cells (marketed by Invitrogen as High Five cells) were infected with COL XII 23, XII-PCR1 or XII-PCR2 viruses together with recombinant viruses encoding the α and β subunits of human prolyl 4-hydroxylase (31). These viruses were a generous gift from Prof. Taïna Pihlajaniemi, University of Oulu, Finland. Cells were cultured at 27°C in serum free medium containing 0.1% pluronic acid, penicillin and streptomycin. For cells cultured in plates, 6 x 10^6 cells were seeded per 100 mm culture dish. For cells cultured in suspension in a spinner system (Cell Spin, Integra Biosciences), 100 ml of medium were inoculated with 10^8 cells in a 250 ml spinner flask to ensure proper aeration, and stirred continuously at 40 rpm until the cells were adapted to suspension culture conditions. Cells were maintained at 10^6 cells/ml by removing the excess of cell suspension and replacing it with fresh culture medium. They were infected when they had reached 98% viability and 18-24 h doubling time. In both culture conditions, cells were infected at a multiplicity of infection 5:1:1 for the recombinant baculoviruses coding for one or an other minicollagen, the α subunit and the β subunit of the prolyl 4-hydroxylase, respectively. Sodium ascorbate was added daily at 50 μg/ml immediately after the replacement of the viral inocula with fresh medium (7 ml) for cells cultured in plates or 1h after inoculation of the viruses for the cells cultured in suspension. Cells and media were collected 48 h after infection. Cell fractions were used either immediately or stored as pellets at -80°C after freezing in liquid nitrogen. Media were centrifuged at 12,000 g for 15 min at 4°C after addition of protease inhibitors (10 mM EDTA, 10 mM NEM and 1 mM PMSF) and frozen at -80°C.
Transient transfection of HeLa cells - Cells (10⁶) were plated in 100 mm culture dishes in DMEM supplemented with 5% heat-inactivated fetal calf serum, penicillin and streptomycin. After cell adhesion and spreading, 5 µg expression vector pRc/CMV XII-PCR1 and 10 µg plasmid carrier DNA were used to transfect the cells using the calcium-phosphate procedure (32). After 16 h of contact with the DNA-calcium phosphate coprecipitate, cells were rinsed and sodium ascorbate (50 µg/ml) was added in 7 ml of fresh medium. After 24 h, medium and cells were collected for immunodetection experiments.

SDS-PAGE and Western blotting analysis - The cells cultured in plates were homogenized in ice cold buffer (0.2 M NaCl, 0.1% Triton-X100, 50 mM Tris-HCl, pH 7.4, containing 10 mM EDTA, 10 mM NEM and 1 mM PMSF, 300 µl /10⁶ cells). Aliquots of homogenates or cell extracts and of the corresponding conditioned media were precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE, followed by Coomassie Blue staining or Western blotting with antibody Myc-9E10.2 (designated here as 9E10). This antibody is a mouse monoclonal antibody directed against the tagging sequences derived from the human c-myc protein present at the N-terminus of minicollagen XII constructs.

Purification of minicollagen XII from medium of infected insect cells cultured in suspension - Conditioned medium containing protease inhibitors was chromatographed on DEAE-cellulose. After rinsing of the gel with 50 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM NEM, elution of minicollagen XII was performed with the same buffer containing 0.5 M NaCl. Minicollagen XII was further purified with an anion exchange FPLC column (HiTrap Q, 5 ml) equilibrated in 10 mM Tris-HCl, pH 7.4, containing 0.1 M NaCl and 5 mM EDTA. A linear gradient of 0.1 to 0.7 M NaCl in 50 min was applied at a flow rate of 1 ml/min. In this gradient, species which migrate as single bands with an apparent molecular mass corresponding to
monomers in SDS-gels elute earlier than trimers. These fractions were separately injected to a Superose 6 FPLC column equilibrated in 0.15 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 7.4, at 0.4 ml/min. This gel filtration allows preparation of two fractions corresponding to trimers and monomers which elute after 12 and 16 ml, respectively.

*Pepsin digestion of minicollagen XII* - Minicollagen XII (about 700 µg) purified from the medium of infected insect cells cultured in suspension as described above (except that the gel filtration step was omitted) was adjusted to pH 3 with acetic acid and incubated in the presence of pepsin (20 µg) for 18 h at 4°C. Fresh pepsin was added (30 µg), and after 90 min at 20°C digested minicollagen XII was purified by gel filtration on Superose 6.

*Purification of XII-PCR1 and XII-PCR2 from insect cell extracts* - XII-PCR1: Infected High Five cells were homogenized (3.4 x 10⁶ cells/ml) in cold extraction buffer (0.2 M NaCl, 0.1% Triton X-100, 50 mM Tris-HCl, pH 7.4, containing 10 mM EDTA, 10 mM NEM and 1 mM PMSF). After 30 min incubation on ice, the homogenate was centrifuged at 12,000 g for 15 min at 4°C. The extract was diluted two-fold with extraction buffer lacking NaCl and chromatographed on a DEAE-cellulose column equilibrated in 0.1 M NaCl, 50 mM Tris-HCl, 5 mM EDTA, 5 mM NEM, pH 7.4. Elution was performed by raising the NaCl concentration to 0.5 M. XII-PCR1 was further purified by HiTrap Q chromatography (a 100 min linear gradient of 0.1 to 0.5 M NaCl in 10 mM Tris-HCl pH 7.4 at 1 ml/min) followed by gel filtration on Superose 6 performed as described for minicollagen XII.

XII-PCR2: Infected High Five cells were homogenized, and the extract was subjected to DEAE-cellulose chromatography as described for XII-PCR1. Monomers elute in the flow through whereas dimers elute with the 0.5 M NaCl containing buffer. Monomers were further purified by HiTrap Q chromatography using a 0.1 M to 0.3 M NaCl gradient. For the analysis of the XII-
PCR2 hydroxylation state, an additional reverse phase chromatography on C18 was performed before trypsin digestion.

*Trypsin/chymotrypsin digestions* – The fractions (3-6 µg) equilibrated for 5 min at the digestion temperature were incubated 3 min at 21°C unless otherwise indicated in the presence of a mixture of trypsin (1-1.5 µg) and chymotrypsin (3-4.5 µg).

*Purification of the COL1 domain of minicollagen XII* - An extract corresponding to 25 x 10⁶ infected insect cells cultured in plates was adjusted to pH 2-2.5 with HCl before digestion with 1.5 mg pepsin for 2 h at 20°C. After centrifugation for 15 min at 12000 g, the supernatant was neutralized with NaOH, filtered, and applied to a HiTrap Q column. The unbound fraction was dialyzed against 50 mM Na-acetate, pH 5.1, and batch incubated with CM-cellulose. Pepsinized minicollagen was eluted with 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4, and chromatographed on Superose 6 as described for the full-length minicollagen XII. An aliquot of pepsinized minicollagen XII (0.4 mg) was further digested using a mixture of trypsin (20 µg) and chymotrypsin (50 µg) for 15 min at 25°C, and then 30 min at 20°C. The digest was injected to a HiTrap sulfopropyl column, and the trimeric disulfide bonded COL1 domain was eluted using a 0 to 0.25 M NaCl gradient in Bis-Tris-HCl buffer, pH 6.7. Further purification was achieved by gel filtration on a Superose 12 column equilibrated in 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4, at 0.4 ml/min.

*Labeling of cysteine residues with MPB* – Isolated T, Mt and Mm forms of minicollagen XII (0.1 mg/ml) were reacted with 120 µM MPB for 35 min at 30°C either directly or after incubation for 15 min at 45°C in the presence of 5 mM DTT followed by removing of DTT by reverse phase chromatography. The MPB labeling was stopped by addition of reducing Laemmli sample
buffer. Aliquots (0.1 µg) were analyzed by SDS-PAGE followed by electroblotting. MPB modified proteins were detected after incubation with avidin-conjugated alkaline phosphatase (ExtrAvidin, Sigma) using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Biorad).

**Analysis of XII-PCR2 hydroxylation state** – XII-PCR2 (120 µg) in 0.15 M NaCl, 50 mM Tris-HCl, pH 8, was incubated 20 h at 37°C in the presence of 10 µg trypsin (sequencing grade, Promega). The digest was chromatographed on a C18 reverse phase column (Supelcosyl LC-318, 25 cm x 4.6 mm, 5 µ, Supelco) using an aqueous acetonitrile gradient (0-45%) over 60 min in 0.1% trifluoroacetic acid at 1.2 ml/min. An aliquot of each peak was tested by a standard ELISA for its recognition by a monoclonal antibody (17-8H11) whose epitope is located within the 22 first residues of the NC1 domain of chicken collagen XII (unpublished results). The major immunoreactive peaks were analyzed by electrospray mass spectrometry and N-terminal sequencing.

**Determination of GSSG concentration in GSH solutions** - GSSG concentrations were determined by a coupled assay as glutathione reductase-dependant oxidation of NADPH (33). Briefly, samples (5-200 µl) were assayed for GSSG in a final volume of 1 ml of a 20 mM Tris-HCl, 0.1 M NaCl buffer (pH 8) containing 0.2 mM NADPH by addition of 0.6 units of yeast glutathione reductase (120 U/ml) and monitoring the absorbance decrease at 340 nm. NADPH consumption was quantified using an extinction coefficient of 6.34 mM⁻¹ cm⁻¹.

**Other procedures** – SDS-PAGE analysis was performed according to Laemmli (34) unless otherwise noted. Alternatively, the procedure of Schägger and von Jagow (35) was used. Amino acid sequence analysis was performed by automated Edman degradation on an Applied
Biosystems 473A protein sequenator using the trifluoroacetic acid conversion program provided by the manufacturer. Mass spectrometry analysis was performed on an electrospray apparatus API 165 Sciex (Perkin Elmer) with an ion spray source.
RESULTS

Characterization of non-disulfide bonded trimers of minicollagen XII with intrachain disulfide bonds - The formation of disulfide bonded trimers of minicollagen XII depends on the activity of prolyl 4-hydroxylase (27). Therefore, insect cells have been infected with a baculovirus recombinant for minicollagen XII and with a virus coding for the α and β subunits of the enzyme. To obtain large volumes of conditioned medium, infected cells have been cultured in suspension in a spinner device, yielding about 40 mg/l of minicollagen XII. As judged by SDS-PAGE, only two species are observed under nonreducing conditions (Fig. 1A). The upper band represents disulfide bonded trimers. The faster migrating band represents non-disulfide bonded minicollagen XII in which the cysteines of the COL1/NC1 junction appear to be involved in intrachain disulfide bonds as judged by the different electrophoretic mobility under reducing and under nonreducing conditions.

Minicollagen XII was purified from culture medium by DEAE-cellulose followed by HiTrap Q and gel filtration chromatography. SDS-PAGE revealed that monomeric species elute earlier from anion exchange gels than disulfide bonded trimers. The two corresponding pools were prepared and separately injected into a Superose 6 column. Trimers and monomers elute after 12 and 16 ml, respectively (not shown). The final preparations are shown in Fig. 1B. Whereas monomers (lane 2) are a homogenous population of non-disulfide bonded species (called Mm), on SDS-gels, the trimers appear mainly as a mixture of disulfide bonded trimers (T) and non-disulfide bonded trimers of minicollagen XII (Mt, lane 1). Then, monomers seen in figure 1A (called M) represent a mixture of single chain monomers (Mm) and of non-disulfide bonded trimers (Mt). To show that the trimer fraction consists of disulfide and non-disulfide bonded material, it was analyzed by gel filtration and SDS-PAGE with or without prior heating in the presence or the absence of a reducing agent. Under non-denaturing conditions a single peak is observed which contains disulfide bonded and non-disulfide bonded trimers (Fig. 2A, a). After
heating in the absence of DTT, the three chains of non-disulfide bonded trimers dissociate and elute in the monomer position (Fig. 2A, b, peak 3). In the presence of DTT, only monomers are seen (Fig. 2A, c). This shows that the trimer preparation contains a mixture of disulfide and non-disulfide bonded trimers that can not be separated without denaturation. A schematic representation of the different forms is shown in Fig.2B. Mt and Mm are represented with intrachain disulfide bonds.

The redox state of the cysteinyl residues in the different species was analyzed using the sulfhydryl reagent MPB. Disulfide and non-disulfide bonded trimers were separated by reverse phase chromatography. Under such denaturing conditions, the non-disulfide bonded trimers dissociate and elute as monomers before the disulfide bonded trimers. Trimers, Mt or Mm monomers were incubated in the presence of MPB with or without prior reduction by DTT at 45°C. These experiments showed that none of the unreduced species reacted with MPB, but all were labeled by the biocytin coupled sulfhydryl reagent after reduction. This demonstrates that their cysteinyi residues were in an oxidized form (interchain disulfide bonds for T, intrachain disulfide bonds for Mt and Mm; data not shown).

Triple-helices are resistant to degradation by proteolytic enzymes. To determine whether the COL1 domain is in a triple-helical conformation, samples were digested with pepsin and/or a mixture of trypsin/chymotrypsin. As similar results were observed with or without previous pepsin treatment (data not shown), only those obtained after trypsin/chymotrypsin digestion are presented in Fig. 3A. Proteolytic digestion leads to the removal of the non-collagenous parts of minicollagen XII, and the production of material containing the COL1 domain including the two cysteines present at the COL1/NC1 junction. Whereas disulfide bonded trimers produce disulfide bonded COL1 domain (αCOL1), material corresponding to single chains is also present (αCOL1), confirming that Mt species are trimers with a COL1 domain in a triple-helical conformation with the three chains in register (lane 2). As a control, Mm monomers were treated in the same manner. As expected, no material was detected in SDS-gels after their digestion by a
mixture of trypsin and chymotrypsin (Fig. 3A, lane 3). Under reducing conditions, the band corresponding to single chains (αCOL1) is increased by the presence of αCOL1 resulting from the reduction of (αCOL1)₃ (Fig. 3A, lane 5).

To estimate the thermal stability, pepsinized trimers were treated with a mixture of trypsin/chymotrypsin at various temperatures. The Tm value for the triple-helix of disulfide bonded trimers and non-disulfide bonded trimers is between 35°C and 40°C, and between 30°C and 35°C, respectively, demonstrating that interchain disulfide bonds stabilize the triple-helix (Fig. 3B).

To verify that the three chains of the non-disulfide bonded trimers are in register, various combinations of reduced/oxidized glutathione (GSH/GSSG) were tested, and their ability to rearrange the disulfide bonding pattern of the trimers of minicollagen XII was analyzed by SDS-PAGE under nonreducing conditions after reaction with an excess of iodoacetamide to block all free sulfhydryl groups. As these experiments revealed that the same effect was observed at 4°C or 20°C in the presence or absence of GSSG (not shown), Fig. 3C shows the results obtained after incubation of minicollagen XII trimers at various temperatures in the absence or presence of 10 mM reduced glutathione. At temperatures below the Tm of the triple-helix (T ≤ 30°C), an increase of the band corresponding to disulfide bonded trimers at the expense of the band corresponding to non-disulfide bonded trimers was observed in the presence of 10 mM GSH. This indicates that interchain disulfide bonds are not reduced under these conditions whereas the intrachain bonds rearrange to form interchain disulfide bridges even in a reducing medium. The GSH/GSSG ratio in the solution used for this experiment was determined to be 465. This emphasizes the strong tendency of the cysteines of the COL1/NC1 junction to form interchain bonds when the minicollagen XII is in a triple-helical structure. Furthermore, the complete interchain bonding observed after incubation with GSH proves that the three chains in the non-disulfide bonded trimer are effectively in register. As NEM was present during the purification of minicollagen XII to block free sulphydryl groups, it also confirms that non-disulfide bonded
trimers had their cysteines in an oxidized form. In contrast, when the temperature is above the Tm of the non-disulfide bonded trimer (Fig. 3C, 35°C), the disruption of the triple-helix prevents the intra- to interchain rearrangement, and a mixture of unreduced disulfide bonded trimers and of reduced monomers is observed. At 40°C, the triple-helix of the disulfide bonded trimers is denatured and only reduced monomers are visible, which migrate more slowly than oxidized monomers.

The characterization of the minicollagen XII has allowed us to reveal the occurrence of large amounts of non-disulfide bonded trimers in which the cysteines are involved in intrachain disulfide bridges. These trimers contain a triple-helix with the three chains in register and a disulfide exchange between intra- and interchain bonding is observed under reducing conditions providing that the triple-helix is maintained. These results suggest that the triple-helix can form first and that, providing the redox conditions are favourable, interchain bonding occurs which stabilizes the molecule.

In vitro reassociation of minicollagen XII and of its COL1 domain - To analyze the role of the two cysteines per chain at the COL1/NC1 junction in the assembly process, in vitro reassociation studies using monomers of minicollagen XII or monomers of its COL1 domain have been performed.

Minicollagen XII - Minicollagen XII monomers with oxidized cysteines (Mm) were heated to 45°C for 15 min, in the absence or presence of GSH or DTT. After 19 or 42 h incubation at 4°C, the reassociation is followed by gel filtration chromatography. SDS-PAGE under nonreducing conditions is used to analyze the samples before injection on the Superose column.

SDS-PAGE of the unfractioned samples at various incubation times is shown in Fig. 4A. Before boiling in nonreducing sample buffer, aliquots were treated with iodoacetamide to block all free sulphhydryl groups. At time zero, after a 15 min treatment at 45°C, the reducing agents have been effective in disrupting the intrachain disulfide bonds present in Mm as indicated by
the slightly reduced electrophoretic mobilities. After incubation at 4°C for 19 h to allow refolding of the different species, the cysteines of unreduced Mm remain oxidized and the samples reduced in the presence of DTT are still reduced. Three bands are observed in the case of the samples treated with the weaker reducing agent GSH, corresponding in electrophoretic mobility to one chain (reduced), to two disulfide bonded chains, and to three disulfide bonded chains, respectively. Samples analyzed after 42 h incubation at 4°C, show essentially the same results. For the GSH reduced samples, the bands corresponding to disulfide bonded trimers increase concomitently with a decrease of the bands representing monomers and disulfide bonded dimers. Gel filtration analysis after 19 h incubation shows similar relative amounts of trimers and monomers (Fig. 4B) indicating that trimeric assembly of minicollagen XII in vitro can take place even when the two cysteinyl residues are involved in intrachain disulfide bonds, or are fully reduced. The tendency of the monomers containing either oxidized or reduced cysteines to form trimers increases with time to the detriment of monomers (Fig. 4B). As gel filtration does not indicate the presence of any free dimers, which are observed in SDS-PAGE (Fig. 4A), they most probably are associated with monomeric chains. In all cases, the three chains constituting the triple-helical domain of the trimers are in register as judged by their resistance to proteolytic attack (Fig. 4C). As an additional control, the trimeric peak collected from the Superose column after 42 h incubation in the presence of DTT was analyzed by SDS-PAGE. As expected, only disulfide bonded trimers are present, confirming that the interchain disulfide bridges have formed after the elimination of the reducing agent during gel filtration (data not shown). In addition, under reducing conditions, monomeric peaks migrate slightly faster than trimeric peaks, suggesting that the monomers of minicollagen XII used for these in vitro reassociation experiments may contain some underhydroxylated molecules (data not shown). In favor of this hypothesis, when the same experiments are performed with the DTT reduced trimers of minicollagen XII, higher amounts of trimers are obtained relative to those presented in Fig. 4B.
These results demonstrate that the formation of the triple-helix is independent of the prior formation of interchain disulfide bonds. Thus, the formation of interchain disulfide bonds is not required for the trimeric association of the α chains, and the nucleation and the triple-helical folding of the collagen XII molecule.

COL1 domain - To verify that the NC1 part of minicollagen XII is not responsible for the trimerization observed in vitro, minicollagen XII was pepsinized, treated with a mixture of trypsin/chymotrypsin and the resulting (αCOL1), fragment was purified. After treatment of the sample with DTT at 45°C for 15 min, only monomers are seen by gel filtration (Fig. 5A). After 22 h incubation at 4°C, a substantial amount of trimers is observed (Fig. 5B), showing that the COL1 domain and the COL1/NC1 junction contain all the informations necessary for the trimerization of the minicollagen XII and the nucleation of its triple-helix. As found for the complete minicollagen XII, SDS-PAGE analysis of the peak corresponding to trimers demonstrates that the three chains constituting the reassociated COL1 domain are disulfide bonded (data not shown).

Sequence regions of COL1 involved in the trimeric association of minicollagen XII and the nucleation of the triple-helix - To determine more precisely the sequence regions involved in the assembly and/or the minimal size of COL1 necessary to promote this process, new constructs coding for minicollagens XII with a shortened COL1 domain have been prepared. The corresponding proteins have been produced in insect cells cultured in plates, detected by Western blotting both in cell homogenates and culture medium and finally biochemically characterized after purification.

XII-PCR1 - XII-PCR1 contains the entire NC1 domain and the thirty C-terminal amino acids of COL1, representing one third of the COL1 domain and covering the two C-terminal clusters of hydroxyprolines (-GPOGPOGRO- and -GPOGPO-) (Fig. 6). As shown on the Western blot (Fig. 7A), in crude cell homogenates large amounts of disulfide bonded trimers of XII-PCR1 are
detected by antibody 9E10. Disulfide bonded dimers and monomers are also visible. In the medium, 9E10 detects four bands corresponding to disulfide-bonded trimers and two bands corresponding to monomers. This result is not surprising as we have shown that minicollagen XII is partially processed in the medium of insect cells cultured in plates, resulting in the removal of the last eight or nine amino acids of zero, one, two or three chains, and in the appearance of four disulfide bonded trimers in SDS gels. The high amount of disulfide bonded trimers observed for XII-PCR1 (Fig. 7A) suggests that the removal of 2/3 of the COL1 domain of minicollagen XII does not impair its assembly properties.

XII-PCR1 trimers have been purified from the insect cell homogenate and digested with a mixture of trypsin/chymotrypsin at increasing temperatures. The analysis of the digested samples by SDS-PAGE on a Tris/Tricine gradient gel demonstrates that the short COL1 domain of XII-PCR1 has a triple-helical conformation (Fig. 7B). In addition, it can be seen that XII-PCR1 is partially resistant to trypsin/chymotrypsin even at 40°C. At this temperature, no COL1 domain resistant to trypsin/chymotrypsin is observed for the entire minicollagen XII (Fig. 3B). This difference might reflect the destabilization of the full length minicollagen XII resulting from the presence of two imperfections in the entire minicollagen XII (underlined in Fig. 6) which are absent in XII-PCR1.

To determine whether XII-PCR1 has the ability to fold at 37°C, we have transfected mammalian cells with a minigene coding for XII-PCR1. Western blotting with antibody 9E10 confirms the presence of disulfide bonded trimers of XII-PCR1 in HeLa cell homogenates (data not shown).

These results show that 1/3 of the COL1 domain is sufficient to promote the nucleation and propagation of the triple-helix and the formation of disulfide bonded trimers, and that the informations required for these processes are contained within this part of the COL1 domain.

XII-PCR2 - In XII-PCR2, we further limited the COL1 sequence from the N-terminus to the fifteen C-terminal amino acids. The sequence contains the remaining cluster of hydroxyprolines
located close to the cysteines responsible for the interchain disulfide bonds (Fig. 6). Both in cell homogenate as well as in the culture medium, the antitag antibody does not detect disulfide-bonded trimers (Fig. 8A). In cell homogenates, only dimers and monomers are detected by antibody 9E10. In culture medium, the dimers have been processed since their apparent molecular mass is decreased. As for the entire minicollagen XII and XII-PCR1, two monomers probably representing unprocessed and processed forms are revealed by Western blotting.

We have previously shown that the formation of disulfide bonded trimers of minicollagen XII depends on the hydroxylation of prolyl residues by prolyl 4-hydroxylase (26, 27). Therefore, the absence of disulfide linked trimers of XII-PCR2 could be due to the absence of hydroxylation of the remaining prolyl residues due to the short size of its COL1 domain. It has been shown that the affinity of this enzyme for short peptides is significantly lower than for longer collagenous sequences (37). To determine if the two prolyl residues in position Y of the two GPP triplets located close to the cysteines are hydroxylated, monomers of XII-PCR2 were purified and digested with trypsin. Trypsin was chosen because an arginine is present adjacent to the two GPP triplets (Fig. 6). The resulting peptides were separated on a C18 reverse phase column and tested in ELISA for their recognition by the antibody to the N-terminal part of NC1 (17-8H11). The two major immunoreactive peaks (L and O in Fig. 8B) were analyzed by mass spectrometry. The major peak (O) has a mass of 5625.4, which corresponds to molecules with two hydroxylated prolyl residues, and the two cysteines blocked by the NEM used during the purification of XII-PCR2. Its identity and the hydroxylation of the two prolines in Y position was further confirmed by N-terminal sequencing. Peak L has a mass of 5373.8, which corresponds to molecules with two hydroxylated prolyl residues and two cysteines involved in intrachain disulfide bonds. These data demonstrate that XII-PCR2 is a good substrate for prolyl 4-hydroxylase, and that the absence of disulfide bonded trimers is not due to a lack of hydroxylation. In addition, as XII-PCR2 monomers are mainly in reduced form before homogenization of cells as judged by the formation of ethylsuccinimidyl cysteiny1 residues, the
absence of disulfide bonded trimers of XII-PCR2 does not result from the formation of intrachain disulfide bonds and more likely reflects the absence of trimeric association.

In order to unambiguously verify that XII-PCR2 is not a trimer formed by a non-covalent association between chains, the purified molecule was submitted to gel filtration, directly or after heating 15 min at 45°C (Fig. 8C). XII-PCR2 elutes at the same position in both conditions, demonstrating its monomeric nature. However, it can be seen in the blot (Fig. 8A) that significant amounts of disulfide bonded dimers of XII-PCR2 are also present. Do they really correspond to dimers, or do they belong to trimers in which only two chains are linked? To address this question, the fractions containing these species were further purified and analyzed by gel filtration chromatography. These experiments showed that the disulfide bonded dimers form a single peak upon gel permeation, well separated from monomeric species both with or without previous heating (data not shown).

In summary, the data show that XII-PCR2 consisting of the entire NC1 domain of collagen XII, the junction between NC1 and COL1 with the two cysteines, and the 5 adjacent GXY repeats cannot form trimers. The same construct, containing 5 additional triplets at its N-terminus (XII-PCR1), however, folds into a trimer with three chains in register and interchain disulfide bonded.

DISCUSSION

The experiments reported here demonstrate that the COL1 domain is the key element in the assembly of minicollagen XII. The characterization of recombinant minicollagen XII produced in insect cells cultured in suspension shows the presence of a mixture of correctly folded trimers in which the cysteines of the COL1/NC1 junction are inter- or intrachain linked. Their trimeric, triple-helical structure, with the three chains in register has been demonstrated. The intrachain bonds rearrange to form interchain disulfide bonds under reducing conditions, providing that the
triple-helix is maintained. In contrast, the disruption of the triple-helix prevents the intra- to interchain bonds rearrangement. *In vitro* reassociation studies of monomers of minicollagen XII or of its COL1 domain show that the triple-helix forms to the same degree whatever the redox state of the cysteines is, involved in intrachain disulfide bonds or totally reduced.

These results show that interchain disulfide bond formation is not a prerequisite for the association of the \( \alpha \) chains, nucleation and folding of the collagen XII molecules in trimers with a triple-helical COL1 domain. They are similar to those previously described for the fibrillar collagen III (38) and are in favour of a mechanism in which the collagenous part has first to fold into a triple-helical conformation before interchain disulfide bond formation occurs. As the triple-helix formation is the rate limiting step, when the redox conditions are too oxidative, the cysteines of monomers form intrachain disulfide bonds. In this respect, the COL1/NC1 junction of collagen XII produced in cells cultured in suspension shows the same behaviour as a synthetic peptide containing the COL1/NC1 junction of collagen XIV, \((GPO)_3GYCDPSSCAG\), in reassociation studies. Indeed, air oxidation of this peptide mainly leads to formation of an intrachain disulfide bond (36). This could explain why in the case of minicollagen XII produced in insect cells cultured in suspension, significant amounts of non-disulfide bonded trimers are observed. On the opposite, when the redox conditions are less oxidative (cells cultured in plates), interchain disulfide bonding occurs as soon as the triple-helix has formed, as very few non-disulfide bonded trimers are observed\(^2\). *In vitro* reassociation data also show that the COL1 domain and the COL1/NC1 junction contain all the information necessary for trimerization of minicollagen XII and nucleation of its triple-helix. These results confirm those previously obtained in HeLa cells transfected with a minigene of collagen XII coding only for the COL1 domain and the COL1/NC1 junction, which showed that triple-helical disulfide bonded trimers were produced (28).

To determine the respective roles of COL1 and of the junction in assembly, two shortened minicollagens XII, XII-PCR1 and XII-PCR2, constituted of the complete NC1 domain and of a
COL1 domain reduced to its 1/3 or 1/6 C-terminal extremity, respectively, have been produced in insect cells. We show that the presence of 1/3 of the COL1 domain is sufficient to promote the formation of interchain disulfide bonded trimers in which the ten remaining GXY triplets of COL1 have folded into a triple-helix. This result is also true at 37°C, as shown by transfection experiments in HeLa cells. In contrast, XII-PCR2 does not form trimers neither disulfide bonded nor non-disulfide bonded. As XII-PCR2 still contains the five C-terminal GXY triplets of COL1, a difference in reactivity of the cysteines of the junction due to a different environment is unlikely. Neither the hydroxylation state of the two prolyl residues in the Y position of the two remaining GPP triplets, nor the redox state of the cysteines are responsible for the inability of the α chains of XII-PCR2 to associate into trimers as we have shown that the two prolyl residues are hydroxylated and that the cysteines are mainly reduced. These results show that the COL1/NC1 junction is not sufficient to promote the association of the three chains, and that COL1, or a part of it, is required to ensure trimerization. A similar conclusion has emerged from studies using synthetic peptides corresponding to the NC1 sequences of collagen IX (39) or to the sequence of the COL1/NC1 junction of collagen XIV (36). The sequence of COL1 is crucial for the trimeric assembly of minicollagen XII. Taking into account the natural tendency of the GXY triplets to fold into a triple-helix, it is tempting to speculate that the nucleation of the helix is the driving force for the assembly process. In this case, the collagenous part of XII-PCR2 is probably too short to form a stable triple-helix. Indeed, the thermal stability of triple-helices has been reported for various length (GPO)_n peptides and the melting temperatures are <2°C when n=5, reaching 44.5°C when n=8 (40, 41). Having in view that GPO is known to contribute the most to the formation and to the stability of a triple-helix (42, 43), it is likely that additional hydroxyprolines present in the COL1 domain are important for association and/or nucleation and stabilization of the triple-helix. This may explain the need for prolyl 4-hydroxylase activity for the correct folding of collagen XII, previously reported in cells expressing this molecule (44) and in recombinant approaches using the C-terminal parts of this molecule (26, 27). In contrast to what
is observed for XII-PCR2, the proper folding of XII-PCR1 and the high Tm (above 40°C) of its
triple-helical part reveal a region with triple-helix formation propensity comparable to host-guest
peptides constituted of (GPO)_3GXY(GPO)_4 sequences (45, 46). This region, perhaps including
the cysteines of the junction which are responsible for interchain disulfide linkage, contains a
cluster of three GXO triplets, and is to our knowledge the shortest collagenous sequence able to
associate and nucleate a stable triple-helix using a recombinant approach in cells. These results
are different from those described for fibril forming collagens in which association events and
nucleation are clearly separated. Indeed, in these collagens, it has been shown that the non-
collagenic C-terminal part of the molecules, the C-propeptides, are responsible for the initial
association of the three α chains, as shown by their ability to fold, to associate, and to form
disulfide bonds in the absence of any GXY triplets (23, 24, 47). In addition, molecular
recognition sequences have been determined for collagen I and III, showing that the C-
propeptides are also responsible of type specific assembly of these collagens (24, 48-50).
Nucleation of the triple-helix takes place in a hydroxyproline-rich region located near the
junction between the triple-helix and the C-telopeptides, as shown by deletion experiments
conducted in this part of the molecule in collagen type III (24).

To describe the assembly process of collagen XII, we propose the following sequence of
events:1) association/nucleation of the triple-helix through informations totally contained in the
C-terminal first part of the COL1 domain or shared with the COL1/NC1 junction. 2) propagation
of the triple-helical folding from the C- to the N-terminus of the molecule. Interchain disulfide
bonding occurs then either between step 1 and 2 or after step 2 to stabilize the triple-helix, as
shown by the higher Tm of interchain disulfide bonded trimers in comparison to the Tm of non-
disulfide bonded trimers.
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REFERENCES


FOOTNOTES

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1 The abbreviations used are: bp, base pairs; DTT, dithiothreitol; FACIT, Fibril-Associated Collagen with Interrupted Triple-helices; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; MPB, 3-(N-maleimidylpropionyl)biocytin; NEM, N-ethylmaleimide; O, hydroxyproline; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; Tm, melting temperature.

2 E. Aubert-Foucher, D. Goldschmidt, M. Jaquinod, and M. Mazzorana, submitted for publication.
Fig. 1. **SDS-PAGE of minicollagen XII produced in insect cell medium.** A, Analysis of insect cell conditioned medium. High Five cells cultured in suspension were coinfectected with viruses coding for minicollagen XII and for the α and β subunits of prolyl 4-hydroxylase. After 48 h of infection, the medium was collected, 50 µl (corresponding to 5 x 10⁴ cells) were precipitated with 8% trichloroacetic acid, and redisolved in Laemmli buffer in the presence (lane 1) or absence (lane 2) of 2-mercaptoethanol. T, disulfide bonded trimer of minicollagen XII; M, non-disulfide bonded minicollagen; BSA, bovine serum albumin. Markers (lane 3) are reduced. Gel 10%T. B, Trimers (lane 1) and monomers (lane 2) purified from insect cell conditioned medium are analyzed by SDS-PAGE under nonreducing conditions (13%T gel). T, disulfide bonded trimers; Mt, non-disulfide bonded trimers; Mm, monomers. Both gels were stained with Coomassie Blue.

Fig. 2. **Gel filtration of minicollagen XII trimers.** A, Purified trimers of minicollagen XII (4 µg) were injected into a Superose 6 column equilibrated in 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4 without prior denaturation (a) or after heating for 15 min at 45°C in the absence (b) or in the presence (c) of 5 mM DTT. Flow rate, 0.4 ml/min. In d, the different peaks (numbered 1 to 4) are analyzed by SDS-PAGE in nonreducing conditions. B, Schematic representation of different forms of minicollagen XII. The putative conformation of disulfide linked trimers (T), non-disulfide bonded trimers (Mt) and monomers (Mm) are shown. The N-terminal tagging sequence and the NC1 domain have been omitted. The assumed assymetric disulfide bonding corresponds to that shown for collagen XIV (36).

Fig. 3. **Characterization of purified minicollagen XII.** A, triple-helical conformation of the COL1 domain of minicollagen XII. Purified trimers (lanes 2 and 5) and monomers (lanes 3 and
6) were incubated in the presence of a mixture of trypsin and chymotrypsin and analyzed under nonreducing (lanes 1-3) or reducing (lanes 4-6) conditions. Lanes 1 and 4 show the mixture of enzymes. \((\alpha \text{COL1})_3\), trimeric disulfide bonded COL1 domain; \((\alpha \text{COL1})_3\), isolated chains dissociated from trimeric non-disulfide bonded COL1 domain. B, evaluation of the thermal stability of the COL1 triple-helix. One \(\mu\)g of pepsinized minicollagen XII trimers has been treated as in A at the indicated temperatures with a mixture of trypsin/chymotrypsin and analyzed under nonreducing conditions. E, proteolytic enzymes. C, effect of reduced glutathione on disulfide bonding of trimeric minicollagen XII. Two \(\mu\)g of trimers are heated for 15 min at the indicated temperatures in the absence (GSH-) or presence (GSH+) of 10 mM GSH. Samples are then treated 30 min at ambient temperature with 50 mM (final concentration) iodoacetamide before boiling in nonreducing sample buffer.

Fig. 4. **In vitro reassociation of minicollagen XII.** Purified Mn monomers of minicollagen XII (1 mg/ml) are heated at 45°C for 15 min in the absence (Mm) or presence of 9 mM GSH (Mm+GSH) or 5 mM DTT (Mm+DTT). After the indicated times of incubation at 4°C, the samples are analyzed by SDS-PAGE (A) or by gel filtration (B). In C, the resistance to trypsin/chymotrypsin is tested. A, SDS-PAGE analysis. Samples are analyzed in nonreducing conditions after 30 min treatment at ambient temperature with 50 mM iodoacetamide. B, gel filtration analysis of trimerization of oxidized, and GSH- or DTT-reduced monomers. At the indicated times of incubation at 4°C, aliquots are diluted in 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4 and injected into a Superose 6 column equilibrated in the same buffer. Flow rate, 0.4 ml/min. C, control of triple-helical conformation of the COL1 domain of the reassociated samples. Aliquots of oxidized, and GSH- or DTT-reduced monomers after 42 h of incubation at 4°C are treated with trypsin/chymotrypsin and analyzed by SDS-PAGE under reducing conditions. The
arrow indicates isolated chains dissociated from the resistant (αCOL1) domain. The mixture of proteolytic enzymes without minicollagen XII is shown in the first lane.

Fig. 5. *In vitro* reassociation of the COL1 domain of minicollagen XII. The trimeric disulfide bonded COL1 domain (0.6 mg/ml in 0.15 M NaCl, 20 mM Tris/HCl, pH 7.4) is first heated to 45°C for 15 min in the presence of 5 mM DTT to separate the three chains (A) and then incubated for 22 h at 4°C (B). Aliquots are injected to a gel filtration column as described for Fig. 4B. 5 µl (A) and 15 µl (B) have been injected.

Fig. 6. Diagram representing the different minicollagens XII. In XII-PCR2 and XII-PCR1, the entire NC1 domain is present but the COL1 parts contain only the 15 and 30 C-terminal amino acids of the COL1 domain of collagen XII, respectively. The amino acids belonging to the so-called COL1/NC1 junction are boxed. The two cysteines involved in interchain disulfide bridges are printed in bold. Black dots indicate hydroxylated prolyl residues. The two imperfections within the GXY repeat of COL1 are underlined. The two large shaded zones at the left and right of the figure represent the common N-terminal and C-terminal sequences, respectively, of the three proteins. The signal peptide (SP) is absent in the mature proteins. The tagging sequences (TAG 1 and TAG 2) consist of the repeat of a short sequence coding for a small part of the human c-myc protein.

Fig. 7. XII-PCR1 produced by insect cells. A, Western blot of insect cell culture medium (conditioned by 10^4 cells) or homogenates (corresponding to 500 cells) as revealed by antibody 9E10 after electrophoresis under nonreducing conditions. T and M represent the different forms of disulfide bonded trimers and monomers, respectively (see text for details), D represents disulfide bonded dimers. B, Triple-helical conformation of the COL1 domain of purified disulfide bonded trimers of XII-PCR1. Purified XII-PCR1 trimers have been incubated at the
indicated temperatures with a mixture of trypsin/chymotrypsin. SDS-PAGE has been performed on a 10 to 20% Tris/Tricine gradient gel under nonreducing conditions (35). COL1-PCR1, resistant COL1 domain of PCR1. Undigested XII-PCR1 is presented as a control.

Fig. 8. **XII-PCR2 produced in insect cells.** A, Western blot of the culture medium (conditioned by 2000 cells) and cell homogenate (100 cells) revealed by antibody 9E10. B, HPLC reverse phase chromatographic profile of XII-PCR2 after digestion with trypsin. The region corresponding to the peaks recognized by monoclonal antibody 17-8H11 was selected. The sequence of the N-terminal part of the fragments, including the two GPP and the two cysteines of the COL1/NC1 junction has been determined by mass spectrometry and N-terminal sequencing, and is indicated by the two main peaks L and O. Black dots indicate hydroxylated prolyl residues. NEM refers to modified cysteinyl residues. C, gel filtration analysis of purified XII-PCR2 monomers before and after heat treatment. XII-PCR2 monomers (0.85 mg/ml) were injected into a Superose 6 column run as described for Fig. 4B before (upper chromatogram) or after (lower chromatogram) treatment at 45°C for 15 min.
Mazzorana et al. Figure 1
Mazzorana et al. Figure 2
Figure 3

A

non reduced reduced

1 2 3 4 5 6

(αCOL1)₃

αCOL1

B

T(°C) 20 25 30 35 40 20

(αCOL1)₃

E

αCOL1

C

GSH- GSH+

T(°C) 4 30 35 40 4 30 35 40

Mt with oxidized Cys

Mt with reduced Cys

Mazzorana et al. Figure 3
Mazzorana et al. Figure 5
Mazzorana et al. Figure 6
Mazzorana et al. Figure 7
Figure 8: Mazzorana et al.

A. Western blot analysis showing dimers and monomers in medium and cells.

B. Chromatogram with absorbance at 214 nm showing NEM treated samples.

C. Chromatogram with relative absorbance at 220 nm.
Collagenous sequence governs the trimeric assembly of collagen XII
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