

Large and Small Splice Variants of Collagen XII: Differential Expression and Ligand Binding

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Abstract. Collagen XII has a short collagenous tail and a very large, three-armed NC3 domain consisting primarily of fibronectin type III repeats. Differential splicing within this domain gives rise to a large (320 kD) and a small (220 kD) subunit; the large but not the small can carry glycosaminoglycan. To investigate whether collagen XII variants have distinct expression patterns and functions, we generated antibody and cDNA probes specific for the alternatively spliced domain. We report here that the large variant has a more restricted expression in embryonic tissue than the small. For example, whereas the small variant is widespread in the dermis, the large is limited to the base of feather buds. Distinct proportions of mRNA for the two variants were detected depending on the tissue. Monoclonal antibodies allowed us to separate collagen XII variants,

and to show that homo- and heterotrimers exist. Collagen XII variants differ in ligand binding. Small subunits interact weakly with heparin via their COOH-terminal domain. Large subunits have additional, stronger heparin-binding site(s) in their NH₂-terminal extra domain. In vivo, both large and small collagen XII are associated with interstitial collagen. Here we show biochemically and ultrastructurally that collagen XII can be incorporated into collagen I fibrils when it is present during, but not after, fibril formation. Removal of the collagenous domain of collagen XII reduces its coprecipitation with collagen I. Our results indicate that collagen XII is specifically associated with fibrillar collagen, and that the large variant has binding sites for extracellular ligands not present in the small variant.

A group of fibril-associated collagens with interrupted triple helices (FACIT; types IX, XII, XIV)¹ has recently been discovered (for review see Mayne and Brewton, 1993; Shaw and Olsen, 1991; Van der Rest et al., 1990). The prototype FACIT is collagen IX, a heterotrimeric molecule with four noncollagenous (NC1 to NC4) and three triple-helical domains (Col1 to Col3) (Ninomiya and Olsen, 1984). The $\alpha 2$ (IX) chain carries chondroitin sulfate on the NC3 domain (McCormick et al., 1987). Collagen IX is covalently bound to the surface of collagen II fibrils in cartilage (Vaughan et al., 1988; Wu et al., 1992). Its function might be to regulate the diameter of collagen II fibrils, and to link them to other collagen fibrils, extra-

cellular matrix proteins, or cells (Shaw and Olsen, 1991). A mouse strain lacking the $\alpha 1$ (IX) chain developed a severe degenerative joint disease (Fässler et al., 1994).

Two other members of the FACIT subfamily, collagens XII and XIV, have been identified by homology to collagen IX (Gordon et al., 1987), and are thought to be bound to the surface of interstitial collagen fibrils (Keene et al., 1991). In contrast to collagen IX that has three different chains, the subunits of collagen XII and XIV are each encoded by a single gene. Both molecules have two collagenous (Col1 and Col2) and three noncollagenous domains (NC1-NC3). Whereas the collagenous and the NC1 and NC2 regions are short, the NH₂-terminal NC3 is a huge trimeric domain containing a globe homologous to NC4 of the $\alpha 1$ (IX) collagen chain, and three extended arms with many fibronectin type III (FN III) and two to four von Willebrand factor A (vWF A) repeats (Yamagata et al., 1991; Gerecke et al., 1993; Wälchli et al., 1993). Collagen XIV is localized in the chick embryo at places where collagen I is also present (Castagnola et al., 1992; Wälchli et al., 1994). It is believed that collagen XIV bind via its NC1 and Col1 domains to collagen fibrils, whereas the Col2 and the NC3 domains are thought to extend into the

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1. *Abbreviations used in this paper:* Col, collagenous domain; DIG, digoxigenin; FACIT, fibril-associated collagen with interrupted triple helices; FN III, fibronectin type III; NC, noncollagenous domain; vWF A, von Willebrand Factor A.

extracellular space and to interact with other matrix proteins or cells (Shaw and Olsen, 1991). However, there is no direct evidence for this hypothesis so far. Collagen XIV from human placenta displayed no cell attachment activity, and binding to collagen VI and heparan sulfate, but not to collagen I, III, or V could be observed by solid phase assays (Brown et al., 1993). Decorin, a proteoglycan associated with collagen I fibrils (Fleischmajer et al., 1991), interacts with collagen XIV (Font et al., 1993), and hence might be a linking molecule.

Collagen XII was isolated from embryonic tendon and cartilage (Dublet et al., 1989; Watt et al., 1992) and is also found in bone, dermis, and smooth muscle (Oh et al., 1993; Wälchli et al., 1994). Two vastly different variants of collagen XII were found that arise by alternative mRNA splicing (Yamagata et al., 1991; Trueb and Trueb, 1992). The protein isolated from fetal tendon or skin consisted mainly of "small" subunits (220 kD), whereas the form with large subunits (320 kD) was later purified from cell culture media (Koch et al., 1992; Lunstrum et al., 1992). The two variants differ in their NC3 domains, the larger containing eight more FN-III and two more vWF A repeats. In addition, large collagen XII is a proteoglycan because glycosaminoglycan is attached to its extra domain (Koch et al., 1992).

So far, much of the interest in collagens XII and XIV has focussed on their fascinating structure. Their collagenous domains comprise a few percent of the total mass, whilst the disulfide-linked arms of their NC3 domains relate them to fibronectin, tenascin, and von Willebrand factor (Engel, 1991). Alternative mRNA splicing generates even more structural complexity. The function of the two very distinct forms of collagen XII remains a mystery. Part of the problem in studying collagen XII function is its molecular heterogeneity. We therefore sought ways to distinguish between large and small variants and to separate them. A prerequisite was the generation of antibodies and cDNA probes specific for the differentially spliced domain. These reagents enabled us to investigate whether large and small collagen XII subunits are expressed differentially during embryonic development. We could also test whether the extra NC3 domain of the large collagen XII subunit contains ligand-binding sites absent in the small variant. Finally, we asked how collagen XII molecules might become incorporated into interstitial collagen fibrils, to provide evidence for a bridging function of this FACIT.

Material and Methods

SDS-PAGE and Immunoblotting

SDS-PAGE on gradient gels of 3–15% polyacrylamide, and two-dimensional SDS-PAGE (nonreduced vs. reduced) were done as described (Koch et al., 1992). Gels were processed for immunoblotting as described (Towbin et al., 1979; Koch et al., 1992). For detection the ECL™ (Amersham Life Sciences, Switzerland) system was used.

Preparation of Monoclonal Antibodies and Affinity Columns

Generation of a hybridoma library against chick collagen XII and the resulting mAb C19 have been described before (Koch et al., 1992). The same hybridoma library was rescreened for mAbs that on immunoblots

recognize only the large subunit variant. Two appropriate mAbs, SE14 and SC20, were purified and coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden).

Isolation of the Large Collagen XII Variant from Fibroblast Conditioned Medium

Collagen XII was purified from culture media of skin fibroblasts from 11-d-old chick embryos by mAb C19 affinity chromatography as described (Koch et al., 1992). To separate large from small collagen XII variant, the eluate from the C19 column was dialyzed against TBS and applied to either a mAb SE14 or SC20 affinity column. Molecules with large subunits were retained on the column and eluted with 0.1 M triethylamine, pH 11.5. Fractions were immediately neutralized with 1 M Tris/HCl, pH 8.

Isolation of the Small Collagen XII Variant from Adult Chick Skin

Collagen XII extracted from adult chick skin was performed as described by Lunstrum et al. (1991) with the following modifications: the skin of the wings was removed and homogenized in wash buffer (20 mM Tris-HCl, pH 7.4; 1 mM PMSF, 2.5 mM NEM). After centrifugation the pellet was homogenized in extraction buffer (200 mM NaCl, 10 mM EDTA, 25 mM Tris-HCl, pH 7.8; 1 mM PMSF, 2.5 mM NEM) and extracted at 4°C overnight. The supernatant was filtered, DEAE-cellulose (20 g per 300 ml extraction buffer) was added, and the mixture stirred for 1 h. After centrifugation, the supernatant was applied to CL-4B-Sepharose and gelatine-Sepharose precolumns (Pharmacia), respectively, followed by the mAb C19 affinity column. After washing with 0.1% Triton X-100, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.6, collagen XII was eluted with high pH as described above. To remove the large variant, the preparation was neutralized and absorbed on a mAb SE14-Sepharose column. Collagen XII in the flow through consisted of pure small subunits.

Preparation of Polyclonal Antiserum

Rabbit antisera 522 against chick fibroblast collagen XII and 245 against chick laminin have been characterized before (Brubacher et al., 1991; Koch et al., 1992). Affinity-purified polyclonal antibody 522 is specific for type XII collagen and does not cross-react with collagen XIV (Wälchli et al., 1994). A specific antiserum against the large subunit variant of collagen XII was generated by digesting with α -chymotrypsin (20 μ g/ml) for 1 h at 37°C. Fragments were passed first over the mAb C19 affinity column and then over the mAb SE14 column. A major band on a Coomassie stained blot (54 kD, see Fig. 4 *i*, arrow) reacting with mAb SE14 was cut out and a rabbit antiserum was prepared as described (Koch et al., 1992). Antiserum 155 was absorbed on small collagen XII coupled to Sepharose, and then affinity-purified on large collagen XII-Sepharose.

Immunofluorescence Staining

Cryosections of chick embryos of the appropriate stage (11–18-d-old) were labeled with affinity-purified polyclonal antibody (6 μ g/ml) as described (Brubacher et al., 1991).

Electron Microscopy

Protein samples were rotary shadowed as described (Koch et al., 1992). For immunoelectron microscopy, collagen I fibrils were prepared in the presence or absence of collagen XII as described below, collected by centrifugation and washed with 90 mM NaCl, 30 mM Na/K-phosphate, pH 7.4, for 5 h. The collagen pellets were fixed in 4% paraformaldehyde in PBS for 1 h and washed with PBS. Pellets were incubated with affinity-purified polyclonal anti-collagen XII antibody 522 (4 μ g/ml in PBS containing 0.3% BSA) for 6 h followed by several washes. Gold-labeled goat anti-rabbit IgG (10-nm colloidal gold particles; diluted 1:30 in PBS containing 0.3% BSA; Biocell Research Ltd., Cardiff, GB) was applied for 6 h, and after washing the pellets were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde in PBS for 1 h. Pellets were postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon. Ultrathin sections were cut with a Sorvall Ultratom (Du Pont Co., Newton, CT) and stained with uranyl acetate and lead hydroxide.

Ultrathin frozen sections were prepared from chicken skin fixed in 4% formaldehyde, 0.5% glutaraldehyde in PBS for 16 h at 4°C. Samples were infiltrated with 2.1 M sucrose in PBS for 1 h on ice and frozen in liquid nitrogen. Sections were cut at -110°C with a diamond knife (Diatome, Ltd.,

Bienne, Switzerland) in a FC-4 cryoattachment unit on a Ultracut E microtome (Reichert Jung, Vienna, Austria). They were mounted on Formvar/carbon coated copper grids. Ultrathin cryosections were floated on 0.1 M NH₄Cl in PBS for 5 min, then on 1% BSA and 1% ovalbumin (Boehringer, Mannheim, Germany) in PBS for 10 min. Staining was performed with polyclonal antibody 155 (10 µg/ml in 1% BSA, and 1% ovalbumin in PBS) and gold-labeled goat anti-rabbit IgG as above. Sections were contrasted with 0.2% uranyl acetate and embedded in 2% Methocell (25cP; Fluka AG, Buchs Switzerland) according to Griffith et al. (1984).

Cloning of cDNA Specific for the Large Form of Type XII Collagen by PCR

A cDNA fragment specific for the large splice variant of chick type XII collagen was amplified by PCR from a λgt11 cDNA library of 10-d-old chick embryos (Clontech, Palo Alto, CA). The sequence of the primers was taken from Yamagata et al. (1991; EMBL accession number D00824; 5' primer, nucleotides 2271–2295, 3' primer, nucleotides 3798–3820). With these primers the desired cDNA fragment (1.5 kb) was amplified with the Taq polymerase kit (Boehringer Mannheim). The cDNA fragment XII(lg) was purified and subcloned into pBluescript[®] KS+ vector (Stratagene Inc., La Jolla, CA) yielding plasmid pKXII(lg). The construct was sequenced to confirm identity and orientation.

Northern Blotting

Total RNA from various chick tissues of embryonic day 11, 14, and 18 was isolated as described (Chomczynski and Sacchi, 1986). 10 µg of total RNA per sample were separated on a gel and transferred to Zeta-Probe[®] GT membranes (Bio-Rad Labs., Richmond, CA) according to Sambrook et al. (1989). The plasmids pKXII(lg) and pKXII(2B) (Trueb and Trueb, 1992; subcloned into pBluescript[®] vector) were linearized. Synthesis of anti-sense RNA and labeling with digoxigenin-dUTP, hybridization on the membranes with 50 ng/ml labeled RNA, and detection of mRNA were performed with the DIG RNA labeling and DIG luminescence detection kit from Boehringer Mannheim.

In Situ Hybridization

Cryosections (10 µm) were prepared as described above. After fixation (4% paraformaldehyde in PBS, 20 min) followed by washes in PBS, the sections were digested with 2 µg/ml proteinase K (Serva Biochemicals, Paramus, NJ) in 20 mM EDTA, 0.1 M Tris-HCl, pH 8, for 30 min. Digestion was stopped with 0.2 mg/ml glycine in PBS for 1 min, and slides were fixed with 4% paraformaldehyde in PBS for 30 min. Sections were acetylated for 10 min in 0.25% acetic anhydride, 0.1 M triethanolamine, pH 8.0. The slides were prehybridized with 350 µl hybridization buffer (50% formamide, 4× SSC, 2× Denhardt's [Sambrook et al., 1989], 4% dextran sulfate, and 1 mg/ml *Escherichia coli* tRNA) in a moist chamber for 5 h at 20°C.

Labeled RNA probes (see Northern blotting) were hydrolyzed to a final length of 0.1 kb (Cox et al., 1984). 40 µl probe (200 ng/ml; denatured at 80°C for 3 min; chilled on ice) was applied per slide, covered with a coverslip, sealed, and incubated at 68°C for 24 h. Slides were washed in 0.2× SSC at 60°C for 30 min followed by digestion with RNase A (10 µg/ml) in 2× SSC for 5 min at 37°C. Slides were submerged with 50% formamide in 2× SSC for 30 min and then equilibrated in 0.2× SSC at 20°C.

For detection of the DIG labeled RNA, the DIG DNA labeling and detection kit of Boehringer Mannheim was used. To achieve low background, slides were washed overnight. After development, sections were washed with water, dehydrated, and mounted in DePeX (BDH Chemicals Ltd., Pool3e, England).

Binding of Type XII Collagen to Heparin

Large collagen XII, small collagen XII, and the corresponding collagenase treated samples (Koch et al., 1992; further purified on a mAb C19 affinity column) were dialyzed against 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, and applied onto a 500-µl heparin-Sepharose CL-6B column (Pharmacia). After three washes with loading buffer (each 500 µl), the column was eluted stepwise with increasing salt concentration (0.2, 0.3, 0.4, and 0.5 M NaCl in 20 mM Tris-HCl, pH 7.4; each 500 µl).

Sequencing of Heparin-binding Peptides

Heparin-binding fragments were isolated from a chick skin collagen XII preparation that was partially degraded by endogenous proteases during

storage at 4°C for 2 mo. The sample was passed over heparin-Sepharose. Retained fragments were eluted with 0.5 M NaCl, 20 mM Tris-HCl, pH 7.4, lyophilized, separated by SDS-PAGE, and blotted onto Immobilon[™] membrane. A 60- and 20-kD fragment were cut out from the membrane. The NH₂-terminal amino acid sequence of the two fragments was determined by automated repetitive Edman degradation by Dr. Paul Jenö at the Biocenter facility.

Collagen Fibrillogenesis Assay

Collagen I from rat tail (Serva collagen R; 2 mg/ml stock solution in 0.1% acetic acid) was used and checked for contaminations as follows. First, bacterial collagenase completely digested all bands visible on Coomassie-stained SDS-PAGE, including γ-components and larger oligomers. Second, no glycosaminoglycan could be detected by alcian blue staining. Third, no reactivity was found with anti-bovine decorin antiserum obtained from Dr. D. Heinegard, Uppsala (data not shown). The fibrillogenesis assay was performed at a scale of 150 µl in Eppendorf tubes precoated with 3 mg/ml BSA in PBS. Type XII collagen and laminin were dialyzed against buffer (90 mM NaCl, 30 mM Na₂HPO₄/KH₂PO₄, pH 7.4) and centrifuged for 10 min at 15,000 g before use. Buffer and proteins were mixed to give the following final concentrations: 50–180 mM NaCl, 30 mM Na/K phosphate buffer, pH 7.4; 270 µg/ml collagen I; 100 µg/ml BSA; and 30 µg/ml collagen XII or EHS laminin (Paulsson, 1987). Fibrils were allowed to assemble at 30°C for 6 h. In some experiments, collagen XII was added to preformed collagen I fibrils for 6 h. The mixtures were centrifuged at 15,000 g for 10 min and pellets and supernatants were analyzed by nonreducing SDS-PAGE followed by Coomassie staining. For quantification of collagen XII, Coomassie stained gels were scanned with a densitometer (300A; Molecular Dynamics, Sunnyvale, CA). The uppermost collagen XII band (homotrimers of the large subunits) was measured. The background was determined for each lane in an area above the selected protein band and subtracted. For each band the integrated density was determined six times and averaged. The combined integrated protein density in pellet plus supernatant never differed by more than 10% between samples.

Results

Monoclonal Antibodies Specific for the Large Subunit of Collagen XII

Previously we characterized a large collagen XII variant (subunit size 320–350 kD depending on glycosylation) from chick fibroblast conditioned medium (Koch et al., 1992; for a model of variants see Fig. 1). In addition, a minor amount of small collagen XII variant with subunits of 220 kD is synthesized by these cells in culture. The mAb C19, which detects both splice variants of chick collagen XII (Koch et al., 1992; see Fig. 1), produced a complicated staining pattern on an immunoblot of fibroblast conditioned medium. Under nonreducing condition mAb C19 labeled four bands (Fig. 2 a), the composition of which is explained below (see Fig. 3). In the presence of reducing agent at least five bands could be distinguished (Fig. 2 b). These are the small variant with a molecular mass of 220 kD, the large glycosaminoglycan free variant (320 kD), the large subunit with glycosaminoglycan side chains (350 kD), as well as nonreducible dimers of small and of large subunits (440–700 kD), respectively (Koch et al., 1992; Lunstrum et al., 1992).

To investigate the different expression and function of the two major collagen XII splice variants, we needed a mAb specific for the extra domain of the large variant. The screening of a previously obtained hybridoma library (Koch et al., 1992) revealed two mAbs named SE14 and SC20 (Fig. 1), which reacted with all bands containing large collagen XII subunits (Koch et al., 1992), but did not

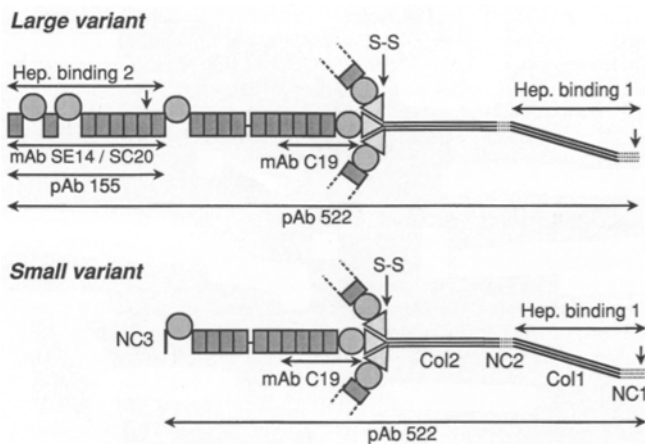


Figure 1. Model of the two splice variants of collagen XII as predicted from their cDNAs (Yamagata et al., 1991; Trueb and Trueb, 1992). The NH₂ terminus is to the left. Collagenous (*Coll*, and *Col2*) and noncollagenous (*NC1*, *NC2*, and *NC3*) domains are indicated. Homologies to vWF A domains (circles), to FN III repeats (squares), to NC4 domains of collagen IX (triangles), and the triple-helical collagenous domains (triple lines) are drawn. The location of interchain disulfide bridges in NC3 is shown (S-S). Chondroitin sulfate side chains can be attached to the extra domain in NC3 of the large collagen XII variant. Binding regions of the polyclonal (*pAb 522* and *pAb 155*) as well as of the monoclonal antibodies (*mAb C19*, *mAb SE14*, and *mAb SC20*) are marked. In addition, the heparin-binding regions (*Hep. binding 1* and *2*) are depicted.

detect the small 220-kD variant on a Western blot (Fig. 2 *d*; SC20 not shown), in contrast to mAb C19 (Fig. 2 *b*, lower arrow). Without reducing agent, only three instead of four bands were visible with mAb SE14 (Fig. 2 *c*; for explanation see Fig. 3).

Heterotrimeric Composition of Collagen XII

On immunoblots under non-reducing conditions, mAb C19 recognizes four distinct collagen XII bands, whereas mAb SE14 detects the three larger of the four bands (Fig.

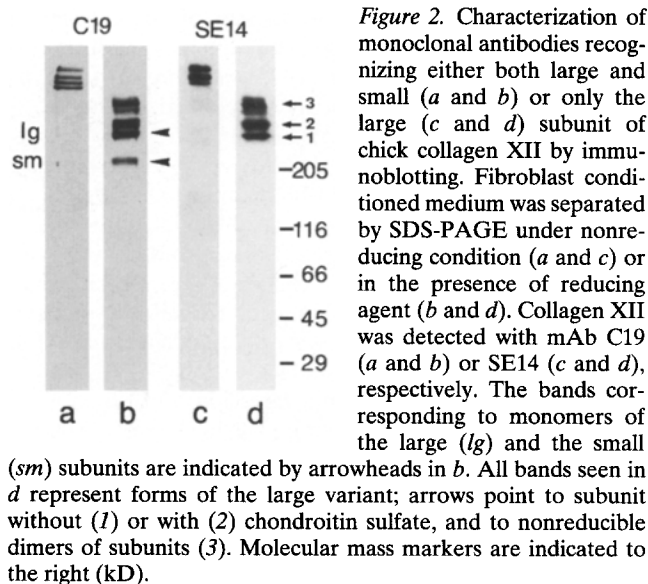


Figure 2. Characterization of monoclonal antibodies recognizing either both large and small (*a* and *b*) or only the large (*c* and *d*) subunit of chick collagen XII by immunoblotting. Fibroblast conditioned medium was separated by SDS-PAGE under nonreducing condition (*a* and *c*) or in the presence of reducing agent (*b* and *d*). Collagen XII was detected with mAb C19 (*a* and *b*) or SE14 (*c* and *d*), respectively. The bands corresponding to monomers of the large (*Ig*) and the small (*sm*) subunits are indicated by arrowheads in *b*. All bands seen in *d* represent forms of the large variant; arrows point to subunit without (*1*) or with (*2*) chondroitin sulfate, and to nonreducible dimers of subunits (*3*). Molecular mass markers are indicated to the right (kD).

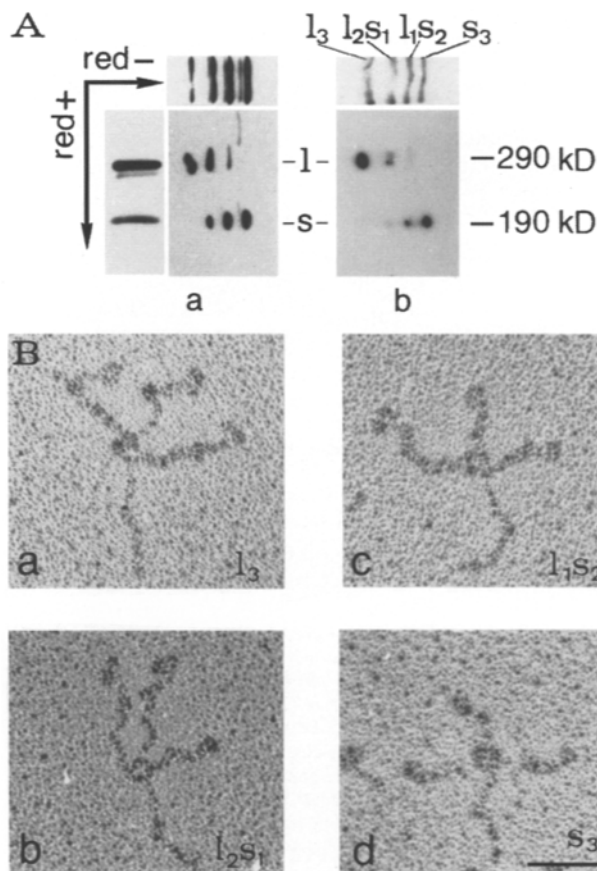


Figure 3. Homo- and heterotrimeric composition of chick collagen XII as detected by two-dimensional SDS-PAGE and electron microscopy. Fibroblast conditioned medium (*A, a*) and salt extract of embryonic gizzard (*A, b*) were treated with collagenase and chondroitinase and separated by SDS-PAGE in the first dimension under nonreducing conditions. The four bands stained with mAb C19 on an immunoblot (*A, top*) fell apart after reduction in the second dimension into various proportions of small (*s*) and large (*l*) subunits (*A, bottom*). The putative subunit compositions of trimers are indicated at the top of *A, b* (*l*₃, three large subunits; *l*₂*s*₁, two large and one small subunit; *l*₁*s*₂, one large and two small subunits; *s*₃, three small subunits). Affinity-purified collagen XII from fibroblast conditioned medium was viewed in the electron microscope (*B*). Four types of molecules were identified that represent various combinations of large and small subunits. In *B*, a large homotrimer (*a*) and a small homotrimer (*d*) are shown. Heterotrimers with two large and one small subunit (*b*) and with one large and two small subunits (*c*), respectively, are depicted as well. (Bar, 50 nm).

2, *a* and *c*). What is the exact nature of these four species? Because glycosaminoglycans are attached to a fraction of large subunits, the four bands might differ in glycosylation. Alternatively, they might be due to the formation of heterotrimers between large and small subunits. To avoid the problems of covalent dimer formation and glycosaminoglycan heterogeneity, collagen XII samples were digested with chondroitinase ABC and collagenase. Nonreducible covalent bonds of dimers are lost after collagenase digestion while interchain disulfide bridges are maintained (Koch et al., 1992). After enzyme treatment, samples were separated by two-dimensional SDS-PAGE (nonreduced vs. reduced) and noncollagenous, glycosaminoglycan-free

NC3 domains of collagen XII variants were identified by immunoblotting with mAb C19. Under nonreducing condition four disulfide linked, trimeric collagen XII bands were detected (Fig. 3 A, a). The four nonreduced trimeric bands fell apart into the NC3 domains of their subunits after reduction in the second dimension (Fig. 3 A, a). The heaviest and the lightest band consisted of only large (290 kD) or small (190 kD) NC3 domains, respectively, whereas the intermediate two bands were built up from combinations of large and small subunits. Collagen XII molecules are therefore partially subunit homotrimers and partially heterotrimers. To exclude a culture artifact, proteins were isolated from chick embryo tendons by salt extraction and analyzed. The same result was found as for fibroblast conditioned medium (Fig. 3 A, b). For confirmation collagen XII molecules purified from fibroblast culture were viewed by electron microscopy. Rotary shadowed three-armed particles showed heterogeneous but interpretable composition. In Fig. 3 B, a, a large collagen XII molecule is visible with three long flexible arms (NC3 domains) and a thinner collagenous domain with a kink. At lower frequency (depending on the preparation) molecules were found with three shorter NC3 domains, i.e., with only small subunits (Fig. 3 B, d). In addition, heterotrimeric molecules were also identified that had two large and one small (Fig. 3 B, b) or one large and two small (Fig. 3 B, c) subunits, respectively.

Separation of Collagen XII into Large and Small Variants and Generation of a Polyclonal Antibody Specific for the Large Variant

Because the content of small collagen XII variant in fibroblast conditioned medium is relatively low, chick skin was selected as a richer source. Salt extract was applied to a mAb C19 affinity column, and mainly small collagen XII variant was eluted. The minor fraction of large variant was removed by passing the protein samples over a mAb SC20 or SE14 affinity column. The flow through contained isolated small variant of collagen XII consisting of a homogeneous population of subunit trimers as revealed by electron microscopy (not shown) and on nonreducing SDS-PAGE, although it had an anomalously slow migration rate (Fig. 4 c). In the presence of reducing agent, the trimers resolved into bands of 220 kD (monomers) and of 440 kD (nonreducible dimers) (Fig. 4 d). On an immunoblot, this material reacted only with mAb C19 (Fig. 4 f) and not with mAb SE14 (Fig. 4 h), confirming that it was composed of only small subunits. A preparation of collagen XII consisting of mainly large subunit variant was isolated from chick fibroblast conditioned medium with the aid of mAb C19. To remove contaminating small variant, the protein was applied to a mAb SC20 affinity column. The eluate was mainly composed of large subunits (Fig. 4 b; monomers and dimers), but still contained a minor amount of small 220-kD variant (see previous paragraph, Fig. 3).

The two mAbs SE14 and SC20 specific for large collagen XII did not give satisfactory results when used for immunofluorescence. Therefore a polyclonal antibody directed against a specific proteolytic fragment of the large variant (Fig. 4 i) was generated (see Materials and Methods). When tested on immunoblots, polyclonal antibody

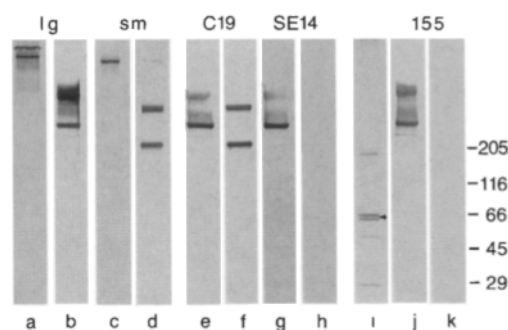


Figure 4. Analysis of affinity purified collagen XII on silver stained SDS-PAGE gels (a–d) and by immunoblotting (e–h; j, k) and preparation of fragments from the extra domain of the large collagen XII variant used for immunization (i). Large (lg) collagen XII variant isolated from fibroblast conditioned medium yields three bands under nonreducing (a) and one sharp and two more fuzzy bands under reducing conditions (b). Small (sm) collagen XII variant from skin exhibits one single band under nonreducing (c) and two bands under reducing conditions (d). For testing the purity of both proteins, large (e and g) and small (f and h) variants were probed after reduction on an immunoblot with mAb C19 (e and f) or mAb SE14 (g and h), respectively. Fragments derived from the extra domain of the large collagen XII variant were purified on a mAb SE14 column (i, silver stained). Bands indicated by an arrow were chosen as antigen (see Materials and Methods). On an immunoblot, the obtained polyclonal antibody 155 recognizes only the large (j) but not the small (k) collagen XII variant. Molecular mass markers are indicated to the right (kD).

155 reacted with the large (Fig. 4 j) but not with the small variant (Fig. 4 k).

Differential Expression of the Collagen XII Splice Variants

Expression of the two collagen XII mRNA splice variants in different tissues during development was investigated by Northern blotting. Total mRNA was isolated from skin, gizzard, and breast muscle of day 11, 14, and 18 embryos. Collagen XII mRNAs were detected with a chick collagen XII antisense probe that hybridizes to common sequences. Two bands of 10 and 7 kb (Fig. 5 A) represent the two splice variants of collagen XII (Wälchli et al., 1994). The 10 kb but not the 7 kb mRNA band was also detected with an antisense probe specific for the extra sequences found only in the large variant (Fig. 5 A, 11*). In skin of chick embryos mainly the small form is expressed, and with development the signal decreases (Fig. 5 A). The ratio between mRNAs for the small and the large variant was measured to be 3:1 in this tissue (Fig. 5 B). In gizzard both collagen XII mRNA variants are expressed, but in contrast to skin the signal increases at later stages (Fig. 5 A), and the two mRNAs are present at a ratio of 1:1 (Fig. 5 B). Collagen XII mRNA is also more abundant in breast muscle of 18- than of 11-d-old chick embryos (Fig. 5 A), and the variant ratio is intermediate (Fig. 5 B). The large mRNA variant was never more predominant than the small; its highest proportions were found in embryonic gizzard (Fig. 5 B) and tendon (not shown).

To look at the tissue distribution of collagen XII protein and mRNA, immunofluorescence labeling and in situ hy-

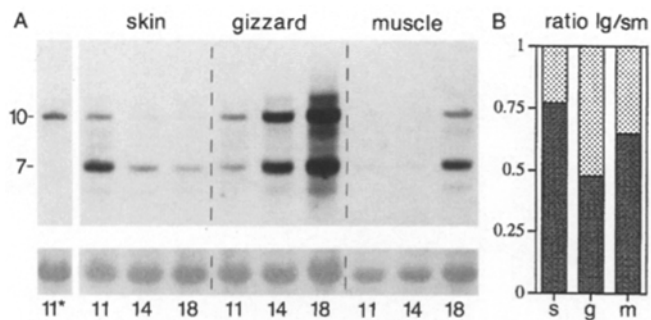


Figure 5. Northern blot analysis of collagen XII mRNA in chick embryonic tissues. Total RNA from skin, gizzard, and muscle of different stages (11, 14, and 18 d of development) was resolved on an agarose gel and transferred to nylon membranes. Blots were hybridized with digoxigenin labeled antisense RNA. In the first lane of *A* (11*), RNA from 11 day old embryonic chick skin was hybridized with probe XII(1g) specific for the extra domain of the large collagen XII splice variant; only the 10 kb mRNA is detected. The other lanes in *A* were hybridized with probe XII(2B) recognizing both splice variants. At the bottom, methylene blue stained ribosomal 18S RNA in each lane is shown, indicating that comparable amounts of total RNA were loaded. The size of collagen XII mRNAs relative to markers are indicated to the left (kb). In *B*, the mRNA ratio for the two splice variants was determined by densitometric scanning of the blots shown in panel *A*. The upper part of the bars represents the relative amount of mRNA for the large, and the lower of mRNA for the small form (*s*, skin; *g*, gizzard; *m*, muscle; 18 d of incubation).

bridization were performed on 14-d-old chick embryonic dorsal skin and 18-d-old embryonic breast muscle. In 14-d-old embryonic skin the feather buds have developed. Total collagen XII protein is concentrated in the upper layer of the dermis and also in the subcutis that links the dermis to the back muscles. By immunofluorescence with antibody 522, fibrillar components in the upper layer of the dermis, underneath feather buds, and in the subcutis were stained (Fig. 6 *b*). The distribution of the large form of the protein as detected with antibody 155 is more restricted since it was found mainly at the base of the feather buds (Fig. 6 *a*).

In 18-d-old skeletal muscle, total collagen XII protein was detected in tendon, perimysium (around muscle fiber bundles), and in blood vessel walls (Fig. 6 *e*). Immunofluorescence for the large variant was restricted to tendon and blood vessels whereas in the perimysium no labeling was observed (Fig. 6 *d*). By in situ hybridization with antisense RNA, collagen XII mRNA was found mainly in fibroblasts in a similar distribution as the protein (Fig. 6, *g* and *h*). Again, the small (Fig. 6 *h*) but not the large (Fig. 6 *g*) mRNA variant was found in perimysium fibroblasts. Other tissues and stages (6-d-old embryos; bone, tendon, gizzard, and heart of 11–18-d-old embryos) were also examined, but the distribution was found to be similar for both variants.

Differential Binding of the Collagen XII Variants to Heparin

Heparin-binding regions are putative interaction sites of ECM proteins with proteoglycans. To test whether collagen XII variants bind to heparin, isolated large and small forms were dialyzed against 100 mM NaCl and applied to

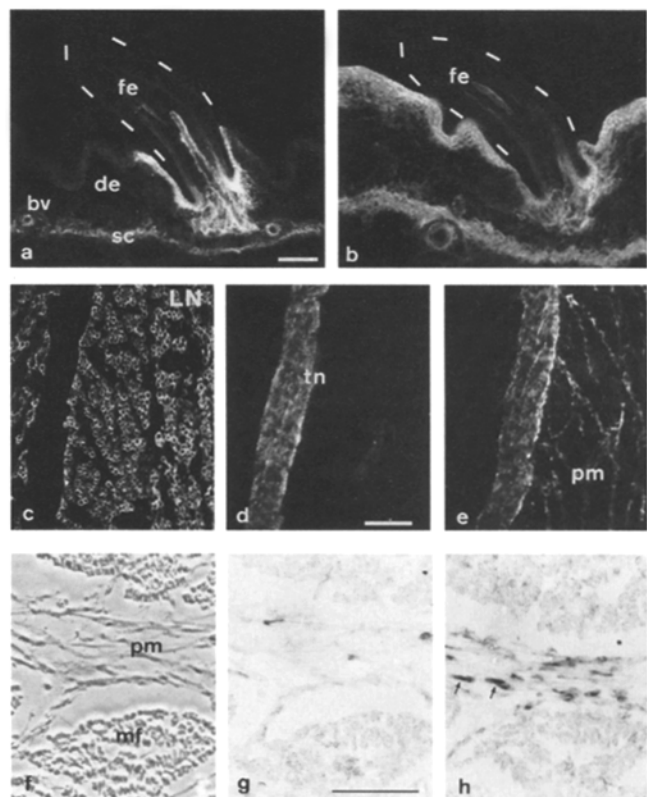


Figure 6. Immunofluorescence staining and in situ hybridization of chick embryonic tissues with probes specific for collagen XII. Serial sections of 14-d feather buds (*a* and *b*) and 18-d breast muscle (*c*–*h*) were prepared. The sections (*b* and *e*) were stained with antibody 522 that recognizes both subunit variants of collagen XII. Antibody 155 is used to localize the large variant of collagen XII (*a* and *d*). In picture *c* the section was stained with anti-laminin antiserum 245 as control. This antibody stains the muscle fiber basement membranes (*c*). In situ hybridization performed with antisense RNA probe recognizing both collagen XII splice variants, XII(2B), is shown in *h*. The antisense RNA probe XII(1g) was used as a probe specific for the mRNA of the large variant (*g*). In addition a phase contrast picture of *g* is shown (*f*). *fb*, feather bud; *de*, dermis; *sc*, subcutis; *mf*, muscle fibers; *tn*, tendon; *bv*, blood vessel; *pm*, perimysium. Bar, 100 μ m.

a heparin–Sepharose column. Both collagen XII variants quantitatively bound to the column under these conditions (Fig. 7, *A* and *B*). However, the small variant was eluted already at 200 mM NaCl, whereas 300–400 mM NaCl was needed to release the large variant from the column. To localize binding sites in the small collagen XII variant, proteolytic fragments were applied to a heparin–Sepharose column in 100 mM NaCl. Two ~140-kD bands appeared in the flow through, whereas a 50-kD fragment eluted from the column with 200 mM NaCl (Fig. 7 *C*). The NH₂-terminal sequence of this fragment was determined to be XATSTXPLIYL, i.e., amino acids 2507–2517 of the chick collagen XII sequence (Yamagata et al., 1991). A smaller heparin-binding fragment of 20 kD (not shown in Fig. 7) was also sequenced. Its NH₂ terminus was NQGPXG-PXGP (amino acids 2943–2953), locating it within the larger 50-kD fragment and at the COOH terminus (Col1 and NC1) of collagen XII. A simple way to test whether this is the only heparin-binding region in small collagen

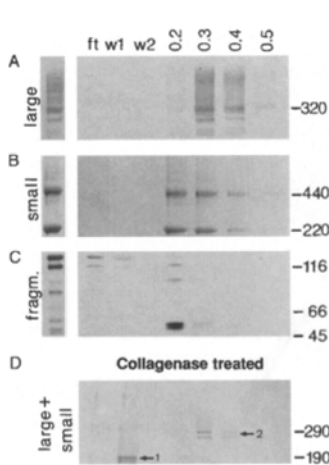


Figure 7. Differential binding of collagen XII variants to heparin. Affinity-purified large collagen XII variant (A), small variant (B), and proteolytic fragments of the small variant (C) were applied to a heparin-Sepharose column in 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4. After collecting the flow through (ft) and two washes (w1 and w2) the columns were eluted by increasing the salt concentration stepwise from 0.2 to 0.5 M NaCl (0.2–0.5). Eluted fractions were analyzed on silver stained SDS-PAGE. Molecular masses are indicated to the right (kD). In D a mixture of small and large collagen XII was digested with collagenase and passed over heparin-Sepharose. Truncated small variant (1) appeared in the wash, while digested large variant (2) was eluted with 0.3–0.4 M NaCl.

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XII was to digest the protein with bacterial collagenase, before applying it to heparin-Sepharose. Collagenase-treated small collagen XII did not bind to the column in 100 mM NaCl and was found in the flow through (Fig. 7 D, 1). This result confirms that one heparin-binding site is located within 20 kD from the COOH terminus of the molecule and within the region common to both sequenced peptides. In contrast to the truncated small variant, collagenase treated large variant still bound to heparin (Fig. 7 D, 2) and eluted at the same salt concentration (300–400 mM) as undigested large collagen XII. Fig. 8 D shows an experiment where a mixture of digested small and large variant was passed over the heparin column, but results were also confirmed by applying either collagenase-treated variant alone. This indicated the presence of additional heparin-binding sites within the extra NC3 domain of the large subunit.

Interaction between Collagen XII and Collagen I Fibrils

Collagen XII and XIV have been shown to be located on collagen fibrils in vivo by immunogold labeling of tissue before embedding and sectioning (Keene et al., 1991). This technique can potentially lead to artifacts like redistribution of antigen during antibody incubation. Therefore, we directly stained ultrathin cryosections of embryonic chick tendon and skin with antibodies against collagen XII. Confirming the published results, gold particles were found on collagen fibrils with antibody 522 (not shown). To localize the large form of collagen XII, polyclonal antibody 155 was used and showed a labeling pattern similar to antibody 522 (Fig. 8, a and b). The nature of the interaction between collagen XII and collagen I fibrils in tissues is unknown. To test whether collagen XII could coassemble with interstitial collagens during fibrillogenesis, we allowed acid soluble collagen I to aggregate in the presence of collagen XII. In a second experiment collagen XII was incubated with preformed collagen I fibrils. For analysis of incorporation, collagen I fibrils were washed to remove unbound collagen XII, collected by centrifugation

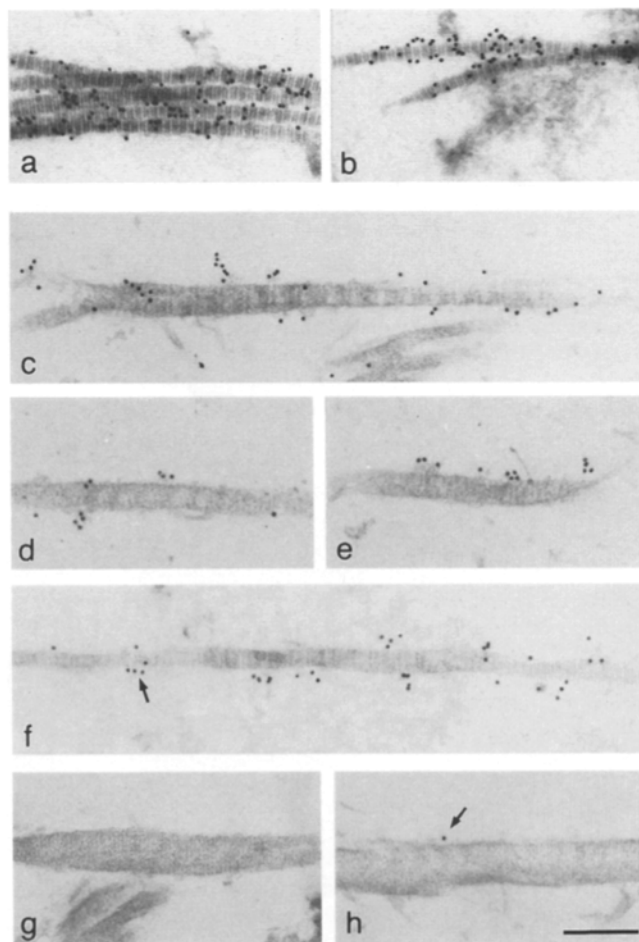


Figure 8. Colocalization of collagen XII with collagen I fibrils in embryonic skin in vivo (a and b) and after copolymerization in vitro (c–h). Ultrathin cryosections were prepared from skin of a 14-d-old chick embryo and labeled with antibody 155 followed by gold-labeled secondary antibody. The gold particles on the fibrils reveal the localization of large collagen XII (a and b). In vitro, soluble collagen I was allowed to polymerize into fibrils in the presence of collagen XII. After washing, the fibrils were labeled with antibody 522 and gold labeled secondary antibody, imbedded into Epon and sectioned (c–f). In a second experiment, collagen XII was incubated with preformed collagen I fibrils that were then washed and processed as above (g and h). Arrows point to gold particles associated with fibrils. Bar, 200 nm.

and labeled with antibody 522 followed by gold-labeled secondary antibody. Fibrils formed in the presence of collagen XII were found to be stained with gold particles (Fig. 8, c–f). However, when collagen XII was added to preformed collagen I fibrils, virtually no staining could be detected (Fig. 8, g and h).

Coassembly of collagen XII with collagen I into fibrils was further investigated by biochemical methods. First its salt dependence was determined. Fig. 9 A shows an experiment where collagen XII was mixed with collagen I before fibril formation. After incubation at different NaCl concentrations, the samples were centrifuged, and supernatants as well as precipitates were analyzed (Fig. 9 A). In the absence of collagen I, all collagen XII protein remained in the supernatant. At the lowest salt concentration (90 mM NaCl, 30 mM phosphate), 57% of the applied

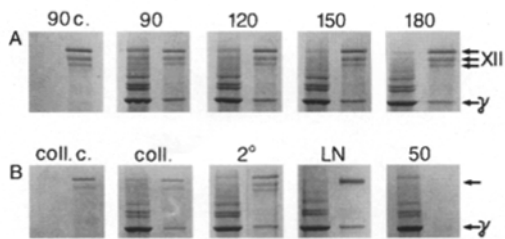


Figure 9. Coprecipitation of collagen XII with assembling collagen I fibrils. Affinity-purified collagen XII from fibroblast conditioned medium was incubated with collagen I during fibrillogenesis in 30 mM Na/K-phosphate, pH 7.4, containing varying concentrations of NaCl. After 6 h at 30°C samples were centrifuged, supernatant and pellet were analyzed by SDS-PAGE under nonreducing conditions, and gels were stained with Coomassie blue. For each sample, the left lane corresponds to the pellet and the right lane to the supernatant. Arrows at right of *A* indicate the three bands of collagen XII homo- and heterotrimers (XII) and the γ -components of collagen I (*g*). The upper arrow in *B* points to either collagen XII or laminin bands. *A* shows the salt dependence of collagen XII incorporation into polymerizing collagen I gels in a single experiment. The first sample is a control with collagen XII alone at a salt concentration of 30 mM phosphate and 90 mM NaCl (*A*, 90 *c.*). In the other four samples, collagen I was present during incubation. The NaCl concentration was increased from 90 mM (90) to 120 mM (120), 150 mM (150), and 180 mM (180). In *B*, all samples except the last were incubated at a salt concentration of 90 mM NaCl and 30 mM phosphate. In the first two samples, collagenase digested collagen XII was incubated either alone (*coll. c.*) or in the presence of collagen I (*coll.*). In the third sample, collagen XII was added to preformed collagen I fibrils and incubated (2°). In the fourth sample of *B*, laminin instead of collagen XII was added to collagen I during assembly (*LN*). The last sample shows coprecipitation of collagen XII with collagen I under low salt conditions, i.e., in 50 mM NaCl, 20 mM Na/K-phosphate, pH 7.4 (50).

collagen XII coprecipitated with collagen I as determined by densitometric scanning of the SDS gel. In an independent experiment, a value of 53% precipitated collagen XII was found. With increasing NaCl concentration (all in 30 mM phosphate), coprecipitated collagen XII decreased to 33% (120 mM NaCl), 23% (150 mM NaCl), and 16% (180 mM NaCl), respectively (Fig. 9 *A*). At lower salt, the percentage of collagen XII coprecipitated with collagen I increased to 82% (65 mM NaCl, 20 mM phosphate; not shown) and 87% (50 mM NaCl, 20 mM phosphate; Fig. 9 *B*), respectively, while without collagen I all collagen XII still remained soluble. In contrast, when collagen XII was added to preformed collagen I fibrils, only about 12% of the protein was coprecipitated in 90 mM NaCl, 30 mM phosphate (Fig. 9 *B*).

To exclude that coprecipitation of collagen XII with collagen I fibrils was an artifact due to physical trapping, mouse laminin was chosen as control, an extracellular matrix protein of similar size and shape. When mixed with aggregating collagen I, almost all laminin remained in the supernatant (Fig. 9 *B*). Furthermore, collagen XII was treated with collagenase to investigate the role of its collagenous domain for fibril association. When incubated with aggregating collagen I under standard conditions, only 20% truncated collagen XII was detected in the pellet in one, and 14% in a second experiment (Fig. 9 *B*). These

experiments argue for a specific interaction between intact collagen XII and collagen I during fibril assembly.

Discussion

The fascinating domain structure of collagen XII suggests multivalent functions in cell and matrix binding. In analogy to the related collagen IX in cartilage (Vaughan et al., 1988), it has been proposed that the small collagenous domains of collagens XII and XIV interact with the surface of interstitial collagen, whereas the very large NC3 domains reach out to interact with other ECM components and cell surfaces (Shaw and Olsen, 1991). However, there is little published evidence so far to support this model. For the more extensively studied collagen XIV, no cell binding activity could be detected (Brown et al., 1993). Moreover, binding to collagen VI but not to collagens I, III, and V was found in solid phase binding assays (Brown et al., 1993). These results were in contrast to the reported decoration of interstitial collagen fibrils in fetal skin by antibodies to collagen XII and XIV as observed with the electron microscope (Keene et al., 1991). Since the latter findings were obtained by incubating the tissue with antibody before embedding and sectioning, one could argue that collagen XII and XIV were redistributed during sample preparation. To eliminate such doubts, we used here a postembedding labeling technique by incubating ultracryosections with antibody, and demonstrate that both large and small collagen XII are true fibril-associated collagens *in vivo*.

The discrepancy between the *in vivo* and the *in vitro* data concerning the interaction of collagens XII and XIV with fibrillar collagens might be explained in two ways. First, a third component could mediate binding. Indeed, the NC1/Col1 domain of collagen XIV was shown to interact with the small proteoglycan decorin (Font et al., 1993), which itself is attached to interstitial collagen fibrils (Fleischmajer et al., 1991). A second, not mutually exclusive possibility is that collagens XII and XIV are able to coassemble with interstitial collagen monomers into fibrils, but that they exhibit little affinity to preformed collagen fibrils. The results presented here support this possibility: when mixed with collagen I monomers at neutral pH and close to physiological ionic strength, a considerable proportion of collagen XII became incorporated into assembling collagen fibrils. This could be observed both biochemically and ultrastructurally. Collagen XII was not simply trapped during fibril formation because the interaction was very salt sensitive, and because other proteins of similar size and shape such as laminin were not coprecipitated with fibrils. Collagen XII lacking its collagenous tail, which makes up only a few percent of its mass, was hampered in its incorporation into collagen I fibrils, indicating a specific interaction via this domain. It seems puzzling that a slight increase in the salt concentration abolishes the association between collagen XII and collagen I; however, this is in accordance with the fact that collagen XII (at least the small form) can be extracted from tissues with only 200 mM NaCl (Dublet et al., 1989). It is known that interstitial collagen fibrils are assembled in tight pockets on the cell surfaces of fibroblasts (Birk et al., 1989). Presumably, the assembly conditions are tightly controlled by the cells in

vivo, allowing incorporation of collagen XII and XIV into nascent collagen fibrils. Like other minor collagens (Birk et al., 1990), FACIT collagens might influence fibril parameters such as their diameter. We consistently observed that collagen I fibrils formed in the presence of collagen XII had a smaller diameter (see Fig. 9).

Whereas both large and small variants of collagen XII associate with interstitial collagen fibrils, their structure suggests distinct interactions with other extracellular ligands. Separation of collagen XII variants for such studies was complicated by our finding that not only homo-, but also heterotrimers of subunits exist, i.e., there are four instead of only two protein variants. Using antibodies specific for the alternatively spliced domain, we obtained pure homotrimers of small subunits, whereas preparations of large variant were always "contaminated" with a minor fraction of small subunits. Heterotrimers could be identified by two-dimensional SDS-PAGE, and molecules with smaller NC3 domains were observed in preparations of large collagen XII by electron microscopy here and earlier (Koch et al., 1992; Watt et al., 1992). It seems unlikely that the observed heterotrimers arise proteolytically from the large homotrimers. By digestion of large collagen XII with various proteases, we have never observed a stable fragment of the size of the small subunit. Instead, a major cleavage site is found within the constant domain, producing a 150-kD fragment (Koch et al., 1992). Although heterotrimers of collagen XII subunits clearly exist, homotrimers are more abundant, however. This indicates that either homotrimers are preferentially assembled by cells synthesizing both subunit variants, or that individual cells synthesize mostly one variant at a time. Splice variants of the related extracellular matrix protein, tenascin, almost exclusively form subunit homo-oligomers (Chiquet et al., 1991).

Several extracellular matrix proteins such as fibronectin (Bober Barkalow et al., 1991), laminin (Yurchenko et al., 1993), and tenascin (Fischer et al., 1995) use specific sites to bind to the glycosaminoglycan heparin, and to the related heparin sulfate chains of certain matrix and cell surface proteoglycans (see Salmivirta et al., 1991). Heparin binding was therefore chosen to reveal possible differences in ligand binding between collagen XII variants. Interestingly, we found one heparin-binding region common to both large and small collagen XII subunits, whereas a second binding site is specific for the large variant. NH₂-terminal sequencing of heparin-binding fragments indicated that the binding region shared between variants must be located near to the COOH terminus of the collagen XII subunit, presumably in the NC1 and/or the Col1 domain. Affinity of this site for heparin seems to be relatively low because binding is abolished by increasing the salt concentration to only 0.2 M NaCl. In collagen XIV, a sequence near to the COOH terminus, -FRRKIAKRS-, fits a postulated heparin-binding consensus motif of basic flanked by hydrophobic amino acids (Cardin and Weintraub, 1989). This region has been implicated in binding of collagen XIV to heparin and heparan sulfate proteoglycan (Brown et al., 1993), as well as to the dermatan sulfate chain of decorin (Font et al., 1993), which in turn is found on the surface of interstitial collagen fibrils (Fleischmajer et al., 1991). A similar basic sequence, -SRKAKRKP, is

found at the very COOH terminus of the collagen XII subunit. Whilst the basic COOH-terminal sequences in collagen XII and XIV probably bind heparin *in vitro*, they might be involved in the direct or indirect interaction with collagen fibrils *in vivo*.

We found that collagenase treatment of small collagen XII completely abolished its interaction with heparin. The same has been reported for collagen XIV (Brown et al., 1993). In contrast, the large variant of collagen XII bound strongly to heparin whether or not it contained an intact collagenous tail. The additional heparin-binding site that must be located in the extra domain of large collagen XII resists higher salt concentrations (0.3–0.4 M NaCl) for activity, indicating an affinity comparable to the specific sites in other extracellular matrix proteins (Bober Barkalow and Schwarzbauer, 1991; Fischer et al., 1995). We have not yet determined the exact location of this site in the large collagen XII subunit, but will focus on the sequence -LKRLKPRT- in the second most COOH-terminal FN III repeat of the extra domain, (Yamagata et al., 1991), the only other sequence to comply with a putative heparin-binding consensus motif (Cardin and Weintraub, 1989). To date, this is the first evidence for a difference in function between large and small collagen XII splice variants. Ligands in the extracellular matrix or on cell surfaces for the additional heparin-binding site of large collagen XII remain to be identified.

The more restricted expression pattern compared to the small variant is an additional indication for a specialized function of the large collagen XII subunit. The concentration of large variant at the base of feather buds in embryonic dermis is intriguing since it very much resembles the distribution of tenascin (Jiang and Chuong, 1992), an extracellular matrix protein expressed during epithelial-mesenchymal interactions in morphogenesis (Chiquet-Ehrismann et al., 1991). Tenascin synthesis in fibroblasts is induced when they are cultured in a stressed collagen gel (Chiquet-Ehrismann et al., 1994); we observed that synthesis of the large collagen XII variant is also increased under these conditions (see Koch et al., 1992; Chiquet-Ehrismann et al., 1994). The base of a feather bud can be regarded as a dermal joint around which the appendix rotates. Collagen fibrils in this location of the dermis are expected to experience high strain and shear forces. Compared to the small variant, large collagen XII is more extended, has an extra heparin-binding site that is likely to bind proteoglycans, and is itself a proteoglycan. It is tempting to speculate that these additional features make large collagen XII especially suitable as a "shock absorber" between collagen fibrils, similar to proteoglycans in cartilage. The probes specific for large collagen XII that we have generated should allow us to test such a hypothesis.

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