Collagenous Sequence Governs the Trimeric Assembly of Collagen XII*

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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 favorable redox conditions interchain bonding occurs to stabilize the molecule. This hypothesis is verified by 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 collagenous domain that is too short 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.

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 their 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 FACITs1 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 (collagen types I, II, III, V, and XI) is a complex process in which three chains must fold into a triple-helix. This results from chain association, nucleation, and propagation of the folding occurring from the C to N termini 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 (EC 1.14.11.2) 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, and 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 Chemical Co. NADPH and yeast glutathione reductase (EC 1.6.4.2) were from Roche Molecular Biochemicals. Express Five medium, pluronc acid, Dubelco's modified Eagle's medium, fetal calf serum, penicillin, and streptomycin were from Life Technologies, Inc.

**Construction of the Vectors and Generation of Recombinant Viruses**

Generation of baculoviruses recombinant for the minigenic 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 pReC/MV COL XII 23 as a template (28). PVL-XII-PCR1 was generated using a 5′-oligonucleotide primer H1 (5′-GAAGCTTCCTGGGCTCAGATCGAGG-3′) complementary to the sequence downstream of the second imperfection of the COL1 domain (an XhoI site has been introduced in this oligonucleotide to facilitate subsequent subcloning) and a 3′-oligonucleotide primer H2 (5′-AGGACAGCTGGAGG-3′) complementary to the sequence of the pRe/MV vector (Invitrogen) located 50 nucleotides downstream of the XhoI site of the polylinker. PCR yielded a 415-bp fragment (PCR1), which was cut by XhoI and XbaI 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 1 chain of human collagen I and two tagging sequences, each consisting of a short fragment of human c-Myc protein. BKS XII-PCR1 is then digested by HindIII and XbaI, and the resulting fragment is then cloned in the mammalian expression vector pReCMV, giving rise to pReCMV XII-PCR1. Concurrently, BKS XII-PCR1 and the baculovirus transfer vector pVL 1392 (PharMingen) are linearized by HindIII and NotI, respectively, and filled with Klenow. Both are then digested with XbaI. The HindIII-filled/XbaI fragment of BKS XII-PCR1 is then ligated with pVL 1392 containing a NotI-filled extremity and a cohesive XbaI extremity. pVL XII-PCR2 has been prepared in the same way using a 5′-oligonucleotide primer I (5′-GACGCTCTGAGGGATCCAGG-3′) complementary to the sequence coding for the last 15 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 amplification, the recombinant vibrovl vectors, 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* Tb-SBI-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. Taina Pihlajaniemi, University of Oulu, Finland. Cells were cultured at 27 °C in serum-free medium containing 0.1% pluronc acid, penicillin, and streptomycin. For cells cultured in plates, 6 × 10⁶ 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 was inoculated with 10⁶ 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 10⁶ 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- to 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 the α subunit of any one of the minicollagens, 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 1 h 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 μg EDTA, 10 μg NEM, and 1 μg PMSF) and frozen at −80 °C.

**Transient Transfection of HeLa Cells**

Cells (10⁶) were plated in 100-mm culture dishes in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal calf serum, penicillin, and streptomycin. After cell adhesion and spreading, 5 μg of expression vector pReCMV XII-PCR1 and 10 μg of 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 immunoassay 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 X-100, 50 mM Tris-HCl, pH 7.4, containing 10 mM EDTA, 10 mM NEM, and 1 mM PMSF, 300 μl/10⁹ 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 mM NaCl. Minicollagen XII was further purified with an anion exchange fast-liquid chromatography on 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 that 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 fast-protein liquid chromatography 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 adjusted to 3 by HiTrap Q chromatography 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 × 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 is diluted 2-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-ml 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 after 50 μl NaCl-containing buffer. Monomers were further purified by HiTrap Q chromatography using a 0.1 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 5 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 × 10^6 infected insect cells cultured in plates was adjusted to pH 2–2.5 with HCl before digestion with 1.5 mg of papain 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 sodium acetate, pH 5.1, and batch-incubated with CM-cellulose. Pepsinized minicollagen was eluted with 0.15 mM NaCl, 20 mM Tris-Cl, 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 sulfonylpropyl column, and the trimeric disulfide-bonded COL1 domain was eluted using a 0 to 0.25 mM NaCl gradient in Bis-Tris-Cl buffer, pH 6.7. Further purification was achieved by gel filtration on a Superose 12 column equilibrated in 0.1 mM NaCl, 20 mM Tris-Cl, 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 removal 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 (Bio-Rad).

Analysis of XII-PCR2 Hydroxylation State

XII-PCR2 (120 μg) in 0.15 mM NaCl, 50 mM Tris-Cl, pH 8, was incubated 20 h at 37 °C in the presence of 10 μg of trypsin (sequencing grade, Promega). The digest was chromatographed on a C18 reverse phase column (Supelcosyl LC-318, 25 cm × 4.6 mm, 5 μm, 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 enzyme-linked immunosorbent assay for its recognition by a monoclonal antibody (17-SH11) whose epitope is located within the 22 first residues of the NC1 domain of chicken collagen XII. The major immuno reactive 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-dependent 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-Cl, 0.1 M NaCl buffer (pH 8) containing 0.2 mM NADPH by 2-mercaptoethanol. T, disulfide-bonded trimer of minicollagen XII; Mt, non-disulfide-bonded minicollagen; BSA, bovine serum albumin. Markers (lane 3) are reduced. Gel was 10% T, B, trimer (lane 1) and monomers (lane 2) purified from insect cell-conditioned medium were analyzed by SDS-PAGE under non-reducing conditions (13% T gel). T, disulfide-bonded trimers; Mt, non-disulfide-bonded trimers; Mm, monomers. Both gels were stained with Coomassie Blue.

RESULTS

Characterization of Non-disulfide-bonded Trimmers 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/liter minicollagen XII. As judged by SDS-PAGE, only two species are observed under non-reducing 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 non-reducing 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 eluted 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 (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 Fig. 1A (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 cannot 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 sulphydryl reagent MPB. Disulfide and non-disulfide-bonded trimers were separated by reverse phase chromatography. Under such denaturing condi-
tions, the non-disulfide-bonded trimers dissociate and elute as monomers before the disulfide-bonded trimers. Trimers or 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 cysteinyl 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. 3 A. 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)₃, 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). 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. 3 A, 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. 3 A, 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. 3 B).

To verify that the three chains of the non-disulfide-bonded trimers are in register, various combinations of reduced/oxi-
dized 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 non-reducing 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 (Tm 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 disulfide bridges 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. Because NEM was present during the purification of minicollagen XII to block free sulfhydryl 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 intrato 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 intra-chain 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 favorable, interchain bonding occurs that 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 (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 was tested. A, SDS-PAGE analysis. Samples were analyzed in non-reducing conditions after 90-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 were 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 were treated with trypsin/chymotrypsin and analyzed by SDS-PAGE under reducing conditions. The arrow indicates isolated chains dissociated from the resistant (aCOL1)3 domain. The mixture of proteolytic enzymes without minicollagen XII is shown in the first lane.

Fig. 4. In vitro reassociation of minicollagen XII. Purified Mm monomers of minicollagen XII (1 mg/ml) were 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 was tested. A, SDS-PAGE analysis. Samples were analyzed in non-reducing conditions after 90-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 were 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 were treated with trypsin/chymotrypsin and analyzed by SDS-PAGE under reducing conditions. The arrow indicates isolated chains dissociated from the resistant (aCOL1)3 domain. The mixture of proteolytic enzymes without minicollagen XII is shown in the first lane.
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 concomitantly 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 cysteiny1 residues are either involved in intrachain disulfide bonds or fully reduced. The tendency of the monomers containing either oxidized or reduced cysteines to form trimers increases with time to the detriment of monomers, 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 trimers 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 and treated with a mixture of trypsin/chymotrypsin and the resulting (αCOL1b) 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 information 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 30 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, because 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 two-thirds 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.

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The tagging sequences (TAG 1 and TAG 2) represent the common N- and C-terminal sequences, respectively, of the three proteins. The signal peptide (SP) is insect cell culture medium (conditioned by 10^4 cells) or homogenates so-called COL1/NC1 junction are 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 Y repeat of COL1 are prolyl residues. The two imperfections within the GPPP triplets (Fig. 6). The resulting peptides were separated on a C18 reverse phase column and tested in enzyme-linked immunosorbent assay for their recognition by antibody to the N-terminal part of NC1 (17–8H11). The two major immunoreactive peaks (O and L) 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, because XII-PCR2 monomers are mainly in reduced form before homogenization of cells as judged by the formation of ethylsuccinimidyl cysteinyl 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.

To unambiguously verify that XII-PCR2 is not a trimer...
BPCR2 monomers (0.85 mg/ml) were injected into a Superose 6 column purified XII-PCR2 monomers before and after heat treatment. XII-NEM cells) revealed by antibody 9E10. culture medium (conditioned by 2000 cells) and cell homogenate (100 main peaks spectrometry and N-terminal sequencing and is indicated by the two cysteines of the COL1/NC1 junction has been determined by mass N-terminal part of the fragments, including the two GPP and the two by monoclonal antibody 17-8H11 was selected. The sequence of the gestion with trypsin. The region corresponding to the peaks recognized XII-PCR2 after di- chromatogram treatment at 45 °C for 15 min.

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, high performance liquid chromatography reverse phase chromatographic profile of XII-PCR2 after di-gestion 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.

formed by a non-covalent association between chains, the pu-rified 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 signif-icant 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 mono-meric 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 five adjacent GXY repeats, cannot form trimers. The same construct, containing five additional triplets at its N terminus (XII-PCR1), however, folds into a trimer that has three chains in register and is 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 tri-meric, triple-helical structure, with the three chains in regis-ter, has been demonstrated. The intrachain bonds rearrange to form interchain disulfide bonds under reducing conditions, pro-viding that the triple-helix is maintained. In contrast, the disruption of the triple-helix prevents the intra- to interchain bond 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 independent of whether the redox state of the cysteines is involved in intrachain disul-fide bonds or totally reduced.

These results show that interchain disulfide bond formation is not a prerequisite for the association of the α chains, nucleation, and folding of the collagen XII molecules in trimers with a tripehelical COL1 domain. They are similar to those previously described for the fibrillar collagen III (38) and are in favor of a mechanism in which the collagenous part has first to fold into a triple-helical conformation before interchain disulfide bond formation occurs. Because the triple-helix formation is the rate-limiting step, when the redox conditions are too ox-idative, 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 be-havior as a synthetic peptide containing the COL1/NC1 junc-tion 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 ex-plain why, in the case of minicollagen XII produced in insect cells cultured in suspension, significant amounts of non-disul-fide-bonded trimers are observed. However, when the redox conditions are less oxidative (cells cultured in plates), interchain disulfide bonding occurs as soon as the triple-helix has formed, because very few non-disulfide-bonded trimers are ob-served. 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 ob-tained 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 one-third or one-sixth C-terminal
Trimeric Assembly of Collagen XII Molecule

extremity, respectively, have been produced in insect cells. We show that the presence of one-third 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. Because 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 is responsible for the inability of the α chains of XII-PCR2 to associate into trimers, because 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 (GPO) 3 GXY(GPO) 4 sequences (45, 46). This región, perhaps including the cysteines of the junction that are hydroxylated and that the cysteines are mainly reduced.

To describe the assembly process of collagen XII, we propose the following sequence of events: 1) association/nucleation of the triple-helix through information 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 termini 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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