COLLAGEN XII INTERACTS WITH AVIAN TENASCIN-X THROUGH ITS NC3 DOMAIN*

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Large oligomeric proteins often contain several binding sites for different molecules and can therefore induce formation of larger protein complexes. Collagen XII, a multi-domain protein with a small collagenous region, interacts with fibrillar collagens through its C-terminal region. However, no interactions to other extracellular proteins have been identified involving the non-collagenous N-terminal NC3 domain. To further elucidate the components of protein complexes present close to collagen fibrils, different extracellular matrix proteins were tested for interaction in a solid phase assay. Binding to the NC3 domain of collagen XII was found for the avian homologue of tenascin-X that in humans is linked to Ehlers-Danlos disease. The binding was further characterized by surface plasmon resonance spectroscopy and supported by immunohistochemical co-localization in chick and mouse tissue. On the ultrastructural level, detection of collagen XII and tenascin-X by immunogold labeling confirmed this finding.

The integrity of extracellular matrix is maintained by supramolecular networks assembled by a large variety of matrix macromolecules. Among those is the group of 28 different collagen types so far described in the literature. Collagens are further subdivided into several families reflecting their assembly forming properties (1). As a common feature, fibril-associated collagens with interrupted triple helices (FACITs) comprise at least two collagenous domains interrupted by non-collagenous domains. Collagen XII is a member of the FACIT subfamily and its three chains are encoded by a single gene. The two collagenous domains (Col1 and Col2) are interrupted and flanked by three non-collagenous domains (NC1 to NC3), of which the large trimeric NC3 domain contains up to 90% of the molecular mass of collagen XII. The amino-terminal NC3 part of each polypeptide chain consists of two to four von Willebrand factor type A (VWA) domains, several fibronectin type III (FNIII) repeats, and a thrombospondin N-terminal (TSPN) domain (2,3). The size of the protein is variable due to two alternative splice sites located at the 5'- and 3'-end of the collagen XII-mRNA. Especially remarkable is the splicing mechanism which produces mRNA encoding for NC3 domains that differ about 100 kD in mass, denoted as XIIA for the large and as XIIB for the small form (4,5). The other alternative splicing generates two NC1 domains of similar size, termed –1 or –2, which results in the nomenclature of the four different collagen XII isoforms: XIIA-1, XIIA-2, XIIB-1 and XIIB-2, respectively (for overview of the domain structure please refer to Fig 2A) (6). The isoforms differ in their histological and developmental distribution: the large forms XIIA-1 and XIIA-2 are preferably expressed during embryonic stages whereas the expression of the small forms persists in adult tissues (7). Furthermore, the small isoform XIIB-1 predominantly occurs in ligaments and tendons, whereas the XIIB-2 shows a more widespread expression (6). The isoforms also differ in their biochemical properties. The large forms contain an additional heparin binding site in the 7th FNIII domain and, in addition, covalently linked glycosaminoglycan chains are attached to them (4,8), whereas alternative splicing in the NC1 domain leads to an additional heparin binding site in the XIIA/B-1 form (9). Collagen XII also interacts with decorin (10) and is a component of collagen I containing fibrils (8,11).
Tenascins are a distinct family of ECM proteins with four members in vertebrates (TN-C, TN-R, TN-W, and TN-X). They all exhibit a similar domain structure with a N-terminal oligomerization domain, a series of epidermal growth factor (EGF) modules followed by several FNIII domains and a large globular fibrinogen-related domain (FReD) (for review see (12)). Mammalian tenascin-X can associate with collagen I containing fibrils through binding to the glycosaminoglycan chains of the small fibril-associated proteoglycan decorin (13-15). In addition, cells in culture adhere to tenascin-X through interaction with integrin receptors (16). The first indication for the function of tenascin-X in vivo was derived from the finding that human patients lacking the protein develop a connective-tissue disorder - Ehlers-Danlos syndrome (17). Today, the suggestion is widely accepted that tenascin-X is a regulator of collagen deposition in vivo, consistent with a reduced density of collagen fibrils in the skin of tenascin-X null mice (18). However, the molecular mechanism behind these findings still remains elusive (19).

A closely related gene and protein has been identified in the chick (20,21). The avian protein is most homologous to mammalian tenascin-X in its C-terminal region and shares with it a serine/proline-rich domain, but has smaller subunits with only one (instead of 18) complete EGF repeats, and with FNIII modules not related in sequence to any other avian or vertebrate tenascin. At the time of discovery, these considerable differences justified a new name, tenascin-Y, for the avian protein (20-22). However, recent phylogenetic studies of the tenascin gene family in chordates revealed that chick tenascin-Y belongs to the tenascin-X branch, and that it is more closely related to mammalian than to Xenopus tenascin-X (Tucker et al., submitted'). The human tenascin-X gene is located in the human MHCIII locus on chromosome 6q21.3, a locus that, according to comparative mapping information, corresponds to chicken chromosome 16 (23). At this location, the chicken tenascin-X-gene is in immediate synteny with the complement C4, TAP1, TAP2 and MHC-II like genes that represent orthologues of the genes present in the human MHCIII locus next to the human tenascin-X gene, proving a common evolutionary origin for this genomic region (Tucker et al., submitted'). For these reasons, it is justified to abandon the former name tenascin-Y and to call the protein “avian tenascin-X” instead.

For collagen XII, the function as a modulator of tissue biomechanical properties by bridging collagen I containing fibrils to other ECM components has been suggested (24,25). Our findings reported here support this concept. Avian tenascin-X interacts with the NC3 domain of avian collagen XII thereby establishing a mechanical coherence of banded collagen fibrils with their extrafibrillar environment.

**EXPERIMENTAL PROCEDURES**

*Antibodies and Antibody production* - Antibodies against mouse collagen XII were produced as described previously (26). Briefly, cDNA coding for the fibronectin type III domains 14 to 18 of the mouse collagen XII was amplified by PCR using primers that introduced a NheI restriction site at the 5' end and a BamHI site at the 3' end (M193, forward, CACGCTAGCAGAGGACTGTCAAGAAACATCC and M194, reverse, TTGGGATCCTAGGTCTGTTCTTTGATGGGACA). The cDNA was cloned into a modified pPET (EMD Biosciences) vector carrying a His-6 tag with a thrombin cleavage site. Upon transformation with the recombinant plasmid, *E. coli* cells (Bl21, EMD Biosciences) were induced with 1 mM IPTG and grown for 16 h at 30°C. The cells were harvested (15 min, 5000 x g, 4°C) and resuspended in TBS pH 8.0, containing 7 M urea. The bacteria were sonicated, followed by removal of insoluble cell debris by centrifugation (30 min, 20000 x g, 4°C). After twofold dilution with H2O the supernatant was applied to a Ni-chelating Sepharose column (GE Healthcare) and eluted with binding buffer containing 40-80 mM imidazole. Following removal of urea by dialysis, thrombin cleavage was performed over night at room temperature (5 mM CaCl2, 1 U/mg thrombin, Sigma-Aldrich) and the cleaved His-6 tag was removed by passing the solution again over a Ni-chelating Sepharose column.

The recombinant protein was used to immunize a rabbit from which the antiserum was purified by affinity chromatography on a column with antigen coupled to CNBr-activated Sepharose (GE Healthcare). The specific antibody, termed KR33, was eluted with 150 mM NaCl, 0.1 M
triethylamine, pH 11.5, and the eluate neutralized with 1 M Tris-HCl, pH 6.8.

Polyclonal rabbit antibody against chick collagen XII (522) (8), polyclonal chick antibody against mouse tenascin-X (KX3), polyclonal rabbit antibody against chick tenasin-X (KX8) (21,27), and polyclonal rabbit antibody against chick tenasin-C (TN474) (28) have been characterized before.

**SDS-Polyacrylamide Gel Electrophoresis and Determination of Protein Concentration** – SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (29). 1.3 µg protein per lane was separated on 3-10% SDS-PAGE gradient gels under either reducing or non-reducing conditions and visualized with a silver staining kit (Invitrogen) according to the manufacturer’s instructions. Protein concentrations were determined using a bicinotic acid assay kit (Uptima) following the manufacturer’s instructions.

**Solid-phase Binding Assay** – The production of recombinant avian tenasin-X (formerly tenasin-Y) has been described previously (20). Tenasin-C and collagen XII were purified from chick embryo fibroblast conditioned medium by mAb affinity chromatography following established procedures (8,30). To calculate the molar concentrations of the proteins utilized for titration measurements, the molecular masses of the monomeric forms of avian tenasin-X (205 kD), avian tenasin-C (200 kD) and the mean value of the small and large splice variant of avian collagen XII, estimating a molar ratio of 1:1, (274.5 kD) were used. Purified proteins were diluted in TBS, pH 7.4 and 5 µg/ml (250 ng/well) were coated onto 96-well plates (Nunc Maxisorb) at 4°C over night. After washing with TBS, unspecific binding sites were blocked at room temperature with 5% skimmed milk powder in TBS for 2 h. Ligands were diluted in blocking buffer to concentrations from 0.03 to 300 nM for the tenasins or 0.023 to 230 nM for collagen XII and incubated for 1.5 h. For competition experiments the competitor was added to the ligand solution before incubation. After removing excess ligand by washing twice with TBS, bound ligand was fixed with 2.5% (v/v) glutaraldehyde for 10 min. Bound ligands were detected with specific affinity purified antibodies rabbit against chick collagen XII (522), chick tenasin-X (KX8) or chick tenasin-C (TN474) followed by swine anti-rabbit horseradish peroxidase coupled IgG (Dako Cytomation). For enzymatic reaction, wells were incubated with 50 µl 0.25 mM tetramethylbenzidine and 0.005% (v/v) H₂O₂ in 0.1 M sodium acetate (pH 6.0) for 10 min. The reaction was stopped with 50 µl/well 2.5 M H₂SO₄ and the absorbance was measured at 450 nm using a microplate reader (Labsystems Multiscan MS). For analysis, measurements of wells treated equally, except for the addition of ligand, were subtracted as blank values. All buffers contained 2 mM CaCl₂.

**Surface-Plasmon-Resonance Spectroscopy** – Surface plasmon resonance spectroscopy was performed using a Biacore 2000 (BIACore AB) system. Avian tenasin-X, full-length avian collagen XII and the purified collagen XII NC3 domain were coupled in 25 mM sodium acetate, pH 4.7 with a flow rate of 5 µl/min to a CM5 chip. The chip was previously activated with N-Hydroxysuccinimide and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. After coupling the required amount of protein (~ 1000 RU), unbound reactive groups were saturated with 1 M ethanolamine hydrochloride, pH 8.5. Experiments were carried out using different concentrations (3 nM, 10 nM, 30 nM and 100 nM) of avian tenasin-X and full-length collagen XII diluted in running buffer (20 mM Hepes; 150 mM NaCl; 2 mM CaCl₂; 0.005% P20). The analyte was passed over the sensor chip with a constant flow rate of 30 µl/min for 120 sec, dissociation was measured over 350 sec. Fittings of the data, overlay plots and calculation of $K_D$ values were done with BIAevaluation software 3.2.

**Collagenase digestion** – To generate avian collagen XII molecules consisting only of the trimeric NC3 domain, i.e. lacking their collagenous parts as well as the NC1 and NC2 domains, the protein was subjected to collagenase digestion. A solution of 77 nM collagen XII was incubated with 100 U/ml highly purified collagenase (CLSPA, Worthington Biochemicals) in TBS containing 5 mM CaCl₂ and 1 mM AEBSF (Roche) for 4h at 37°C. Collagenase treated collagen XII was immediately used for SDS-PAGE and solid phase binding assay. Alternatively, the NC3 domain was separated from collagenase and digestion fragments by running the sample over a gel-filtration column (Superose 12 HR 10/30, GE Healthcare).

**Immunohistochemistry** – Immunohistochemistry was performed on cryosections of embryonic chick (E14.5) and newborn mouse (P1). The frozen sections were
preincubated in ice cold methanol for 2 min, blocked for 1 h with 5% normal goat serum in PBS containing 0.2% Tween 20 and incubated with the primary antibodies against collagen XII (KR33 or 522) overnight at 4°C followed by Cy3-conjugated goat anti rabbit IgG (Dako Cytomation). For co-staining of mouse sections a chick antibody against tenascin-X (KX3) was used followed by Cy2-conjugated donkey anti chick IgG (Dako Cytomation). For co-staining of chick sections, the 522 antibody was biotinylated using Biotin-X-NHS (Calbiochem) according to the manufacturer’s instructions. Co-staining was accomplished using sequential incubation first with KX8 antibody for detection of tenascin-X followed by incubation with Cy2-conjugated affinity purified Fab fragment goat anti-rabbit IgG (Dianova), and second with biotinylated 522 antibody followed by Cy3-conjugated anti-biotin antibody (Sigma). Stained sections were analyzed and pictures were taken with a confocal laser scanning microscope (Leica TCS SL) using two lasers in parallel with the excitation wavelengths 488 nm for Cy2 and 543 nm for Cy3.

**Immuno-electron microscopy** - Fragments of native collagen fibrils were isolated form newborn mouse skin, placed on grids, immunostained with KR33, KX3 and colloidal gold-labeled secondary antibodies, and analyzed by transmission electron microscopy as previously described (31). PreEmbedding immunogold labeling of mouse skin was carried out as previously described (32). Skins were lightly prefixed in 0.1% glutaraldehyde, 4% paraformaldehyde in 0.1 M cacodylate buffer pH 7.4, immunolabeled en bloc by immersing in primary antibody, rinsed extensively in PBS, immersed in goat anti-rabbit 1 nm gold conjugate (Amersham Pharmacia Biotech) diluted 1:3, and rinsed extensively in PBS. The 1 nm gold particles in some instances were enhanced using the Nanoprobe GEEM gold enhance kit (Nanoprobes, Inc., Yaphank, NY). This was followed by fixation in 1.5% glutaraldehyde, 1.5% paraformaldehyde containing 0.05% tannic acid in 0.1 M cacodylate buffer and fixation in 1% osmium tetroxide before embedding in Spurrs epoxy (Ladd Research, Williston, VT). Ultrathin sections (0.2 µm) were mounted on 0.7% formvar-coated grids, stained with uranyl acetate and lead citrate, and examined with a Philips EM 208 transmission electron microscope.

**RESULTS**

*Interaction between collagen XII and tenascin-X* – In a solid-phase binding screen for possible interaction partners of avian collagen XII, we discovered avian tenascin-X as a possible candidate. Titration experiments showed saturable binding using either collagen XII or tenascin-X as the soluble ligand. Because of its similar domain structure (33), tenascin-C was used as a negative control (Fig. 1A and B). To calculate the molar protein concentrations for the titration measurements, the molecular masses of the monomeric forms of avian tenascin-X (205 kD) and a mean value of the small and large splice variant of avian collagen XII, estimating a molar ratio of 1:1, (274.5 kD) were used. In the titration experiment, half-maximal saturation was reached at a concentration of soluble collagen XII of 2.5 ± 0.5 x 10^-8 M. When tenascin-X was the soluble binding partner, half-maximal saturation was found at 1.5 ± 0.5 x 10^-8 M, i.e. at a closely comparable concentration. The small discrepancy might originate from different avidities of the oligomeric forms of the two proteins.

To validate the results, surface plasmon resonance spectroscopy was performed and the associations and dissociations of the obtained binding curves were analyzed in a Langmuir 1:1 binding model (Fig. 1C and D). Apparent Kd values for collagen XII as soluble interaction partner of 1.34 x 10^-8 M and for soluble tenascin-X of 6.44 x 10^-9 M were calculated, which is in good agreement with the solid phase binding data.

The purity of the isolated proteins was checked by reducing and non-reducing SDS-PAGE (Fig. 2B). Avian tenascin-X occurs in trimeric and hexameric forms (20) containing at least three or six binding sites for collagen XII. Upon reduction the oligomeric forms dissociate into monomers. A similar pattern can be observed for tenascin-C whose splice variants result in three bands on reducing gels (28,33). Collagen XII can exist as homo- or heterotrimeric combinations of the small and the large splice variant, which represent the four major bands on non-reducing SDS-PAGE. Upon reduction, the polypeptide pattern is complex but entirely explainable (8). The trimeric forms dissociate into large and small monomers and into various non-reducible dimers. Additional bands on reducing SDS-PAGE are due to glycosaminoglycan side chains attached to the large isoform of collagen XII (4).
The NC3-domain of collagen XII contains the binding site for tenascin-X – Collagen XII interacts with collagen I containing fibrils through its collagenous domain (8). Additionally two interaction sites for heparin are known, one at the very end of a C-terminal splice variant (6,9), and the other within the 7th fibronectin type III domain of the large form of collagen XII (8). To exclude the heparin binding sites being involved in binding of collagen XII to tenascin-X, competition experiments were performed. Even in 130 fold molar excess, heparin had no effect on the binding of collagen XII to tenascin-X (Fig. 3A). To study the contribution of the collagenous region (consisting of domains NC1, Col1, NC2, and Col2) of collagen XII in the binding process, collagenase digestion was performed. Treatment of collagen XII with highly purified bacterial collagenase simplified the complex pattern in reducing SDS-PAGE to two major bands which represent the NC3-domains of the small and large splice variants of the protein (Fig. 3B). The NC3-domains was purified via gel filtration chromatography and since the cysteines are not removed the NC3 remains a trimeric molecule. (Fig. 3B lane 4). In solid phase binding assays, collagenase treated collagen XII showed a similar interaction with immobilized tenascin-X compared to the intact protein (Fig. 3C). To confirm those results the purified NC3 domains were measured in surface resonance spectroscopy interaction experiments and from the obtained curves the apparent $K_D$ for the binding of soluble tenascin-X to the fragment is $5.90 \times 10^{-9}$ M (Fig. 3D), i.e. very similar as to intact collagen XII (cf. Fig. 1D). Taken together these results indicate that the binding site(s) for tenascin-X reside within the NC3 domain of collagen XII.

Partial co-distribution of collagen XII with tenascin-X in chick skin and muscle - The extracellular matrix of skeletal muscle is arranged in three levels: the individual muscle fibers are surrounded by the endomysium, bundles of muscle fibers are encased by the perimysium and the complete muscle is embedded in the epimysium. It is known that chick tenascin-X is a component of muscle extracellular matrix and is primarily situated in the epimysium and perimysium of developing muscles, whereas tendons are negative for tenascin-X, both at the mRNA and the protein level (20,21). Collagen XII is a component of the skeletal muscle extracellular matrix as well. The protein and the mRNA are located in the epimysium, perimysium and, unlike tenascin-X, also in tendon (8,34). By double immunofluorescence we could show that the two proteins co-localize, seemingly on the same interstitial fibrils, in the epimysium and perimysium of developing chick muscles, whereas tendon is only positive for collagen XII (Fig. 4B, C).

In the developing chick skin, tenascin-X is located throughout the dermis with increasing concentration in its lower parts, and in the connective tissue layers surrounding small blood vessels in the dermis (20,21). In contrast, collagen XII is more concentrated in the upper part of the dermis, in the subcutis and also in the wall of blood vessels containing smooth muscle cells (4,8). Overlay of immunofluorescence stainings of the two proteins revealed co-localization in the subcutis, and in a defined zone of the middle dermis layer (Fig. 4A).

To analyze the less well-characterized spatial expression of collagen XII in mouse embryos, we raised the polyclonal antibody KR33 which recognizes both the small and large splice forms of mouse collagen XII. We found that collagen XII is expressed throughout the developing dermis with increasing concentrations in the sub-epithelial layer. In the developing mouse skeletal muscle collagen XII is situated in the epimysium and perimysium, but the endomysium is only weakly positive for collagen XII (Fig. 5A-C). Similar to the findings in chick (34), there is a strong expression of collagen XII in tendon, perichondrium and periosteum (35). Tenascin-X is expressed throughout the dermis of mouse embryos and the staining decreases towards the epidermis. In mouse skeletal muscle, tenascin-X is present in the connective tissue along the muscle fibers (27), Overlay analysis revealed a co-localization of collagen XII with tenascin-X in the epimysium and perimysium of the developing skeletal muscle (Fig. 5B, C), and in a broad but defined zone of the reticu lar dermis (Fig 5A). Taken together, the spatial organization of collagen XII and tenascin-X in mouse embryos resembles closely the distribution of the two proteins in chick with well-defined areas of co-localization.

Ultrastructural analysis of the spatial expression of collagen XII and tenascin-X – By immunofluorescence staining, the fibrillar distribution pattern for collagen XII and tenascin-X is clearly visible. Based on ultrastructural analysis, a fibril-associated localization has been suggested for both proteins (8,13). On ultrathin
immunogold labeled sections of the embryonic chick skin, collagen XII and tenascin-X were located directly on interstitial fibrils or in electron dense material attached to the fibrils in the same area of the dermis (Fig. 6A, B). We made the same observation for collagen XII and tenascin-X in the mouse dermis (Fig. 6C, D), whilst, consistent with immunofluorescence analysis, chick tendon showed only a fibril-associated labeling for collagen XII but not for tenascin-X (data not shown).

To further support the co-localization of both proteins in the same ultrastructure, double labeling for collagen XII and tenascin-X was performed on fragments of matrix suprastructures extracted from newborn mouse skin under native conditions. Immunogold labels for both proteins were clearly located together on electron dense material. A gallery of such mats together with a larger overview of doubly labeled suprastructures is shown in Fig. 7. 223 gold particles corresponding to an immunolabel for collagen XII (18 nm gold particles) were arbitrarily chosen and the distances to the nearest particle indicating tenascin-X-labeling (12 nm gold particles) was determined. Colocalization was considered to occur if the gold particles could be identified within the same electron-dense structure and if the distance between collagen XII and tenascin-X-gold labels was less than 100 nm. This distance is comparable to the dimensions of the non-collagenous domains of collagen XII. By these criteria, 82% of collagen XII-labels were associated with those of tenascin-X. However, tenascin-X-labeling also occurred in structures which apparently lacked collagen XII, indicating that tenascin-X also occurred in suprastructures in which collagen XII had no part.

**DISCUSSION**

Collagen XII belongs to the subfamily of collagens designated as FACITs. It has been proposed that FACITs are surface components of banded collagen fibrils with their C-terminal, collagenous FACIT-domains anchored into the fibril body. In this model (25), exemplified by the cartoon shown in figure 8, their N-terminal domains can reach out into the perifibrillar matrix to interact with other ECM components and cell surfaces.

Collagen IX, another FACIT, appears to form such bridges between collagen II containing fibrils and non-collagenous cartilage matrix components, including matrilins and COMP (36-39). Also there is evidence that collagen XII too can bind to interstitial fibrils through its collagenous domains (8,11). In addition, a glycosaminoglycan dependent binding of decorin to collagen XII outside the NC3 domain was shown (10). However, for the large NC3 domain, which comprises up to 90% of the molecular mass, no interaction partners other than heparin have been described (8). This large multidomain part of collagen XII consists of fibronectin type III domains, von Willebrand factor A domains and a single thrombospondin N-terminal domain. VWA domains, which are found in a variety of proteins, are known to mediate protein-protein interactions. For example, the VWA domains present in the integrin α1β1 and α2β1 receptors are responsible for the interaction with fibrillar collagens (40).

The present study describes a novel interaction between collagen XII and tenascin-X, which is saturable and has an apparent Kd in the range of 10 nM. This binding is not sensitive to treatment of collagen XII with collagenase and for the purified NC3 domain similar binding parameters were obtained compared to the full-length molecule. Therefore the binding site is located within the non-collagenous NC3 domain. Previously, a heparin binding site was located in the 7th fibronectin type III domain of the large form of collagen XII (8). However, the heparin-binding site, saturated with soluble heparin, does not influence the binding to tenascin-X and, therefore, does not overlap with the tenascin-X-binding region in the NC3 domain.

Tenascin-X also binds to heparin (14,21,27) and binding to collagen I containing fibrils was demonstrated (41). Furthermore, in a different study this interaction was suggested to be mediated by decorin (15). In addition, evidence for tenascin-X mediating cell attachment through binding to integrin receptors was presented (16).

Taking published information together with the new interaction studies presented here results in a more comprehensive picture of the protein network associated with fibrils containing collagens I and XII, which presumably contributes to the integrity of the extracellular matrix and facilitates cellular interactions. The components involved in these interactions are schematically visualized in the cartoon shown in figure 8.

Immunofluorescence analysis of tissue sections shows a partial co-localization of collagen XII with tenascin-X. Especially in the reticular dermis and in the epimy whole page text here.
the muscle exact co-staining of both proteins was seen whereas, in other tissues, predominantly one of the proteins is present, e.g. collagen XII in the papillary dermis and in tendon. These results were confirmed by ultrastructural analysis, which revealed association of both proteins with banded fibrils in the dermis and demonstrated co-localization on fibrils extracted from mouse dermis. In particular, collagen fibrils are tightly surrounded by a remarkable, electron dense material, which could be labeled for collagen XII as well as for tenascin-X, and might represent complexes of several proteins held together by the numerous interactions as indicated in figure 8.

In mice lacking tenascin-X as well as in patients suffering from Ehlers-Danlos Syndrome because of the absence of tenascin-X, it has been shown that there is less collagen I in the skin and that the packing of the fibrils is less tight (18). Therefore, it has been suggested that tenascin-X regulates collagen fibril deposition and spacing through multiple interactions with collagen I or interactions with other proteins which in turn themselves interact with collagen I fibrils (19). In the absence of tenascin-X, as in the aforementioned cases, the biomechanical tissue stability is compromised because crucial interactions between fibrils and the perifibrillar matrix are abrogated. Interestingly, fibroblasts derived from tenascin-X -/- mice show a decreased collagen XII mRNA expression level compared to wild-type fibroblasts (42).

Recently, it has been demonstrated that collagen IX, the FACIT prototype, mediates the interactions between fibroblasts and banded collagen fibers through integrins (43). For collagen XII, no direct interaction with cell surface receptors could be shown so far. Because of the integration of collagen XII into protein complexes linking the interstitial fibrils to other ECM components and cell surfaces, collagen XII might function as a modulator of tissue biomechanical properties (24). It is tempting to speculate that the restricted spatial co-localization of collagen XII and tenascin-X plays a role in generating specific mechanical properties in those tissues, through collagen XII acting in concert with tenascin-X as a regulator of fibril deposition and spacing. Further studies are necessary to elucidate the composition and function of such protein complexes.

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FOOTNOTES

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The abbreviations used are: Col, collagenous domain; EGF, epidermal growth factor; FACIT, fibril-associated collagen with interrupted triple helices; FNIII, fibronectin type III; FReD, fibrinogen-related domain; His, histidine; IPTG, isopropyl thiogalactoside; NC, non-collagenous domain; Ni, nickel; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; TBS, tris buffered saline; TN-C, tenascin-C; TN-X, tenascin-X; TSPN, thrombospondin N-terminal; VWA, von Willebrand factor type A.

FIGURE LEGENDS

Fig. 1. Concentration dependence of the chick collagen XII – tenascin-X interaction determined in a solid phase binding assay and by surface plasmon resonance spectroscopy. A, Different concentrations of tenascin-X were incubated with collagen XII or tenascin-C coated onto microtiter plates. Binding was revealed in an ELISA style manner with an affinity purified polyclonal antibody against chick tenascin-X (KX8). The resulting saturation curve was used for calculation of an apparent K_D value. B, Chick tenascin-X was coated onto the plates and different concentrations of soluble chick collagen XII were added. The bound collagen XII was detected with an affinity purified antibody (522). ∆E, measured extinction minus blank value. C and D, Surface plasmon resonance spectroscopy was performed with tenascin-X (C) or collagen XII (D) as soluble analyte. The amount of interacting analyte (3 nM, 10 nM, 30 nM and 100 nM) was monitored by measuring the variation in the plasmon resonance angle as function of time and expressed in terms of response units. The background signal has been subtracted from each curve; the curves are shown in ascending order depending on the analyte concentration used. Fittings and overlay plots were done with the Biaevaluation software version 3.2. The continuous black lines represent the fitted curves.

Fig. 2. Domain structure (A) and SDS-PAGE analysis (B) of the applied proteins. A, A schematic depiction of the domain structures of the large (XIIA) and small (XIIB) variants of chick collagen XII, of mouse tenascin-X, of chick tenascin-X, and of chick tenascin-C. B, Purified collagen XII (cCol XII) and tenascin-C (cTN-C) from chick fibroblasts as well as purified recombinant chick tenascin-X (cTN-X) were subjected to SDS-PAGE with (+DTT) or without (-DTT) prior reduction. The 3-10% gradient gels were silver stained.

Fig. 3. Heparin competition (A) and influence of the NC3 domain of collagen XII (B, C and D) on the interaction with tenascin-X. A, Solid phase assay was done with or without the addition of 10 µM heparin
Collagenase digestion of purified collagen XII was performed at 37°C for 4 h. The undigested (lane 1), the digested (lane 2) protein, the collagenase alone (lane 3) or the purified NC3 domain (lane 4 +DTT) were reduced and separated by SDS-PAGE followed by silver staining. In addition the capability of the purified NC3 domain to form disulfide-linked trimers was checked on non-reducing SDS-PAGE (lane 4 –DTT). In a solid phase assay, collagenase digested (corresponding to the NC3 domain of the molecule) or undigested collagen XII were incubated with immobilized tenascin-X. In surface plasmon resonance spectroscopy the interaction of soluble tenascin-X with the purified immobilized NC3 domain of collagen XII was evaluated. The curves are shown in ascending order depending on the analyte concentration (3 nM, 10 nM, 30 nM and 100 nM) used. The continuous black lines represent the fitted curves.

Fig. 4. Tissue distribution in chick embryos of collagen XII and tenascin-X, and co-localization of the two proteins. Immunohistochemistry was performed on frozen chick embryo leg sections (E14.5). Co-staining was accomplished using sequential incubations with the antibodies KX8 for detection of chick tenascin-X and 522 for localization of collagen XII. Collagen XII (red) is strongly expressed in the papillary dermis, subcutis, in the connective tissue surrounding blood vessels (A) and in epimysium as well as perimysium and tendon (B, C). Tenascin-X (green) is highly expressed in the reticular dermis and around blood vessels (A), subcutis, epimysium and perimysium (B, C). The two proteins co-localize (yellow) at the border between papillary and reticular dermis, in the subcutis, in the connective tissue surrounding blood vessels (A), in the epimysium and the perimysium (B, C). The bars are 200 µm in (A, B) and 40 µm in (C).

Fig. 5. Tissue distribution in neonatal mice of collagen XII and tenascin-X, and co-localization of the two proteins. Immunohistochemistry was performed on frozen mouse leg sections (P1). Sections were incubated with KR33 antibody for the detection of collagen XII and KX3 antibody for the localization of tenascin-X. Collagen XII (red) is strongly expressed in the papillary and reticular dermis (A) and epimysium as well as perimysium and tendon (B, C). Tenascin-X (green) can be detected in the reticular dermis (A) as well as in the epimysium and perimysium (B, C). The two proteins co-localize (yellow) in a broad zone of the middle dermis (A) as well as in the epimysium and perimysium (B, C). The bars are 200 µm in (A, B) and 40 µm in (C).

Fig. 6. Ultrastructural localization of collagen XII with tenascin-X, respectively. Ultrathin sections of chick dermis (A, B) or mouse dermis (C, D) were incubated with the 522 antibody (A) for labeling of chick collagen XII, the KX8 antibody (B) for labeling of chick tenascin-X, the KR33 antibody (C) for labeling of mouse collagen XII or the KX3 antibody (D) for labeling of mouse tenascin-X followed by 10 nm colloidal gold-labeled secondary antibody (A, B) or 1 nm colloidal gold-labeled secondary antibody followed by enhancement (C, D). Collagen XII in chick dermis (A) or in mouse dermis (C) is located either directly on banded fibrils or integrated in electron dense material, which is attached to the fibrils. Tenascin-X in chick dermis (B) and in mouse dermis (D) shows a localization similar to collagen XII. Bars are 300 nm.

Fig. 7. Immuno-electronmicroscopic analysis of collagen I containing fibril fragments extracted from mouse skin. Native fibril fragments were extracted from mouse dermis and doubly labeled for collagen XII and for tenascin-X with specific antibodies followed by colloidal gold-labeled secondary antibodies (particle size 18 nm for collagen XII and 12 nm for tenascin-X). Bar is 150 nm.

Fig. 8. Model for the supramolecular assembly of interstitial fibrils. Collagen XII and tenascin-X act as adaptor molecules to interconnect collagen I containing fibrils with each other, and therefore might modulate the distances between fibrils. This would in turn influence the biomechanical properties of tissues. Collagen XII directly or indirectly interacts with the fibrils, whereas in case of tenascin-X the interaction is mediated by decorin. The binding of glycosaminoglycans (GAGs) to both proteins suggests
the involvement of other potential interaction partners. In addition tenascin-X is able to bridge the extracellular network to cell surfaces due to the interaction with integrin receptors.
Fig. 1

A

B

C

D

Fig. 1
Fig. 2
Fig. 3
Fig. 5
Fig. 6
Collagen XII containing fibril

Decorin

GAGs

cell attachment via integrin receptors

Collagen I containing fibril

Tenascin-X

Decorin

GAGs

Fig. 8
Collagen XII interacts with avian tenascin-X through its NC3 domain
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