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A model of tenascin-X integration within the collagenous network

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Abstract **Tenascin-X is an extracellular matrix protein whose absence leads to an Ehlers-Danlos syndrome in humans, characterized mainly by disorganisation of collagen and elastic fibril networks. After producing recombinant full-length tenascin-X in mammalian cells, we find that this protein assembled into disulfide-linked oligomers. Trimers were the predominant form observed using rotary shadowing. By solid phase interaction studies, we demonstrate that tenascin-X interacts with types I, III and V fibrillar collagen molecules when they are in native conformation. The use of tenascin-X variants with large regions deleted indicated that both epidermal growth factor repeats and the fibrinogen-like domain are involved in this interaction. Moreover, we demonstrate that tenascin-X binds to the fibril-associated types XII and XIV collagens. We thus suggest that tenascin-X, via trimerization and multiple interactions with components of collagenous fibrils, plays a crucial role in the organisation of extracellular matrices.**

Keywords: Extracellular matrix; Ehlers-Danlos syndrome; Tenascin-X; Collagen

1. Introduction

Tenascin-X (TNX) is an extracellular matrix glycoprotein that belongs to the tenascin family. All tenascins share the same multidomain structure consisting of an N-terminal region involved in oligomerization, a series of epidermal growth factor (EGF)-like repeats, a variable number of fibronectin-type III (FNIII) modules and a C-terminal domain homologous to fibrinogen (Fbg). This complex structure gives rise to multiple interactions with proteins and carbohydrates [1].

Functions of TNX in extracellular matrix network formation and/or stabilization are suggested by in vivo observations. Some patients show an Ehlers-Danlos-like syndrome that is due to TNX deficiency [2,3]. Major clinical symptoms consist of skin hyperextensibility, joint laxity and easy bruising. Ultrastructural analyses reveal abnormalities in collagen fibril networks and elastic fibres [2,4]. Mice deficient in TNX also exhibit connective tissue defects such as skin hyperextensibility. Fibroblasts isolated from TNX−/− mice failed to deposit collagen in cell culture [5]. Considering these alterations of connective tissue structure and the localization of TNX at the surface of collagen fibrils [6], TNX might be involved in collagen fibrillogenesis and/or in the interaction between collagen fibrils. We have previously shown that TNX interacts with decorin and suggested that this small proteoglycan may mediate the interaction between TNX and collagen fibrils [7].

The aim of this study was to test fibrillar and fibril-associated collagens as possible ligands for TNX. To this end, we produced, in mammalian cells, full length TNX and TNX variants with large regions deleted. These proteins were used to test interactions with collagens by solid phase assays. Since no data were available on the oligomerization of TNX, we also analyzed the structure of full length TNX by rotary shadowing.

2. Materials and methods

2.1. Production of recombinant TNX in mammalian cells

The cDNA clones used in this study (Flex-2, Flex-12, Flex-21, PCR2, and TX2) originated from our previous work concerning the full-length sequencing of bovine TNX [8]. The strategy used to make the different construct is shown in Fig. 1. To obtain the four constructs corresponding to recombinant TNX, the *Sac*I–*Eco*RI fragment of cDNA clone TX2 was cloned into pBluescriptSK (Stratagene) (clone 1). The *Bam*HI–*Eco*RI fragment from clone PCR2 was successively inserted into the *Bam*HI and *Eco*RI sites of clone 1 (clone 2). To obtain clone 3, the *Aat*II–*Eco*RI fragment from clone 2 was replaced by a similar DNA insert from cDNA clone Flex-21. Clone 4 consists of the *Sac*I–*Eco*RI fragment of cDNA clone Flex-12 cloned in pBluscriptSK. In a next step, the *Stu*I–*Stu*I–*Eco*RI DNA insert from clone 4 was replaced by the *Stu*I–*Eco*RI fragment from clone Flex-221 (clone 5). To generate clone 6, the *Stu*I–*Stu*I fragment from clone Flex-12 was cloned into the unique *Stu*I site of clone 5 in the correct orientation. The *Nco*I–*Eco*RI fragment from clone 6 was inserted into clone 3 to generate clone 7. To obtain the DNA fragment common to the four constructs, the *Nco*I–*Nco*I fragment from cDNA clone Flex-2 was inserted in the right orientation into the *Nco*I site of clone 7 (clone 8, nucleotides 2751-10001, GenBank accession number Y11915). The two 5′ PCR fragments (P1 and P2) were generated using *Taq* Expand and cloned into the *Sac*I fragment of pBluescript. Clones 9 and 10 were obtained by cloning into the *Sac*I site of clone 8, the *Sac*I fragment of clones P1 and P2, respectively. The two last RT-PCR products, P3 and P4 were cloned into the *Eco*RI–*Eco*RV sites of clones 9 or 10. The final step consisted of cloning of the four final *Hin*dIII–*Eco*RV fragments into the pSec-TagHygroB vector (Invitrogen). All the PCR fragments have been sequenced.

Transfection of HEK 293 cells with the four constructions, selection with hygromycin, cloning and selection of positive cells were carried out as previously described [9]. For the production of recombinant proteins, cells were cultured in serum-free conditions and the medium was collected every 3–4 days. Recombinant proteins were purified from the medium by two chromatographic steps. Since we had previously located a heparin-binding site within the FNIII domains of TNX [9], the first chromatography was performed on heparin. The media were loaded on a heparin-Sepharose column (Pharmacia). After rinsing in PBS, bound proteins were eluted with 0.5 M NaCl in PBS. Fractions

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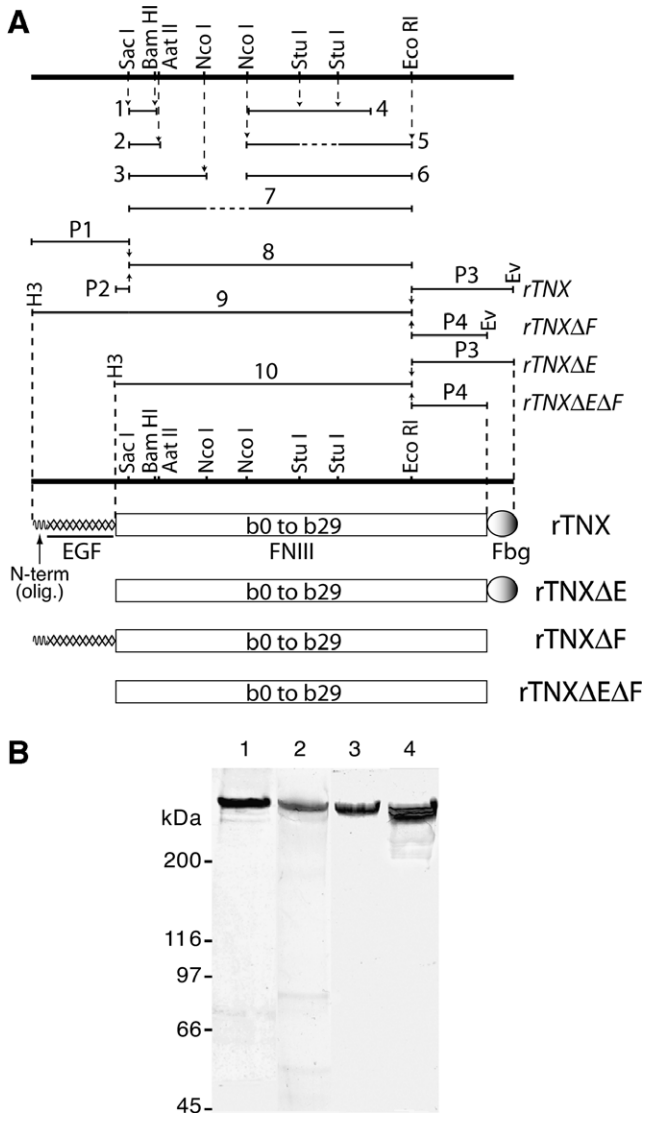

Fig. 1. Recombinant TNX variants produced in HEK293 cells. (A) cDNA fragments (clones 1–10) and PCR products (P1–P4) used for the generation of TNX expression constructs are represented above a schematic drawing of recombinant TNX. rTNX corresponds to the full length TNX, comprising the N-terminal oligomerization region (N-term. (olig.)), 14.5 EGF repeats, 30 FNIII modules and the C-terminal Fbg domain. rTNXΔE is deleted from the EGF-like domains, rTNXΔF is deleted from the Fbg-like domain, and rTNXΔEΔF comprises only the FNIII modules. According to bovine TNX protein (accession number CAA72671), rTNX, rTNXΔE, rTNXΔF and rTNXΔEΔF included residues 23-4135, 745-4135, 23-3910 and 745-3910 respectively. Ev, *Eco*RV; H3, *Hind*III. (B) SDS–PAGE migration profiles under reducing conditions of recombinant TNX molecules after the two purification steps. Lane 1: rTNX, lane 2: rTNXΔF, lane 3: rTNXΔE, lane 4: rTNXΔEΔF. The position of molecular mass standards is shown at the left of the gel.

enriched in recombinant proteins were then dialyzed against 50 mM Tris–HCl, pH 8.0. A second chromatography step was performed on Q-Sepharose (Pharmacia) and elution was achieved by a linear gradient of NaCl in 50 mM Tris–HCl, pH 8.0. Purified proteins were dialyzed against PBS and stored at −80 °C. Protein concentration was determined using the QuantiPro BCA assay kit (Sigma). Electrophoresis was carried out in 6% acrylamide gels in the presence of sodium dodecyl sulfate (SDS–PAGE) and gels were stained with Coomassie blue.

2.2. Solid phase assays

Ninety-six well microtiter plates (Maxisorp, Nunc) were coated overnight at +4 °C with collagens diluted in water. Acid-soluble bovine collagen, mainly consisting of type I collagen was from Coletica (Lyon, France), and types III and V collagens are a generous gift of Dr. M.C. Ronzière (IBCP, Lyon, France). Types XII and XIV collagens were prepared using previously described procedures [10,11]. In some assays, collagen was denaturated by heating the solution for 20 min at 95 °C before being added to the wells. All further steps were done at room temperature. Wells were saturated with T-PBS-BSA (Tween 0.05%, bovine serum albumin 1% in phosphate buffered saline) for 2 h and then incubated with purified recombinant TNX diluted in the same buffer for a further 2 h. Wells were rinsed with T-PBS (Tween 0.05% in phosphate buffered saline), incubated for 1 h with an antibody specific for TNX diluted in T-PBS-BSA (mouse monoclonal clone 8F2 [9], hybridoma supernatant, diluted 1/80, or guinea pig polyclonal serum obtained by immunization with the recombinant Fbg domain, diluted 1/500). Bound antibodies were further revealed with anti-mouse IgG (BioRad) or with anti-guinea pig IgG (Jackson Laboratories) conjugated to peroxidase for 30 min. After a last series of rinses, bound peroxidase was detected with H_2O_2 and 2,2-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) and the absorbance read at 405 nm. Each data point are the means of triplicate determinations and bars represent the standard error of the mean.

2.3. Rotary shadowing

The TNX solution was dialyzed against 0.2 M ammonium bicarbonate, diluted with glycerol (v/v), and spread on fleshly cleaved mica sheets. Samples were shadowed in a Balzers MED10 coating unit and the replicas were observed with a Philips CM120 microscope at the "Centre de Technologie des Microstructures" (Université de Lyon).

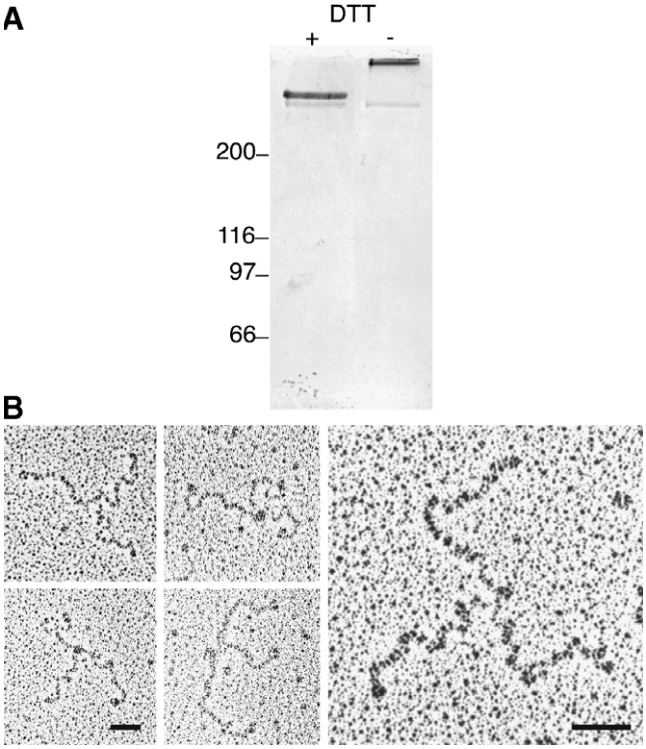

Fig. 2. Purification and rotary shadowing of rTNX. (A) Comparison of SDS–PAGE migration profiles of purified rTNX in the presence (+) or absence (−) of dithiothreitol (DTT). Migration of protein standards is shown at the left of the upper panel. (B) Rotary shadowing micrographs of purified rTNX molecules appearing as trimers (bar = 50 nm).

3. Results and discussion

3.1. Production and characterization of recombinant TNX

In order to study the interaction between TNX and fibrillar collagens, we decided to produce recombinant TNX as a full-length molecule and as variants with large regions deleted. The different proteins are shown in Fig. 1A. The yield was 3 mg of purified protein per litre of culture medium for rTNX and deleted variants exhibited variable production efficiencies comprised between 1 and 30 mg of protein per litre. As shown in Fig. 1B, the proteins purified by the two chromatographic steps appeared homogenous and as a single band compatible with their respective molecular mass. As no data were available on the oligomerization properties of TNX, we compared the migration profiles of rTNX under reducing and non-reducing conditions. Distinct patterns were observed, i.e. the appearance of a single band of high molecular mass located at the upper limit of the separating gel under non-reducing conditions (Fig. 2A). This result indicated that rTNX was produced as disulfide-linked oligomers. In the preparation of full-length TNX submitted to rotary shadowing, the majority of molecules appeared as trimers (Fig. 2B). On each arm, we could observe one terminal globule, a thick flexible region, a thinner short segment and the three arms associated by another globular domain. As shown in the early studies concerning tenascin-C [12,13], these successive domains may respectively correspond to the C-terminal Fbg, the FNIII and the EGF repeats, and the N-terminal oligomerization region. In tenascin-C, which is secreted as hexamers, the oligomerization process has been studied in detail. The first step involves the nucleation of three chains in the region of the heptad repeats. The trimer is stabilized by disulfide intermolecular bonds at the margin of the heptad region. These heptads are common motives found in several extracellular matrix proteins such as laminins, thrombospondins and tenascins [14]. The second step of tenascin-C assembly involves the most N-terminal region and cysteine 64, responsible for the association of two trimers to form a hexamer [15,16]. The trimeric association of TNX might be explained by a structure of its amino-terminal region differing from that of tenascin-C. Hence, the most amino-terminal 20 residues and the equivalent of cysteine 64 are absent in TNX [8,17]. In previous studies, we found that TNX extracted from tissues appeared mainly monomeric [6,8], suggesting that, during the purification process, the heptad-containing region was submitted to partial proteolysis. These rotary shadowing studies of recombinant TNX also showed the efficacy of the system for producing intact correctly folded proteins usable for functional studies.

3.2. Interaction with collagens

The interaction of TNX with collagen was tested by solid-phase interaction assays. Fibrillar collagen molecules were

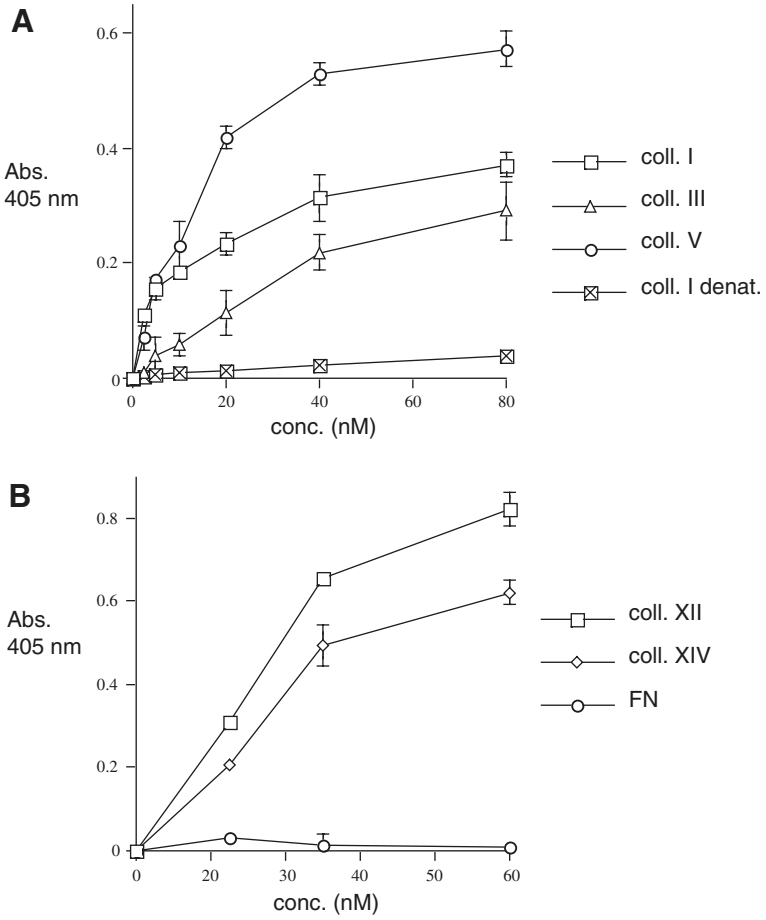

Fig. 3. Solid-phase assay of the interaction between rTNX and fibrillar (A) and fibril-associated (B) collagens. Wells were coated with collagens at 5 μg/mL and interactions were measured with variable concentrations of soluble TNX.

coated on plates in their native or denaturated conformation and the recombinant TNX was added and revealed by one monoclonal antibody whose epitope is located within the FNIII domains. The results are shown in Fig. 3A. Full-length rTNX interacted with collagen types I, III and V in their native state, the best interaction being obtained with collagen V. Heat denaturation of collagens before coating led to the complete abolition of the interaction with rTNX, either with collagen I (Fig. 3A) or with collagens III and V (data not shown). The triplehelical structure of collagen monomers is thus crucial for the interaction with TNX. Other components of the collagenous fibril environment, i.e. fibril-associated collagens XII and XIV, were also shown to interact with TNX, whereas fibronectin was ineffective in this assay (Fig. 3B). Finally, deleted variants were used to map the collagen-binding site on the TNX molecule. As shown in Fig. 4, deletion of the Fbg domain or the region comprising the EGF-like modules led to a strong decrease in the interaction with fibrillar collagen I. Moreover, when the Fbg domain alone was used, no interaction is observed. These observations suggest that the EGF region, and one site including the Fbg domain, co-operate in the interaction with fibrillar collagens. This result is different from that of Minamitani et al., who found an interaction with mutants deleted from these domains [18]. This difference may be due to the recombinant TNX produced, since they used a short form comprising only 24 FNIII domains, compared with our long form encompassing 30 of these modules. Also, the design of their experiment is different since they coated recombinant TNX and added collagen type I diluted in PBS. Under these conditions, collagen might form aggregates that have properties different from monomeric collagen. However, our results are in accordance with the study by these authors who observed that these two regions, namely Fbg domain and EGF-like modules, were involved in the acceleration of in vitro collagen fibrillogenesis induced by TNX [18]. Moreover, we could exclude the possibility that the trimerization of TNX was important for this interaction because when we used monomeric TNX extracted from tissues, an interaction similar to that observed with rTNX was obtained (unpublished data).

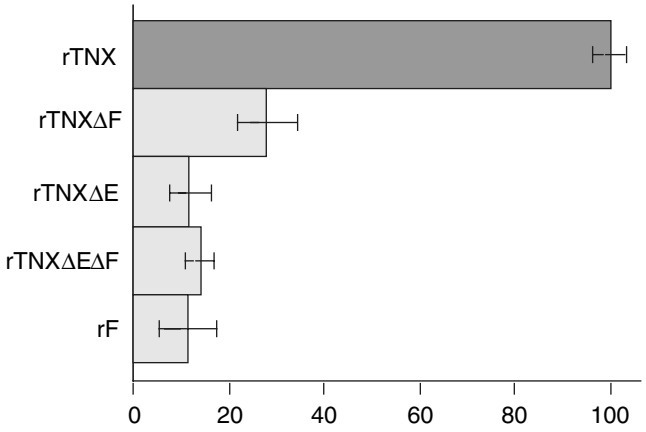

Fig. 4. Mapping of regions involved in the interaction between TNX and type I collagen. Wells were coated with 5 μg/mL of acid-soluble bovine type I collagen. rTNX and its variants were added at a 80 nM concentration. For rTNXΔF, rTNXΔE and rTNXΔEΔF, detection was with the monoclonal antibody 8F2 that is specific for the tenth FNIII domain of TNX. For rF, detection was with an anti-guinea pig serum obtained by immunization with recombinant Fbg domain. Results are given as a percentage of the absorbance measured for the full length rTNX revealed by the same antibody.

3.3. Conclusions

Several studies suggest that TNX acts as a bridge between collagen fibrils. Firstly, we have demonstrated that TNX is located between fibrils when they are organized in bundles [6], and secondly, Bristow et al. have shown that, in the skin of a patient deficient in TNX, the interfibrillar distance is increased [19]. As demonstrated here, fibrillar types I, III, V and fibril-associated XII and XIV collagens, are partners of TNX, and in a previous paper, we have shown that TNX interacts with decorin, a small proteoglycan located on collagen fibrils [7]. Thus, the bridging property of TNX might originate from its multiple interactions with components of the fibrillar surface. Moreover, TNX is secreted as disulfide-linked trimers, a property that is probably important for bridging. Finally, the FNIII domains of TNX might be important in relation to elastic properties of the molecule. It has been demonstrated that these domains are able to partially unfold under stretching conditions and recover their native conformation with relaxation [20]. Consequently, TNX might be important for the compliance of connective tissues. In conclusion, our results reinforce the concept that TNX, by trimerization and interactions with multiple components of the collagenous fibrils, plays a central role in the organisation of connective tissues. It remains to be determined if and how these interactions are involved in the in vivo deposition of collagen networks.

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