Tendon extracellular matrix contains pentameric thrombospondin-4 (TSP-4)

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Abstract In preparations of cartilage oligomeric matrix protein (COMP) from bovine tendon two contaminating polypeptides of 120 and 135 kDa were detected. N-Terminal protein sequencing of these polypeptides showed homology to the N-terminus and to an internal sequence in TSP-4, respectively. TSP-4 was further enriched by heparin affinity chromatography. Electron microscopy of this sample shows primarily five armed particles with globular domains at the periphery connected to a central assembly domain in which smaller N-terminal globular domains can be resolved tightly packed at the center of the particle. We can thereby confirm the pentameric model for TSP-4 proposed by Lawler et al. [(1995) J. Biol. Chem. 270, 2809–2814], on the basis of recombinantly expressed protein. We further show that TSP-4 is abundant in tendon.

Key words: Cartilage oligomeric matrix protein (COMP); Thrombospondin; Extracellular matrix; Bovine tendon

1. Introduction

The thrombospondins (TSP) form a protein family including the originally studied TSP-1 as well as TSP-2 [2], TSP-3 [3], TSP-4 [4] and COMP [5,6,7]. A structural model for the prototype thrombospondin, TSP-1, was postulated on the basis of sequence interpretation [8] and molecular electron microscopy [9]. Each subunit of M_r 127,500 has an N-terminal and a C-terminal globular domain connected by a region of more extended structure. Close to the N-terminal globular domain disulfide bonds connect the subunits to a trimeric structure. The sequence of the major extended portion is made up of stretches of so called TSP type I, type II and type III repeats, in order from the N- to the C-terminus. The type I repeats share homology with a variety of proteins whereas the type II repeats are homologous to epidermal growth factor (EGF). The type III repeats are more unique to the thrombospondin family and are considered to be the Ca^{2+} binding structure in thrombospondin. The larger C-terminal globular domain does not show sequence similarity with any protein outside the thrombospondin family. TSP-2 shows extensive sequence homology with TSP-1 [2], has a very similar domain structure and also forms trimers [10]. The partial sequence known for TSP-3 does not yet allow for prediction of its molecular structure. The sequence for TSP-4 for *Xenopus laevis* [4] and for man [1,11] has been completed, and shows that this variant lacks the type I repeats. Recombinantly expressed TSP-4 molecules were recently prepared and shown by electron microscopy to form pentamers similar to COMP [1]. TSP-4 contains a N-terminal globular domain of about 200 amino acids which has been implicated in heparin binding.

2. Materials and methods

Fresh tendons (280 g wet weight) from adult bovine shoulder joints were obtained from the local slaughterhouse and cut into cubes of about 1 cm^3, homogenized five times for 10 s each using a Polytron homogenizer at full speed in 1000 ml of prechilled 0.15 M NaCl, 0.05 M Tris/HCl, pH 7.4 (TBS), containing 1 mM PMSF and 2 mM NEM. Tissue residue was collected by centrifugation at 17,700 × *g* at 4°C for 20 min. This wash cycle was repeated twice, each time with 15 min of stirring at 4°C. The tissue residue was then suspended in 1000 ml of the same buffer containing, in addition, 10 mM EDTA and extracted for 15 h with stirring at 4°C. The extraction with the EDTA-containing buffer was repeated once. The first EDTA extract was diluted with an equal volume of cold distilled water and applied to a column (35 × 2.6 cm) of DEAE-Sepharose Fast Flow (Pharmacia) equilibrated in 0.05 M Tris-HCl, pH 7.4, containing 1 mM PMSF, 2 mM NEM, and 5 mM EDTA. This and all further purification steps were performed at 4°C. The bound proteins were eluted with a linear gradient (500+500 ml) of 0.075 M to 0.50 M NaCl at a flow rate of 60 ml/h. The proteins of interest were detected by SDS-polyacrylamide gel electrophoresis in fractions eluting between 0.15 and 0.20 M NaCl. The pool containing the 120 kDa and 135 kDa polypeptides was concentrated by ultrafiltration (Amicon YM 10 membrane) to 40 ml, divided into two parts that were separately applied to a column (90 × 2.6 cm) of Sepharose CL4B (Pharmacia) eluted with 0.25 M NaCl, 0.05 M Tris/HCl, pH 7.4, 2 mM PMSF, 2 mM NEM, and 10 mM EDTA at a flow rate of 40 ml/h. Fractions containing the proteins of interest were pooled, dialyzed against TBS, containing 2 mM NEM, 2 mM PMSF, and 10 mM EDTA, and applied to a column (15 × 2.6 cm) of heparin Sepharose equilibrated in the dialysis buffer. After washing, the bound proteins were eluted with 0.5 M NaCl, 50 mM Tris-HCl, pH 7.4, and the fractions enriched in the 135 kDa protein were pooled, aliquoted and stored at −30°C.

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [12] on gradient gels of 4–15% polyacrylamide. The samples were, when desired, reduced with 2.5% (v/v) 2-mercaptoethanol in sample buffer. Sample proteins and molar mass standards were detected by staining with Coomassie Brilliant Blue R. The relative intensity of bands was determined from scans (NIH Image) of video images of the gels.

The 120 kDa and the 135 kDa proteins from bovine tendon were subjected to SDS-polyacrylamide gel electrophoresis after reduction and electroblotted to a Problott PVDF-membrane (Applied Biosystems). The membrane was briefly stained with Coomassie Brilliant Blue R and destained in 40% (v/v) HPLC-grade methanol. The bands of interest were cut out and N-terminal amino acid sequence was obtained using an Applied Biosystems gas phase sequenator.

For electron microscopy, samples were dialyzed against 0.2 M ammonium hydrogen carbonate, pH 7.8, and diluted with an equal volume of 80% glycerol, sprayed onto mica and rotary-shadowed [13].

3. Results and discussion

COMP was isolated from adult bovine shoulder tendon by

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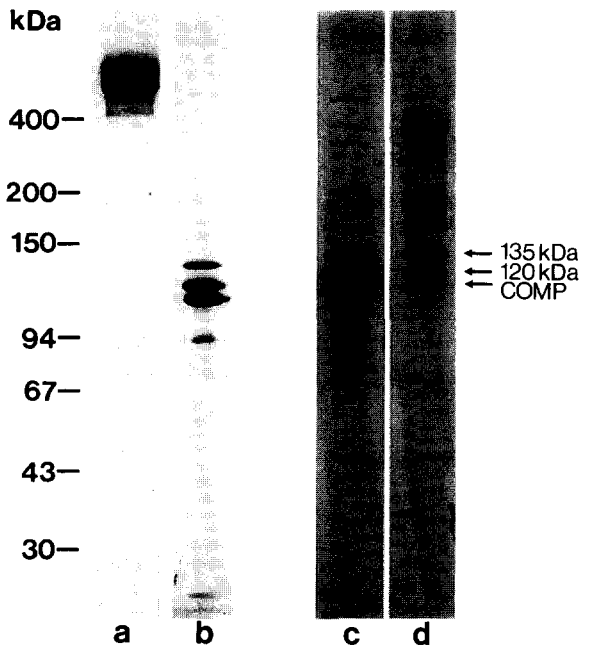


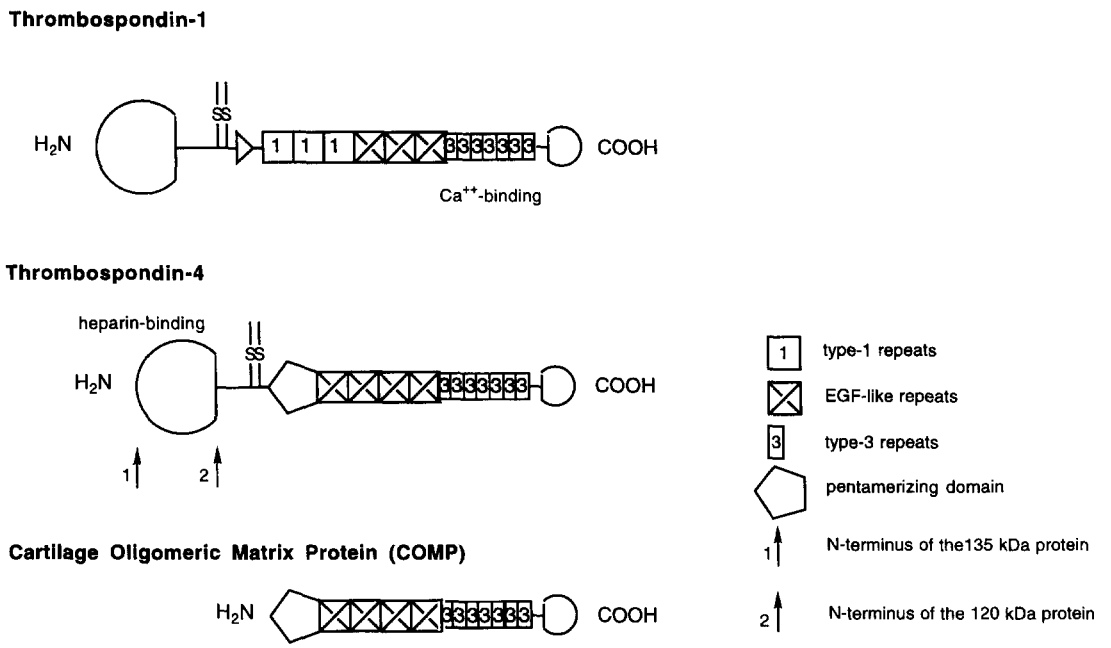
Fig. 1. SDS-polyacrylamide gel electrophoresis of the COMP/TSP-4 sample prior to the heparin Sepharose chromatography (a,b) and of unbound (c) and bound (d) pools after affinity chromatography on heparin Sepharose. The sample in (a) was not reduced while those in (b–d) were treated with 2-mercaptoethanol. The partially purified sample from bovine tendon, containing the 120 kDa and 135 kDa polypeptides and COMP as a major contaminant, was applied to heparin Sepharose. The unbound fraction (c) predominantly contains the 120 kDa polypeptide and COMP. The bound fraction (d), eluted with 0.5 M NaCl, enriched in the 135 kDa protein, contains only small amounts of the 120 kDa polypeptide and to a lesser amount COMP. Other contaminants are likely to be collagens type XII and/or XIV as observed by electron microscopy.

selective extraction with EDTA-containing buffer, ion exchange chromatography on DEAE-Sepharose, and molecular sieve chromatography on Sepharose CL4B [14]. On close analysis, the preparations showed two additional bands in SDS-polyacrylamide gel electrophoresis that under reducing conditions migrated with an apparant molar mass of 120 and 135 kDa, respectively (Fig. 1b). Under non-reducing conditions all proteins migrated close to COMP (524 kDa, [6]). There appeared to be more of the 120 and 135 kDa proteins using this purification scheme from shoulder as compared to the earlier used tendons which cross the fetlock joints [14]. The selective extraction with EDTA-containing buffer points towards a divalent cation-dependent anchorage of the 120 and 135 kDa polypeptides and COMP within the extracellular matrix of tendon. The bulk of the COMP was removed by affinity chromatography on heparin Sepharose, where the 135 kDa polypeptide bound and COMP, which lacks a heparin binding domain, elutes in the flow-through (Fig. 1c,d). The 120 kDa polypeptide which had copurified with COMP and the 135 kDa protein (Fig. 1), also eluted in the flow-through on heparin Sepharose (Fig. 1).

N-terminal sequencing of the 120 and 135 kDa proteins revealed both to be derived from TSP-4 (Fig. 2), which has been sequenced from *Xenopus laevis* [4] and man [1,11]. The 135 kDa protein corresponds to the full-length TSP-4 with a cleavage site for the signal peptide at Ala^3 (Fig. 2) when compared to the sequence and the predicted cleavage site proposed by Lawler et al. [1] for human TSP-4 on the basis of cDNA sequence (Fig. 2). The 120 kDa protein represents a fragment of TSP-4 cleaved between Gln^{186} and Ser^{187} resulting in the removal of the N- terminal globular domain of TSP-4 (Fig. 2). This fragmentation together with the observation that the 120 kDa protein does not bind to heparin Sepharose implies that the heparin binding site must be within the N-terminal globular domain. Therefore the 120 kDa TSP-4 fragment and COMP can be separated from the full-length TSP-4 by heparin affinity chromatography. The results are in agreement with Lawler et al. [1] who detected a fragment in the recombinantly expressed human TSP-4, which is recognized by antibodies to TSP-4, but which was removed by heparin affinity chromatography.

Electron microscopy using the glycerol spraying/rotary shadowing technique of the preparation enriched for full-length TSP-4 showed primarily pentameric particles arranged with the C-terminal globular domains at the periphery followed by a rod-like structure leading to a central assembly domain and the smaller N-terminal globular domains, which are tightly packed in the center of the molecule (Fig. 3). The domain structure of TSP-4 is similar to that of TSP-1, but with five subunits, instead of three, assembled in a manner analogous to the structure of COMP. Analysis of the preparation by densitometry of SDS-polyacrylamide gel electrophoresis revealed that about 40% of the protein was in either intact TSP-4 or COMP. Out of these 40% at least three-quarters were in TSP-4. Thus most of the particles seen by electron microscopy are TSP-4, which can in optimally spread particles be distinguished from COMP by showing globular structures at the center of the pentameric molecules. We can therefore confirm the in vivo relevance of the pentameric model proposed by Lawler et al. [1] on the basis of recombinantly expressed protein. The electron microscopy results are also supported by the similarity in migration of TSP-4 and COMP on SDS-polyacrylamide gel electrophoresis both before (Fig. 1a) and after (Fig. 1b) thiol reduction.

In this study we describe for the first time the isolation of TSP-4 from tissue. In bovine shoulder tendon it is expressed as a pentameric molecule and can be highly enriched under native conditions by selective extraction with EDTA-containing buffer, ion exchange chromatography, molecular sieve chromatography, and finally affinity chromatography on heparin Sepharose. Lawler et al. [1], observed that the resistance of TSP-4 to tryptic digestion is calcium-dependent. This is consistent with the selective extractibility from tendon tissue by the use of EDTA-containing buffer, which implies that interactions with other matrix components require a calcium-dependent structure. The copurification of a prominent fragment of TSP-4 with an apparent molar mass of about 120 kDa after reduction throughout the purification procedure including molecular sieve chromatography shows that this fragment still displays a pentameric structure and is only apparent as a distinct polypeptide after reduction and denaturation. The fact that the N-terminal globular domain is missing in the 120 kDa protein suggests that this part of the molecule is not essential for retaining pentameric structure. It is likely that TSP-4, similarily to COMP, is assembled via a pentameric α-helical coiled-coil structure [15]. The fragmented TSP-4 molecules as well as COMP can be almost quantitatively removed from full-length



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135 kDa protein H_2N-X-Q-A-X-P-Q-V-F-N-L-...

TSP-4 (human) ...-^{1}A-G-A-Q-A-T-P-Q-V-F-D-L-L-P-...

TSP-4 (*X. laevis*) ...-C-Q-A-^{1}Q-P-N-Y-Q-V-F-D-L-L-S-...

120 kDa protein H_2N-S-E-P-L-A-X-X-N-G-G-D-F-N-R-Q-...

TSP-4 (human) ...-L-Q-Q-^{187}S-E-P-L-A-A-T-G-T-G-D-F-N-R-Q-F-L-G-...

TSP-4 (*X. laevis*) ...-M-Q-K-^{184}S-E-A-G-Q-Q- T-G-D-V-S-R-Q-L-I-G-...

Fig. 2. Domains of thrombospondins and identification of the 120 kDa and 135 kDa polypeptides. The domain structures of TSP-1, TSP-4, and COMP are shown together with the identification of the 120 kDa and 135 kDa polypeptides by comparison of N-terminal protein sequences. The 135 kDa and 120 kDa polypeptides from bovine tendon were shown to correspond to full-length and fragmented TSP-4, respectively. The truncated 120 kDa fragment is missing the N-terminal globular domain of TSP-4 which contains the heparin binding site.

TSP-4 by heparin affinity chromatography demonstrating that the heparin binding site of TSP-4 is within the N-terminal globular domain, in analogy with TSP-1 [16]. This result also implies that proteases degrading TSP-4 preferentially cleave all five subunits of a pentameric molecule. Proteolytic cleavage of the N-terminal heparin binding domains must change the properties of the whole pentameric molecule and could well be of physiological relevance.

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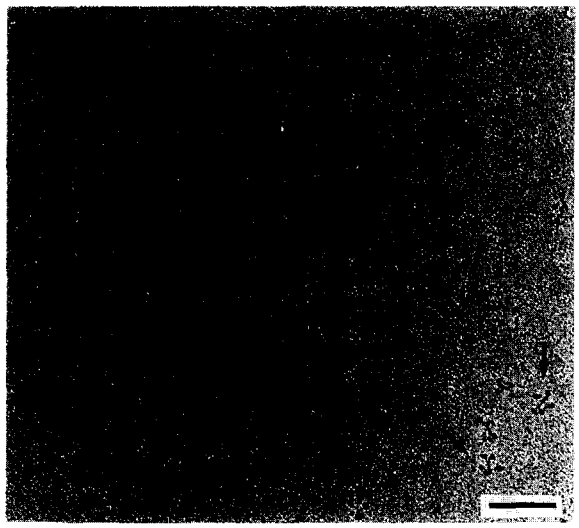

Fig. 3. Rotary shadowing electron microscopy of a protein preparation from bovine tendon, enriched for TSP-4. Most particles show a pentameric structure with globular domains at the periphery and rod-like connections leading to the central assembly domain. In some particles (indicated by arrows), smaller N-terminal globular domains of TSP-4 can be seen tightly packed at the center of the particle. Bar = 50 nm.

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