Trimeric assembly of the C-terminal region of Thrombospondin-1 or Thrombospondin-2 is necessary for cell spreading and fascin spike organisation

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Summary

Thrombospondin-1 (TSP-1) and the highly related protein thrombospondin-2 (TSP-2) are trimeric extracellular molecules that have complex roles in wound healing, angiogenesis and matrix organisation. At the cellular level, TSP-1 supports cell adhesion and migration by the organisation of fascin spike cytoskeletal structures. To define the molecular requirements for assembly of fascin spikes by thrombospondins, we developed a panel of recombinant protein units of TSP-1 and TSP-2; these were designed according to the domain boundaries and included matched monomeric and trimeric units. These proteins were tested for their effects on cell attachment and fascin spike organisation using C2C12 skeletal myoblasts and vascular smooth muscle cells. In monomeric units, cell attachment activity was localised to the type 1 repeats or type 3 repeats/C-terminal globule, and both regions need to be present in the same molecule for maximal activity. On a molar basis, cell-attachment activities with monomeric units were low compared with intact TSP-1, and no monomeric unit induced cell spreading. Trimeric versions of the type 1 repeats were more adhesive but did not induce cell spreading. Strikingly, trimers that contained the type 3 repeats/C-terminal globule of either TSP-1 or TSP-2 supported cell spreading and fascin spike organisation, producing a similar activity to intact TSP-1. We conclude that trimeric assembly of the highly conserved TSP C-terminal region is necessary for organisation of the fascin-based cytoskeletal structures that are needed for thrombospondin-induced cell motility.

Key words: Cell adhesion, Extracellular matrix, Oligomerisation, Actin cytoskeleton, Fascin

Introduction

Interactions between cells and the extracellular matrix (ECM) play a crucial role in tissue organisation and regulate many aspects of cell behavior in multicellular organisms (for reviews, see Adams and Watt, 1993; Boudreau and Bissell, 1998). Thrombospondins (TSPs) are a family of multimeric, secreted glycoproteins that are regulated components of the ECM. On the basis of the properties of the prototypic TSP, TSP-1, these proteins are thought to provide dynamic modulation of matrix organisation, cell adhesion and migratory behavior in developing tissues and in vascular injury and wound repair in adult organisms (for reviews, see Adams et al., 1995; Bornstein, 1995; Lawler, 2000).

Within the TSP family, TSP-1 and TSP-2 are most closely related in terms of their domain organisation and degree of sequence identity (O'Rourke et al., 1992; Laherty et al., 1992). TSP-1 and TSP-2 form a distinct subgroup, subgroup A, because they are the only members of the family to contain a procollagen homology region and TSP type 1 repeats and to be assembled as trimers (Adams and Lawler, 1993). TSP-1 and TSP-2 transcripts and proteins have distinct patterns of expression, most probably because of different mechanisms of transcriptional regulation (Bornstein et al., 1991; Bornstein, 1992; O'Rourke et al., 1992; Iruela-Arispe et al., 1993; Tucker 1993; Tooney et al., 1998). TSP-1-null mice have decreased embryonic viability, are born with lordotic curvature of the spine and have increased numbers of circulating white blood cells. In addition, as neonates they develop chronic inflammation in the lung that leads to their death by pneumonia (Lawler et al., 1998). TSP-2-null mice have connective tissue abnormalities associated with disorganisation of collagen fibrils, increased bone density, increased vascular density in many tissues and a bleeding diathesis (Kyriakides et al., 1998). This multiplicity of phenotypes emphasises the general roles of TSP-1 and TSP-2 in tissue organisation.

The available mechanistic evidence indicates that TSP-1 and TSP-2 may have overlapping effects on cell function in the different tissues in which they are expressed. Deletion of either gene leads to perturbation of skin wound healing and wound healing angiogenesis, and overexpression of TSP-1 suppresses wound healing (Lawler et al., 1998; Kyriakides et al., 1999; Streit et al., 2000). In cell culture assays, TSP-1 and TSP-2 both act as adhesion molecules and are antiangiogenic (Good et al., 1990; Chen et al., 1994; Volpert et al., 1995). TSP-1 also

induces cell migration (reviewed by Adams et al., 1995). Peptides derived from the N-terminal domains of TSP-1 and TSP-2 promote disassembly of focal adhesions when added to preadherent cells (Murphy-Ullrich et al., 1993). The mechanisms by which TSP-1 binds to cells have been studied intensively and involve interactions with cell-surface proteoglycans, integrins, CD47 and the LDL-receptor-related protein, LRP (for reviews, see Lawler, 2000; Adams, 2001). For the limited number of cell types that have been examined, TSP-2 was also found to bind to cells through multiple contacts with integrin and proteoglycan adhesion receptors and LRP (Chen et al., 1994; Chen et al., 1996) (reviewed by Bornstein et al., 2000).

Cell adhesion to TSP-1 is characterised by a unique cytoskeletal organisation in which cells form lamellae with radial spikes and ribs, which contain F-actin and the actin-bundling protein fascin (Adams, 1995; Adams, 1997). These structures are also needed for cell migration on TSP-1 (Adams 1997; Adams and Schwartz, 2000). Induction of spikes by TSP-1 is transduced by syndecan-1 and requires the syndecan-1 core protein and glycosaminoglycan substitutions at residues S45 and S47 (Adams et al., 2001). Skeletal myoblasts form particularly large arrays of spikes and attach to TSP-1 by binding to the type 1 repeats and C-terminal globular domains (Adams, 1995; Adams and Lawler, 1994). However, a monomeric C-terminal domain shows low cell attachment activity and does not support full cell spreading or spike formation (Adams and Lawler, 1994) (J.C.A., unpublished). Thus the mimimal requirements for induction of cell spreading and fascin spikes by TSP-1 are not known. Furthermore, TSP-2-null fibroblasts show impaired spreading on rigid culture substrata, but the direct effects of TSP-2 on the cytoskeleton are not known (Kyriakides et al., 1998).

To date, the mechanisms of action of TSP-1 and TSP-2 have principally been determined by the use of short, linear peptides that correspond to cell-binding sites, or reagents that block particular adhesion receptors, both of which act as inhibitors of cell attachment to intact TSP1 or TSP-2. Many studies have demonstrated functional effects of peptides derived from TSP-1 on cell adhesion and migration (e.g. Guo et al., 1992; Wang and Frazier, 1998; Iruela-Arispe et al., 1999). However, the interpretation of these data is complicated by uncertainties as to the positioning of the peptide motifs within the natural tertiary structures of TSP modules. Preparation of recombinant protein units from TSPs has proved challenging because of the large size of these molecules and the need for validation of the physical structure of an engineered unit against that of the native protein. Although native TSP-1 is trimeric, fragments and monomeric portions are reported to occur in vivo (Rabhi-Sabile et al., 1996; Bonnefoy and Legrand, 2000), and it is not clear whether these moieties also have biologically relevant effects on cell adhesion and migration. To address the important question of the mechanisms by which TSP-1 or TSP-2 use to induce fascin spike cytoskeletal structure, we have prepared a panel of recombinant, physically characterised monomeric or trimeric protein units of TSP-1 and TSP-2. We demonstrate that although certain monomeric protein units have cell-attachment activity, trimeric assembly of the type 3 repeats and C-terminal globule is needed to achieve cell spreading and fascin spike cytoskeletal organisation. The implications of these novel results for the design of TSP-containing matrices for biomedical or tissue engineering applications are discussed.

Materials and Methods

Reagents

C2C12 mouse skeletal myoblasts (ATCC) were grown in DMEM containing 20% fetal calf serum (FCS), and rat aortic vascular smooth muscle cells (VSM cells, the gift of Martin Schwartz, TSRI) were grown in DMEM containing 10% FCS. All cells were maintained in a humidified 5% CO_2 atmosphere. Recombinant full-length human TSP-1 was purified from serum-free conditioned media of baculovirus-infected High Five cells using heparin-agarose chromatography as described previously (Adams et al., 1998). Fibronectin (FN) and vitronectin (VN) were purchased from Calbiochem-Novabiochem (Nottingham, UK). The mouse monoclonal antibody 55k2 to fascin was obtained from Dako (Gostrup, Denmark). Rhodamine-conjugated phalloidin was purchased from Sigma Chemical Company (St Louis, MO). Antibodies to rat integrin subunit α2 (Ha1/29), α4 (MRα4-1), β1 (Ha2/5) and β3 (F11) were purchased from Pharmingen (San Diego, CA). The affinity-purified rabbit polyclonal antibody to human FN receptor was obtained from Life Technologies (Scotland). All other chemicals used were high quality analytical grade reagents from Sigma.

Preparation of monomeric and trimeric TSP recombinant protein units

Constructs containing portions of the coding sequences of human TSP-1 or human TSP-2 were prepared in the plasmid pAcGP67.coco (COCO). The COCO vector is the baculovirus transfer vector pAcGP67A (Pharmingen) modified by the addition of DNA to encode a short linker (AAG) and a six His-tag (HHHHHH) 3′ to the cloning site, with or without an intervening thrombin cleavage site (LVPRGS) (Misenheimer et al., 2000). The cDNA inserts were generated by PCR of appropriate segments of the cDNAs for human TSP-1 or TSP-2 to yield the modules shown in Fig. 1. The inserts were ligated into the COCO vector using standard molecular biology techniques (Sambrook et al., 1993). In the N-terminus of each construct the signal sequence from the acidic glycoprotein gp67 of the AcNPV virus directly precedes the cloning site and targets the protein for secretion. The thrombin cleavage site allows the His-tag to be removed after purification of recombinant proteins that are not themselves susceptible to thrombin cleavage. Recombinant baculoviruses were obtained from Sf9 insect cells (Invitrogen, Netherlands) by co-transfection of the COCO constructs with Baculogold DNA (Pharmingen) according to standard procedures. Individual recombinant virus clones were isolated by plaque purification. After three rounds of viral amplification in Sf9 cells, pass three virus strains were made and tested for protein production by a TSP immunoblot. The highest producing clones were chosen for subsequent use. Serum-free suspension cultures of High Five (Invitrogen) or Sf9 insect cells were infected with pass three virus at multiplicities of infection of two to 10, depending on the construct and its exact protein yield. The conditioned media was harvested between 49 hours and 72 hours post-infection, after which the His-tagged recombinant proteins were purified by nickel-chelation chromatography (matrix from Invitrogen) as described (Meisenheimer et al., 2000). The trimeric or monomeric status of proteins and their purity was assessed by Coomassie blue staining of SDS-polyacrylamide gels run under reducing or non-reducing conditions in conjunction with immunoblotting with antibodies specific to TSP-1 or TSP-2. Protein concentrations were quantified by the Bradford assay (Bradford, 1976) using bovine serum albumin as a standard (BioRad kit). The preparations were stored in portions in TBS containing 0.3 mM $CaCl_2$ at –80°C.

Cell attachment assay

Flat-bottomed 96-well polystyrene plates (Nunc) were coated with 50 nM human plasma FN, 50 nM recombinant TSP-1 or between 50 nM and 1 μM of the TSP recombinant protein units for 16 hours at 4°C. The efficiency of protein coating was confirmed in two ways: by determining the TSP content of SDS-PAGE sample buffer extracts of the proteins from dishes after coating by gel electrophoresis and Coomassie blue staining or immunoblotting using standard procedures (Adams et al., 1999), and by comparison of the protein concentration of the coating solution before and after the adsorption period using the Bradford method (BioRad kit). By both measurements, 80% to 90% of each protein became adsorbed. Protein-coated surfaces were washed twice with Tris-buffered saline (TBS) containing 2 mM $CaCl_2$ and blocked with 1 mg/ml of heat-denatured bovine serum albumin (BSA, Sigma) for 1 hour at 23°C. C2C12 myoblasts or rat aortic VSM cells were trypsinised, washed and suspended at concentrations of 5×10^5 cells/ml, and a total of 50,000 cells were added per well and incubated for 1 hour or for other time periods at 37°C. Non-adherent cells were removed by gentle rinsing, and the number of adherent cells was quantified by measurement of cellular phosphatase activity (Prater et al., 1991). Briefly, the cells were incubated for 90 minutes at 37°C with 100 μl of substrate lysis solution (1% Triton X-100 containing 6 mg/ml p-nitrophenyl phosphate in 50 mM sodium acetate buffer, pH 5). The reaction was stopped by addition of 50 μl of 1 M NaOH, and the optical density was measured at 410 nm in a microplate reader.

In experiments to determine the mechanisms of cell attachment, 5 μg/ml of anti-integrin antibodies, 1 mM GRGDSP peptide, 100 μg/ml heparin, 300 μg/ml chondroitin sulphate A or 1 mM of a synthetic peptide, KRFYVVMWKQVTQS, which corresponds to the CD47-binding motif within the C-terminal globule of TSP-1 (Gao et al., 1996), were added to the cell suspensions at the time of plating. The concentrations used were based on the known sensitivities of other cell types to these reagents and, in the case of the anti-integrin reagents, the maximum levels of inhibition obtainable in pilot titration experiments against cell attachment to their known major matrix ligands. The number of attached cells was quantified as described above. Each experimental condition was carried out in triplicate in a single experiment, and data from three independent experiments were collated for descriptive statistical analysis in Excel worksheets.

Immunofluorescent staining of cells for F-Actin and fascin

Glass coverslips were coated with 50-100 nM FN, VN or recombinant TSP-1, or varying concentrations of the TSP-1 or TSP-2 recombinant protein units for 16 hours at 4°C. The surfaces were blocked, and the cells were prepared for attachment assay as described above. Non-adherent cells were removed by gentle rinsing in TBS. For staining of F-actin, cells were fixed in 3.7% paraformaldehyde for 10 minutes, washed briefly with TBS, permeabilized with O'Neill buffer (O'Neill et al., 1990) and washed again in TBS. Permeabilised cells were incubated with tetramethylrhodamine-conjugated phalloidin (Sigma) for 90 minutes, washed and mounted on slides using Vectashield mounting medium (Vector Laboratories Inc, CA).

For fascin staining, cells were fixed in absolute methanol for 10 minutes, washed with TBS and incubated with 55k2 antibody to fascin for 90 minutes. Cells were washed and incubated with FITC-conjugated anti-mouse IgG (Sigma) for 60 minutes, then washed, mounted and observed under an Axioskop epifluorescence microscope (Zeiss, Germany), and photographs were taken on Kodak T-Max 100 film. In other experiments, digital images were captured by a Hammamatsu C5985 CCD camera controlled by Improvision Openlab 2.2.5 software and processed into Adobe Photoshop 5.5 for the montages presented here.

Results

Characterisation of TSP recombinant protein units

To analyse the functional roles of the different structural domains of TSPs, we prepared a panel of recombinant protein units of TSP-1 and TSP-2 that spans the length of a type A TSP subunit (Fig. 1). The NoC(1) and NoC(2) units included the oligomerisation sequence, o, that comprises heptad repeats which specify the formation of a coiled-coil (reviewed by Beck and Brodsky, 1998). In TSP-1, this region effects trimerisation by assembly of a three-stranded coiled-coil and two interchain disulphide bonds between the subunits (Sottile et al., 1991; Misenheimer et al., 2000). These proteins were secreted as trimers and resolved on SDS-PAGE gels with apparent molecular masses of 125 kDa under non-reducing conditions and 43 kDa under reducing conditions (Fig. 2A) (data not shown). The unit oCP123(2), which included the oligomerisation domain with the procollagen domain and type 1 repeats, was also secreted as a trimer that resolved with an apparent molecular mass of 140 kDa under non-reducing conditions and 43 kDa under reducing conditions (Fig. 2A, lanes 11 and 12). The units DelNo(1), DelNo(2), CP123(1), E123(1), E3Ca(1) and E3Ca(2) did not contain the oligomerisation sequence and were secreted as monomers of apparent molecular mass 97, 97, 34, 20 or 60 kDa, respectively, under reducing conditions (Fig. 2A). These proteins showed small shifts in apparent molecular mass when compared under non-reducing or reducing gel electrophoresis conditions. These shifts are indicative of the presence of intramolecular disulphide bonds (data not shown) (Panetti et al., 1999).

The proteins that lacked the N-terminal domain, N, and included the oligomerisation domain with the entire C-terminal portion of the subunits (designated DelN(1) and DelN(2), Fig.

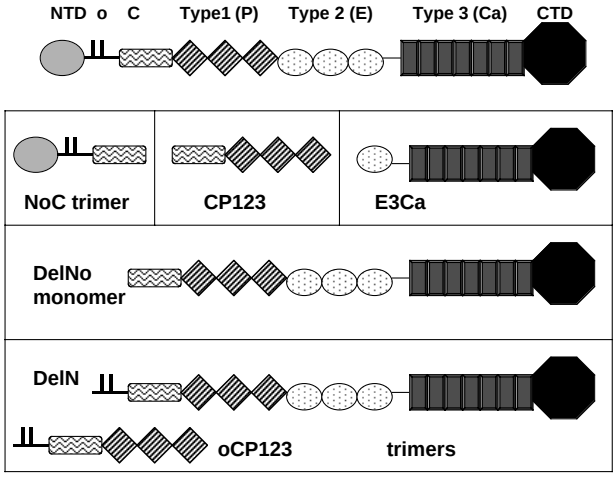

Fig. 1. Schematic representations of the domains of a subgroup A thrombospondin subunit and the recombinant protein units of TSP-1 and TSP-2. Nomenclature: NTD or N, N-terminal domain; o, oligomerisation sequence; C, procollagen module, P, type 1 (properdin) repeat; E, type 2 (EGF-like) repeat; Ca, type 3 repeat/C-terminal globule; CTD, C-terminal globule; Del, deleted. The smaller protein units are designated by the domains they contain. To avoid lengthy terminology, the largest multidomain units are designated by the portions deleted. In the text, each unit is also designated (1) or (2) to indicate whether the protein unit is derived from TSP-1 or TSP-2, respectively.

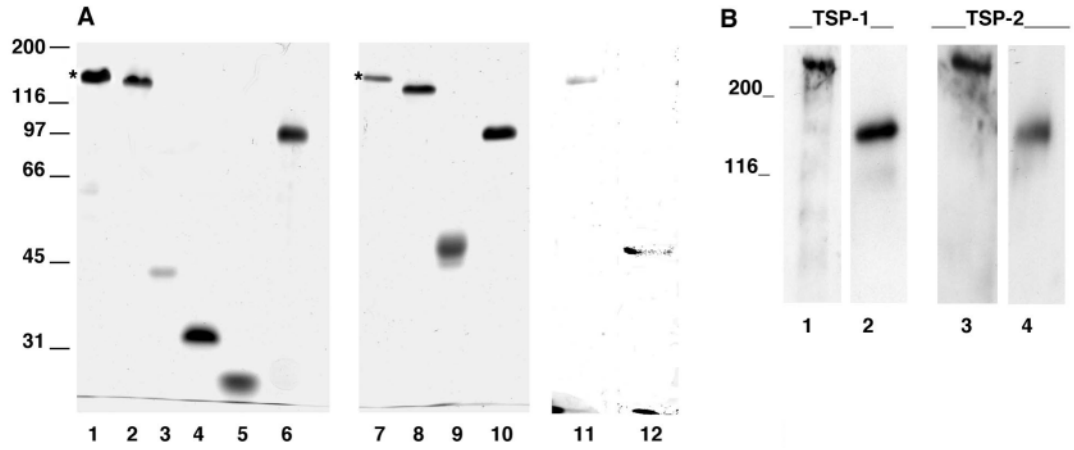

Fig. 2. Preparation of recombinant proteins. (A) The recombinant protein units DelN(1) (lane 1), DelNo(1) (lane 2), NoC(1) (lane 3), CP123(1) (lane 4), E123(1) (lane 5), E3Ca(1) (lane 6), DelN(2) (lane 7), DelNo(2) (lane 8), NoC(2) (lane 9), E3Ca(2) (lane 10) and oCP123(2) (lanes 11 and 12) were resolved by SDS-PAGE under non-reducing (lane 11) or reducing conditions (all other lanes) and stained with Coomassie blue. The asterisk marks the migration position of DelN proteins. (B) Western blots of trimeric DelN(1) (lane 1) and DelN(2) (lane 3), or monomeric DelNo(1) (lane 2) and DelNo(2), (lane 4), resolved under non-reducing conditions, transferred to nitrocellulose and probed with an antibody to TSP-1 (lanes 1,2) or an antibody to TSP-2 (lanes 3, 4). Molecular weight markers are indicated in kDa.

1) were also secreted as stable trimers that resolved on SDS-PAGE gels with apparent molecular masses of 305 kDa under non-reducing conditions and 103 kDa under reducing conditions (Fig. 2B). Detailed physical analysis of the structure of the protein units using resistance to proteolytic digestion, circular dichroism and fluorescence spectroscopy showed that their physical properties were similar to those of intact TSP-1 and TSP-1 fragments derived by limited proteolysis (Panetti et al., 1999; Misenheimer et al., 2000) (D.F.M., unpublished). The physical structure of the N-terminal globular domain does not depend on trimerisation (Misenheimer et al., 2000). The recombinant protein units are thus a complete, defined and structurally appropriate set of reagents with which to probe specific functional properties of TSP domains.

Cell attachment activity is contained within the Type 1 repeats and Type 3 repeat/C-terminal globule recombinant protein units

We first compared the ability of the TSP recombinant protein units to support cell attachment. To ensure that all of the protein activities observed were of general relevance, the assays were carried out on two non-transformed cell types that spread on intact TSP-1:C2C12 myoblastic cells, which were derived from skeletal muscle where TSP-1 and TSP-2 are co-expressed, and rat aortic vascular smooth muscle cells (VSM cells), derived from arterial smooth muscle where TSP-1 is implicated in the migratory phenotype (Majack et al., 1988; Tucker, 1993; Iruela-Arispe et al., 1993; Tooney et al., 1998; Wang and Frazier, 1998). In pilot experiments we found that the monomeric TSP-1 or TSP-2 recombinant protein units, coated at a concentration of 50 nM at which intact TSP-1 or fibronectin (FN) very effectively support cell attachment (Fig. 3A), did not promote cell attachment of either C2C12 myoblasts or VSM cells. We established in control experiments that all the recombinant protein units were adsorbed efficiently to plastic or glass surfaces. A titration experiment using up to 2 μM coating concentration of each protein was carried out to establish whether higher concentrations of the TSP units promote cell attachment. From these experiments, 1 μM coating concentrations for the E3Ca(1) and CP123(1) units from TSP-1 were found to support the attachment of both cell types, with half-maximal attachment occurring at a coating concentration of 500 nM (data not shown). Pilot time-course experiments showed that maximal cell attachment was reached at incubation times of 45 minutes or longer (data not shown). Therefore, the subsequent experiments used 1 μM coating concentrations for the recombinant protein units and a 1 hour time point.

We next quantified the attachment activities of recombinant protein units that contained different structural domains at 1 μM to 2 μM coating concentrations and compared their activities with those of intact TSP-1 and intact FN at 50 nM. Intact TSP-1 promoted approximately 90% attachment of C2C12 myoblasts and VSM cells compared with cell attachment to intact FN (Fig. 3A). Of the units from TSP-1, DelNo(1) promoted 70% to 80% cell attachment (Fig. 3A). The trimeric N-terminal unit, NoC(1), supported attachment of around 10% of the cells, which was not significantly different from background attachment to BSA (Fig. 3A). CP123(1) promoted 50% attachment of skeletal myoblasts and nearly 45% attachment of VSM cells. E3Ca(1) promoted 36% attachment of C2C12 cells and 47% attachment of VSM cells. Of the units of TSP-2, DelNo(2) supported around 70% attachment of both cell types relative to FN, and thus had similar quantitative attachment activity to DelNo(1). Cell attachment to the NoC(2) trimeric unit was not above background (Fig. 3A). E3Ca(2) had almost identical attachment activity to E3Ca(1) for both cell types (data not shown). The number of attached cells did not increase further when 2 μM coating concentrations of the recombinant protein units were used, therefore we conclude that the values obtained represent maximal levels of cell attachment to the recombinant protein units. We also tested 50:50 mixtures of 2 μM DelNo(1) with 2 μM NoC(1), or 2 μM DelNo(2) with 2 μM NoC(2), to examine whether reconstitution of all the domains of a

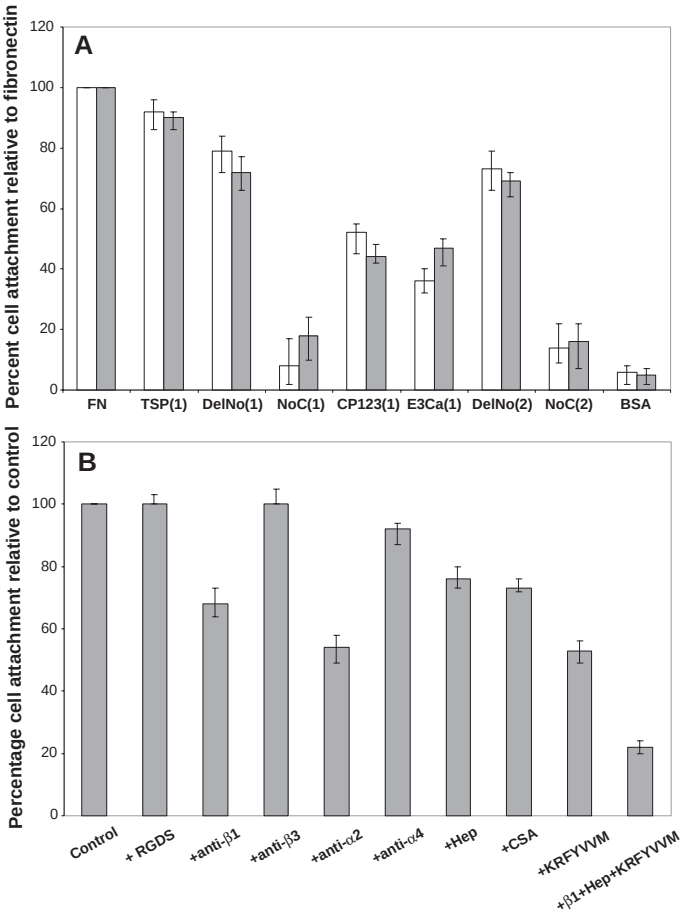

Fig. 3. Cell attachment to TSP recombinant protein units. (A) 5×10^4 C2C12 cells (white bars) or VSM cells (shaded bars) were seeded on microtitre plates coated with 50 nM intact TSP-1, FN or 1 μM of the TSP units, and cell attachment quantified after 1 hour of incubation at 37°C. Attachment to TSP-1 or the modules is expressed as percentage of cell attachment to FN. The bars show the mean±s.e.m. of three independent experiments. (B) VSM cells use multiple adhesion systems to attach to the DelNo(1) unit. Cells were incubated in the presence of 1 mM GRGDS peptide, antibodies to integrin β1, β3, α2 or α4 subunits (all at 5 μg/ml), 100 μg/ml heparin, 300 μg/ml chondroitin sulphate A or 1 mM of CD47-binding peptide, KRFYVVMWKQVTQK, either alone or in combination. Cell attachment was quantified after 1 hour of incubation and is expressed as the percentage of cell attachment relative to untreated controls. Bars show mean values ± s.e.m. from three experiments.

subgroup A TSP subunit in trans would promote higher cell attachment than the individual units. Cell attachment to these mixtures was indistinguishable from attachment to DelNo(1) or DelNo(2) coated alone at 1 μM (data not shown).

To control for the possibility that the protein conformation of the monomeric recombinant protein units was not optimal for cell attachment activity, we performed cell attachment assays with the TSP modules coated in sodium acetate buffer at pH 4. An acidic pH has been shown to improve the adsorption of TSP-1 to tissue culture plastic (Kaesberg et al., 1989). Under these conditions, the percentage attachment of both C2C12 cells and VSM cells was identical to that obtained on the protein units coated in the standard buffer conditions (data not shown). We also extended the attachment assays to 90 minutes or 120 minutes to examine whether longer incubation times were needed to detect cell attachment to certain recombinant protein units. We did not detect any significant quantitative increase in the percentage of cells attached or any change in the morphology of attached cells at longer incubation times on any of the TSP units and therefore continued to use the 60 minute incubation period in further experiments (data not shown). Overall, these experiments show that the major cell attachment sites of subgroup A TSPs are located in the type 1 repeats and type 3 repeats/C-terminal globule. The N-terminal domain and type 2 repeats are essentially non-adhesive. It was of note that the monomeric recombinant protein units that supported cell attachment had 10- to 20-fold lower activity on a molar basis than intact TSP-1.

The mechanism of attachment of VSM cells depends on multiple adhesion receptors

The attachment mechanism of C2C12 myoblasts to TSP-1 was studied previously using domain-specific antibodies and various attachment inhibitors. C2C12 cells interact with the type 1 repeats of TSP-1 and the C-terminal globular domain in a proteoglycan-dependent process that shows little or no sensitivity to anti-integrin reagents (Adams et al., 1998; Adams et al., 2001). We obtained identical results for the mechanisms of C2C12 attachment to DelNo(1) and DelN(1) (data not shown). To characterise the attachment mechanisms of VSM cells, specific inhibitors of candidate cell-matrix adhesion receptors were first tested for perturbation of cell attachment to DelNo(1), the monomer that showed maximal cell-attachment activity (Fig. 3A). Neither GRGDSP peptide nor a function-blocking antibody to the rat β3 integrin subunit blocked attachment, even when tested at a range of concentrations that, at their maximum, reduced cell attachment to VN by 75% (Fig. 3B) (data not shown). In contrast, a function-blocking monoclonal antibody to rat β1 integrin subunit maximally inhibited attachment by 32%. The same concentration of this reagent blocked VSM cell attachment to FN by 92% (Fig. 3B) (data not shown). We also tested a function-blocking polyclonal antibody to human β1 integrin subunit that has broad species cross-reactivity (Adams et al., 1998). At a concentration that maximally inhibited attachment to FN by 95%, this reagent maximally inhibited VSM attachment to DelNo(1) by 41%, and no further increase in inhibition was produced by the use of a two-fold higher concentration (data not shown).

Several β1 integrins have been implicated in the attachment of individual cell types to TSP-1 (Yabkowitz et al., 1993; Wang and Frazier, 1998; Krutsch et al., 1999). We therefore tested the effects of the available function-blocking antibodies to rat integrin α subunits (Mendrick and Kelly, 1993). Antibodies to the α4 or α5 subunits did not inhibit attachment to DelNo(1) at any of the concentrations tested (Fig. 3B) (data not shown), whereas the antibody to the α2 subunit maximally inhibited attachment by 42%, which is equivalent to the level of inhibition obtained with the anti-β1 subunit reagents (Fig. 3B). This α2 integrin antibody maximally inhibited VSM cell attachment to collagen IV by 60% (data not shown). Proteoglycans are important in cellular responses to TSP-1

(e.g. Sun et al., 1989; Mikhailenko et al., 1995; Chen et al., 1996; Shafiee et al., 2000; Adams et al., 2001; Li et al., 2001), and both heparin and chondroitin sulphate A caused approximately 25% inhibition of cell attachment to DelNo(1) (Fig. 3B). The CD47-binding peptide reduced cell attachment by 47% (Fig. 3B).

Since none of the inhibitory reagents blocked cell attachment completely, it appeared likely that multiple adhesion receptors were involved in VSM cell attachment to DelNo(1). We therefore proceeded to test the reagents in combination. The levels of inhibition produced by pairwise combinations of the inhibitors were not significantly different from those achieved using the single reagents (data not shown). In contrast, the triple combination of the antibody to β1 integrin subunit, heparin and the CD47-binding peptide reduced the attachment of VSM cells to DelNo(1) by 80%, a significant increase in inhibition compared with the double combinations (P=0.002 relative to heparin+CD47 peptide, or β1 antibody+heparin, P=0.01 relative to β1+CD47 peptide; Fig. 3B). These results indicate that α2β1 integrin, proteoglycans and CD47 act in combination to support VSM cell attachment to DelNo(1).

Cell-attachment activity depends on trimeric assembly of active domains

Whereas 50-100 nM coating concentrations of intact TSP-1 were sufficient to achieve maximal quantitative cell attachment at a level comparable to that of FN (Adams et al., 1998) (this study), 500 nM to 1 μM coating concentrations of the CP123, E3Ca or DelNo units were needed to bring about maximal cell attachment (Fig. 3A). This led us to consider the effect of subunit trimerisation on cell attachment activity. We compared the attachment activity of 100 nM TSP-1 with 100 nM of the monomeric and trimeric versions of the CP123 and DelN recombinant protein units. For both cell types, the trimeric DelN(1) and DelN(2) proteins showed comparable activity to intact TSP-1, and the trimeric oCP123(1) had 50% attachment activity, whereas at the 100 nM concentration the monomers were non-adhesive (Fig. 4A, shown for VSM cells only). The mechanisms of VSM cell attachment to the trimeric DelN(1) and DelN(2) proteins paralleled attachment to DelNo(1) in that maximal inhibition of attachment required the combination of anti-α2β1 integrin, heparin and CD47 peptide (Fig. 4B). Cell attachment to oCP123 was maximally blocked by a combination of anti-α2β1 integrin and heparin. A previously described recombinant protein corresponding to the procollagen region alone (Misenheimer et al., 2000) had negligible attachment activity, indicating that the binding site for α2β1 integrin is located in the type 1 repeats (data not shown). In accordance with the mapping of the CD47-binding site to the C-terminal globule of TSP-1, the CD47 peptide, alone or in combination, did not affect cell attachment to oCP123 (Fig. 4B) (Gao et al., 1996). Thus, cell attachment to large monomeric or trimeric units from TSP-1 depends on similar mechanisms; the mechanisms of VSM cell attachment to DelN(1) and DelN(2) are similar and in each case the avidity or affinity of interaction is higher on the trimerised modules than on equivalent monomers.

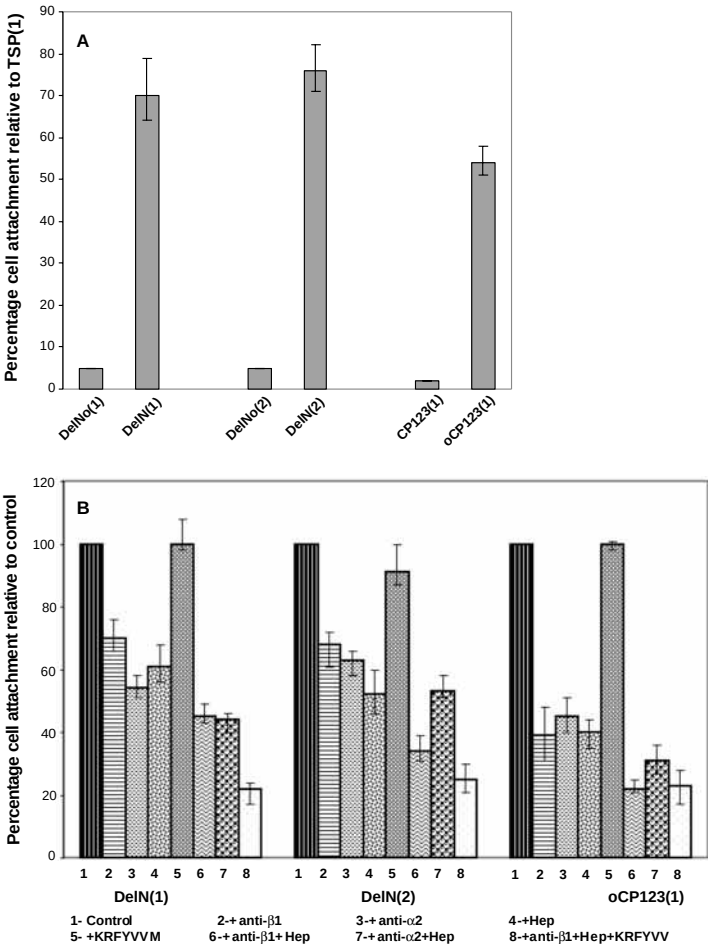

Fig. 4. Cell attachment properties of trimerised TSP recombinant protein units. (A) Attachment activity of trimeric TSP recombinant protein units. VSM cell attachment was quantified on surfaces coated with 100 nM monomeric or trimeric proteins as indicated and compared with the level of cell attachment to 100 nM TSP-1. Bars show mean values ± s.e.m. from three experiments. (B) Mechanisms of VSM cell attachment to the trimeric proteins, DelN(1), DelN(2) and oCP123(1). Cells were incubated in the presence of 1 mM GRGDS peptide, antibodies to integrin β1, β3, α2 or α4 subunits (all at 5 μg/ml), 100 μg/ml heparin, 300 μg/ml chondroitin sulphate A or 1 mM of CD47-binding peptide, KRFYVVMWKQVTQK, either alone or in combination. Cell attachment was quantified after 1 hour of incubation and is expressed as the percentage of cell attachment relative to untreated controls. Bars show mean values ± s.e.m. from three experiments.

Trimerisation of the TSP C-terminal region is needed for induction of cell spreading and fascin spike organisation

The attachment of C2C12 myoblasts or VSM cells to intact TSP-1 results in cell spreading and assembly of fascin spikes, which are also needed in cell migration on TSP-1 (Adams, 1997; Adams and Schwartz, 2000) (J.C.A., unpublished). To determine the effects of the recombinant protein units on fascin spike formation, we first examined cell spreading and cytoskeletal organisation in cells attached to monomeric DelNo(1) or DelNo(2) used at concentrations that showed similar cell-attachment activity to 100 nM TSP-1 (Fig. 3). Both cell types spread on FN, assembled actin microfilament bundles and had a diffuse distribution of fascin (Fig. 5).

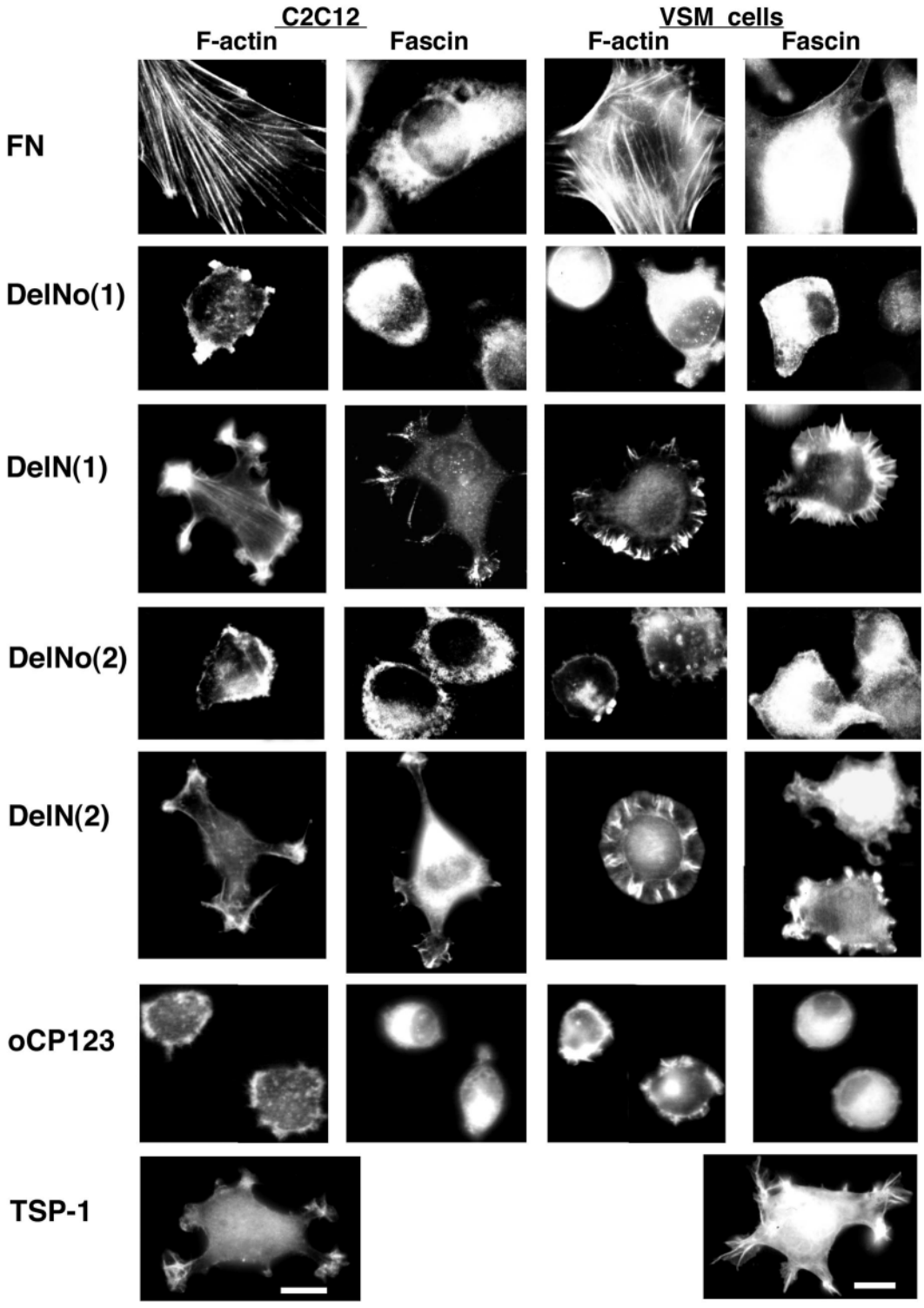

Fig. 5. The trimeric DelN(1) and DelN(2) units support cell spreading and fascin cytoskeletal organisation. C2C12 cells (left) or VSM cells (right) were seeded onto coverslips coated with 50 nM FN, TSP-1, 1 μM of the DelNo(1) or DelNo(2) units or 50 nM of the DelN(1), DelN(2) or oCP123(2) units, then fixed and stained for F-actin or for fascin. Bars, 10 μm.

However, neither DelNo(1) nor DelNo(2) supported effective cell spreading of either cell type. The cells on DelNo(1) and DelNo(2) were rounded with an irregular cell periphery or, in some cells within the population, small marginal actin ruffles. Actin microfilament bundles were not assembled, fascin distribution was diffuse and no fascin-containing structures were formed at cell margins (Fig. 5). Vinculin distribution was also diffuse (data not shown). We also examined F-actin and fascin organisation in cells on CP123(1) or E3Ca(1). On CP123(1), cells remained round. On E3Ca(1), cell margins were more irregular but the extent of spreading was no more than on the DelNo(1) units, and fascin spikes were not apparent (data not shown).

We also examined spreading and cytoskeletal organisation of C2C12 cells and VSM cells plated on a 50:50 equimolar mixed matrix of DelNo(1) and NoC(1), or DelNo(2) and

NoC(2). The mixtures did not support spreading or cytoskeletal organisation of either cell type (data not shown). We tested whether NoC(1) or NoC(2) in soluble form could affect F-actin organisation in C2C12 myoblasts or VSM cells adherent on FN. No reproducible effects on cytoskeletal organisation were apparent in these cell types (data not shown). Thus, even under conditions of maximal cell attachment, the DelNo units do not induce cell spreading, F-actin cytoskeletal organisation or assembly of matrix contacts.

We proceeded to test the capacity of the trimeric adhesive molecules to induce cytoskeletal organisation. Strikingly, both DelN(1) and DelN(2) supported cell spreading to a level comparable with that documented on intact TSP-1 when tested at equivalent molarity. On either module, C2C12 and VSM cells adopted angular, protrusive morphologies with multiple lamellipodial regions enriched in F-actin. The assembly of fascin spikes and ribs was clearly apparent in both cell types on DelN(1) (Fig. 5). C2C12 cells adherent with DelN(2) also assembled arrays of fascin spikes; however in VSM cells the organisation of fascin into radial spikes was less pronounced. The cells tended to form marginal ruffles, in which fascin was enriched at the cell peripheries but was not tightly organised into bundles like those seen in cells on DelN(1) (Fig. 5). In contrast, the trimeric oCP123(2) protein did not support cell spreading or induce fascin spikes (Fig. 5). These results demonstrate that monomers and trimers that include the C-terminal type 3 repeats/C-terminal globule show a fundamental, concentration-independent distinction in activity for the induction of cell spreading and cytoskeletal organisation.

Discussion

TSP-1 and -2 are multidomain, multimeric proteins that are regulated components of the extracellular matrix. Their interactions with cells underlie their physiological roles in tissue organisation, homeostasis and wound-healing and tumor angiogenesis. Precise definition of the molecular mechanisms involved with regard to the native structure of TSPs is a crucial step for potential experimental regulation and therapeutic modulation of the biological effects of TSPs. Here, use of a panel of TSP recombinant protein units, designed to respect the boundaries of self-folding structural modules, has enabled a new view of mechanistic requirements for primary effects of TSPs on cell adhesive behaviour. Importantly, we uncovered evidence that the trimeric assembly of the C-terminal regions of TSP subunits is necessary for the induction of cell spreading and fascin cytoskeletal organisation in adherent cells. Clearly, trimerisation per se did not induce cell spreading, because trimers of the N-terminal and procollagen domains were non-adhesive, and trimers of the procollagen domain and type 1 repeats supported cell attachment but not spreading. The spreading activity of the DelN(1) and DelN(2) trimers thus specifically relates to trimerisation of the C-terminal region, which interestingly is the most highly conserved region across all the TSP family members (reviewed by Adams, 2001).

Little is known about the adhesive effects of TSP-2, and our data provide new insights into its effects on the cytoskeleton. The matched recombinant reagents enabled direct comparisons to be made between the properties and mechanisms of action of TSP-1 and TSP-2. For both molecules, cell-attachment activity is located in the CP123 and E3Ca units and maximal cell-attachment activity is supported by these domains in combination. Both DelN(1) and DelN(2) supported cell spreading and actin cytoskeletal organisation and had a similar potency to intact TSP-1. A direct comparison with intact TSP-2 could not be made because of poor production of the recombinant protein.

Multimerisation of other ECM molecules has been documented to affect other aspects of cell function. The proliferation and migration of vascular smooth muscle cells was inhibited on polymerised collagen I (Koyama et al., 1996; Ichii et al., 2001), whereas the presence of proteolytic fragments of collagen I stimulated cell rounding and focal adhesion disassembly (Carragher et al., 1999). Lymphocytes require activation signals in order to attach to monomeric FN and fibrinogen, yet they attached and initiated intracellular signals without activation on polymerised forms of these matrix molecules (Stupack et al., 1999). Experiments in which the same mean concentration of RGD peptide is presented to cells in clusters or as a uniform sheet have directly demonstrated that clusters of ligand are more effective at inducing cell spreading and motility than monomers (Maheshwari et al., 2000). In the case of TSPs, our data provide the first direct demonstration of a necessity for TSP trimer assembly for the induction of fascin spikes. This accords with the recent demonstration that antibody-mediated clustering of syndecan-1 is sufficient to induce bundling of F-actin and fascin in spikes (Adams et al., 2001).

The trimerisation of subunits is likely to be required for a number of the functions attributed to TSP-1 or TSP-2 that depend on cell spreading and thus on the presence of an organised actin cytoskeleton. These activities include the regulation of cell-cell interactions and cell migration during wound healing or the motility of smooth muscle cells in atherosclerotic lesions (reviewed by Adams, 2001). Our results also raise the possibility that monomeric TSP moieties that have been documented in tissues (Bonnefoy and Legrand, 2000) could have different effects on cell adhesion behaviour by supporting cell attachment and not cell spreading.

In summary, our results define for the first time activities of TSP cell-attachment sites across a set of appropriately structured recombinant protein units. The data reveal major similarities in the effects on the actin cytoskeleton between TSP-1 and TSP-2. The striking differences in spreading and cytoskeletal organisation obtained on monomeric or trimeric C-terminal protein units demonstrate higher order aspects of TSP function that have implications for the molecular mechanisms of action of TSP-1 and TSP-2 in cell locomotion and tissue-modelling cell interactions. These aspects of function would need to be taken into account when designing potential preventative or therapeutic strategies or producing engineered ECM to regulate and mimic biological effects of TSP-1 and TSP-2 in wound healing, tumour angiogenesis and vascular function.

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