Thrombospondin-4 Binds Specifically to Both Collagenous and Non-collagenous Extracellular Matrix Proteins via Its C-terminal Domains*

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Full-length and truncated forms of rat thrombospondin-4 (TSP-4) were expressed recombinantly in a mammalian cell line and purified to homogeneity. Biochemical analysis revealed a limited proteolytic processing, which detaches the N-terminal heparin-binding domain from the rest of the molecule and confirmed the importance of the heptad-repeat domain for pentamerization. In electron microscopy the uncleaved TSP-4 was seen as a large central particle to which five smaller globules are attached by elongated linker regions. Binding of TSP-4 to collagens and to non-collagenous proteins could be detected in enzyme-linked immunosorbent assay-style ligand binding assays, by surface plasmon resonance spectroscopy, and in rotary shadowing electron microscopy. Although the binding of TSP-4 to solid-phase collagens was enhanced by Zn2⁺, that to non-collagenous proteins was not. The interactions of TSP-4 with both classes of proteins are mediated by C-terminal domains of the TSP-4 subunits but do not require an oligomeric structure. Major binding sites for TSP-4 are located in or close to the N- and C-terminal telopeptides in collagen I, but additional sites are detected in more central regions of the molecule.

The thrombospondins (TSPs) are a family of multidomain extracellular matrix proteins, characterized by containing epidermal growth factor-like TSP type II domains, calcium-binding TSP type III repeats, a highly conserved C-terminal domain, and an assembly domain that allows oligomerization through the formation of a coiled-coil α-helix. At present, five family members fulfill these criteria, i.e. TSP-1 (1–3), TSP-2 (4), TSP-3 (5), TSP-4 (6), and COMP (cartilage oligomeric matrix protein) (7–9). All TSPs except COMP also contain an N-terminal heparin-binding domain, and TSP-1 and TSP-2, in addition, contain procollagen-like domains and properdin-like TSP type I domains. Although TSP-1 and -2 form trimers via their coiled-coil domains, TSP-3, TSP-4, and COMP form pentamer coiled coils.

TSP-1 is the best characterized family member. It is found in blood platelets but also in the extracellular matrix formed by many cell types. It has been proposed to bind other matrix proteins, proteolytic enzymes, growth factors, and cellular receptors (for review see Ref. 10). Mice lacking TSP-1 show a mild skeletal and hematological phenotype together with lung abnormalities and develop pneumonia (11). TSP-2 is present in many kinds of connective tissue, including the walls of blood vessels (12, 13), and mice deficient in this protein show connective tissue abnormalities, increased vascular density, and a bleeding diathesis (14). TSP-3 is mainly expressed in lung, cartilage, and brain (12, 15), but little is known about its functions in those tissues. COMP is strongly expressed in all types of cartilage (7) but is also present in tendon (16). It has been shown to bind specifically to collagen II (17) and might regulate fibril formation or mediate interactions of collagen with other matrix components.

TSP-4 was initially identified and cloned from Xenopus laevis (6). It is first expressed in late gastrulation, with its mRNA being restricted to somitic mesoderm and skeletal muscle (18). Its complete cDNA sequence has been determined also from humans (19) and rat (20). A structural model showing a pentameric structure was proposed based on the characterization of the human recombinant protein (19) and confirmed by analysis of bovine TSP-4 isolated from tendon tissue (21). Some results indicate that in tendon TSP-4 subunits may occur in hybrid pentamers together with COMP subunits (22). In chick its mRNA is found in osteogenic tissues and corneal fibroblasts, but not in somitic mesoderm or skeletal muscle (23) as in frog and humans, showing that the pattern of tissue-specific expression may vary between species. Arber and Caroni (20) isolated rat TSP-4 cDNA from a subtractive cDNA library prepared from denervated skeletal muscle. Using a polyclonal antibody raised against a synthetic peptide derived from the C-terminal domain, they could detect TSP-4 in the interstitial cells in both skeletal and heart muscle. The expression of TSP-4 was elevated after denervation of skeletal muscle, with the signal intensity increasing specifically in the interstitial cells. Using in situ hybridization as well as immunofluorescence localization of TSP-4 combined with α-bungarotoxin labeling, they could show that TSP-4-expressing cells were in the immediate vicinity of the neuromuscular junction. In addition, the expression of TSP-4 by a variety of neuronal cells was detected, and in vitro assays showed that TSP-4 possesses a neurite outgrowth-promoting activity (20).

Our identification of TSP-4 as an abundant non-collagenous protein in tendon extracellular matrix (21) prompted us to investigate whether TSP-4 plays a role in extracellular matrix structure and assembly. In the present work we have recombinantly expressed full-length and truncated forms of TSP-4 in mammalian cells, characterized their structure, and used them in interaction studies to demonstrate that TSP-4 binds with...
high affinity to both collagenous and non-collagenous extracellular matrix proteins via its C-terminal region and that the former interaction is enhanced by Zn^{2+} ions.

**MATERIALS AND METHODS**

**RNA Preparation and Reverse Transcription-Polymerase Chain Reaction**—Total RNA was isolated from the skin of 4-day-old rats by the guanidinium isothiocyanate method. Reverse transcription-polymerase chain reaction was performed with Expand-Polymerase using the following primers: primer 1, 5'-TTAACATGACGCGGGCCCAACCACG-3'; primer 2, 5'-GTAAGGCTTCAACGCGACACAGC-3'; primer 3, 5'-GGCTAGCCGAGCTGCTTGAGG; primer 4, 5'-GCGAAGTCTTGGAGTGACTCC; primer 5, 5'-GGACTAGTGGTGTGTGAGATTG-3'.

**cDNA library construction**—The library was constructed using the primer pair 5/6 for CT and CT* fragments introduced a novel NcoI site, respectively, at the 3'-end and a stop codon, a NotI or a BamHI site, respectively, at the 3'-end. The products were restriction-digested with NheI and NotI/BamHI and, after purification by agarose gel electrophoresis, were inserted in-frame with the BM40 signal peptide in the expression vector pCEP-Pu (24) digested with the same enzymes. DNA sequencing was performed on an ABI 373 instrument (Amersham Pharmacia Biotech) using the Cy5TM Auto Read sequencing kit (Amersham Pharmacia Biotech).

**Expression and Purification of Recombinant TSP-4**—The recombinant plasmids were introduced by electroporation into the human embryonic kidney cell line 293-EBNA (Invitrogen). The transfected cells were selected with 0.5 μg/ml purineycin and grown to confluency. Secreted CT and CT* proteins were purified by affinity chromatography on heparin-Sepharose CL4B (Amersham Pharmacia Biotech) equilibrated in 75 mM NaCl, 15 mM Tris-HCl, pH 7.4 and applied to a column of DEAE-Sepharose FF. For the analogous purification of the heparin-binding domain, final concentration was achieved by stepwise elution from a small column of DEAE-Sepharose FF. The purification of the full-length protein. For both fragments only the 5'-end was changed by using the primer pair 5/6 or 5/56 for CT and stepwise elution from a small column of DEAE-Sepharose FF. The pure full-length protein was isolated from cell culture medium by dialysis against TBS containing 0.5 mM acetate and the non-collagenous proteins from 0.15 mM NaCl, 50 mM Tris, pH 7.4 (TBS). After rinsing with 0.04% Tween 20 in TBS for 1 h the wells were blocked with 200 μl of 1 mg/ml ovalbumin in TBS at room temperature. After rinsing as above, the coated wells were incubated for 1 h at room temperature with 10 μg/ml TSP-4 diluted in TBS containing either 1.25 mM CaCl2, 0.22 mM ZnCl2, 0.9 mM MgCl2, 0.02 mM MnCl2, or 10 mM EDTA. In separate experiments the zinc concentration was varied between 0 and 1 mM. For dose-response curves full-length TSP-4 was added at different concentrations between 0 and 4 μg/ml. Bound full-length TSP-4 and the recombinant fragments CT and CT+CC were detected using the polyclonal antibodies against full-length TSP-4. For the detection of the N-terminal TSP-4 fragments, the polyclonal antibodies against the fragment NT were used.

**RESULTS**

Cloning and Sequencing of TSP-4 cDNA from Rat Skin—cDNA was isolated from the skin of 4-day-old rats, and cDNA coding for TSP-4 was synthesized by reverse transcription. The primers used were based on the sequence derived from rat brain (20), which lacks the 5'-region coding for the signal peptide. For this reason the amplified sequence started with the N-terminal, heparin-binding domain of TSP-4. Two overlapping cDNAs with lengths of 1305 and 1617 base pairs were amplified and ligated to obtain the full-length cDNA for TSP-4.

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Sequencing revealed four discrepancies to the published sequence of rat TSP-4 (20). A deletion of three nucleotides 1308GGC1310 and two single nucleotide alterations G 1961C and C1978T occurred in our sequence. These changes were confirmed by repeated cDNA synthesis and sequencing. The deletion is also found in sequences for TSP-4 previously determined for X. laevis and humans (6, 19). The single base changes produced nucleotide triplets encoding amino acid residues conserved at these sites in other species. Similarly, the sequence 1311GCG1313 instead of GAC was found repeatedly and appeared plausible, because it is identical with that in X. laevis and conserves the acidic nature of the amino acid residue coded for in the human sequence. A silent mutation G1115T was not further investigated.

Expression of Full-length and Truncated TSP-4 and Structural Characterization of the Recombinant Proteins—The full-length and four truncated cDNAs were inserted into the pCEP-Pu vector, utilizing the signal sequence of BM40 (24). The constructs represented the full-length TSP-4 (TSP-4), the C-terminal domains together with the α-helical coiled-coil region (CT+CC), the C-terminal domains alone (CT), the N-terminal heparin-binding domain together with the coiled-coil region (NT+CC), and the N-terminal heparin-binding domain (NT) (Fig. 1). The plasmids were introduced into the human embryonic kidney 293-EBNA cell line, where they were stably maintained in episomal form. The secreted recombinant proteins were purified chromatographically from serum-free culture media. All proteins were purified in a buffer containing 2 M urea to increase solubility and purification yields. To demonstrate that the use of 2 M urea does not cause irreversible structural or functional changes, smaller amounts of the full-length TSP-4 were purified by a procedure avoiding all potentially denaturing agents and retaining divalent cations. All recombinant proteins were analyzed by automated amino acid sequencing, and in each case yielded a major sequence confirming the expected cleavage of the BM40 signal peptide.

The TSP-4 proteins were subjected to SDS-PAGE under both reducing and non-reducing conditions (Fig. 2). In each case the reduced protein product, when compared with reference proteins of known mass, yielded a molecular mass equal to or somewhat higher than that predicted from the amino acid sequence (Fig. 2B). The full-length TSP-4 gave a major band at 135 kDa and a minor one at 120 kDa. When not reduced, those proteins containing the coiled-coil domain migrated to positions compatible with the expected pentameric structure (Fig. 2A).

The purified, full-length TSP-4 was also analyzed by both negative staining and rotary shadowing electron microscopy (Fig. 3). The particles seen were comparatively homogenous with most showing up to five small globules extending via a thinner segment from a central accumulation of mass. Based on the proposed structure of TSP-4 (19), each of the small external globules corresponds to the C-terminal part of one subunit and the central mass is made up by the five N-terminal...
heparin-binding domains, which are held at close distance from each other by the coiled coil and are not individually resolved. The accumulation of stain at the center of the particles was more extensive than in earlier images of bovine TSP-4 (21).

Screen for Matrix Protein Ligands for TSP-4 and Requirement of Divalent Cations for Interactions—A variety of purified extracellular matrix proteins were coated to plastic and after saturation of free binding sites, a solution of 10 μg/ml of full-length recombinant TSP-4 was incubated in the wells. Bound TSP-4 was detected by use of a specific antiserum raised against the full-length protein. TSP-4 was incubated in the wells. Bound TSP-4 was detected by use of an antiserum to TSP-4 and a secondary antibody coupled to horseradish peroxidase yielding a colored product measured by its absorbance at 450 nm.

Because many extracellular matrix proteins, including COMP with its close structural relationship to TSP-4 (17), are known to have specific requirements for divalent cations when binding ligands, the assay was performed in the presence of a variety of such ions. Zn$^{2+}$ enhanced binding of TSP-4 to collagens, in agreement with previous observations for COMP (17), whereas binding to non-collagenous proteins did not show a strong requirement for Zn$^{2+}$ (Fig. 4) or other divalent cations that were used at their physiological plasma concentrations. In further ELISA-style assays the binding of TSP-4 to the various collagens was determined in the presence of varying concentrations of Zn$^{2+}$. A plateau of maximal binding was reached in the range of 0.1–0.5 mM (results not shown).

The interaction between TSP-4 and collagen I was investigated in greater detail. Incubation of increasing concentrations of TSP-4 with immobilized collagen I in the presence of Zn$^{2+}$ showed a saturable binding with an apparent $K_D$ of 2 mM (Fig. 5A). Independent determination of the color yield of TSP-4 coated directly to plastic allowed estimation that, at half-saturation, 18% of all TSP-4 molecules added were bound to collagen I. A sample that had been purified in the absence of urea and in the presence of divalent cations, dialyzed into the buffer with 2 M urea used for most purifications, and then back into a physiological buffer, gave a binding curve identical to that obtained for TSP-4, which had never been exposed to urea (Fig. 5A). For both kinds of samples, binding occurring in the presence of 1 mM Zn$^{2+}$ was abolished when this cation was removed and replaced by 1 mM Ca$^{2+}$ or 1 mM EDTA (Fig. 5B).

Analysis of the Interactions of TSP-4 with Collagens and Laminin-1 Using Surface Plasmon Resonance Spectroscopy—Collagens I–IV were assayed for binding to full-length TSP-4 that had been immobilized to a BiACore CM5 sensor chip (Fig. 6A). The collagens were used at 10 μg/ml in a buffer containing 0.5 mM Zn$^{2+}$. Collagens I–III showed binding, in agreement with the ELISA-style binding assays, whereas collagen IV again showed no or only weak association. The dissociation was biphasic for all bound collagens. Dissociation rate constants $k_{\text{diss}}$ were separately fitted for the fast and slow phases. The resulting $k_{\text{diss}}$ values differed between the two phases by a factor of less than seven. This difference was correspondingly also found in the calculated equilibrium dissociation constants $K_D$. Different binding sites for TSP-4 are present on collagens (see below) and could be the reason for the biphasic dissociation. Nonetheless, we give mean $K_D$ values for the binding of TSP-4 to collagens, because other causes of biphasic dissoci-
Fig. 5. Comparison of binding of differently prepared TSP-4 samples to collagen I (A) and the effect of divalent cations on the two types of preparation (B). Collagen I was coated to plastic at 2.5 μg/ml and TSP-4 isolated in the absence of urea (open circles, open bars) or after treatment with 2 mM urea and renaturation (filled circles, filled bars) was incubated in the blocked wells in the presence of 1 mM ZnCl₂ (A) or 1 mM CaCl₂ (B). TSP-4 concentration in B was 8 μg/ml (77 nM). The extent of binding was determined as described in Fig. 4.

The interaction between immobilized full-length TSP-4 and soluble laminin-1 was analyzed by surface plasmon resonance in an analogous manner (results not shown) and yielded a biphasic dissociation with a mean \( K_d \) of 0.2 ± 0.1 mM.

Localization of Ligand Binding Sites in TSP-4 by Use of Truncated Recombinant Proteins—Full-length and truncated recombinant versions of TSP-4 were tested in an ELISA-style ligand binding assay for binding to matrix proteins coated to plastic. Each TSP-4 protein was incubated at 10 μg/ml in the precoated wells in the presence of Zn²⁺ and binding detected by specific antibodies to TSP-4 (Fig. 7). Because the antiserum raised against the full-length protein favors C-terminal epitopes, a second antiserum raised against the N-terminal domain was used to detect constructs lacking the C terminus. The two antisera were used at dilutions that had been titrated to give equal binding to the full-length TSP-4. All fragments containing the C-terminal arm of the molecule bound all protein ligands, whereas those containing only the N-terminal heparin-binding domain or this domain together with the coiled-coil region showed low or no affinity (Fig. 7), indicating that the C-terminal arm contains the major binding site(s).

The full-length TSP-4 and the monomeric C-terminal fragment CT were also used in the fluid phase to study their binding to collagen I immobilized on a CM5 sensor chip by surface plasmon resonance (Fig. 8). The full-length TSP-4 showed a biphasic dissociation (Fig. 8A) similar to that seen for collagen I in the inverse assay (Fig. 6A). The overall dissociation gave a \( K_d \) of 8.1 ± 4.5 mM in good agreement to the value of 8.5 ± 6.5 mM obtained for the same interaction when collagen I was used in the fluid phase. The monomeric fragment CT showed a monophasic dissociation with a \( K_d \) of 3.6 mM (Fig. 8B).

Analysis of the Interaction of TSP-4 with Collagen I by Rotary Shadowing Electron Microscopy—Collagen I and full-length recombinant TSP-4 were dialyzed against 0.2 mM ammonium formate, containing 1 mM ZnCl₂, mixed, and studied by rotary shadowing electron microscopy (Fig. 9). A large proportion of all TSP-4 particles was bound to collagen molecules. They were most frequently seen at the N- and C-terminal extremes of the collagen (Table I) and, when samples were sprayed from more concentrated solutions, often connected two or more collagen molecules in an end-to-end manner (Fig. 9, inset). The images indicate that the major binding sites on collagen are in or close to the telopeptides, but a smaller proportion of TSP-4 particles were bound to more central regions of the collagen molecule (Table I). When the images were magnified an association of the external smaller globules, corresponding to the C-terminal globular domain, of TSP with the collagen thread was seen, in good agreement with the results obtained in binding assays using recombinant TSP-4 fragments (Figs. 7 and 8). When COMP was incubated with collagen I under the same conditions, binding was seen with a similar frequency and localization along the collagen molecule as for TSP-4 (Table I). Treatment with EDTA did not abolish binding of TSP-4 to collagen I as observed with this method. A similar proportion of all collagen molecules carried TSP-4, but in EDTA-treated samples a large proportion of TSP-4 particles bound to central regions of the collagen and not to the ends (Table I).

**Discussion**

Most extracellular matrix proteins contain multiple domains, and many also form oligomers with identical or non-identical subunits. This complex structure often allows them to interact with multiple extracellular and cell surface ligands. In some cases, like laminin, fibronectin, and TSP-1, such molecules have been dissected in great detail and the various biological activities localized to single domains or even sequence motifs. Very little specific information is available on the molecular interactions of TSP-4 and the nature of the correspond-
Fig. 7. Domain specificity of the interaction of TSP-4 with its extracellular matrix ligands studied in an ELISA-style ligand binding assay. Various collagenous and non-collagenous proteins were coated to plastic and the binding of full-length and truncated forms of TSP-4 detected in the presence of 0.22 mM ZnCl2 as described in the legend to Fig. 4. The designations for TSP-4 proteins are those given in the legend to Fig. 1.

Fig. 8. Surface plasmon resonance measurements of the binding of full-length TSP-4 and the monomeric C-terminal fragment CT to immobilized collagen I. Collagen I was immobilized on a CM5 sensor chip. A, full-length TSP-4 (50 μg/ml) and B, the fragment CT (25 μg/ml) were passed over the chip using a flow rate of 50 μl/min in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5 mM ZnCl2.

binding under the conditions used (Fig. 4). Similar results were obtained irrespective of whether TSP-4 was used in the solid or the fluid phase (results not shown). The non-collagenous ligands, laminin-1, fibronectin, and matrilin-2, showed strong binding both in the presence and absence of divalent cations, while the binding to collagens was markedly enhanced through the presence of Zn2+ but not by the other divalent cations tested. Our results are in this regard similar to those of Rosenberg et al. (17) who showed that the interactions of COMP with collagen I and II depend on the presence of either Zn2+ or Ni2+. They further indicate that the interactions of TSP-4 with collagenous and non-collagenous proteins differ in their mechanism of binding. The interactions observed in ELISA-style assays could be confirmed and quantitatively analyzed by surface plasmon resonance spectroscopy. Binding of collagens to immobilized TSP-4 in the presence of Zn2+ gave rise to biphasic dissociation curves typically giving 7-fold differences in Ka, when this constant was separately calculated for the fast and slow dissociation phase. The underlying cause of this effect is not known and to allow comparison between different ligands,
average dissociation constants were calculated for both phases together. Values in the range of 4–40 nM were obtained for the binding of collagens I, II, and III to TSP-4 in the presence of Zn\(^{2+}\) (Fig. 6A) and were independent of collagen concentration (Fig. 6B). The lowest dissociation constants were within the same order of magnitude as the 1.5 nM determined for the interaction of COMP with collagens I and II in the presence of Zn\(^{2+}\) (17). Laminin-1 bound to TSP-4 with a \(K_d\) of 0.24 nM, showing that binding to non-collagenous ligands may be at least as strong as to collagens.

The use of the truncated forms of recombinant TSP-4 in ELISA-style ligand binding assays with immobilized matrix proteins showed that both the collagenous and non-collagenous ligands bound to all TSP-4 proteins that contain the C-terminal arm, and not to those containing only the N-terminal domain or this domain together with the coiled coil (Fig. 7). Protein binding, therefore, occurs to sites in TSP-4 distant from the heparin binding site. When the full-length TSP-4 was compared with the monomeric C-terminal fragment CT for binding to collagen I in surface plasmon resonance assays (Fig. 8), the apparent dissociation constants were in the same order of magnitude, but the full-length TSP-4 showed a more complex, biphasic dissociation behavior.

Electron microscopy confirmed the involvement of the C-terminal parts of TSP-4 in the binding to collagen I and further showed that, in the presence of Zn\(^{2+}\), binding occurred mainly at sites on the very N- and C-terminal ends of the collagen molecules (Fig. 9). When samples were sprayed at higher concentration, often a particle of TSP-4 was seen to connect the ends of two collagen molecules, a phenomenon that could be of relevance for collagen fibril assembly. Parallel experiments with mixtures of the TSP-4 homolog COMP and collagen I showed a very similar frequency and topography of binding, and our results are in good agreement with previous work on COMP-collagen interactions (17). TSP-4 was also incubated with collagen I in the presence of EDTA, which efficiently chelates divalent cations. EDTA did not abolish the binding of TSP-4 to collagen, but shifted the bound TSP-4 particles from terminal to more central positions on the collagen molecules (Table I). This is surprising, because no or minimal interaction between TSP-4 and collagen I can be detected by ELISA-style binding assays performed in the presence of EDTA (Fig. 5B). In related studies of the interaction of COMP with collagens I and II Rosenberg et al. (17) also observed a more random binding with a decreased preference for terminal sites when Zn\(^{2+}\) was removed by EDTA complexation, but in their studies the redistribution was accompanied by an overall decreased binding. TSP-1 has been shown to bind specifically to the ends of collagen molecules (32), but this interaction differs from those of COMP and TSP-4 in that it is inhibited, not enhanced, by Zn\(^{2+}\).

We can at present not fully explain the differences in Zn\(^{2+}\) dependence seen for the TSP-4/collagen I interaction when studied by electron microscopy and by ELISA-style ligand binding assays, although a possibility is that the solid phase assay favors detection of a Zn\(^{2+}\)-dependent binding to terminal sites on the collagen molecules due to the way the molecules are presented when bound to a surface.

The multiple, strong interactions of TSP-4 with other matrix

| Table I Binding of TSP-4 and COMP to collagen I determined by electron microscopy |
|---------------------------------------------------------------|
| TSP-4 (Zn\(^{2+}\)) | TSP-4 (EDTA) | COMP (Zn\(^{2+}\)) |
|---------------------|-------------|------------------|
| Total number of collagen I molecules | 247 | 235 | 197 |
| Collagen I molecules with bound ligand | 87 | (35.2%) | 47 |
| Collagen I molecules with ligand bound to end | 78.2% | 51.1% | 76.6% |
| Collagen I molecules with ligand bound to internal site | 26.4% | 52.2% | 31.9% |

* A portion of the collagen I molecules carry ligands bound to more than one terminal or internal site.
macromolecules makes this protein a candidate for a role as an adaptor protein in extracellular matrix assembly. Future work will be aimed at analyzing the interactions of TSP-4 in situ and at determining the exact localization and nature of the protein binding sites in the C-terminal part of TSP-4.

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