Published in final edited form as:

Matrix Biol. 2008 May ; 27(4): 339–351.

Calcium indirectly regulates immunochemical reactivity and functional activities of the N-domain of thrombospondin-1

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Abstract

Conformational changes induced in thrombospondin-1 by removal of calcium regulate interactions with some ligands of its N-modules. Because calcium binds primarily to elements of the C-terminal signature domain of thrombospondin-1, which are distant from the N-modules, such regulation was unexpected. To clarify the mechanism for this regulation, we compared ligand binding to the N-modules of thrombospondin-1 in the full-length protein and recombinant trimeric thrombospondin-1 truncated prior to the signature domain. Three monoclonal antibodies were identified that recognize the N-modules, two of which exhibit calcium-dependent binding to native thrombospondin-1 but not to the truncated trimeric protein. These antibodies or calcium selectively modulate interactions of fibronectin, heparin, sulfatide, $\alpha 3 \beta 1$ integrin, tumor necrosis factor- α -stimulated gene-6 protein, and, to a lesser extent, $\alpha 4 \beta 1$ integrin with native thrombospondin-1 but not with the truncated protein. These results indicate connectivity between calcium binding sites in the C-terminal signature domain and the N-modules of thrombospondin-1 that regulates ligand binding and functional activities of the N-modules.

Keywords

Thrombospondin-1; integrin recognition; conformational epitopes; calcium binding; cell adhesion; extracellular matrix

1. Introduction

Many developmental and pathophysiological processes in multicellular organisms require cells to regulate adhesive interactions with their surrounding extracellular matrix. Integrins are bidirectional signaling molecules that play a major role in both static adhesion and the regulated binding of cells to matrix components required for cell motility (Hynes, 2002). Integrins receive context-specific information from extracellular matrix ligand binding that is transmitted into the cell. Conversely, integrins respond to signals from inside the cell to attach or detach from specific matrix ligands. Both signaling processes involve conformation changes in the integrins, and the latter result in alterations of the affinity or avidity of integrins for binding to their respective matrix ligands (Hughes and Pfaff, 1998).

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In addition to this dynamic regulation of adhesion receptors, processing of their extracellular matrix ligands can also modulate binding. Supramolecular assembly, covalent modifications, and limited proteolysis are known to modulate integrin binding activities of specific matrix proteins (reviewed in (Silverstein *et al.*, 1984; Davis *et al.*, 2000)). Some integrin ligands may also be subject to conformational regulation of activity similar to that described for their receptors. For example, conformational regulation of fibronectin controls its ability to assemble into fibrils (Mao and Schwarzbauer, 2005; Tomasini-Johansson *et al.*, 2006).

A conformation change in thrombospondin-1 (TSP1)¹ induced by removing calcium or binding to fibronectin regulates exposure of an $\alpha 3\beta 1$ integrin binding site on the protein (Rodrigues *et al.*, 2001), suggesting that conformation similarly regulates some functions of TSP1. Each TSP1 subunit contains 11–12 exchangeable calcium binding sites and additional nonexchangeable calcium binding sites (Misenheimer and Mosher, 1995). Analysis of constructs of TSP2, a paralog of TSP1, indicates that the exchangeable sites are in the type 3 repeats (Misenheimer *et al.*, 2003). Recent crystal structures of C-terminal constructs based on TSP1 and TSP2 identified a total of 30–31 calcium binding sites, one in the second EGF repeat, 26 in the so called wire modules, and three or four in the lectin-like G module (Kvansakul *et al.*, 2004; Carlson *et al.*, 2005). Removal of calcium alters the hydrodynamic properties and circular dichroism spectrum of TSP1 (Lawler and Simons, 1983; Vuillard *et al.*, 1991), alters its structure as visualized by rotary shadowing electron microscopy (Galvin *et al.*, 1985; Lawler *et al.*, 1985), and produces local conformational changes in the wire modules as assessed by electron spin resonance (Slane *et al.*, 1988), intrinsic fluorescence (Hannah *et al.*, 2003), and exposure of calcium-dependent epitopes recognized by two TSP1 antibodies recognizing epitopes in the wire module, A6.1 and D4.6 (Dixit *et al.*, 1986; Annis *et al.*, 2006) (Annis *et al.*, 2007). Removing calcium markedly enhances binding of TSP1 to type V collagen (Galvin *et al.*, 1987).

Recent studies of a polymorphism in human TSP1 also suggest that calcium-induced conformation changes regulate some of its physiological functions. A single nucleotide polymorphism converts Asn-700 to Ser. The Ser polymorphism is associated with familial premature coronary artery disease and is located in the first calcium-binding wire module (Topol *et al.*, 2001). In vitro studies demonstrated that recombinant TSP1 containing the Ser polymorphism bound calcium less avidly and was more sensitive to thermal denaturation (Hannah *et al.*, 2003; Hannah *et al.*, 2004).

Although the extracellular milieu contains abundant Ca^{2+} , conformations characteristic of calcium-depleted TSP1 may occur in this environment. The TSP1 antibody F18 1G8 binds preferentially to the calcium-depleted conformation of TSP1 through an unmapped epitope (Rodrigues *et al.*, 2001). This conformation was not predicted to exist in the calcium-rich extracellular milieu, but the 1G8 antibody recognizes TSP1 on the surface of some cells, where it is found in a complex with fibronectin. Direct binding of TSP1 to fibronectin induces the same conformation change as removal of divalent cations, as assessed by exposure of the 1G8 epitope (Rodrigues *et al.*, 2001).

Remarkably, calcium-induced conformation changes in TSP1 have been associated with altered interactions of TSP1 with three ligands of its N-module: $\alpha 3\beta 1$ integrin (Krutzsch *et al.*, 1999), TSG-6 (Kuznetsova *et al.*, 2005), and versican (Kuznetsova *et al.*, 2006). The calcium depleted conformation of TSP1 that is stabilized by 1G8 or fibronectin is preferentially recognized by $\alpha 3\beta 1$ integrin (Rodrigues *et al.*, 2001). Conversely, removal of calcium decreases binding of TSG-6 and versican to TSP1 (Kuznetsova *et al.*, 2005; Kuznetsova *et al.*

¹Abbreviations: DPBS, Dulbecco's phosphate buffered saline; ECM, extracellular matrix; NoC1, trimeric N module-oligomerization domain-von Willebrand C domain of thrombospondin-1; TSP, thrombospondin.

al., 2006). Because the calcium binding sites in TSP1 are in the signature domain comprising the EGF-like modules, wire modules, and lectin-like modules (Kvansakul *et al.*, 2004; Carlson *et al.*, 2005), these results suggest that a conformation change in the signature domain sterically or allosterically influences binding of these ligands to the N-module of TSP1.

We have examined this hypothesis using three novel TSP1 antibodies that preferentially recognize TSP1 bound to the surface of activated platelets. We show here that their epitopes are located in the N module of TSP1, but the calcium-dependence for two of these epitopes requires the presence of the signature domain. We further show that these antibodies regulate recognition of the N-module of TSP1 by $\alpha 3\beta 1$ integrin and differentially modulate binding of several ligands to this region of TSP1.

2. Results

2.1 Identification of additional adhesion-stimulating TSP1 antibodies

We previously showed that recognition of TSP1 by $\alpha 3\beta 1$ integrin is enhanced by the conformation-dependent TSP1 antibody 1G8 (Rodrigues *et al.*, 2001). 1G8 recognizes an as yet undefined calcium-dependent epitope in TSP1 (Rodrigues *et al.*, 2001). Our attempts to map this epitope using proteolytic digests of platelet TSP1 or recombinant TSP1 domains were unsuccessful (data not shown). Therefore, we searched for antibodies exhibiting similar effects on $\alpha 3\beta 1$ -mediated adhesion from a panel of monoclonal antibodies that, like 1G8, were selected to be specific for activated versus resting platelets. Three antibodies, 4B6, 2D11, and 5H9, markedly enhanced adhesion of unstimulated MDA-MB-231 cells to TSP1 (Fig. 1A). 1G8 had little effect on adhesion of unstimulated MDA-MB-231 cells. The effect on $\alpha 3\beta 1$ -mediated adhesion was specific in that $\alpha 4\beta 1$ -mediated adhesion of unstimulated Jurkat cells was not increased by any of the antibodies (Fig. 1A).

Because $\alpha 3\beta 1$ and $\alpha 4\beta 1$ in these cells are only partially activated, we also examined adhesion in the presence of the $\beta 1$ integrin activating antibody TS2/16 (Fig. 1B). 5H9, 2D11, 4B6, and 1G8 further enhanced $\alpha 3\beta 1$ -mediated attachment of MDA-MB-231 cells on TSP1. 2D11 and 5H9 also increased $\alpha 4\beta 1$ -mediated adhesion of activated Jurkat cells, but the response was much less than for $\alpha 3\beta 1$ -mediated adhesion.

2.2 Antibodies 5H9, 2D11, and 4B6 recognize epitopes in the N-modules of TSP1

Using recombinant proteins representing various regions of TSP1 and TSP2 (Fig. 2A), the epitopes for antibodies 2D11, 5H9, and 4B6 were localized to NoC1, which contains the N-terminal and von Willebrand factor-C (vW-C, C) modules of TSP1 (Fig. 2B). The antibodies bound weakly to recombinant C module but not to trimeric DelN or oCP123, both of which contain the C module but lack the N-module (Fig. 2B and results not shown). Conversely, all three antibodies bound with similar dose dependencies on a subunit molar basis to monomeric recombinant N module and to native trimeric TSP1 (Fig. 2C). Thus, the epitopes for all three antibodies are in the N module of TSP1, and monomeric N module is sufficient for binding. None of these antibodies bound to the corresponding region of TSP2 (NoC2) or to any other recombinant TSP2 construct tested (Fig 2B and data not shown). The three antibodies competed reciprocally for binding to TSP1, indicating that their epitopes are in close proximity or overlapping (Fig.2D).

2.3 Divalent cation-dependence for antibody binding to TSP1

As with 1G8 (Rodrigues *et al.*, 2001), binding of soluble TSP1 to immobilized 5H9 and 2D11 was significantly enhanced in the absence of calcium (Fig. 3A and B). Antibody 4B6, did not show a significant divalent cation preference in binding soluble TSP1 (Fig. 3C). These results

suggest that 5H9 and 2D11 recognize epitopes that are negatively regulated by calcium in the NoC region of TSP1, whereas 4B6 recognizes a distinct calcium-independent epitope.

2.4 Divalent cation-dependence is not a local effect

Binding sites in TSP1 that mediate adhesion via $\alpha 3\beta 1$ and $\alpha 4\beta 1$ have been localized to the N-module (Chandrasekaran *et al.*, 1999; Krutzsch *et al.*, 1999; Li *et al.*, 2002). The previously identified calcium binding sites of TSP1, however, are all in the C-terminal signature domain of TSP1, as described in the Introduction. Therefore, finding calcium-dependent antibodies that recognize the N-module suggested that 5H9 and 2D11 binding to the N module is sterically or allosterically regulated by divalent cation-induced conformational changes in the signature domain or that unidentified calcium binding sites are present in the NoC region.

We tested these possibilities using NoC1, which is trimeric like native TSP1 but lacks the known Ca-binding sites in the signature domain (Kvansakul *et al.*, 2004; Carlson *et al.*, 2005). If the Ca-dependence is due to local effects of calcium binding on TSP1 conformation, NoC1 should show similar cation-dependence for antibody binding. However, soluble ^{125}I -NoC1 showed no significant calcium-dependence for binding to any of the three TSP1 antibodies (Fig. 2D–F). Since all three antibodies bound well to NoC1 in the presence of divalent cations, their epitopes appear to be constitutively exposed on NoC1, whereas binding of calcium to the signature domain in intact TSP1 may limit exposure of the epitopes for 2D11 and 5H9 either sterically or allosterically. Consistent with this conclusion, 4B6, 2D11, or 5H3 did not further enhance the robust adhesion of cells to NoC1 (Fig 1C).

2.5 Reversibility of affinity modulation by calcium

K_d values for binding to TSP1 for the three antibodies were assessed using self displacement assays with immobilized antibody and labeled TSP1 in solution. Antibody 4B6 bound to TSP1 with a K_a value of $4.3 \times 10^8 \text{ M}^{-1}$. Consistent with the increased binding of calcium-depleted TSP1 to 2D11 in Fig. 3, titration of calcium into calcium depleted TSP1 progressively decreased the affinity of TSP1 binding to this antibody (Fig. 4A, B). TSP1 depleted of calcium bound to 2D11 with $K_a = 2.5 \times 10^9 \text{ M}^{-1}$. This decreased to $9 \times 10^8 \text{ M}^{-1}$ in the presence of 2 mM calcium. 5H9 showed a more complex dependence on calcium (Fig. 4C, D). Pre-incubation of calcium-depleted TSP1 with 0.5 mM or 1 mM calcium for 30 min increased its affinity for binding to 5H9 approximately 2-fold. In both cases this was reversed by addition of an excess of EDTA prior to measuring 5H9 binding. However, increasing the calcium concentration to 2 mM decreased the affinity as expected based on Fig. 3. Combined with the results of Fig. 3 these data demonstrate reversibility of the divalent cation effects on binding of 5H9 and 2D11.

Further experiments were performed using dialysis against Chelex resin to more completely remove bound calcium from the TSP1. However, such TSP1 showed further changes in binding that were not reversible by readdition of calcium (results not shown). Thus, reversibility of the conformation changes in TSP1 detected by these antibodies occurs only within calcium concentrations close to physiological concentrations. The irreversible change resulting from exhaustive calcium depletion may be associated with isomerization of disulfides in the signature domain due to the unpaired cysteine in the lectin-like domain (Speziale and Detwiler, 1990; Sun *et al.*, 1992; Kvansakul *et al.*, 2004).

2.6 Effects of mAbs on sulfated glycoconjugate binding to the N module in the absence of calcium

Because several ligand-binding sites have been mapped to the N module of TSP1, we examined effects of 5H9, 2D11, and 4B6 on ligand interactions with TSP1 to further map their epitopes. The major high affinity heparin-binding site of TSP1 is located in the N module (Dixit *et al.*, 1984; Yu *et al.*, 2000). None of these TSP1 antibodies inhibited TSP1 binding to heparin (Fig.

5A). This is consistent with previous reports that heparin binding does not require calcium (Dixit *et al.*, 1984) and demonstrates that the epitopes in the N-module for all three antibodies are distinct from the heparin binding site and do not sterically interfere with heparin binding. Instead, we observed a significant enhancement of TSP1 binding to heparin in the presence of 4B6 and 2D11 relative to TSP1 alone or TSP1 complexed with a control TSP1 antibody ahTSP (HB8432), which binds to an epitope in the EGF-like repeats (Annis *et al.*, 2006; Annis *et al.*, 2007). Consistent with the finding that the heparin binding site overlaps with a sulfatide-binding site in the N-module (Yu *et al.*, 2000), antibodies 4B6 and 2D11 and, to a lesser extent, 5H9 enhanced TSP1 binding to sulfatide (Fig. 5B). As observed for TSP1 binding to heparin, enhancement was specific in that the control antibody ahTSP1 had no effect. These results demonstrate, therefore, that 4B6 and 2D11 enhance heparin binding and all three enhance sulfatide binding.

Because the avidity of heparin or sulfatide binding to intact TSP1 depends on multivalent interactions, we could not exclude the possibility that the increased TSP1 binding to these ligands in the above experiment is an artifact of antibody-mediated dimerization of the labeled TSP1. This potential artifact could be circumvented by using a reverse assay in which the TSP1-antibody complexes were immobilized and binding of a labeled heparin conjugate was assessed (Fig. 5C). Labeled heparin binding to TSP1 was significantly enhanced when the TSP1 was complexed with 5H9 and 2D11, and to a lesser degree with 4B6 antibody (Fig. 5C). The enhancement was specific in that 1G8 and ahTSP did not enhance heparin binding in this assay. Importantly, heparin binding to immobilized NoC1 was comparable in the absence or presence of the same TSP1 antibodies (Fig. 5D). These results parallel those obtained using TSP1 in solution and imply that these antibodies induce a conformation change in TSP1 that enhances binding to heparin or sulfatide. Lack of enhancement of the antibodies on heparin binding by NoC1 could be explained if the conformation of NoC1 is freed of restraints from the other parts of TSP1 and resembles that the conformation induced in the same region of TSP1 by antibody binding.

2.7 Effects of TSP1 antibodies on TSP1 binding to Link_TSG-6 and fibronectin

Divalent cations modulate TSP1 binding to both full length and the Link module of TSG-6 (Kuznetsova *et al.*, 2005). Antibodies 5H9, 4B6, and 2D11 partially inhibited TSP1 binding to immobilized Link_TSG6 (Fig. 6A). This effect was specific in that the antibody ahTSP and another antibody specific for the N module, Ab-9, did not inhibit TSP1 binding to the same ligand.

Fibronectin binds to at least one site in the N-terminal regions of TSP1 (Dardik and Lahav, 1989). In contrast to heparin and TSG-6 binding, 5H9, 2D11, and 4B6 decreased binding of ¹²⁵I-TSP1 to immobilized fibronectin (Fig. 6B). Inhibition was dose-dependent, but complete inhibition was not achieved even at the highest concentrations of antibodies. These results were also reproduced using NoC1 (Fig. 6C). The partial reduction in fibronectin binding suggests that the antibodies cause steric interference with fibronectin binding rather than directly interacting with an epitope involved in fibronectin binding. Divalent cations also modulate fibronectin binding to TSP1, but because fibronectin interacts with several domains of TSP1, we could not specifically assign these responses to the N module (results not shown). The differential effects of the antibodies on TSG-6 and heparin versus fibronectin binding demonstrate that these effects are ligand-specific.

2.8 Divalent cations indirectly enhance ligand binding to the N-terminal region of TSP1

Binding of soluble TSP1 to heparin was significantly enhanced in the presence of divalent cations (Fig. 7A). This does not result from a direct effect of calcium on heparin binding to the N-terminal domain of TSP1, because binding of NoC1 to heparin was comparable in the

absence or presence of calcium (Fig. 7B). Thus, C-terminal elements of TSP1, likely the signature domain, are required for divalent cation binding to perturb ligand binding to the N domain and further suggest a steric or allosteric mechanism.

We also considered the possibility that divalent cations increase TSP1 binding by altering the conformation of heparin. However, binding of soluble TSP1 to sulfatide was similarly enhanced in the presence of calcium (Fig 7C). It is unlikely that calcium interactions with these structurally distinct ligands would have the same effect on binding of TSP1.

3. Discussion

Conformation-specific antibodies have become valuable reagents for studying the regulation of integrin activation. Antibodies that recognize only activated integrins, also known as ligand-induced binding site (LIBS) antibodies, provide a means to assess the extent of integrin activation on live cells (Frelinger *et al.*, 1991; Newham *et al.*, 1998). Conversely, some antibodies that inhibit integrin function preferentially recognize and stabilize an inactive conformation of the integrin (Mould *et al.*, 1996). We have found similar conformation-dependent epitopes on TSP1. As in integrins, these epitopes are modulated by binding of ligands to sites in TSP1 that are distant from the induced epitope (Fig. 8). We previously found that this conformational regulation alters interactions of TSP1 with one of its cell surface receptors, $\alpha 3 \beta 1$ integrin (Rodrigues *et al.*, 2001). Conformation changes associated with the N⁷⁰⁰S polymorphism in TSP1 were also reported to alter heparin and fibrinogen binding (Narizhneva *et al.*, 2004). Here we show that modulation of cell surface receptor binding is selective, in that recognition of a second sequence in the N module by a second integrin, $\alpha 4 \beta 1$, is less sensitive to this conformation change. Likewise, the conformation change differentially modulates binding of extracellular ligands for TSP1 to this region of the protein. Binding of heparin, TSG-6, and fibronectin are affected in the calcium depleted conformation. In the case of fibronectin, the conformational change induced by calcium chelation increases TSP1 binding. In contrast, calcium chelation decreases heparin and TSG-6 binding to TSP1 (Kuznetsova *et al.*, 2005).

Based on these data, we propose that conformational changes in TSP1 coordinately regulate its cell and ECM interactions. Several TSP1 ligands show preferences for binding to specific conformations of TSP1 and conversely modulate the binding of other TSP1 ligands by stabilizing or destabilizing their preferred conformations (Fig. 8). Binding of TSP1 to both fibronectin and type V collagen appears to favor the calcium-depleted conformation, and the latter interaction is preferentially inhibited by TSP1 antibodies that exclusively bind to the calcium-depleted conformation (Galvin *et al.*, 1987; Rodrigues *et al.*, 2001). Such functional modulation may help to explain the complex biological activities of this matricellular protein (reviewed in (Bornstein, 2001)).

Occupancy of the predicted 31 calcium binding sites in the signature domain of TSP1 likely causes some rearrangement of the EGF-like modules, wire module, and the lectin-like module, which in the calcium-replete structure interact extensively with one another (Carlson *et al.*, 2005). These C-terminal domains must come apart to explain observations that upon chelation of calcium TSP1 has a lengthened stalk and diminished C-terminal globular structure in rotary shadowing images (Galvin *et al.*, 1985; Lawler *et al.*, 1985). To explain the rotary shadowing results and studies of conformation-sensitive antibodies to the signature domain, a model has been proposed in which the wire module, which is rigid in the calcium-replete structure, and relaxes in the absence of calcium with loss of interactions between the lectin-like module and the third EGF-like module, (Annis *et al.*, 2007); such events are depicted in Fig. 8. We envision two ways that such conformational changes may affect the N modules. The intrinsic affinity of the TSP1 N modules for their ligands and receptors may be allosterically altered (Fig.8A).

Alternatively, the affinity may be unchanged, whereas the avidity for ligands is modulated by the absence or presence of steric hindrance that impedes ligand binding to the N-module (Fig. 8B). NoC1 appears to exist in a constitutively active conformation that is insensitive to divalent cations or to the TSP1 antibodies (Fig. 8C). The first model would require that changes in the signature domains be transmitted through fairly rigid stalks, which appears less likely. The second model would require that the stalks be flexible. Examination of a model of TSP1 gleaned from structures of the component modules reveals few regions in which the stalk is likely to be flexible (Carlson *et al.*, 2008). The behavior of NoC1 is more easily explained by the second model (Fig. 8B). Additional structural data for the N-terminal domains in a trimeric configuration and of calcium-replete and -depleted trimeric TSP1 in solution will be needed to distinguish between these models.

TSP1 is a ligand for at least 5 integrins (Calzada *et al.*, 2004; Calzada and Roberts, 2005), and recognition of TSP1 by two of these is conformation-dependent. $\alpha 3\beta 1$ recognition is reversibly regulated by the conformational equilibrium described in this paper and suggested by previous studies of the 1G8 antibody (Rodrigues *et al.*, 2001), whereas $\alpha 4\beta 1$ recognition is relatively insensitive to the same conformational change. However, we cannot strictly exclude an alternative mechanism in which the antibody-induced conformation change selectively alters $\alpha 3\beta 1$ -mediated adhesion via the type 1 or EGF repeats (Calzada *et al.*, 2004). In contrast, $\alpha v\beta 3$ integrin recognition of an RGD sequence in the type 3 repeats is regulated by conformational changes controlled by disulfide bond isomerization (Sun *et al.*, 1992). Protein-disulfide isomerase catalyzes the interconversion of active and inactive TSP1 isomers (reviewed in (Hogg, 2003)).

Although initial studies indicated that TSP1 binding to heparin-Sepharose does not require divalent metal ions (Dixit *et al.*, 1984), several subsequent studies provide evidence that divalent cations do influence heparin and sulfatide binding. Altered susceptibility to proteolysis suggested that TSP1 in complex with fibronectin or heparin adopts the Ca^{2+} -replete conformation in the absence of Ca^{2+} (Dardik and Lahav, 1999). Calcium influenced TSP1 binding to perlecan (Vischer *et al.*, 1997), and divalent cations enhanced TSP1 binding to sulfatide (Roberts *et al.*, 1985). Immobilization in the presence of Ca^{2+} also enhanced proteoglycan-dependent Chinese hamster ovary cell adhesion on TSP1 (Kaesberg *et al.*, 1989). All of these results are consistent with the present data and can now be explained as indirect effects on the N-module of calcium binding to the TSP1 signature domain.

TSP1 has two binding sites for fibronectin (Dardik and Lahav, 1989). Binding to the first site at the N-terminal domain induces a conformational change that makes possible the binding to the second site on the type 1/2 repeats. (Dardik and Lahav, 1999). Conversely, binding of fibronectin to TSP1 modulates its interactions with $\alpha 3\beta 1$ integrin (Rodrigues *et al.*, 2001). Therefore, binding of TSP1 to cell surface receptors can be regulated by the extracellular matrix context. Because the conformation-sensitive epitopes that define these conformations of TSP1 are specifically induced following platelet activation, such context-dependent regulation of TSP1 clearly occurs in a biological context.

Several TSP1 antibodies are now known to preferentially recognize TSP1 associated with the surface of activated platelets (Huang *et al.*, 1997; Rodrigues *et al.*, 2001). D4.6 preferentially recognizes TSP1 bound to platelets (Huang *et al.*, 1997) and recognizes a conformation-dependent epitope in the calcium wire module (Annis *et al.*, 2006). 1G8 also binds preferentially to the conformation of TSP1 induced by removing divalent cations and recognizes TSP1 on the surface of activated platelets (Rodrigues *et al.*, 2001). However, we have been unable to localize the epitope for 1G8 using recombinant forms of the protein (unpublished results). The antibodies described here were all selected for preferential binding to activated platelets, but they differ from D4.6 in that their conformational epitopes are located

in the N module of TSP1. These results indicate that association of TSP1 with platelets results in global changes in its conformation.

Evidence is accumulating that interactions of TSP1 with other extracellular matrix proteins change its conformation and may sterically or allosterically regulate specific biological functions of TSP1. Such differences between free and complexed TSP1 may help to explain certain functional differences between TSP1 presented to cells in different contexts. For example, TSP1 is a pro-angiogenic protein when immobilized (Chandrasekaran *et al.*, 2000) but an anti-angiogenic protein when in solution (Good *et al.*, 1990; Tarabozetti *et al.*, 1990). Conformational changes on TSP1 may also be relevant in other biological functions of TSP1, since a single polymorphism in the calcium-binding site at the TSP1 is associated with a coronary heart disease (Topol *et al.*, 2001; Hannah *et al.*, 2003). Based on the present data, this mutation and additional disease-associated mutations in the type 3 repeats of other thrombospondins may have global effects on the conformations and biological activities of these proteins.

4. Experimental Procedures

4.1 Proteins

TSP1 and plasma fibronectin were purified from human platelets and plasma, respectively, obtained from the National Institutes of Health Blood Bank (Akiyama and Yamada, 1985; Roberts *et al.*, 1994). Heparin was obtained from Sigma. Monomeric and trimeric recombinant regions of thrombospondin-1 as depicted in Fig. 1 and NoC2 from TSP2 were prepared as described (Misenheimer *et al.*, 2000; Anilkumar *et al.*, 2002; Annis *et al.*, 2006). Monomeric Recombinant N module containing residues 1 to 250 of mature TSP1 was expressed using the Invitrogen FastBac1 baculovirus vector. p6SXTE containing a full length TSP1 cDNA was amplified by PCR using forward primer CTCCGGTACACACAGGATCCCTGCTG and reverse primer TATGAATTCATGATGATGATGATGATGGCCGGCGGCTTGCAAGTCCTTTG. The resulting PCR product containing the TSP1 leader sequence and AHHHHHH appended on the C-terminus was ligated into the pCR-Blunt vector (Invitrogen). After verifying by sequencing, the insert was excised by EcoRI digestion and ligated into the pFastBac vector. Generation of the recombinant baculovirus and protein expression were done according to the Invitrogen Bac-to-Bac procedure using Sf-21 insect cells. Medium from baculovirus infected Sf-21 cells was harvested and dialyzed into 20 mM Tris, 350 mM NaCl, 20 mM imidazole, pH 7.5, and then passed through a Ni-NTA agarose column (Sigma). Bound N module was eluted from the column with 20 mM Tris, 350 mM NaCl, 300 mM imidazole, pH 7.5. The isolated protein was dialyzed into 20 mM Tris, 20 mM NaHCO₃, 350 mM NaCl, pH 8.5, and stored at -70°C. Recombinant Link_TSG6 containing residues 36–133 of TSG-6 preprotein was prepared as described (Day *et al.*, 1996; Kahmann *et al.*, 1997). Proteins were labeled with ¹²⁵I using Iodogen (Pierce) as described previously (Guo *et al.*, 1992).

4.2 Antibodies

TSP1 antibodies F9 4B6 (IgG_{2a}), F12 2D11 (IgG_{2a}), F12 5H9 (IgG_{2b}), and F18 1G8 (IgG₁), abbreviated as 4B6, 2D11, 5H9 and 1G8 respectively, were secreted by hybridomas derived from BALB/c mice immunized with formalin-fixed, thrombin-activated human platelets and selected for preferential binding to activated platelets (Rodrigues *et al.*, 2001). Flow cytometry analysis for 2D11 and 5H9 showed approximately 10% positive cells using unstimulated platelets, which increased to 65.6 and 72.3%, respectively, for 2D11 and 5H9 following activation with 0.1 U/ml thrombin (Table I). TSP1 antibody ahTSP was purified from conditioned medium of the hybridoma HB8432, obtained from the American Type Culture Collection (Manassas, VA), by affinity chromatography on immobilized protein A. TSP1

antibody A6.1 was provided by Dr. William Frazier, Washington University, St. Louis, MO. TSP1 antibody Ab9 (clone MBC 200.1) was from Lab Vision Corporation.

4.3 Solid Phase Binding Assays

Solid phase binding assays were used to analyze the effect of conformational changes on TSP1 for binding to specific ligands. To analyze the changes on TSP1 conformation after depleting calcium, we used three different conformation-dependent TSP1 antibodies. Desired antibody concentrations were diluted in 50 μ l of DPBS or DPBS without divalent cations and containing 2.5 mM EDTA and incubated overnight at 4 °C on Immulon 2HB Removawell strips (Dynex Technologies, Chantilly, VA). Unbound antibody was removed, and the wells were blocked for 1 h with 1% BSA in DPBS. 50 μ l of 125 I-TSP1 or 125 I-NoC1 (0.25 μ g/ml) were incubated for 2 h at room temperature in DPBS containing 2.5 mM EDTA or 0.9 mM CaCl_2 and 0.5 mM MgCl_2 . Wells were washed four times with 0.05% Tween-20 in DPBS, and total binding was quantified with a gamma counter (Packard Instruments). Competition experiments were analyzed to calculate the dissociation constant.

We also studied the effect of depleting calcium or the effect of the antibodies in TSP1 binding to other ligands. For this purpose Immulon 2HB Removawell strips were coated overnight at 4 °C with TSP1 (20 μ g/ml), NoC1 (10 μ g/ml), fibronectin (30 μ g/ml) or heparin-BSA (0.05 μ g/ml) in PBS, or sulfatide (0.2 μ g/ml) in 50% methanol containing 50 ng phosphatidylcholine and 30 ng of cholesterol (Roberts *et al.*, 1986). Wells were incubated alone (in the absence or presence of 2.5 mM EDTA) or mixed with the TSP1 antibodies at the indicated concentrations. After blocking for 1 h. with 1% BSA/DPBS, 50 μ l of 125 I-protein (TSP1, NoC1, fibronectin, or heparin-BSA) at 0.25 μ g/ml were added to the wells and incubated for 3 h at room temperature. The wells were washed four times, and the binding was quantified using a gamma counter.

To assess reversibility of calcium effects on TSP1 binding, antibody 5H9 or 2D11 was coated on Immulon 2HB Removawell strips at 3 μ g/ml in TBS (10 mM Tris, pH 7.6, 150 mM NaCl) overnight at 4°C. Experiments using Chelex treated TBS are indicated. After aspiration and one hour blocking with 1% BSA-TBS, the strips were rinsed three times with TBS with 0.05% Tween 20. 125 I-TSP1 was diluted to 0.5 μ g/ml in TBS with 0.1% BSA with the indicated calcium concentrations, and non-labeled TSP1 was serially diluted from 10 to 0.625 μ g/ml in TBS with 0.1% BSA with the indicated calcium concentrations. After 30 minute incubation at room temperature EDTA, pH 7.6, was added at the indicated concentration before adding equal volumes of the labeled and non-labeled TSP1 to the Immulon 2HB Removawell strips. After three hour incubation at room temperature, the strips were washed three times with TBS/Tween 20, and the bound radioactivity was quantified. Binding analysis was done using Ligand software.

4.4 Enzyme-linked Immunoassay for TSP1 Antibody Binding to Immobilized Proteins

Wells in a 96-well Immulon 2HB flat bottom microtiter plate (Dynex) were coated overnight at 4°C with fibronectin (20 μ g/ml), heparin-BSA (0.02 μ g/ml) or various TSP1 fragments (10 μ g/ml) diluted in either DPBS or 50 mM sodium bicarbonate, pH 8.4. After removing unbound protein, the plates were blocked for 1 h with 1% BSA/DPBS. TSP1 antibodies (2 μ g/ml) were incubated for 1 h at room temperature. Wells were washed four times and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG, from Kirkegaard & Perry Laboratories (Gaithersburg, MD), at 1:2500 for 1 h at room temperature. The wells were washed again, and binding was detected using *o*-phenylenediamine substrate (Sigma).

4.5 Cell Adhesion Assay

TSP1 (12.5 µg/ml) or NoC1 (10 µg/ml) alone or in combination with TSP1 antibodies 4B6, 2D11 or 5H9 (2 µg each) in DPBS were adsorbed (triplicates of 8 µl drops) onto polystyrene dishes (Falcon 1008) by incubating overnight at 4 °C. The drops were removed, and the dishes were blocked with 1% BSA/DPBS for 30 min. MDA-MB-231 cells were dissociated with 2 mM EDTA/DPBS and resuspended in RPMI/0.1% BSA at 2.5×10^5 cells/ml. Jurkat cells were resuspended in the same medium at the same concentration. For activation, TS2/16 antibody was used at 5 µg/ml. After incubation for 1 h at 37°C in 5% CO₂, the dishes were washed three times with DPBS and fixed for 30 min with 1% glutaraldehyde/DPBS. Cells were stained with Diff-Quik and counted microscopically in 0.25-mm² fields for each triplicate analysis.

4.6 Data analysis

Curve fitting and data analysis for the ligand binding experiments with the different antibodies were calculated using Scafit version 2.4 of the Ligand program (Munson and Rodbard, 1980).

Acknowledgments

We are grateful to Dr. Harvey Gralnick for generating and interesting us in the panel of platelet antibodies used for these studies and for providing the flow cytometry data. This paper is written in tribute to him. We thank Dr Anthony Day for providing recombinant TSG-6. This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research and NIH grant HL54462 to D. F. M.

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Fig. 1. Selective enhancement of $\alpha 3\beta 1$ integrin-mediated adhesion by TSP1 antibodies
MDA-MB-231 ($\alpha 3\beta 1$, solid bars) or Jurkat ($\alpha 4\beta 1$ striped bars) cell adhesion to TSP1 (12.5 $\mu\text{g}/\text{ml}$) was determined in the absence or presence of the indicated TSP1 antibodies (2 $\mu\text{g}/\text{ml}$ each) using $2\text{--}2.5 \times 10^5$ cells/ml. MDA-MB-231 cell adhesion was assessed after 1 h incubation at 37°C and 5% CO_2 by washing to remove unattached cells, fixing, staining, and counting microscopically. Jurkat cells were incubated for 20 min. (A) Adhesion of unstimulated cells is expressed as a percent of the respective control without added antibodies (MDA 1.0 ± 0.5 cells/ mm^2 , Jurkat 11.7 ± 1.2 cells/ mm^2), mean $\pm$ SD, $n = 3$. (B) Adhesion of cells stimulated with TS2/16 (5 $\mu\text{g}/\text{ml}$) is expressed as a percent of the respective controls (MDA 6.0 ± 0.6 cells/ mm^2 , Jurkat 97 ± 10 cells/ mm^2). (C) MDA-MB-231 adhesion to NoC1 (10 $\mu\text{g}/\text{ml}$) with

TS2/16 (5 $\mu\text{g/ml}$) activation was assessed in the presence of TSP1 antibodies (5 $\mu\text{g/ml}$ each). Results are expressed as the number of cells/ $\text{mm}^2 \pm \text{SD}$, from three different experiments.

Fig. 2. Mapping of epitopes for TSP1 antibodies

Wells in 96-well plates were coated overnight at 4° with 10 µg/ml of TSP1 or the indicated recombinant proteins (A) diluted in 50 mM sodium bicarbonate, pH 8.4. (B) After removing unbound protein, blocking for 1 h with 1% BSA, and washing, the indicated antibodies at 2 µg/ml were incubated for 1 h at room temperature. Bound antibody was quantified using HRP-conjugated anti-mouse IgG and o-phenylenediamine substrate. Absorbance quantified at 490 nm is presented as mean $\pm$ S.D. (n=3). (C) Binding of the indicated antibodies (2 µg/ml) to wells coated using the indicated concentrations of immobilized platelet TSP1 (expressed on a subunit basis, closed symbols) or recombinant N module (open symbols) was determined in triplicate, mean $\pm$ S.D. (D) Cross-competition experiments were carried out using wells coated

with 2 $\mu\text{g/ml}$ of 4B6 (solid bars), 2D11 (hatched bars) or 5H9 (open bars). The unbound antibody was removed, and the wells were blocked with 1% BSA and then incubated with ^{125}I -TSP1 for 3 h. in the presence of 4B6 (1 nM), 2D11 (2 nM) or 5H9 (1 nM) in solution as competing antibodies. The wells were washed, and bound ^{125}I -TSP1 was quantified. Net binding is presented as mean $\pm$ S.D., n=3.

Fig. 3. Divalent cation dependencies of the TSP1 N-module antibodies

Immulon 2HB Removawell strips were coated with the indicated concentrations of 5H9 (A, D), 2D11 (B, E), or 4B6 (C, F) diluted in PBS with 2.5 mM EDTA (dotted lines) or 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} (solid lines). After removing unbound antibody and blocking for 1 h with 1% BSA, the wells were incubated with ^{125}I -TSP1 (A–C) or ^{125}I -NoC1 (D–F) at 0.25 $\mu\text{g/ml}$ for 3 h in the presence of EDTA or 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} . The wells were washed, and bound radioactivity was counted. Net binding is presented as mean $\pm$ S.D, n = 3.

Fig. 4. Reversibility of the calcium-induced modulation of TSP1 antibody affinity

(A) Representative self-displacement curves for ¹²⁵I-TSP1 binding to immobilized antibody 2D11. Binding was assessed in TBS, pH 7.6, in the absence of divalent cations or TBS containing 2 mM Ca²⁺. Each point represents the mean of 3 replicates, and the curves were calculated by nonlinear regression analysis using Ligand software. (B) Association constants for TSP1 binding to immobilized 2D11 were determined at the indicated calcium concentrations by nonlinear regression analysis of self-displacement curves. Error bars = SD calculated from the regression fit. (C) Representative self-displacement curves showing reversibility of the increase in ¹²⁵I-TSP1 binding to immobilized 5H9 stimulated by 0.5 mM Ca²⁺ following addition of 1 mM EDTA. (D) Association constants for ¹²⁵I-TSP1 binding to immobilized 5H9 were determined in the presence of the indicated concentrations of calcium (w/o EDTA) or following addition of a 2-fold molar excess of EDTA at each calcium concentration (+EDTA).

Fig. 5. N-module antibodies enhance TSP1 but not NoC1 binding to sulfated glycoconjugates in the absence of calcium

(A) ^{125}I -TSP1 binding to immobilized heparin-BSA (0.05 $\mu\text{g/ml}$) in the presence of varying concentrations of TSP1 antibodies in solution. (B) ^{125}I -TSP1 binding to immobilized sulfatide (0.2 $\mu\text{g/ml}$) in the presence of varying concentrations of antibodies. Results are normalized to positive controls in the absence of antibodies and are expressed as mean $\pm$ S.D for triplicate determinations. (C) TSP1 (20 $\mu\text{g/ml}$) or NoC1 (10 $\mu\text{g/ml}$) alone or mixed with 4 $\mu\text{g/ml}$ of the indicated antibodies were coated overnight at 4°C. Antibodies alone were coated as a control. After removing unbound protein and blocking for 1 h with 1% BSA, the wells were incubated with ^{125}I -heparin-BSA. Binding was assessed after 3 h. at room temperature in 2 mM EDTA/PBS. The plates were washed and the binding quantified. Error bars=SD, n = 3.

Fig. 6. TSP1 N-module antibodies in solution decrease TSP1 binding to TSG-6 and fibronectin
 (A) TSP1 binding to immobilized Link_TSG6. Wells were coated with 5 $\mu\text{g/ml}$ of Link_TSG6, blocked with 3% BSA and incubated with ^{125}I -TSP1 in buffer containing 5 mM EDTA for 3 hours at 37°C in the presence of indicated concentrations of antibodies. Results are normalized to net binding measured in the absence of antibody (2792.0 ± 142.9 cpm). (B) ^{125}I -TSP1 binding to immobilized fibronectin (30 $\mu\text{g/ml}$) in the presence of varying concentrations of antibodies in solution. Binding was assessed for 3 h, at room temperature in 2 mM EDTA/PBS. The plates were washed and the binding quantified. (C) ^{125}I -NoC1 binding to fibronectin (30 $\mu\text{g/ml}$) in the presence of varying concentrations of TSP1 antibodies in solution. Results are expressed as mean $\pm$ S.D n= 4.

Fig. 7. Divalent cations modulate ligand binding to the N-module of TSP1 but not of NoC1
 TSP1 binding to different ligands was quantified in the presence or absence of calcium. Immunolon-2 wells coated with heparin-BSA (A, B), or sulfatide (C) at the indicated concentrations were incubated with ^{125}I -TSP1 (A, C) or ^{125}I -NoC1 (B) in PBS containing 2.5 mM EDTA (dotted lines) or 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} (solid lines). After incubating for 3 h, total binding was quantified. Bound radioactivity is presented as mean $\pm$ S.D., $n=3$.

Fig. 8. Models for modulation of TSP1 conformation and function

Binding of Ca^{2+} to the signature domain of TSP1, which may neutralize the negative charge on this domain, and certain antibodies recognizing the N-modules induce conformational changes that alter its interactions with several ligands that bind to the N modules. Altered binding may result from allosteric changes in the intrinsic binding affinity of the N-modules for these ligands (A) or from global conformation changes that either sterically mask certain N-terminal binding sites or alter the avidity by reducing probability that the N-modules on more than one subunit can simultaneously engage multivalent ligands (B). Recombinant NoC1 (C) is not regulated in this manner and may bind ligands similarly in the presence and absence of calcium because it lacks the steric or allosteric constraints imposed by the signature domain

of TSP1. The box around the calcium binding site positioned between E1 and E2 serves as a reference point. In the calcium replete model, 5 of the calciums (one in the EGFs, and four bound to the G module) are depicted as black spheres. A schematic of an antibody molecule is presented in C to illustrate its size relative to TSP1.

Table 1TSP1 antibodies 2D11 and 5H9 recognize epitopes on thrombin-activated platelets^a.

Antibody	% Positive cells		
	Fresh platelets	Fixed platelets	Thrombin-activated fixed platelets
2D11	9.2	10.1	65.6
5H9	7.7	10.1	72.3
10E5	83.4	79.7	84.5
T4	0.4	0.5	0.4

^a Platelets were purified using platelet rich plasma fraction from citrate/EDTA anti-coagulated blood by centrifugation on an arabinogalactan gradient. Where indicated, platelets were activated with 0.1 U/ml thrombin for 15 min and the reaction was stopped with hirudin. 10E5 is an integrin α IIb β 3 antibody (Coller *et al.*, 1983) and was used as a positive control. T4 (Coulter) was used as an irrelevant antibody control. The percentage of positive cells was determined by flow cytometry versus a negative control without primary antibody (Marti *et al.*, 1988).