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Binding of tenascin-X to decorin

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Abstract Tenascin-X (TN-X) is an extracellular matrix protein whose absence results in an alteration of the mechanical properties of connective tissue. To understand the mechanisms of integration of TN-X in the extracellular matrix, overlay blot assays were performed on skin extracts. A 100 kDa molecule interacting with TN-X was identified by this method and this interaction was abolished when the extract was digested by chondroitinase. By solid-phase assays, we showed that dermatan sulfate chains of decorin bind to the heparin-binding site included within the fibronectin-type III domains 10 and 11 of TN-X. We thus postulate that the association of TN-X with collagen fibrils is mediated by decorin and contributes to the integrity of the extracellular network.

Key words: Extracellular matrix; Ehlers–Danlos syndrome; Proteoglycan; Tenascin-X

1. Introduction

Tenascin-X (TN-X) is an extracellular matrix molecule that belongs to the tenascin family, comprising five members, i.e. TN-C, TN-R, TN-X, and the more recently characterised TN-Y and TN-W. All are multidomain proteins consisting of an N-terminal region involved in oligomerisation, a series of epidermal growth factor-like repeats, a variable number of fibronectin-type III (FNIII) modules and one C-terminal domain homologous to fibrinogen (Fbg). This complex structure gives rise to multiple interactions with proteins and carbohydrates [1].

Some biological properties of TNs are mediated by their binding to proteoglycans. Both extracellular and cell surface proteoglycans have been shown to interact with TN-C. Perlecan, a basement membrane heparan sulfate proteoglycan, interacts with FNIII domains 3–5 of TN-C [2]. Some data suggest that heparan sulfates present on the cell surface of fibroblasts [3] or on hematopoietic cells [4] may be involved in the interaction with the Fbg domain. Syndecan from embryonic mesenchyme [5] and glypican [6], two heparan sulfate receptors, have also been found to interact with TN-C using their glycosaminoglycan (GAG) chains. Other proteoglycans may interact via N-linked oligosaccharides (phosphacan/RPTPζ/β) or their core proteins (neurocan) [7–9].

Interaction of TN-X with extracellular proteoglycans has not been demonstrated until now. This protein has the typical arrangement of modules characteristic of TNs [10–13] and a widespread expression during embryonic and adult life, where it appears more specifically in striated muscles, tendon and ligament sheaths, dermis, adventitia of blood vessels, peripheral nerves and digestive tract [11,12,14–16]. By immunoelectron microscopy, we have shown that TN-X is associated with collagen fibrils in the dermis and in the mesangium of kidney glomeruli [14]. The major functions of TN-X are not clearly understood at present, though some hypotheses have emerged with the identification of one patient deficient in TN-X [17], suffering from a connective tissue disorder known as Ehlers–Danlos-like syndrome. Symptoms consist of skin and joint hyperextensibility, vascular fragility and poor wound healing. Ultrastructural analysis of the patient's skin has shown a normal shape but reduced diameter of collagen fibrils, the presence of electron dense elastin bodies beneath the dermal–epidermal junction, an increased perivascular matrix and abnormal packing of the myelin sheath laminae in cutaneous nerves [17]. Considering these alterations of connective tissue structure and biomechanical properties, TN-X might be involved in extracellular matrix network formation.

The aim of our study was to identify extracellular matrix molecules interacting with TN-X. As a screening procedure, we performed overlay blotting experiments in which skin extracts were transferred to membranes and probed with recombinant proteins comprising FNIII domains 10 and 11 of bovine TN-X. We have previously shown that such recombinant proteins contain a heparin-binding site [18]. We have found that decorin is a binding partner for TN-X and that the dermatan sulfate GAG chains of this proteoglycan are involved in the interaction.

2. Materials and methods

2.1. Proteins and antibodies

TN-X was extracted from bovine foetal skin and immunopurified on a monoclonal antibody affinity column using a previously described procedure [14].

Polyclonal antibody against the FNX 9–10 recombinant protein was raised by immunising guinea pigs. The antigen, a recombinant protein corresponding to bovine FNIII domains 9 and 10, was produced in bacteria as previously described [19] and immunisation was done at the Valbex Centre (Institut Universitaire de Technologie, Université Claude Bernard, Lyon I, France). Immunoglobulins were purified on protein G-Sepharose (Amersham/Pharmacia Biotech, Saclay, France), following the manufacturer's instructions.

Bovine skin decorin and cartilage biglycan were provided by B. Font (IBCP, Lyon, France), who also provided us with the monoclonal antibody (clone DS-I) specific for the core protein of bovine decorin. These reagents were given to our laboratory by H.U. Choi and L.C. Rosenberg (Montefiore Medical Center, New York, USA) [20]. Bovine type XIV collagen was a generous gift from E. Aubert-Foucher (IBCP, Lyon, France).

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2.2. Preparation of tissue extracts

Bovine foetal tissues were powdered in a freezer mill (SPEX industries, Metuchen, NJ, USA). The powder was suspended in extraction buffer composed of 0.5 M NaCl diluted in phosphate-buffered saline (PBS), in the presence of a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM EGTA, 2 mM EDTA and 10 mM *N*-ethyl maleimide). The extraction was continued for 5 h at 4°C. After centrifugation (15 min at 20 000 × *g*), the supernatant was collected and stored at −20°C.

2.3. Chondroitinase digestions

Prior to digestion, tissue extracts or purified proteoglycans were dialysed against 50 mM Tris–HCl, 60 mM sodium acetate, pH 8.0, 2 mM EDTA, and were then digested for 2 h at 37°C with 25 mU of chondroitinase ABC (chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris*, Boehringer Mannheim, Meylan, France).

2.4. Overlay blot experiments

Binding of TN-X recombinant fragments to molecules transferred to membranes was performed according to the method of Font et al. [21]. Tissue extracts or purified proteoglycans were loaded on polyacrylamide gels and transferred to PDVF membranes (Immobilon, Millipore, Saint-Quentin-en-Yvelines, France) using standard procedures [14]. The membrane was saturated for 2 h at room temperature with 1% bovine serum albumin (BSA) diluted in PBS. Then, incubation with soluble recombinant TN-X fragments (5 μg/ml, diluted in PBS–BSA) was carried out overnight at 4°C. Control membranes were incubated with PBS–BSA. Bound recombinant protein was detected by successive incubations in polyclonal antibody against FNX 9–10 and peroxidase-conjugated anti-guinea pig IgG (Biosys, Compiègne, France). Further development was performed using a chemiluminescence kit (NEN Life Science, Paris, France).

2.5. Solid-phase assays

Ninety-six well microtiter plates (Maxisorp, Nunc) were coated overnight at 4°C with proteins diluted in PBS. All further steps were done at room temperature. Wells were saturated with T-PBS–BSA for 2 h and then incubated with purified decorin for a further 2 h. Wells were rinsed with PBS, incubated for 1 h with monoclonal antibody specific for decorin (ascites fluid diluted 1/1000) and then for 30 min with anti-mouse IgG conjugated to peroxidase (Biosys). After a final set 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 is the mean of triplicate determinations and bars represent the standard error of the mean.

For inhibition experiments, incubation with GAGs was performed for 30 min before addition of decorin and visualisation of bound decorin was performed as described above.

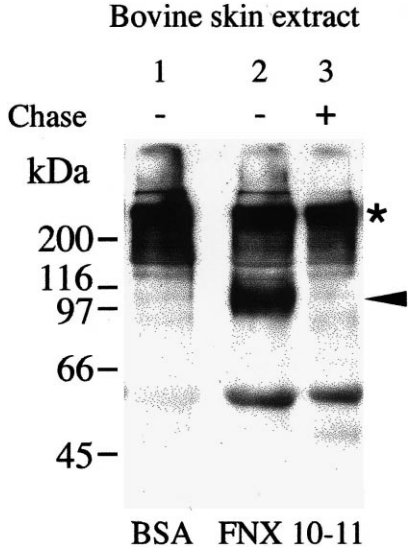

Fig. 1. Interaction of recombinant FNX 10–11 protein with bovine skin extracts by overlay blotting experiments. Bovine skin extracts were loaded on a 10% acrylamide gel and transferred to a PVDF membrane. Lanes 1 and 2: untreated skin extract; lane 3: chondroitinase-digested skin extract. Membrane corresponding to lane 1 was incubated with BSA and lanes 2 and 3 were incubated with recombinant FNX 10–11 protein. Visualisation of the bound protein was performed as described in Section 2.

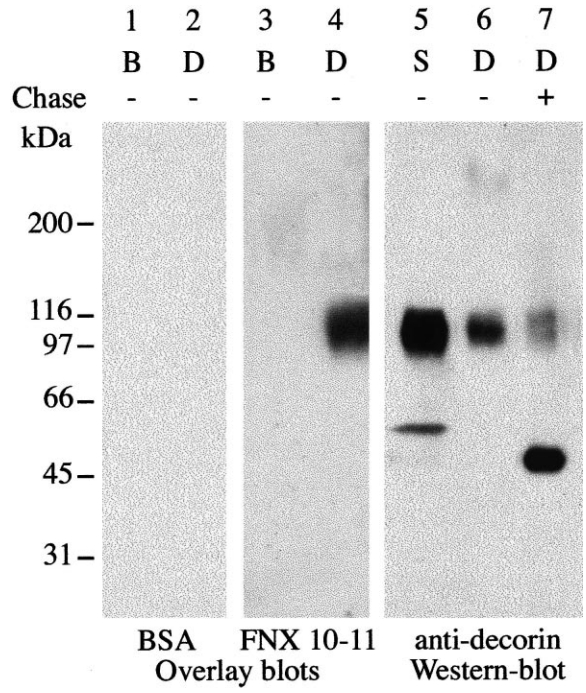

Fig. 2. Interaction of recombinant FNX 10–11 protein with purified biglycan and decorin. Purified biglycan (B), decorin (D) or skin extract (S) were resolved on 4–15% acrylamide gels and transferred to PVDF. Lanes 1–4: overlay blot incubated with BSA (lanes 1 and 2) or with FNX 10–11 (lanes 3 and 4) and revealed as described above. Lanes 5–7: Western blot revealed with monoclonal antibody against decorin. Lanes 1–7: no enzymatic treatment; lane 7: chondroitinase digestion.

3. Results

As the first approach to identifying extracellular ligands of TN-X, we performed overlay blot experiments on skin extracts. The blots were probed with a recombinant protein comprising FNIII domains 10 and 11 of bovine TN-X. This protein was tested because we have previously demonstrated that this region is responsible for heparin-binding activity and is able to bind cell surface heparan sulfates [18]. We postulated that extracellular matrix proteoglycans may also bind to TN-X via this region. In Fig. 1, we can observe that in the blots incubated with FNX 10–11 protein, a fuzzy band of approximately 100 kDa was revealed. This band was not found in the control blot incubated with BSA. The high molecular mass bands that appeared in all blots represent the TN-X present in the extract, which is recognised by the polyclonal antibody against FNX 9–10. After chondroitinase digestion of the extract, the 100 kDa band was no longer present. These results indicate that a 100 kDa proteoglycan from skin interacts with the FNX 10–11 protein and that the GAG chains are crucial for this interaction. Two candidate proteoglycans having this molecular mass, namely biglycan and decorin [22], were tested using the same overlay assay. The results, shown in Fig. 2, clearly demonstrate that purified

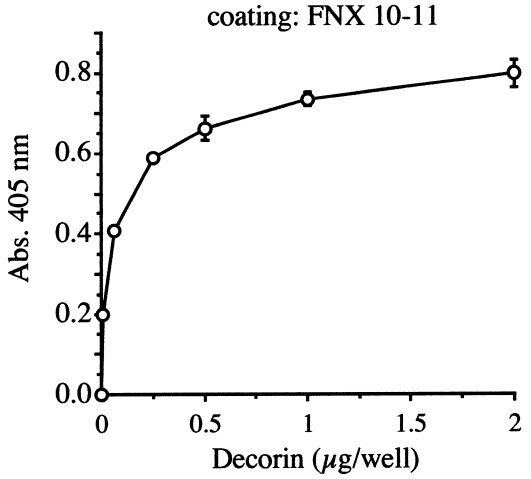

Fig. 3. Solid-phase assay of the interaction between FNX 10–11 and purified decorin. Wells were coated with 0.5 μg FNX 10–11 per well. After saturation with BSA, various amounts of decorin were added. Bound decorin was detected by monoclonal antibody.

decorin interacted with the FNX 10–11 protein, whereas biglycan did not. Also, in this experiment digestion by chondroitinase abolished the binding. The integrity of the core protein of decorin after this treatment was verified by an immunoblotting using the anti-decorin monoclonal antibody. A single band migrating at 40–48 kDa was revealed (Fig. 2), which was consistent with the molecular characteristics of decorin [22]. On this immunoblot, we also verified that the migration of undigested decorin in the skin extract was in the same position as the band revealed on the overlay blot with FNX 10–11.

Further characterisation of this interaction between decorin and TN-X was performed by solid-phase assays. The interaction also occurred when FNX 10–11 was used as the immobilised substrate with decorin in the soluble phase. As shown in Fig. 3, interaction was dose-dependent and saturable. In the previous study, dealing with the heparin-binding site, we demonstrated that it was conformational and involved both domains 10 and 11. For the interaction with decorin, a similar situation was observed; in Fig. 4, the results clearly indicate that both domains 10 and 11 must be present to obtain an interaction. By solid-phase assays, we also showed that the whole TN-X molecule, purified from skin, was able to interact with decorin (Fig. 4). Finally, we attempted to identify the GAG chains of the proteoglycan decorin that were responsible for interaction with TN-X. The results are given in Fig. 5. Chondroitinase digestion of decorin resulted in a strong inhibition of the interaction. Competition with soluble GAG chains had a similar inhibitory effect whether heparin, heparan or dermatan sulfate were used; in contrast, no inhibition was observed when incubating with chondroitin sulfate chains.

4. Discussion

In this study, we have been able to identify decorin as a ligand for TN-X. The site of interaction is closely related or similar to the heparin-binding site that we have previously characterised. This site is conformational, located at the interface between FNIII domains 10 and 11 of bovine TN-X, as determined using recombinant proteins, and is effective in the whole TN-X protein [18]. Using the same set of recombinant proteins, we performed ligand blotting experiments and detected an interaction with decorin. Using solid-phase assays, we observed that interaction with decorin has the same requirements as heparin-binding, i.e. the individual domains 10 or 11 do not interact while proteins bearing contiguous domains 10 and 11 (FNX 10–11 and FNX 9–10–11) do and the interaction is inhibited by soluble GAG heparin but not by chondroitin sulfate. Digestion of decorin with chondroitinase ABC abolished the interaction. Taken together, these results suggest that the heparin-binding site of domains 10 and 11 of TN-X is responsible for binding to decorin.

Competition experiments performed with GAGs demonstrate that heparin, heparan sulfate and dermatan sulfate have a strong inhibitory effect whereas chondroitin sulfate is ineffective. This inhibition profile suggests that the presence of iduronate-rich domains, present in heparin, heparan sulfate and dermatan sulfate but absent in chondroitin sulfate, may be important for this interaction. A similar pattern of interaction has been described for heparin cofactor II [23] and

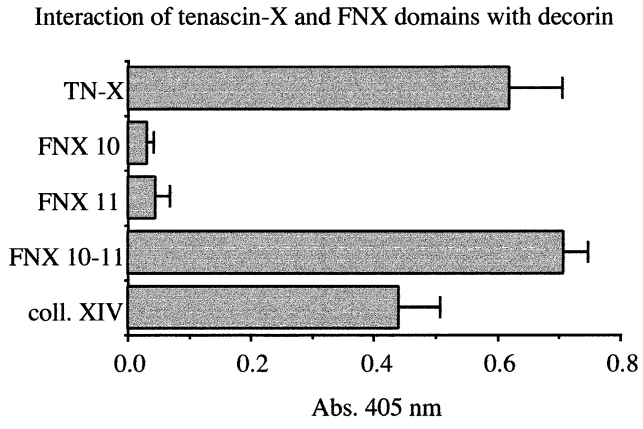

Fig. 4. Interaction of TN-X and FNX domains with decorin by solid-phase assays. Wells were coated with 3 μg TN-X, 1 μg collagen XIV (positive control) or 1 μg per well of FNX 10, FNX 11 or FNX 10–11 recombinant proteins. Interaction was performed with 1 μg decorin per well and visualisation was done as described in Section 2.

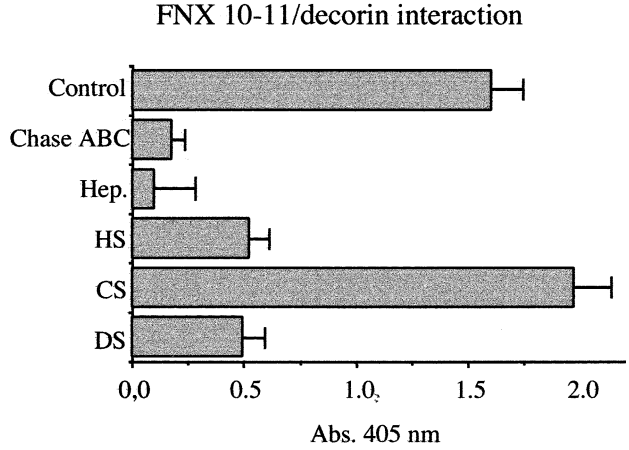

Fig. 5. Influence of GAG chains on the interaction between FNX 10–11 and decorin. Wells were coated with 1 μg of recombinant FNX 10–11 protein. After saturation with BSA, soluble GAG chains were added at 10 μg per well. Further incubation with decorin and visualisation were done as described above.

hepatocyte growth factor/scatter factor [24], both of which are able to bind both heparan and dermatan sulfates.

In a previous study we showed, by immunoelectron microscopy, that TN-X is located around collagen fibrils in dermis [14]. Since decorin is known to interact with fibrillar collagens via its core protein [25], we propose that this small proteoglycan may act as a link between collagen fibrils and TN-X in tissues. This hypothesis is corroborated by the observation of an alteration of skin tensile strength in the absence of decorin in knock-out mice [26] or in the absence of TN-X in a patient suffering from Ehlers–Danlos syndrome [17]. This property of TN-X to interact with decorin may also interfere with some functions of decorin that are due to GAG chains, such as maintenance of interfibrillar spacing [27], interaction with fibril-associated type XII and XIV collagens [21,28] or interaction with heparin cofactor II [29]. In conclusion, the interaction between TN-X and decorin demonstrated in this paper might have important biological implications in terms of building extracellular networks leading to the mechanical properties of connective tissues in normal and pathological situations.

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