Extracellular matrix retention of thrombospondin 1 is controlled by its conserved C-terminal region

Josephine C. Adams[1,2,*], Amber A. Bentley[1], Marc Kvansakul[3,‡], Deborah Hatherley[4] and Erhard Hohenester[3]

[1]Department of Cell Biology, Lerner Research Institute, and [2]Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine, Cleveland Clinic Foundation, Cleveland, OH 44195, USA
[3]Division of Cell and Molecular Biology, Imperial College London, Biophysics Section, Blackett Laboratory, London, SW7 2AZ, UK
[4]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE, UK

*Author for correspondence (e-mail: adamsj@ccf.org)
‡Present address: Structural Biology Division, The Walter and Eliza Hall Institute, 3050 Parkville, Victoria, Australia

Accepted 3 December 2007
J. Cell Sci. 121, 784-795 Published by The Company of Biologists 2008
doi:10.1242/jcs.021006

Summary

Thrombospondins (TSPs) are an evolutionarily ancient family of extracellular calcium-binding glycoproteins. The five mammalian TSPs collectively have important roles in angiogenesis and vascular biology, synaptogenesis, wound repair and connective tissue organisation. Their complex functions relate to the multiple postsecretion fates of TSPs that can involve endocytic uptake, proteolysis or retention within the extracellular matrix (ECM). Surprisingly, the molecular and cellular mechanisms by which TSPs become retained within the ECM are poorly understood. We hypothesised that the highly conserved TSP C-terminal domain mediates ECM retention. We report that ECM incorporation as insoluble punctate deposits is an evolutionarily conserved property of TSPs. ECM retention of TSP1 is mediated by the C-terminal region in trimeric form, and not by C-terminal monomer or trimers of the N-terminal domain or type 1 repeats. Using a novel mRFP-tagged TSP1 C-terminal trimer, we demonstrate that ECM retention involves the RGD site and a novel site in the L-lectin domain with structural similarity to the ligand-binding site of cargo transport proteins. CD47 and β1 integrins are dispensable for ECM retention, but β1 integrins enhance activity. These novel data advance concepts of the molecular processes that lead to ECM retention of TSP1.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/6/784/DC1

Key words: Thrombospondins, Extracellular matrix, Integrins, L-type lectin, Red fluorescent protein

Introduction

The thrombospondins are an evolutionarily ancient family of extracellular, calcium-binding glycoproteins with roles in cell interactions. The five TSPs of mammals, (TSP1-TSP5), have important roles in cell interactions with extracellular matrix (ECM), angiogenesis and vascular biology, synaptogenesis, immune response, wound repair, and connective tissue organisation (reviewed by Bornstein et al., 2004; Adams, 2001; Lawler and Detmar, 2004; Posey et al., 2004). TSPs are complex multidomain and multimeric proteins. In their C-terminal regions, all family members have the same domain organisation that consists of three or four EGF-like domains, a series of calcium-binding, thrombospondin type 3 repeats and a globular C-terminal domain with structural homology to L-lectin domains (Adams and Lawler, 1993; Kvansakul et al., 2004; Carlson et al., 2005) (as schematised in Fig. 2A). The N-terminal regions of individual family members have lower sequence identity and show variations in domain organisation. The five TSPs of land vertebrates divide into two structural subgroups – A and B – according to their assembly as trimers or pentamers (Adams and Lawler, 1993).

The diverse functions of TSPs depend on their presence in the extracellular milieu. However, extracellular TSPs can undergo several fates, which include uptake, proteolysis or retention within ECM. Interactions of the laminin-G-like N-terminal domain with cell-surface heparan sulphate proteoglycans and LDL-receptor-related protein (LRP) provide a mechanism for rapid endocytic uptake and intracellular proteolysis of intact TSP1 or fragments containing the N-terminal domain (Elzie and Murphy-Ullrich, 2004). Multiple TSPs bind to or are substrates of extracellular proteases (Hogg et al., 1994; Bonnefoy and Legrand, 2000; Agah et al., 2005; Lee et al., 2006). The resulting context-dependent extracellular TSP fragments can have activities distinct from the parent protein (Lee et al., 2006). The third fate of retention within the ECM appears to relate to cell phenotype and environmental conditions. Many proliferative cells accumulate extracellular TSP1 in pericellular patches (Raugi et al., 1982; Mosher et al., 1982; Jaffe et al., 1983; Vischer et al., 1985; Mumby et al., 1984). The retention of TSP1 in vascular smooth muscle cell ECM is increased under inflammatory stimulation (Kuznetsova et al., 2006). Surprisingly, despite intensive research into their structure and biological roles, there is relatively little understanding of the cellular and molecular mechanisms by which TSPs are retained within ECM. This question motivated our study. We have examined the hypothesis that the highly conserved TSP C-terminal region has a role in ECM retention.

In vivo, the presence of TSPs in ECM is well documented during mouse embryonic development and in adult human tissues. For example, TSP1 is present in epidermal basement membrane, epiretinal membranes and arterial microfibrils (Raugi et al., 1987; Wight et al., 1985; Hiscott et al., 1992; Fauvel-Lefeve et al., 1996).

TSP2 is present in cartilage and various connective tissues (Bornstein et al., 2004). TSP4 is present in the ECM of the nervous system, at neuromuscular junctions, in the developing retina, adult eye and tendon (Arber and Caroni, 1995; Hauser et al., 1995; Stenina et al., 2003; Dunkle et al., 2007). TSP5 or COMP (cartilage oligomeric matrix protein) is a component of ECM in cartilage, synovium and tendon, which associates with collagen fibrils and binds collagens II and IX, matrilin-3 and fibronectin in vitro (Hedbom et al., 1992; DiCesare et al., 1995; DiCesare et al., 1997; DiCesare et al., 2002; Sodersten et al., 2005). In vivo models and human diseases provide evidence that TSPs contribute functionally to ECM organisation and can modulate the adhesive or structural properties of ECM. For example, TSP2-null mice have aberrant assembly of collagen fibrils because of reduced cell surface tissue transglutaminase, resulting in lax tendons and decreased tensile strength of the skin (Kyriakides et al., 1998; Agah et al., 2005). Interaction of *Drosophila* TSP (D-TSP) with αPS2 integrin on muscle cells is needed for stable tendon and muscle attachment during development (Chanana et al., 2007; Subramanian et al., 2007). Polymorphisms in the coding sequences of human *THBS1* and *THBS4*, which predispose to cardiovascular disease, enhance platelet aggregation in the case of TSP1 and affect endothelial cell adhesion in the case of TSP4 (Stenina et al., 2007).

In vitro binding assays with purified proteins have demonstrated that TSPs can bind to multiple structural ECM components and to various growth factors that may themselves bind ECM (reviewed by Adams, 2001). These findings indicate targets for binding by extracellular TSPs, but do not address the molecular processes by which TSPs come to be retained within ECM. Knowledge of these processes is important with regard to the clear biological significance of TSPs in connective ECM, tumour biology, immune response, synaptogenesis and vascular function. Prospective therapeutic modulations or construction of synthetic ECM would also benefit from this knowledge. Until recently, the ability to analyse these mechanisms was hampered by a lack of understanding of the complex structure of TSP polypeptides, particularly the large and highly conserved C-terminal region. In recent years, structures for all the major domains of TSP1 and TSP2 have been solved at the atomic level (Tan et al., 2002; Tan et al., 2006; Kvansakul et al., 2004; Carlson et al., 2005). We demonstrate here that ECM retention is a conserved property of TSPs and present data that outline the mechanism with regard to TSP1.

Results

An experimental system to analyse extracellular matrix retention of TSP1

Consistent with the presence of extracellular TSP1 in tissues, one fate of TSP1 secreted by cultured cells is to be retained extracellularly as cell-associated patches, short fibrils or granules (Raugi et al., 1982; Jaffe et al., 1983; Vischer et al., 1988; Adams and Lawler, 1994; Kuznetsova et al., 2006). However, molecular mechanisms by which TSPs are retained in ECM remain poorly understood. Experiments to date have relied on the use of fixed cells or exogenously added TSP1 protein and these procedures do not reliably distinguish ECM-incorporated proteins from proteins loosely associated with the cell surface.

To address this question, we set up experimental conditions for the quantifiable analysis of ECM retention of TSP1. To identify appropriate conditions, dense cultures of C2C12 skeletal myoblasts or COS-7 cells were grown on glass coverslips for 4 days, to allow the secretion and assembly of ECM, and then the cell layers were removed by several classic methods for ECM isolation (Carter, 1982; Robinson and Gospodarowicz, 1984). The effectiveness of each method in completely removing the cell layer was assessed by DAPI staining to visualise cell nuclei and FITC-phalloidin staining to visualise F-actin-containing cellular fragments. Whereas residual cellular fragments remained after extraction of cell cultures with 2 M urea, either 8 M urea or 20 mM NH_4OH was efficient in removing all traces of cellular debris (Fig. 1A). For both cell types, the ECM layer remaining after 20 mM NH_4OH extraction was examined by staining with Oregon-Green-conjugated gelatin, which binds to multiple ECM components (Hsieh et al., 1980). The ECM of C2C12 cells contained distinct fibrils whereas the ECM of COS-7 cells was deposited in patches (Fig. 1B). Immunostaining for fibronectin demonstrated that the fibrils of C2C12 ECM contained fibronectin, whereas little fibrillar fibronectin was detected in COS-7 ECM (Fig. 1B). However, fibronectin was readily detectable in both ECMs by immunoblotting the ECM after solubilisation in hot SDS sample buffer (Fig. 1C). Skeletal myoblasts secrete abundant TSP1 that is deposited pericellularly (Adams and Lawler, 1994; Adams, 1997), and indeed TSP1 was detected in the ECM of C2C12 cells but not in COS-7 cells (Fig. 1B). The insoluble TSP1 consisted of small (0.1-0.3 μm diameter) granular patches deposited in closely spaced arrays (Fig. 1B and high-magnification inset). This pattern of deposition was reminiscent of the pericellular TSP1 patches identified previously in association with fixed cells.

To establish whether recombinantly expressed TSP1 incorporates into ECM with the same characteristics as endogenous TSP1, we heterologously expressed human TSP1 in COS-7 cells and analysed its localisation after 4 days with antibodies specific to human TSP1. The human TSP1 was present in cells at high levels in the secretory pathway (Fig. 1D, left panel) and in the NH_4OH-insoluble ECM as clustered arrays of small punctate patches (Fig. 1D, right panel; examples of individual puncta are arrowed). The areas of these arrays (mean area 1838±290 μm^2, n=15) were similar to the areas of well-spread single cells (mean area 1226±246 μm^2, n=10), indicative that the puncta were deposited underneath transfected cells. Diameters of individual puncta were in the range 0.1-0.3 μm, as for endogenous TSP1. The ECM retention of heterologously expressed TSP1 as arrays of puncta reproduced the pattern of deposition of endogenous TSP1 and was distinct from the general patterning of the ECM layer of COS-7 cells (compare the COS-7 OG-gelatin-stained ECM in Fig. 1B with COS-7 TSP1 ECM in Fig. 1D). Immunoblotting of the ECM from COS-7 transfectants established that full-length TSP1 polypeptide was present within the ECM (Fig. 1C). These results demonstrated the suitability of this assay to examine the molecular processes that regulate ECM retention of TSPs. The results obtained with 20 mM NH_4OH or 8 M urea extraction have been consistently comparable and we have routinely used extraction with 20 mM NH_4OH for the experiments described below.

ECM retention properties of TSPs are conserved between trimers and pentamers

TSPs have been well conserved in metazoan evolution (Adams et al., 2003; Adams, 2004) and we wished to test whether the property of ECM incorporation is conserved across the TSP family. We approached this question with regard to three TSPs from phylogenetically diverse animals and with respect to family members that have different numbers of TSP subunits per molecule (Fig. 2A). For consistency of detection, the proteins were expressed in V5-epitope-tagged forms and detected with antibody to the V5

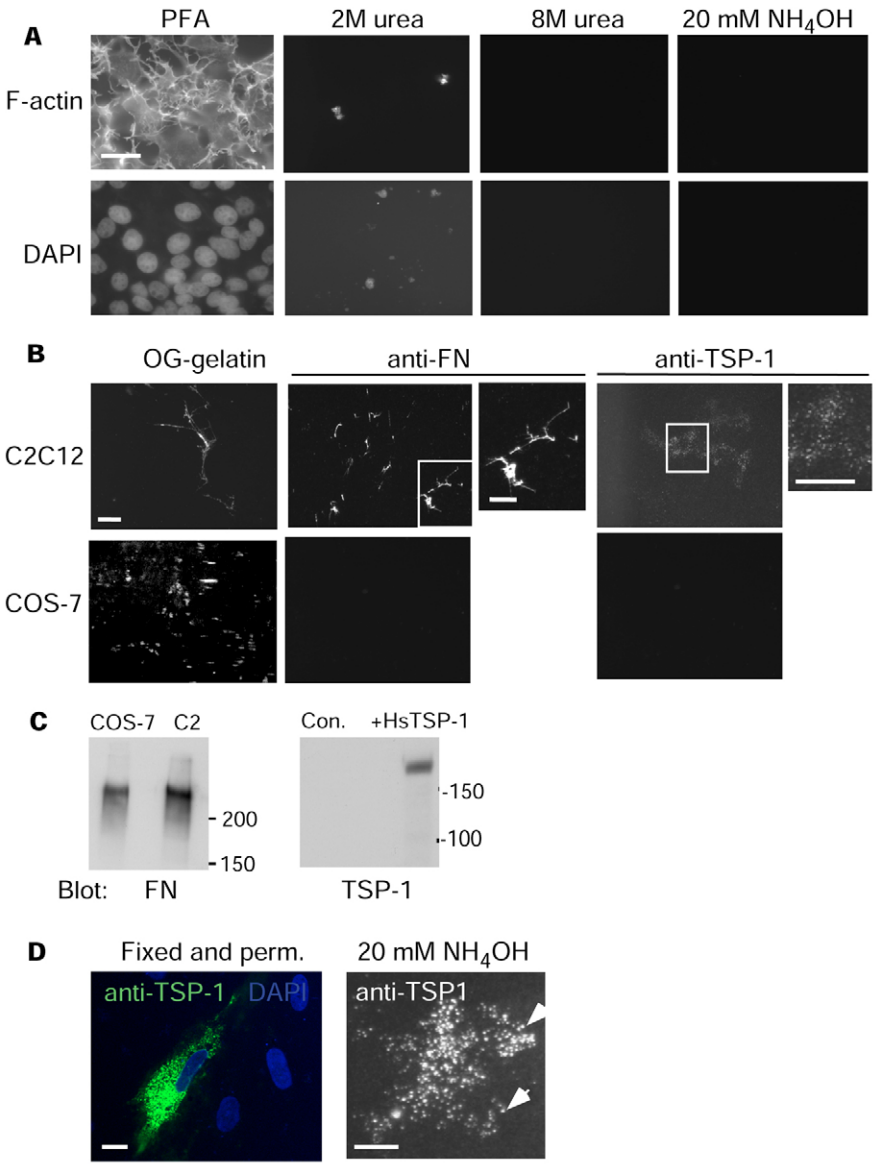

Fig. 1. Retention of endogenous and heterologously expressed TSP1 in the ECM of cultured cells. (A) Analysis of ECM isolation conditions. Confluent 4-day cultures of COS-7 cells were fixed in 2% paraformaldehyde and permeabilised in 0.5% Triton X-100 (PFA), or extracted as indicated, then stained with FITC-phalloidin to visualise F-actin or DAPI to visualise DNA. (B) Production of endogenous ECM by C2C12 and COS-7 cells. Confluent 4-day cultures were extracted with 20 mM NH_4OH and stained as indicated for components of the insoluble ECM. (C) Biochemical analysis. NH_4OH-insoluble ECM from 4-day cultures of the indicated cells was solubilised in hot SDS-PAGE sample buffer containing 100 mM DTT, resolved on 10% polyacrylamide gels and analysed by immunoblotting for fibronectin (FN) (COS-7 and C2C12 cells) or TSP1 (control COS-7 cells and COS-7 transfected to express human TSP1). (D) COS-7 cells were transfected with human TSP1, cultured for 3 days and then fixed in 2% paraformaldehyde, permeabilised and stained for human TSP1 to visualise the intracellular pool, or extracted with 20 mM NH_4OH and the insoluble ECM stained for human TSP1. Right panel shows a single array of puncta; arrows indicate examples of individual puncta. Scale bars: 25 μm (A); 10 μm (B,D).

tag (see Materials and Methods). *Ciona intestinalis* TSPA has a very similar domain architecture to TSP1 and 49% overall sequence identity to human TSP1 (Fig. 2A). *Ciona* TSPA is an unusual TSP that lacks a coiled-coil domain and multimerises as a dimer (Adams et al., 2003) (our unpublished results). Nevertheless, *Ciona* TSPA was retained in ECM in the form of punctate deposits (Fig. 2B). *Drosophila melanogaster* TSP (D-TSP) is a pentamer with a distinct domain architecture (Fig. 2A). The type 3 repeats and L-lectin domain have 41-44% and 53-57% identity, respectively, to the homologous domains of the five TSPs of tetrapods (Adams et al., 2003). D-TSP was also incorporated into the ECM as arrays of fine and very dense puncta (Fig. 2B). Quantification of the percentage of V5-expressing cells in fixed and permeabilised 4 day COS-7 cell cultures established that D-TSP and *Ciona* TSPA were expressed very similarly to TSP1 (Fig. 2C), yet both were retained in ECM to a greater extent than TSP1 (Fig. 2D). COMP/TSP5 has a short, 19 residue N-terminal domain and essentially comprises a pentamer of the C-terminal region (Fig. 2A) (Posey et al., 2004). Human COMP/TSP5 and human TSP1 have 54% identity across the C-terminal region (i.e. comparing EGF domain 2 with the C-terminus of COMP and EGF domain 1 to the C-terminus of TSP1). COMP/TSP5 was also deposited into ECM by COS-7 cells as arrays of circular deposits, with similar morphology to the TSP1 deposits, although the individual puncta tended to be more widely spaced than those of the other TSPs tested (Fig. 2B). Although expressed by a higher proportion of transfected cells, the retention of COMP in ECM was quantitatively lower than for other TSPs (Fig. 2C,D). An unrelated V5-tagged protein, secreted MAEA, was expressed similarly to the TSPs (Fig. 2C) and was completely negative for ECM retention (Fig. 2D). These results establish that ECM retention as punctate deposits is a well-conserved activity within the TSP family. Distinctions in the efficiency of retention and density of ECM puncta indicate a level of specificity in the properties of different family members.

The trimeric C-terminal region mediates retention of TSP1 in ECM

The overall similarities in the ECM retention properties of phylogenetically remote TSPs led us to hypothesise that the C-terminal region, which is the most conserved region between TSP family members, has a major role in mediating ECM retention. Indeed, for several TSP family members, domains or motifs within this large conserved region bind to structural components of the ECM (Rosenberg et al., 1998; Holden et al., 2001; Narouz-Ott et al., 2000) (reviewed by Adams, 2004). To understand which domains of TSP1 are required for its incorporation into ECM, we analysed the activities of a set of deletion proteins. The coiled-coil oligomerisation domain of TSP1 is located adjacent to the N-terminal domain, which can thus be expressed conveniently in trimeric form as a truncation mutant (No, Fig. 3A) (Sottile et al., 1991). To examine the specific activities of the conserved C-terminal region, we built on new structural knowledge of the C-

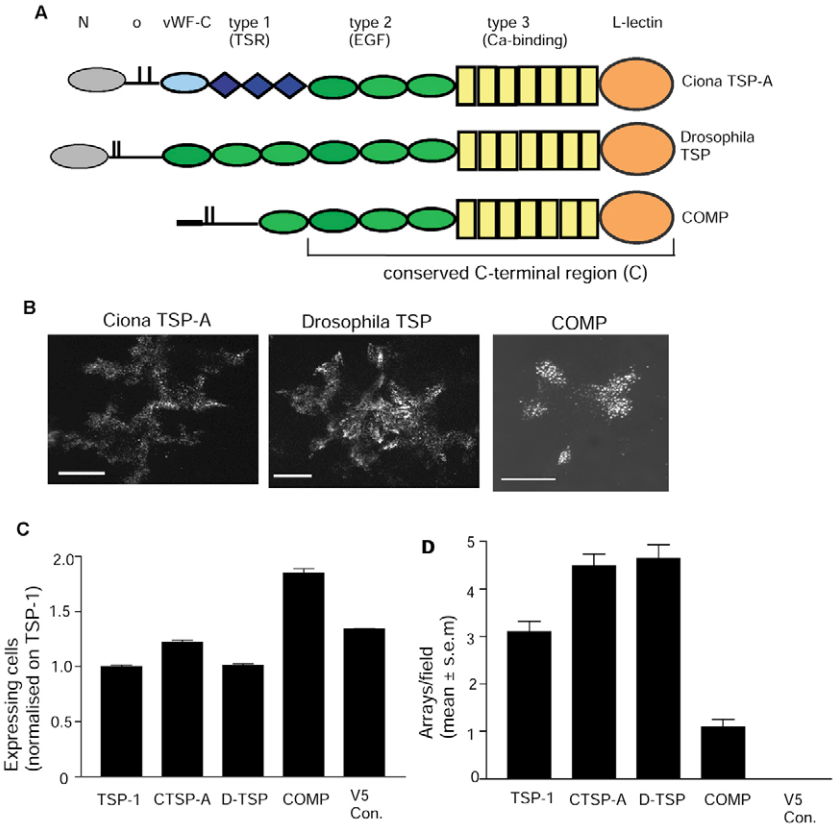

Fig. 2. Evolutionary conservation of ECM retention activity in trimeric and pentameric TSPs. (A) Schematic diagrams of *Ciona* TSPA, *Drosophila* TSP and COMP/TSP5. (B) Retention of the indicated TSPs in the ECM of COS-7 cells. Experimental conditions were as for Fig. 1B; TSPs were expressed with V5-epitope tags and detected with FITC-tagged V5 antibody. Scale bars: 20 μm. (C,D) Quantification of the relative expression (C) and ECM retention (D) of TSPs. Each column represents the mean of three to five experiments; bars represent s.e.m.

terminal regions of TSP1 and TSP2 (Kvansakul et al., 2004; Carlson et al., 2005) to engineer novel, trimeric C-terminal recombinant proteins (Fig. 3A). These were prepared in two forms: (1) as a deletion of the von Willebrand factor_C domain (vWF_C) and type 1 repeats, by fusing the encoding sequence for the signal peptide, N-terminal domain and coiled-coiled domain of human TSP1 (aa 1-297) in-frame with the encoding sequence for the C-terminal region (aa 531-1152) (NoTSP1C, Fig. 3A), and (2) as a chimaeric, fluorescently tagged protein in which the TSP1 signal peptide and N-terminal domain sequence was substituted by the signal peptide of BM40 and the encoding sequence for monomeric red fluorescent protein (mRFP), fused in frame to the encoding sequence of the coiled-coil domain and vWF_C domain (aa 241-360) and C-terminal region (aa 531-1152) of human TSP1 (mRFPovTSP1C, Fig. 3A). The vWF_C domain was included in this construct because its deletion has been found to affect trimer assembly (Lawler et al., 1992). An mRFP-tagged construct was similarly prepared for expression of the trimeric vWF_C domain and type 1 repeats (TSR) (aa 241-530) (mRFPovTSR, Fig. 3A). Constructs for expression of secreted mRFP, secreted mRFP fused to the coiled-coil domain of TSP1 (mRFPo) and mRFP-tagged C-terminal region monomer (mRFPTSP1C) were generated as controls (Fig. 3A). All the proteins were secreted efficiently. Proteins containing the TSP1 oligomerisation domain trimerised as expected (Fig. 3B,C). The mRFPo protein showed reduced fidelity of trimerisation, with higher order oligomers also secreted (Fig. 3C).

The No protein was essentially negative for ECM retention, although rare examples of small deposits of widely spaced puncta were detected (Fig. 4A and quantified in Fig. 4B,C). By contrast, NoTSP1C was retained in ECM as dense arrays of puncta very similar to those formed by intact TSP1 (Fig. 4A, quantified in Fig. 4B,D). A previously characterised monomeric C-terminal fragment corresponding to type 3 repeats 5 to 7 and the L-lectin domain (type$3_{5\text{-}7}$GCT) (Kvansakul et al., 2004) (Fig. 3A) was not deposited into ECM (Fig. 4A; quantified in Fig. 4B,D). Within the set of mRFP-tagged proteins, no incorporation of mRFP, mRFPo, mRFPovTSR or mRFP-TSP1C monomer into ECM was detected, even though these proteins were all well secreted (Fig. 4A and data not shown, quantified in 4B,D, see also Fig. 3C). By contrast, trimeric mRFPovTSP1C was strongly retained in the ECM as arrays of puncta (Fig. 4A,C,E). The appearance of these puncta, detected by fluorescent excitation of RFP, was indistinguishable from the arrays of puncta formed by intact TSP1 or NoTSP1C, detected by indirect immunofluorescence with a monoclonal antibody to human TSP1 (Fig. 4A and Fig. 1). When ECM-incorporated mRFPovTSP1C was analysed by dual imaging for RFP signal and by staining with an antibody reactive with an epitope close to the C-terminus of human TSP1, the two signals colocalised, indicating that the full-length mRFPovTSP1C protein was present in the puncta (data not shown). Immunoblotting of the ECM confirmed that the full-length mRFPovTSP1C subunit was present (Fig. 4F). Lower molecular mass fragments were also detected, although it cannot be excluded that fragmentation of mRFPovTSP1C occurred during ECM solubilisation.

We confirmed the ECM retention activity of mRFPovTSP1C in a second cell type. A549 lung carcinoma cells also deposited mRFPovTSP1C into ECM as arrays of punctate patches. The formation of these patches was specific to mRFPovTSP1C and was not detected upon expression of the mRFPo control protein (supplementary material Fig. S1). Together, these results establish that the C-terminal region in trimeric form is sufficient to mediate ECM retention and that this is a general property of cells from different tissue sources.

Deposition of mRFPovTSP1C into ECM was examined in more detail by time course experiments. ECM retention of mRFPovTSP1C became established between 1 and 2 hours after plating. At this time, cells had attached but only partially respread and the areas of deposition of mRFPovTSP1C puncta were correspondingly small and sparse (Fig. 4G). Between 4 and 8 hours after plating, in correlation with cell spreading, the arrays of punctate mRFPovTSP1C deposits increased in number and size (Fig. 4G). Between 8 and 24 hours, the area of the arrays continued to increase. With incubation for up to 7 days, the density of puncta continued to increase within the arrays, such that puncta sometimes appeared merged together as amorphous regions (Fig. 4G, arrowed, shown for the 3 day time point). At 3 days, confocal *Z*-stack images demonstrated that mRFPovTSP1C was present throughout the

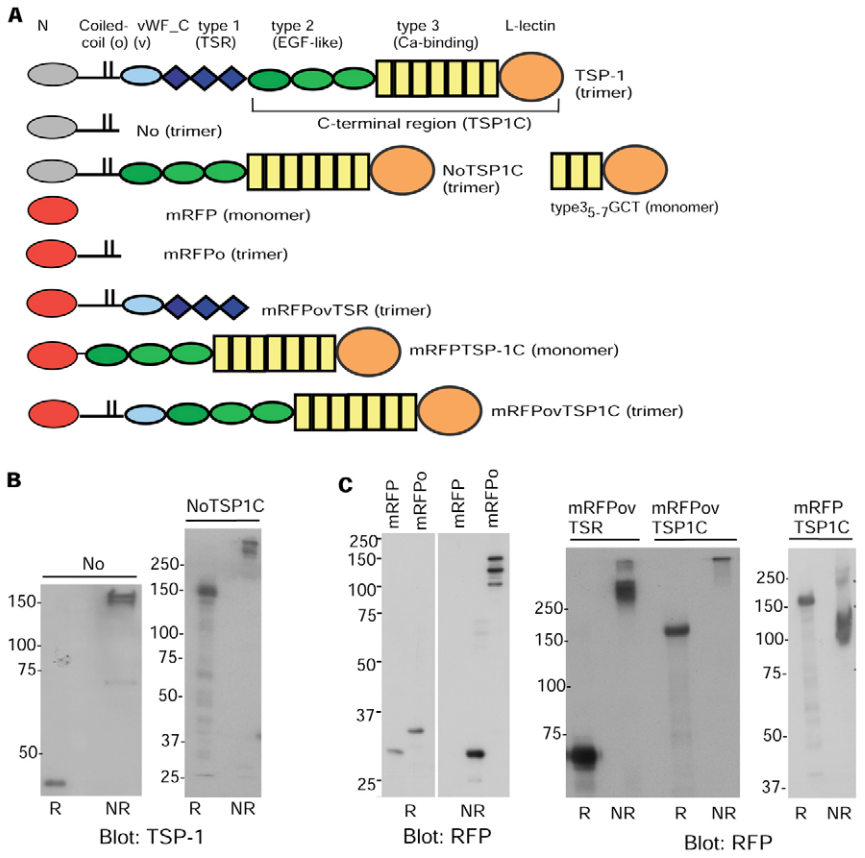

Fig. 3. TSP1 domain constructs. (A) Schematic diagrams of the TSP1 domain proteins. (B,C) Proteins were collected from conditioned medium of COS-7 cells on heparin-Sepharose (B) or Talon metal affinity resin (C) and analysed by SDS-PAGE and immunoblotting under reducing (R) or non-reducing (NR) conditions. Molecular mass markers are in kDa.

secretory pathway of intact cells (Fig. 4G). Examination of confocal *X-Z* sections confirmed the presence of intracellular perinuclear mRP-containing vesicles in intact cells and the presence of discrete punctate deposits in the insoluble ECM after NH_4OH extraction (Fig. 4H).

ECM retention of TSP1 C-terminal trimer depends on the RGD site and a novel site in the L-lectin domain

We next wished to identify specific motifs within the TSP1 C-terminal region that are important for ECM retention. For other families of ECM macromolecules that directly contribute to ECM structure, ECM assembly depends on cell-surface interactions (Colognato and Yurchenco, 2000; Mao and Schwarzbauer, 2005). Although TSP1 is not a regular structural component, we hypothesised that ECM retention would involve its cell attachment properties. The seventh type 3 repeat of TSP1 contains a conserved RGD, integrin-binding motif that mediates calcium-dependent interaction with αvβ3 integrin (Lawler et al., 1988). Interactions with β1 integrins have also been reported (reviewed by Adams, 2004). The C-terminal L-lectin-like domain is also known as a locus for cell interactions, but the molecular basis for these is currently not well understood. Two peptide motifs previously characterised to bind the cell-surface glycoprotein CD47 are located on largely buried β-strands in both the TSP1 and TSP2 crystal structures (Gao et al., 1996; Kvansakul et al., 2004; Carlson et al., 2005) (Fig. 5A) and thus are unlikely to be active for receptor binding under normal physiological conditions.

With regard to the structural homology of the TSP1 C-terminal globular domain to animal L-type lectins, such as ERGIC-53 (Velloso et al., 2002) and VIP36 (Satoh et al., 2007), we noticed that the calcium-dependent carbohydrate-binding site of VIP36 coincides with the double calcium-binding site in the TSP1 L-lectin domain (Fig. 5A). The L-lectin domains of TSP1 and VIP36 share only 10% sequence identity, but 164 Cα atoms can be superimposed with a root-mean-square deviation of 2.7 Å (program SSM) (Krissinel and Henrick, 2004). The two calcium ions in TSP1 are bound by a DDD motif (Kvansakul et al., 2004) that is conserved in all TSPs identified to date (Adams, 2004), suggesting an important function. This face of the L-lectin domain is not involved in interactions with the type 3 repeats in the TSP2 structure (Carlson et al., 2005) and is likely to be available for ligand binding in all TSPs (Fig. 5A). We hypothesised that the cell-attachment site of the TSP L-lectin-like domain may involve the calcium ion-binding DDD motif and the adjacent surface-exposed loop between the seventh and eighth β-strands (Fig. 5A).

To test the hypothesis, we first introduced point mutations of the RGD or DDD motifs into the monomeric TSP1 C-terminal fragment, type3$_{5-7}$GCT, of known structure (Fig. 5A). The RGD motif was mutated to VGD because valine is frequent at the equivalent position in other type 3 repeats. The DDD motif provides the only negatively charged metal ligands at the double-calcium site (Kvansakul et al., 2004) and mutation to AAA is predicted to abolish calcium binding at this site. Importantly, the two mutant proteins were efficiently secreted and their secondary structure and oligomeric status in the presence of 2 mM calcium ions were indistinguishable from wild-type type3$_{5-7}$GCT (supplementary material Fig. S2). Thermal unfolding of all three proteins, as monitored by the CD signal at 215 nm, occurred at ~50°C (data not shown). Thus, the mutations did not affect the folding of TSP1 C-terminal region.

Next, the set of proteins were tested for cell attachment activity under calcium-replete conditions, as a known activity of wild-type type3$_{5-7}$GCT. The type3$_{5-7}$GCT fragment supported attachment of C2C12 and human arterial smooth muscle cells (HASMC) (Fig. 5B). In the calcium-replete state, the attachment of C2C12 cells to intact TSP1 or type3$_{5-7}$GCT is not inhibited by RGD peptide (Adams and Lawler, 1994; Kvansakul et al., 2004). We found that C2C12 cells attached to type3$_{5-7}$GCT/VGD protein almost as well as to wild-type type3$_{5-7}$GCT (Fig. 5B). By contrast, HASMC attached poorly to type3$_{5-7}$GCT/VGD (Fig. 5B). The attachment of both cells to type3$_{5-7}$GCT/AAA was significantly less than to the wild-type protein, indicating that the DDD motif participates in an attachment activity that is distinct from the RGD motif (Fig. 5B). To investigate whether cell attachment to type3$_{5-7}$GCT depends on CD47, we compared attachment of fibroblasts from strain-matched wild-type or CD47-null mice (Lindberg et al., 1996). The CD47-null cells

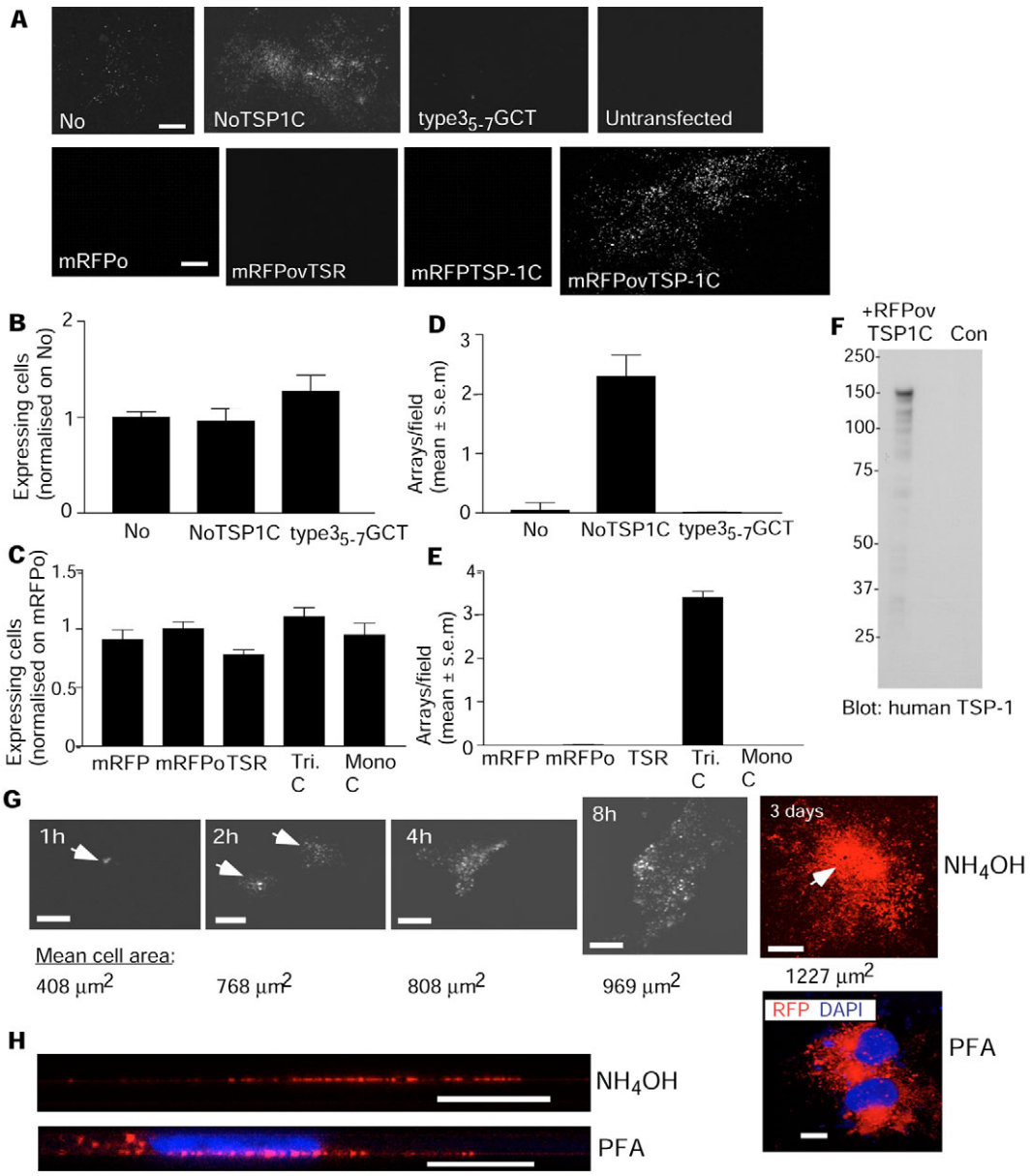

Fig. 4. Retention of TSP1 in ECM depends on the C-terminal region in trimeric form. (A) Retention of the indicated TSP1 domain deletion proteins in NH_4OH-insoluble ECM of COS-7 cells detected by indirect immunofluorescence with antibodies to human TSP1 (upper panels) or direct fluorescence of mRFP (lower panels). (B-E) Quantification of the relative expression (B,C) or ECM retention (D,E) of TSP1 domains, as detected by immunofluorescence (B,D) or mRFP (C,E). Each column represents the mean from 3-16 independent experiments, bars represent s.e.m. (F) Biochemical analysis of mRFPovTSP1C from COS-7 ECM. (G) Time course of ECM incorporation of mRFPovTSP1C. mRFPovTSP1C was expressed in COS-7 cells for 18 hours, then cells replated and extracted with 20 mM NH_4OH from 1 hour to 3 days later. Arrows indicate initial deposits of mRFPovTSP1C. The mean cell area at each time point, as measured from ten phalloidin-stained cells in each sample, is given below each panel. Colour panels show *X-Y* confocal projections of ECM puncta (upper panel; arrow indicates area of merged puncta) or intracellular distribution of mRFPovTSP1C after 3 days (lower panel). (H) Confocal *X-Z* sections show distribution of mRFPovTSP1C in ECM (upper panel) or paraformaldehyde-fixed cells (lower panel) at 3 days. Scale bars: 10 μm.

attached equally as well as wild-type cells to type3$_{5-7}$GCT. Furthermore, both wild-type and CD47-null fibroblasts had significantly reduced attachment to type3$_{5-7}$GCT/VGD and type3$_{5-7}$GCT/AAA (Fig. 5C). Thus, CD47 is not essential for cell attachment to type3$_{5-7}$GCT. This is consistent with previous data that CD47 is dispensable for assembly of fascin protrusions in response to intact TSP1 (Adams et al., 2001).

We introduced the same point mutations into mRFPovTSP1C. The mutant proteins were expressed, secreted and trimerised with comparable efficiency to the wild-type mini-trimers (Fig. 5D, supplementary material Fig. S3) yet both point mutant proteins were decreased for ECM retention (Fig. 5E). Thus, both cell attachment sites are implicated in the mechanism of ECM retention of TSP1.

β1 integrins but not CD47 contribute to ECM retention of TSP1

We next examined the role of CD47 and β1 integrins in ECM retention of TSP1 C-terminal region trimers. Wild-type and CD47-null mouse fibroblasts were found to equivalently deposit arrays of

mRFPovTSP1C puncta of similar morphology (Fig. 6A). Endogenous TSP1 was also detected in the ECMs of both cell types (data not shown). The wild-type fibroblasts were severely impaired for deposition of VGD or AAA mutants, to the extent that meaningful comparison of the CD47-null cells with these mutant proteins could not be made (data not shown). For an independent evaluation of CD47 binding to the C-terminal region of TSP1, analysis was made by surface plasmon resonance. CD47 is a well-characterised ligand of signal regulatory protein α (SIRPα) and the interaction of the extracellular domains of CD47 and SIRPα has been extensively established (Seiffert et al., 1999; Vernon-Wilson et al., 2000; Hatherley et al., 2007; Liu et al., 2007). The interaction of previously characterised recombinant proteins corresponding to the extracellular regions of human CD47 and SIRPα (Hatherley et al., 2007) with type3$_{5\text{-}7}$GCT was examined by surface plasmon resonance. As expected, the SIRPα extracellular region as analyte bound to the immobilised extracellular region of CD47 but not to itself (Fig. 6B). Similarly, the CD47 extracellular region bound to immobilised SIRPα extracellular region, but not to itself. The CD47 preparation contains some multimers (Hatherley et al., 2007), accounting for a slow rate of dissociation from SIRPα (Fig. 6C). By contrast, type3$_{5\text{-}7}$GCT as analyte produced the same signal on the control protein SIRPα as on CD47 (Fig. 6D). The increase in response units upon injection of type3$_{5\text{-}7}$GCT was due to the different refractive index of the buffer used for type3$_{5\text{-}7}$GCT compared with the running buffer. Thus, under conditions in which CD47 clearly binds its ligand SIRPα, no interaction with TSP1 L-lectin domain was detected.

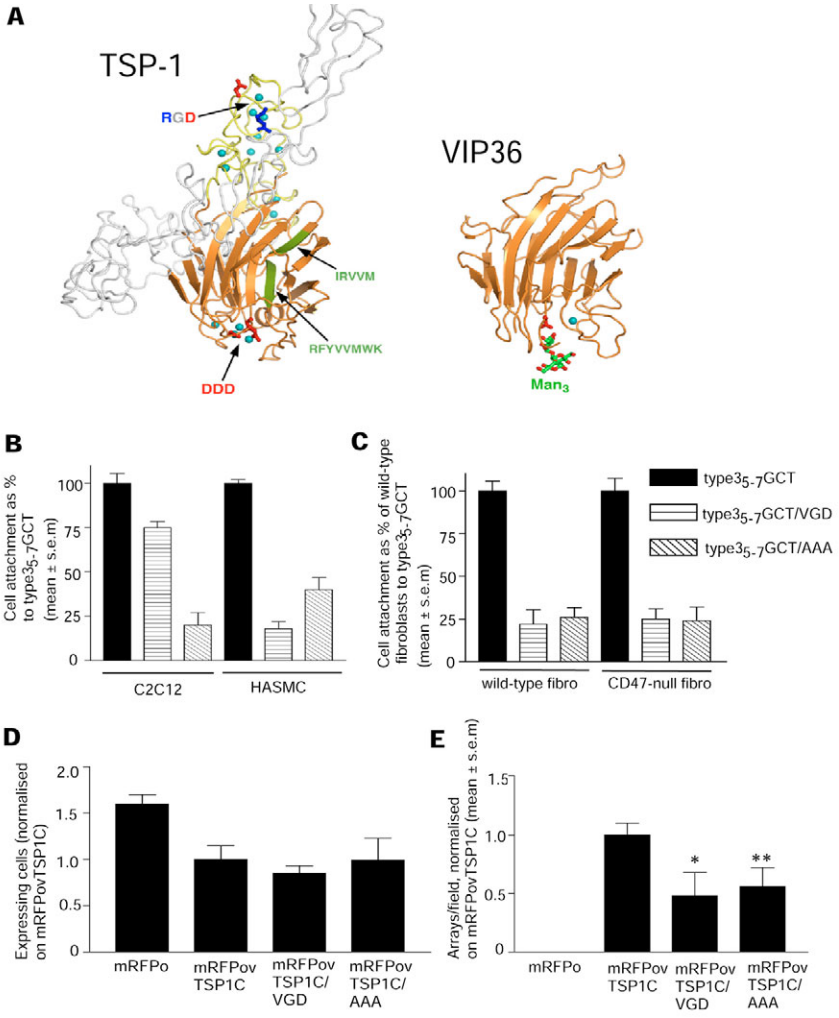

Fig. 5. Contributions of the RGD motif and L-lectin domain to ECM deposition. (A) Structural similarity of TSP1 C-terminal globular domain to L-type lectins. Left, TSP1 type3$_{5\text{-}7}$GCT crystal structure (PDB1ux6) (Kvansakul et al., 2004) with the type 3 repeats in yellow, the L-lectin domain in orange and calcium ions as blue spheres. The remaining type 3 repeats and two EGF domains in light grey were modelled from the homologous TSP2 structure (PDB1yo8) (Carlson et al., 2005). The RGD and DDD motifs in TSP1 (see text) are shown in atomic detail. The putative CD47-binding motifs (Gao et al., 1996) are in green. Right, crystal structure of VIP36 carbohydrate-recognition domain in complex with calcium (blue sphere) and a mannose trisaccharide (carbon and oxygen atoms shown in green and red, respectively) (PDB2e6v) (Satoh et al., 2007). The side chain of Asp131 (see text) is shown in atomic detail. (B,C) Cell attachment to wild-type or mutant TSP1 C-terminal monomer fragments. C2C12 and HASMC (B), or strain-matched wild-type or CD47-null mouse fibroblasts (C) were adhered to surfaces coated with 1 μM of each protein for 1 hour under serum-free conditions. Each column represents the mean from three experiments, bars=s.e.m. (D,E) Quantification of the relative expression (D) or ECM retention (E) of wild-type and mutant mRFPovTSP1C proteins. Each column represents the mean of three independent experiments; bars represent s.e.m. *P=0.02; **P=0.009.

The contribution of the RGD site in ECM retention suggested that integrins could play a role in the ECM incorporation of TSP1. To test whether β1 integrins are essential, we examined the ability of GD25, β1-integrin-null fibroblasts to retain heterologously expressed mRFPovTSP1C in ECM. These cells incorporated mRFPovTSP1C into ECM puncta, whereas control mRFPo was not deposited (Fig. 7). However, in comparison to GD25 cells re-expressing β1A integrin, fewer punctate deposits were present, even though the percentage of RFP-expressing cells was similar for both cell lines (Fig. 7A,B). Thus, β1 integrins are not essential for ECM retention of mRFPovTSP1C but may increase the efficiency of the process. The major integrin heterodimers on GD25 cells are αvβ3, αvβ5 and, at a lower level, α6β4 (Wennerberg et al., 1996). To establish whether αvβ3 has an essential role, we examined ECM retention by CHO cells, which express αvβ5 but not αvβ3 or αvβ1 integrins (Triantafilou et al., 2000). CHO cells also assembled ECM puncta of mRFPovTSP1C, demonstrating that αvβ3 integrin is not essential for the process (data not shown).

Fibronectin is a known ECM binding partner of both TSP1 and certain β1 integrin heterodimers (Homandberg and Kramer-Bjerke, 1987; Dardik and Lahav, 1989; Sottile and Hocking, 2002; Hynes, 2002; Mao and Schwarzbauer, 2005). GD25β1A cells establish a denser fibrillar fibronectin ECM than GD25 cells (Wennerberg et al., 1996). To investigate the hypothesis that increased ECM retention of mRFPovTSP1C by GD25β1A cells is due to fibronectin acting as a bridge between β1 integrins and mRFPovTSP1C, we compared the ECMs of GD25 and GD25β1A cells for possible colocalisation of fibronectin and mRFPovTSP1C. The ECMs of both cell lines contained examples of either nonoverlapping or colocalised mRFPovTSP1C and fibronectin. The frequency of colocalised deposits was not significantly increased in GD25β1A

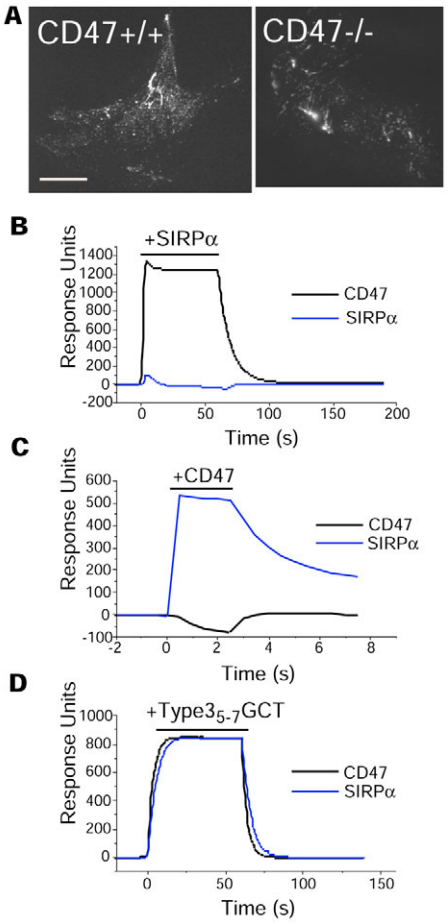

Fig. 6. Analysis of the role of CD47 in TSP1 C-terminal region interactions. (A) CD47 is not essential for ECM retention of mRFPovTSP1C. NH_4OH-insoluble ECMs were analysed after 4 days. Scale bar: 10 μm. (B-D) Surface plasmon resonance analysis of CD47 interaction with SIRPα (B,C) or type3$_{5-7}$GCT (D).

cells. There was no obvious correlation between colocalisation with fibronectin and the density of mRFPovTSP1C deposits (representative examples from GD25β1A cells are shown in Fig. 7C). We conclude that TSP1 can be retained in ECM in proximity to fibronectin, but the deposition of fibronectin is not essential. Colocalisation of mRFPovTSP1C and laminin was not observed (data not shown).

Discussion

We present novel data that advance concepts of the mechanism by which secreted TSPs are retained within ECM. Importantly, we demonstrate that ECM retention is an evolutionarily conserved property of TSPs, shared by both trimeric and pentameric TSPs. Thus ECM retention appears likely to be an ancestral activity of TSPs. This is borne out by the recently reported functional role of the single TSP of *Drosophila*, D-TSP, in the ECM of muscle-tendon attachment sites and the localisation of a prawn TSP to specialised ECM structures – oocyte cortical rods (Yamano et al., 2004; Chanana et al., 2007; Subramanian et al., 2007).

Studies of cell-attachment activities of TSP1 and TSP2 have relied on purified TSPs coated onto glass or plastic surfaces, where they probably form a uniform layer of individual trimers. Using classic procedures for isolation of insoluble ECM, we demonstrate that TSP1 and other TSPs are retained in ECM in the physical form of small, closely spaced puncta. This is consistent with descriptions of punctate, granular or amorphous deposits of TSP1 around fixed cultured cells (Jaffe et al., 1983; Vischer et al., 1988; Raugi et al., 1982; Adams and Lawler, 1994; Kuznetsova et al., 2006). For the cell types tested here, association of TSP1 puncta with fibrillar ECM did not appear obligatory, although partial colocalisation with fibronectin was detected (Fig. 7). Associations of TSP1 with elastin microfibrils and several ECM proteoglycans have been documented (Vischer et al., 1997; Kuznetsova et al., 2006). These interactions have all been linked to TSP1 N-terminal domain and endocytosis of TSP1. The literature contains little evidence for self-association of TSP1 molecules, which suggests that a bridging molecule such as fibronectin might be involved. Alternatively, post-secretion cell-surface interactions might provide conditions suitable for TSP1 self-association. Prior studies indicated multiple binding sites for fibronectin on TSP1 (Dardik and Lahav, 1989; Sipes et al., 1993), but the role of the C-terminal region in fibronectin binding remains unclear and further analysis will be needed. ECM retention of TSP1 may be a complex process involving more than one component of the structural ECM.

By investigating the mechanism of ECM retention for TSP1, we provide novel evidence that the trimeric C-terminal region is necessary and sufficient for ECM retention activity. Recent advances in understanding the structures of TSPs have revealed that there are extensive interactions between the EGF domains, type 3 repeats and L-lectin-like domain, such that the whole multidomain region should be considered a single structural unit (Kvansakul et al., 2004; Misenheimer and Mosher, 2005; Carlson et al., 2005). These findings are corroborated by immunochemical analyses (Annis et al., 2007). For this reason, we chose to develop a recombinant trimer that included all these domains for functional studies. The "mini-trimer" protein is secreted efficiently by cells and should be a valuable general tool for analysing oligomer-dependent functions of the C-terminal region of TSP1 and, in principle, other TSPs.

The two cell-attachment sites within the C-terminal region are located near the C-terminus, in the last type 3 repeat and L-lectin domain. However a TSP1 C-terminal region (aa 531-1152) monomer that includes these sites was not sufficient for ECM incorporation, establishing the importance of trimerisation for ECM retention (Fig. 4). The RGD motif in the last type 3 repeat of TSP1 has a well-documented role in binding integrins (reviewed by Adams, 2004). Our results with a novel VGD point mutation demonstrate that the RGD motif contributes to cell-attachment activity and also contributes to retention of TSP1 C-terminal trimers in ECM. This was corroborated by the demonstration that β1 integrins increase the efficiency of ECM retention. Neither β1 integrins nor αvβ3 were essential, suggesting that several integrin heterodimers can redundantly mediate the role of the TSP1 RGD site in ECM retention. This is consistent with the multiple integrins known to support RGD-dependent cell attachment to TSP1 (reviewed by Adams, 2004). As discussed above, our results do not exclude the possibility of an indirect role for integrins, for example by binding an RGD-containing ligand such as fibronectin, which also binds TSP1.

The L-lectin domain is recognised to have independent cell-attachment activity; however, new structural knowledge has necessitated the reappraisal of the physiological role of previously identified peptide motifs (Gao et al., 1996; Kvansakul et al., 2004; Carlson et al., 2005). A novel candidate functional site was

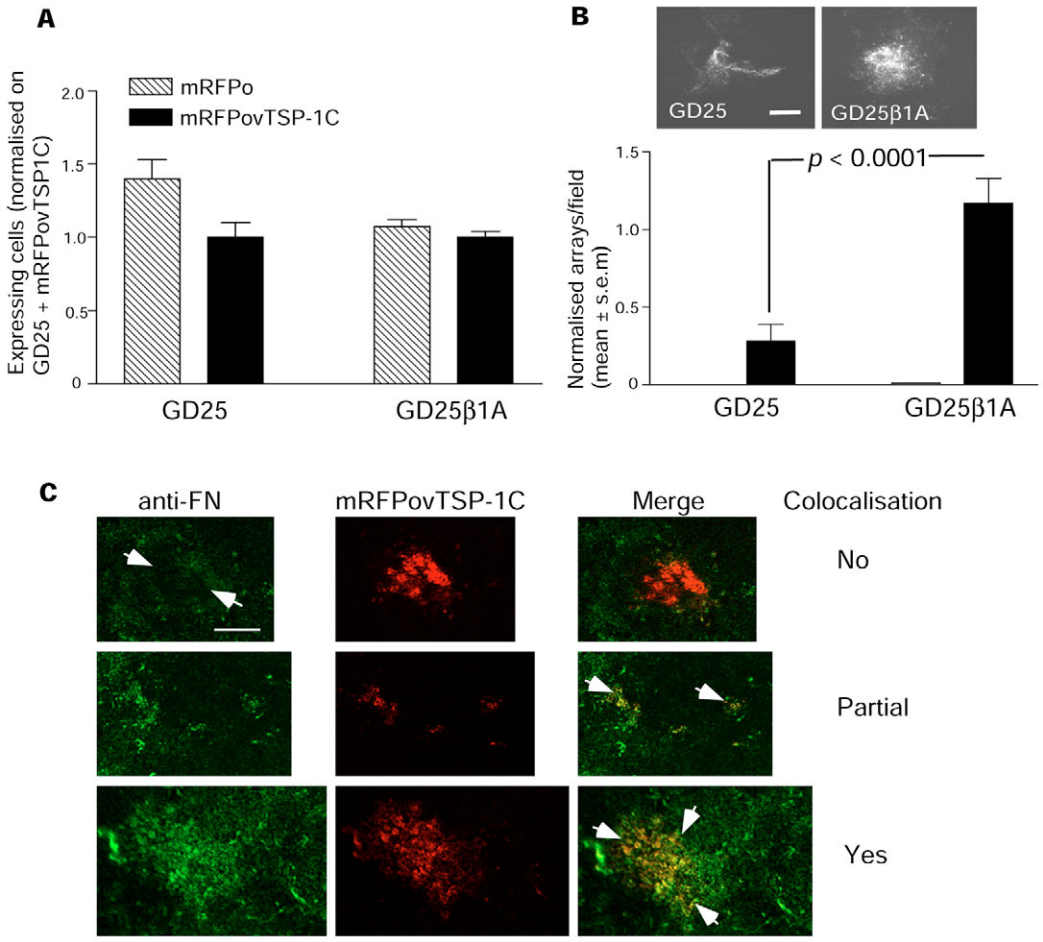

Fig. 7. Role of β1 integrins in ECM retention of mRFPoTSP1C. (A,B) Quantification of relative expression (A) or ECM retention (B) of mRFPo or mRFPovTSP1C by GD25 and GD25β1A cells. Each column represents the mean of three experiments; bars represent s.e.m. Insets in B show representative confocal images of mRFPoTSP1C puncta in the ECMs of GD25 and GD25β1A cells. (C) Partial codistribution of fibronectin and mRFPovTSP1C in the ECM of GD25β1A cells. Top row, arrows indicate absence of colocalised fibronectin. Middle and bottom rows, arrows indicate areas of colocalisation. Scale bars: 10 μm.

suggested by the striking structural homology of TSP L-lectin domain to the calcium-dependent lectins ERGIC53 and VIP36, which bind glycoproteins in the secretory pathway. The well-characterised oligosaccharide binding site of these lectins (Satoh et al., 2007) coincides with a double calcium site that is conserved in all TSPs and suitably positioned for ligand binding (i.e. on the surface and away from the type 3 repeats that wrap around the L-lectin domain; Fig. 5A). Mutation of the DDD motif in TSP1 (AAA mutant) resulted in impaired cell attachment activity for all cells tested and significantly reduced ECM retention. These data provide strong initial evidence that the conserved DDD motif participates in a ligand-binding site of general physiological importance in TSP1 and potentially other TSP family members. Lectins are typically multivalent and the need for trimerisation of the L-lectin domain for ECM retention may reflect a similar requirement for clustering of low-affinity interaction sites to achieve high local avidity (Brewer et al., 2002). It is intriguing that the RGD and DDD motifs are physically well separated (~50 Å distance) (Fig. 5A). Thus, the TSP1 C-terminal region might act to assemble multiprotein complexes, bringing integrins into lateral proximity with other adhesion receptors or ECM components. In our analyses of cell attachment, ECM retention and direct binding we did not obtain evidence for a role of CD47 (Figs 5 and 6). Recent genetic analyses in knockout mice indicate co-dependency of TSP1 and CD47 in endothelial and smooth muscle responses to nitric oxide (Ridnour et al., 2005; Isenberg et al., 2006). Interpretation of these in vivo data is complicated by the multiple roles of CD47 as an integrin-signalling co-receptor and a transcellular ligand for SIRPα, which is itself regulated by integrin activation (Brown and Frazier, 2001; Johansen and Brown, 2007).

The five TSPs of humans are associated with multiple genetic and acquired disease processes. The cellular and molecular roles of the type 1 repeats of TSP1 and TSP2 have been intensively investigated, and this knowledge led to the development of novel anticancer agents currently in clinical trial (e.g. Markovic et al., 2007). Based on recent advances in understanding the structural complexity of the TSP C-terminal region, we establish here for TSP1 that the trimeric C-terminal region is required for ECM retention and that two sites proximal to the C-terminus of TSP1 mediate cell attachment and ECM retention activities. The mRFP-tagged mini-trimers will enable further investigations of the possible significance of ECM retention for other important functions of TSP1, which include modulation of endothelial cell proliferation, vascular wall remodelling and the immune response. The mini-trimers will also

be generally useful novel tools to examine parameters of TSP localization in culture and enable better detection of TSPs in vivo. In summary, knowledge of the cell-mediated mechanisms necessary for ECM retention of TSP1 has important implications for understanding the normal and pathological activities of the TSP C-terminal region.

Materials and Methods

Cell lines and materials

COS-7, A549 and CHO cells were from ATCC. 293-EBNA cells were from Invitrogen. GD25 and GD25β1A cells were from Reinhard Fässler (Wennerberg et al., 1996). Dermal fibroblasts were prepared by trypsinisation from dissected hindlimbs of neonatal wild-type or CD47-null mice, gifts from Frederick Lindberg (Lindberg et al., 1996). pCEP-Pu plasmid was as described (Kvansakul et al., 2004). pcDNA3/D-TSP.V5His was as described (Adams et al., 2003). cDNA encoding mRFP was as described (Campbell et al., 2002). cDNA encoding human COMP (GenBank BC125092) was obtained from Open Biosystems and ligated into pCDNA3.V5His by TOPO cloning. cDNA encoding *Ciona intestinalis* TSPA was obtained from Noriyuki Satoh, Kyoto University (Satou et al., 2002). We sequenced this cDNA in its entirety on both strands by automated DNA sequencing, carried out by the CCF Molecular Biotechnology Core, using vector and cDNA-specific oligonucleotide primers. The complete nucleotide sequence was deposited as GenBank AY465896. Oligonucleotide primers used to generate the various constructs are listed in supplementary material Table S1. All oligonucleotides were synthesised by Sigma-Genosys and oligonucleotides for mutagenesis reactions were HPLC-purified. Mutations in TSP1 C-terminal region were introduced using QuikChange XL kit (Stratagene). FITC-phalloidin was from Sigma. Oregon-Green-gelatin was from Molecular Probes. The following mouse monoclonal antibodies were used: V5 epitope (GKPIPNPLLGLDST) (Southern et al., 1991) (Invitrogen); DsRed (Clontech); clone D4.6 to mouse and human TSP1 (Labvision) (Annis et al., 2006), and MAI and MAII to human TSP1 (Lawler et al., 1985) (a gift from Jack Lawler, Harvard). Rabbit polyclonal antibody to fibronectin was from Sigma and FITC-conjugated goat polyclonal IgG to V5 tag from Abcam. Alkaline-phosphatase-conjugated secondary antibodies were from Applied Biosystems and FITC-conjugated secondary antibodies were from ICN.

Transfection and preparation of cells and ECM

COS-7 cells (2×10^5) were transiently transfected with 2.5 μg of the indicated plasmids using Polyfect transfection reagent (Qiagen) according to manufacturer's instructions. After 18 hours, cells from each transfection were replated onto glass coverslips in two replicate 35-mm dishes and incubated for 3 days to allow for secretion and deposition of ECM. One dish was rinsed in PBS and fixed in 2% paraformaldehyde (Electron Microscopy Sciences). These cells were used to quantify the percentage of transfected cells expressing the protein. The parallel dish was treated with 20 mM ammonium hydroxide for 5 minutes (Robinson and Gospodarowicz, 1984), rinsed in PBS, and fixed in 2% paraformaldehyde. Coverslips from cultures expressing mRFP constructs were mounted directly on slides with Vectashield DAPI mounting medium (Vector Laboratories, Burlingham, CA). Other samples were processed for indirect immunofluorescence before mounting. Treatments with 2 M or 8 M urea for 10 minutes were used as alternative procedures to isolate insoluble ECM (Carter, 1982). To screen for cellular material after the various extractions, samples were stained with FITC-phalloidin to visualize F-actin or DAPI as a nuclear stain. Two coverslips were scored for each sample in each experiment and a minimum of three independent experiments were carried out for each condition.

Fluorescence microscopy and scoring

Samples for fluorescence microscopy were examined under a Leica DMIRE2 inverted microscope (Heidelberg, Germany), equipped with electronically controlled shutters and filter wheel. Images were captured under 63× objective using a Hamamatsu camera controller C4742_95 run by Improvision Openlab software (version 3.1.5). Some samples were examined under a Leica DM RXE(TCS-SP/SP-AOSB) confocal laser scanning microscope with HCX Plan Apo 63× NA 1.4 oil-immersion objective lens, using Leica confocal software version 2.5. Confocal images were acquired as *Z* stacks, *X-Y*, or *X-Z* sections at zoom 1 or 2, at room temperature. Projections of image stacks were prepared in Volocity 3.7.0. In each experiment, the percentage of transfected cells expressing the protein of interest was calculated by direct scoring of cells expressing mRFP-tagged proteins as a fraction of DAPI-stained nuclei, or by permeabilisation in 0.5% Triton X-100 and indirect immunofluorescent staining for the non-RFP proteins. Ten 63× fields were scored per coverslip. Cell area measurements were made from phalloidin-stained samples. To quantify ECM retention, the number of arrays of TSP puncta per 63× field was scored from 30 fields per coverslip. The areas of ten arrays that corresponded to the production of single cells were measured in Improvision Openlab 3.1.5 and a mean area of deposition per single cell was calculated. This mean area was used to calculate the number of cells producing larger arrays, and thereby the total number of single-cell arrays per field.

Preparation of monomeric type$3_{5\text{-}7}$GCT proteins

The TSP1 type$3_{5\text{-}7}$GCT construct with a C974S mutation (Kvansakul et al., 2004) was used as template for strand overlap extension PCR to introduce R908V (VGD mutant) and D1001A/D1002A/D1003A (AAA mutant). Sequence-verified inserts were subcloned into pCEP-Pu vector coding for proteins bearing an N-terminal His-Myc-tag and transfected into 293-EBNA cells using Fugene reagent (Roche). Stable transfectants were selected with 1 μg/ml puromycin. Secreted proteins were purified from conditioned serum-free medium as described (Kvansakul et al., 2004). Gel filtration was carried out using an Äkta chromatography system and a 24 ml S200 column (GE Healthcare). Running buffer was 20 mM Na-HEPES pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$ and the flow rate was 0.5 ml/minute.

Cell attachment

Cell attachment was measured as described (Kvansakul et al., 2004).

Affinity precipitation and immunoblotting

TSP proteins were collected from the conditioned medium of transfected cells using TALON metal affinity resin (Clontech), or heparin-Sepharose (Amersham Biosciences, Sweden) in the case of proteins containing the TSP1 N-terminal domain, according to the manufacturer's instructions. 50 μl packed slurry was incubated with 3 ml medium for 1 hour at 4°C. Beads were collected by centrifugation, washed three times and bead-bound proteins released by boiling in SDS-PAGE sample buffer, without or with 100 mM DTT. Polyacrylamide gel electrophoresis and immunoblotting were carried out as described (Adams et al., 2003).

Characterisation of protein interactions by surface plasmon resonance

Recombinant human SIRPα consisting of the extracellular domain (aa 1-349) followed by the sequence TRHHHHHH was produced as described (Hatherley et al., 2007). Recombinant extracellular regions of human CD47 (residues 1-136) or SIRPα with a biotinylation site and polyhistidine tag were prepared by transient expression in 293T cells using the pEF-BOS vector (Mizushima and Nagata, 1990) and biotinylated with Bir enzyme as described (Brown et al., 1998). CD47 extracellular domain without a biotinylation site was expressed by CHO K1 cells from the pEE14 plasmid (Hatherley et al., 2007). Recombinant proteins were purified by nickel affinity chromatography using chelating Sepharose fast flow (GE Healthcare Life Sciences) and buffer exchanged into 10 mM HEPES pH 7.5, 150 mM NaCl. Protein purity and was as previously described (Hatherley et al., 2007). Protein interactions were analysed using a BIAcore™ 2000 at 25°C, with 10 mM HEPES pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$ as running buffer. Briefly, ~1500 RU of streptavidin were coupled to a CM5 research grade chip using amine coupling. Biotinylated CD47 or SIRPα were bound to separate flow cells (1001.4 and 1134.8 RU, respectively). The following were passed over both flow cells at a flow rate of 5 μl/minute: 5 μl of type$3_{5\text{-}7}$GCT (in 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM calcium acetate) at 70 μM, 5 μl of SIRPα at 10 μM, and then 5 μl of CD47 at 47 μM at 100 μl/minute.

Circular dichroism measurements

Near-UV circular dichroism (CD) spectra were obtained for type$3_{5\text{-}7}$GCT and its VGD and AAA mutants. Proteins were dialysed against 5 mM MOPS pH 7.5, 100 mM NaCl, 2 mM $CaCl_2$ and adjusted to ~5 μM concentration. CD spectra were recorded at 25°C in 0.1 mm quartz cuvettes on an Applied Photophysics Chirascan instrument.

We thank Naa Amponsah for technical assistance, Saffi Hussain for help with protein expression and purification, Yosuke Hashimoto for help with confocal microscopy, the CCF Imaging core for confocal microscopy training and CCF Molecular Biotechnology and Genomics cores for scientific support. We thank Noriyuki Satoh, Kyoto University, and the Academica DNA Sequencing Center, Center for Genetic Resource Information, Japan for providing the *Ciona* TSPA cDNA. Supported by NIH grant GM068073 to J.C.A. and a Wellcome Senior Research Fellowship to E.H. D.H. is supported by the Medical Research Council of Great Britain.

References

Adams, J. C. (1997). Characterization of cell-matrix adhesion requirements for the formation of fascin microspikes. *Mol. Biol. Cell* **8**, 2345-2363.

Adams, J. C. (2001). Thrombospondins: multifunctional regulators of cell interactions. *Annu. Rev. Cell Dev. Biol.* **17**, 25-51.

Adams, J. C. (2004). Functions of the conserved thrombospondin carboxy-terminal cassette in cell-extracellular matrix interactions and signaling. *Int. J. Biochem. Cell Biol.* **36**, 1102-1114.

Adams, J. C. and Lawler, J. (1993). The thrombospondin family. *Curr. Biol.* **3**, 188-190.

Adams, J. C. and Lawler, J. (1994). Cell-type specific adhesive interactions of skeletal myoblasts with thrombospondin-1. *Mol. Biol. Cell* **5**, 423-437.

Adams, J. C., Kureishy, N. and Taylor, A. L. (2001). A role for syndecan-1 in coupling fascin spike formation by thrombospondin-1. *J. Cell Biol.* **152**, 1169-1182.

Adams, J. C., Monk, R., Taylor, A. L., Ozbek, S., Fascetti, N., Baumgartner, S. and Engel, J. (2003). Characterisation of *Drosophila* thrombospondin defines an early origin of pentameric thrombospondins. *J. Mol. Biol.* **328**, 479-494.

Agah, A., Kyriakides, T. R. and Bornstein, P. (2005). Proteolysis of cell-surface tissue transglutaminase by matrix metalloproteinase-2 contributes to the adhesive defect and matrix abnormalities in thrombospondin-2 null fibroblasts and mice. *Am. J. Pathol.* **167**, 81-88.

Annis, D. S., Murphy-Ullrich, J. E. and Mosher, D. F. (2006). Function-blocking antithrombospondin-1 monoclonal antibodies. *J. Thromb. Haemost.* **4**, 459-468.

Annis, D. S., Gunderson, K. A. and Mosher, D. F. (2007). Immunochemical analysis of the structure of the signature domains of thrombospondin-1 and thrombospondin-2 in low calcium concentrations. *J. Biol. Chem.* **282**, 27067-27075.

Arber, S. and Caroni, P. (1995). Thrombospondin-4, an extracellular matrix protein expressed in the developing and adult nervous system promotes neurite outgrowth. *J. Cell Biol.* **131**, 1083-1094.

Bonnefoy, A. and Legrand, C. (2000). Proteolysis of subendothelial adhesive glycoproteins (fibronectin, thrombospondin, and von Willebrand factor) by plasmin, leukocyte cathepsin G, and elastase. *Thromb Res.* **98**, 323-332.

Bornstein, P., Agah, A. and Kyriakides, T. R. (2004). The role of thrombospondins 1 and 2 in the regulation of cell-matrix interactions, collagen fibril formation, and the response to injury. *Int. J. Biochem. Cell Biol.* **36**, 1115-1125.

Brewer, C. F., Miceli, M. C. and Baum, L. G. (2002). Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide-mediated cellular interactions. *Curr. Opin. Struct. Biol.* **12**, 616-623.

Brown, E. J. and Frazier, W. A. (2001). Integrin-associated protein (CD47) and its ligands. *Trends Cell Biol.* **11**, 130-135.

Brown, M. H., Boles, K., van der Merwe, P. A., Kumar, V., Mathew, P. A. and Barclay, A. N. (1998). 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J. Exp. Med.* **188**, 2083-2090.

Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Geoffrey, S., Baird, G. S., David, A. Z. and Tsien, R. Y. (2002). A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA* **99**, 7877-7882.

Carlson, C. B., Bernstein, D. A., Annis, D. S., Misenheimer, T. M., Hannah, B. L., Mosher, D. F. and Keck, J. L. (2005). Structure of the calcium-rich signature domain of human thrombospondin-2. *Nat. Struct. Mol. Biol.* **12**, 910-914.

Carter, W. G. (1982). Transformation-dependent alterations in glycoproteins of extracellular matrix of human fibroblasts. Characterization of GP250 and the collagen-like GP140. *J. Biol. Chem.* **257**, 13805-13815.

Chanana, B., Graf, R., Koledachkina, T., Pflanz, R. and Vorbrüggen, G. (2007). alpha(PS2) integrin-mediated muscle attachment in *Drosophila* requires the ECM protein thrombospondin. *Mech. Dev.* **124**, 463-475.

Colognato, H. and Yurchenco, P. D. (2000). Form and function: the laminin family of heterotrimers. *Dev. Dyn.* **218**, 213-234.

Dardik, R. and Lahav, J. (1989). Multiple domains are involved in the interaction of endothelial cell thrombospondin with fibronectin. *Eur. J. Biochem.* **185**, 581-588.

DiCesare, P. E., Morgelin, M., Carlson, C. S., Pasumarti, S. and Paulsson, M. (1995). Cartilage oligomeric matrix protein: isolation and characterization in human articular cartilage. *J. Orthop. Res.* **13**, 422-428.

DiCesare, P. E., Carlson, C. S., Stollerman, E. S., Chen, F. S., Leslie, M. and Perris, R. (1997). Expression of cartilage oligomeric matrix protein by human synovium. *FEBS Lett.* **412**, 249-252.

DiCesare, P. E., Chen, F. S., Morgelin, M., Carlson, C. S., Leslie, M. P., Perris, R. and Fang, C. (2002). Matrix-matrix interaction of cartilage oligomeric matrix protein and fibronectin. *Matrix Biol.* **21**, 461-470.

Dunkle, E. T., Zaucke, F. and Clegg, D. O. (2007). Thrombospondin-4 and matrix three-dimensionality in axon outgrowth and adhesion in the developing retina. *Exp. Eye Res.* **84**, 707-717.

Elzie, C. A. and Murphy-Ullrich, J. E. (2004). The N-terminus of thrombospondin: the domain stands apart. *Int. J. Biochem. Cell Biol.* **36**, 1090-1101.

Fauvel-Lafeve, F., Arbeille, B., de Romeuf, C., Lemesle, M. and Legrand, Y. J. (1996). Heterogeneity of microfibrils: role of thrombospondin-microfibrils in the thrombogenicity of the subendothelium. *C. R. Acad. Sci. III* **319**, 333-341.

Gao, A. G., Lindberg, F. P., Finn, M. B., Blystone, S. D., Brown, E. J. and Frazier, W. A. (1996). Integrin-associated protein is a receptor for the C-terminal domain of thrombospondin. *J. Biol. Chem.* **271**, 21-24.

Hatherley, D., Harlos, K., Dunlop, D. C., Stuart, D. I. and Barclay, A. N. (2007). The structure of the macrophage signal regulatory protein alpha (SIRPalpha) inhibitory receptor reveals a binding face reminiscent of that used by T cell receptors. *J. Biol. Chem.* **282**, 14567-14575.

Hauser, N., Paulsson, M., Kale, A. A. and DiCesare, P. E. (1995). Tendon extracellular matrix contains pentameric thrombospondin-4 (TSP-4). *FEBS Lett.* **368**, 307-310.

Hedbom, E., Antonsson, P., Hjerpe, A., Aeschlimann, D., Paulsson, M., Rosa-Pimentel, E., Sommarin, Y., Wendel, M., Oldberg, A. and Heinegard, D. (1992). Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. *J. Biol. Chem.* **267**, 6132-6136.

Hiscott, P., Larkin, G., Robey, H. L., Orr, G. and Grierson, I. (1992). Thrombospondin as a component of the extracellular matrix of epiretinal membrances: comparisons with cellular fibronection. *Eye* **6**, 566-569.

Hogg, P. J., Jimenez, B. M. and Chesterman, C. N. (1994). Identification of possible inhibitory reactive centers in thrombospondin 1 that may bind cathepsin G and neutrophil elastase. *Biochemistry* **33**, 6531-6537.

Holden, P., Meadows, R. S., Chapman, K. L., Grant, M. E., Kadler, K. E. and Briggs, M. D. (2001). Cartilage oligomeric matrix protein interacts with type IX collagen, and disruptions to these interactions identify a pathogenetic mechanism in a bone dysplasia family. *J. Biol. Chem.* **276**, 6046-6055.

Homandberg, G. A. and Kramer-Bjerke, J. (1987). Thrombospondin binds to amino-terminal fragments of plasma fibronectin. *Thromb. Res.* **48**, 329-335.

Hsieh, P., Segal, R. and Chen, L. B. (1980). Studies of fibronectin matrices in living cells with fluoresceinated gelatin. *J. Cell Biol.* **87**, 14-22.

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687.

Isenberg, J. S., Ridnour, L. A., Dimitry, J., Frazier, W. A., Wink, D. A. and Roberts, D. D. (2006). CD47 is necessary for inhibition of nitric oxide-stimulated vascular cell responses by thrombospondin-1. *J. Biol. Chem.* **281**, 26069-26080.

Jaffe, E. A., Ruggiero, J. T., Leung, L. K., Doyle, M. J., McKeown-Longo, P. J. and Mosher, D. F. (1983). Cultured human fibroblasts synthesize and secrete thrombospondin and incorporate it into extracellular matrix. *Proc. Natl. Acad. Sci. USA* **80**, 998-1002.

Johansen, M. L. and Brown, E. J. (2007). Dual regulation of SIRPalpha phosphorylation by integrins and CD47. *J. Biol. Chem.* **282**, 24219-24230.

Krissinel, E. and Henrick, K. (2004). Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2256-2268.

Kuznetsova, S. A., Issa, P., Perruccio, E. M., Zeng, B., Sipes, J. M., Ward, Y., Seyfried, N. T., Fieldher, H. L., Day, A. J., Wight, T. N. et al. (2006). Versican-thrombospondin-1 binding in vitro and colocalization in microfibrils induced by inflammation on vascular smooth muscle cells. *J. Cell Sci.* **119**, 4499-4509.

Kvansakul, M., Adams, J. C. and Hohenester, E. (2004). Structure of a thrombospondin C-terminal fragment reveals a novel calcium core in the type 3 repeats. *EMBO J.* **23**, 1223-1233.

Kyriakides, T. R., Zhu, Y. H., Smith, L. T., Bain, S. D., Yang, Z., Lin, M. T., Danielson, K. G., Iozzo, R. V., LaMarca, M., McKinney, C. E. et al. (1998). Mice that lack thrombospondin 2 display connective tissue abnormalities that are associated with disordered collagen fibrillogenesis, an increased vascular density, and a bleeding diathesis. *J. Cell Biol.* **140**, 419-430.

Lawler, J. and Detmar, M. (2004). Tumor progression: the effects of thrombospondin-1 and -2. *Int. J. Biochem. Cell Biol.* **36**, 1038-1045.

Lawler, J., Derick, L. H., Connolly, J. E., Chen, J. H. and Chao, F. C. (1985). The structure of human platelet thrombospondin. *J. Biol. Chem.* **260**, 3762-3772.

Lawler, J., Weinstein, R. and Hynes, R. O. (1988). Cell attachment to thrombospondin: the role of Arg-Gly-Asp, calcium and integrin receptors. *J. Cell Biol.* **118**, 693-701.

Lawler, J., Ferro, P. and Duquette, M. (1992). Expression and mutagenesis of thrombospondin. *Biochemistry* **31**, 1173-1180.

Lee, N. V., Sato, M., Annis, D. S., Loo, J. A., Wu, L., Mosher, D. F. and Iruela-Arispe, M. L. (2006). ADAMTS1 mediates the release of antiangiogenic polypeptides from TSP1 and 2. *EMBO J.* **25**, 5270-5383.

Lindberg, F. P., Bullard, D. C., Caver, T. E., Gresham, H. D., Beaudet, A. L. and Brown, E. J. (1996). Decreased resistance to bacterial infection and granulocyte defects in IAP-deficient mice. *Science* **274**, 795-798.

Liu, Y., Tong, Q., Zhou, Y., Lee, H. W., Yang, J. J., Buhring, H. J., Chen, Y. T., Ha, B., Chen, C. X., Yang, Y. et al. (2007). Functional elements on SIRPalpha IgV domain mediate cell surface binding to CD47. *J. Mol. Biol.* **365**, 680-693.

Mao, Y. and Schwarzbauer, J. E. (2005). Fibronectin fibrillogenesis, a cell-mediated matrix assembly process. *Matrix Biol.* **24**, 389-399.

Markovic, S. N., Suman, V. J., Rao, R. A., Ingle, J. N., Kaur, J. S., Erickson, L. A., Pitot, H. C., Croghan, G. A., McWilliams, R. R., Merchan, J. et al. (2007). A phase II study of ABT-510 (thrombospondin-1 analog) for the treatment of metastatic melanoma. *Am. J. Clin. Oncol.* **30**, 303-309.

Misenheimer, T. M. and Mosher, D. F. (2005). Biophysical characterization of the signature domains of thrombospondin-4 and thrombospondin-2. *J. Biol. Chem.* **280**, 41229-41235.

Mizushima, S. and Nagata, S. (1990). pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* **18**, 5322-5325.

Mosher, D. F., Doyle, M. J. and Jaffe, E. A. (1982). Synthesis and secretion of thrombospondin by cultured human endothelial cells. *J. Cell Biol.* **93**, 343-348.

Mumby, S. M., Abbott-Brown, D., Raugi, G. J. and Bornstein, P. (1984). Regulation of thrombospondin secretion by cells in culture. *J. Cell. Physiol.* **120**, 280-288.

Narouz-Ott, L., Maurere, P., Nitsche, D. P., Smyth, N. and Paulsson, M. (2000). Thrombospondin-4 binds specifically to both collagenous and non-collagenous extracellular matrix proteins via its C-terminal domains. *J. Biol. Chem.* **275**, 37110-37117.

Posey, K. L., Hayes, E., Haynes, R. and Hecht, J. T. (2004). Role of TSP-5/COMP in pseudoachondroplasia. *Int. J. Biochem. Cell Biol.* **36**, 1005-1012.

Raugi, G. J., Mumby, S. M., Abbott-Brown, D. and Bornstein, P. (1982). Thrombospondin: synthesis and secretion by cells in culture. *J. Cell Biol.* **95**, 351-354.

Raugi, G. J., Olerud, J. E. and Gown, A. M. (1987). Thrombospondin in early human wound tissue. *J. Invest. Dermatol.* **89**, 551-554.

Ridnour, L. A., Isenberg, J. S., Espey, M. G., Thomas, D. D., Roberts, D. D. and Wink, D. A. (2005). Nitric oxide regulates angiogenesis through a functional switch involving thrombospondin-1. *Proc. Natl. Acad. Sci. USA* **102**, 13147-13152.

Robinson, J. and Gospodarowicz, D. (1984). Effect of p-nitrophenyl-beta-D-xyloside on proteoglycan synthesis and extracellular matrix formation by bovine corneal endothelial cell cultures. *J. Biol. Chem.* **259**, 3818-3824.

Rosenberg, K., Olsson, H., Morgelin, M. and Heinegard, D. (1998). Cartilage oligomeric matrix protein shows high affinity zinc-dependent interaction with triple helical collagen. *J. Biol. Chem.* **273**, 20397-20403.

Satoh, T., Cowieson, N. P., Hakamata, W., Ideo, H., Fukushima, K., Kurihara, M., Kato, R., Yamashita, K. and Wakatsuki, S. (2007). Structural basis for recognition

of high-mannose type glycoproteins by mammalian transport lectin VIP36. *J. Biol. Chem.* **282**, 28246-28255.

Satou, Y., Yamada, L., Mochizuki, Y., Takatori, N., Kawashima, T., Sasaki, A., Hamaguchi, M., Awazu, S., Yagi, K., Sasakura, Y. et al. (2002). A cDNA resource from the basal chordate *Ciona intestinalis*. *Genesis* **33**, 153-154.

Seiffert, M., Cant, C., Chen, Z., Rappold, I., Brugger, W., Kanz, L., Brown, E. J., Ullrich, A. and Buhring, H. J. (1999). Human signal-regulatory protein is expressed on normal, but not on subsets of leukemic, myeloid cells and mediates cellular adhesion involving its counterreceptor CD47. *Blood* **94**, 3633-3643.

Sipes, J. M., Guo, N., Negre, E., Vogel, T., Krutzsch, H. C. and Roberts, D. D. (1993). Inhibition of fibronectin binding and fibronectin-mediated cell adhesion to collagen by a peptide from the second type I repeat of thrombospondin. *J. Cell Biol.* **121**, 469-477.

Sodersten, F., Ekman, S., Eloranta, M. L., Heinegard, D., Dudhia, J. and Hultenby, K. (2005). Ultrastructural immunolocalization of cartilage oligomeric matrix protein (COMP) in relation to collagen fibrils in the equine tendon. *Matrix Biol.* **24**, 376-385.

Sottile, J. and Hocking, D. C. (2002). Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesions. *Mol. Biol. Cell* **13**, 3546-3559.

Sottile, J., Seleque, J. and Mosher, D. F. (1991). Synthesis of truncated amino terminal trimers of thrombospondin. *Biochemistry* **30**, 6556-6562.

Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W. and Randall, R. E. (1991). Identification of an epitope on the P and V proteins of simian virus 5 that distinguishes between two isolates with different biological characteristics. *J. Gen. Virol.* **72**, 1551-1557.

Stenina, O. I., Desai, S. Y., Krukovets, I., Kight, K., Janigro, D., Topol, E. J. and Plow, E. F. (2003). Thrombospondin-4 and its variants: expression and differential effects on endothelial cells. *Circulation* **108**, 1514-1519.

Stenina, O. I., Topol, E. J. and Plow, E. F. (2007). Thrombospondins, their polymorphisms, and cardiovascular disease. *Arterioscler. Thromb. Vasc. Biol.* **27**, 1886-1894.

Subramanian, A., Wayburn, B., Bunch, T. and Volk, T. (2007). Thrombospondin-mediated adhesion is essential for the formation of the myotendinous junction in Drosophila. *Development* **134**, 1269-1278.

Tan, K., Duquette, M., Liu, J., Dong, Y., Zhang, R., Joachimiak, A., Lawler, J. and Wang, J. (2002). Crystal structure of the TSP-1 type 1 repeats: a novel layered fold and its biological implication. *J. Cell Biol.* **159**, 373-382.

Tan, K., Duquette, M., Liu, J. H., Zhang, R., Joachimiak, A., Wang, J. H. and Lawler, J. (2006). The structures of the thrombospondin-1 N-terminal domain and its complex with a synthetic pentameric heparin. *Structure* **14**, 33-42.

Triantafilou, K., Triantafilou, M., Takada, Y. and Fernandez, N. (2000). Human parechovirus 1 utilizes integrins alphavbeta3 and alphavbeta1 as receptors. *J. Virol.* **74**, 5856-5862.

Velloso, L. M., Svensson, K., Schneider, G., Pettersson, R. F. and Lindqvist, Y. (2002). Crystal structure of the carbohydrate recognition domain of p58/ERGIC-53, a protein involved in glycoprotein export from the endoplasmic reticulum. *J. Biol. Chem.* **277**, 15979-15984.

Vernon-Wilson, E. F., Kee, W. J., Willis, A. C., Barclay, A. N., Simmons, D. L. and Brown, M. H. (2000). CD47 is a ligand for rat macrophage membrane signal regulatory protein SIRP (OX41) and human SIRPalpha 1. *Eur. J. Immunol.* **30**, 2130-2135**.**

Vischer, P., Beeck, H. and Voss, B. (1985). Synthesis, intracellular processing and secretion of thrombospondin in human endothelial cells. *Eur. J. Biochem.* **153**, 435-443.

Vischer, P., Volker, W., Schmidt, A. and Sinclair, N. (1988). Association of thrombospondin of endothelial cells with other matrix proteins and cell attachment sites and migration tracks. *Eur. J. Cell Biol.* **47**, 36-46.

Vischer, P., Feitsma, K., Schon, P. and Volker, W. (1997). Perlecan is responsible for thrombospondin 1 binding on the cell surface of cultured porcine endothelial cells. *Eur. J. Cell Biol.* **73**, 332-343.

Wennerberg, K., Lohikangas, L., Gullberg, D., Pfaff, M., Johansson, S. and Fassler, R. (1996). Beta 1 integrin-dependent and -independent polymerization of fibronectin. *J. Cell Biol.* **132**, 227-238.

Wight, T. N., Raugi, G. J., Mumby, S. M. and Bornstein, P. (1985). Light microscopic immunolocation of thrombospondin in human tissues. *J. Histochem. Cytochem.* **33**, 295-302.

Yamano, K., Qiu, G. F. and Unuma, T. (2004). Molecular cloning and ovarian expression profiles of thrombospondin, a major component of cortical rods in mature oocytes of penaeid shrimp, Marsupenaeus japonicus. *Biol. Reprod.* **70**, 1670-1678.

Table S1. Oligonucleotide primers used in this study (sequence 5′-3′).

Secreted mRFP monomer	
465F	GTACGCTAGCTATGGCCTCCTCCGAGGACGTCATC
473R	GTACCTCGAGTTAGGCGCCGGTGGAGTG
Secreted mRFP trimer	
474R	GTACCTCGAGTTAAGGCCGCCTCAGCTCATT
No	
317F	CCTGCTGGGCACCAACAG
318R	GATCGTTAACGGGCGCCTCAGCTCATTG
VWF_C	
515F	GTACGTTAACCCCCTATGCTATCACAACGGA
115R	GTACCTCGAGGCGTCTCAGGCACCCGGT
TSR	
516R	GTACCTCGAGTTAAATTGGACAGTCCTGCTTGTTGCA
C-term from E1 to C	
445F	GTACGTTAACATTGATGGATGCCTGTCCAAT
446R	GTACCTCGAGTTAGGGATCTCTACATTCGTA
O domain	
461F	GTACAAGCTTATTGGCCACAAGACAAAG
325R	GATCGTTAACGGGGCGCCTCAGCTCATT
VGD MUTANT	
475F	GGCGATGGTGTAGGTGATGCCTGCAAAG
476R	CTTTGCAGGCATCACCTACACCATCGCC
AAA MUTANT	
455F	TTCATCAACACCGAAAGGGCCGCTGCCTATGCTGGATTT
456R	AAATCCAGCATAGGCAGCGGCCCTTTCGGTGTTGATGAA
Ciona TSP-A	
243F	TCTGTGACTTCTGACGCG
244R	CGTGTCTTTACACATGTA
COMP/TSP-5	
510F	CCCGCCACCGCCATGGTC
511R	GGCTTGCCGCAGCTGATG