Conformational Regulation of the Fibronectin Binding and α3β1 Integrin-mediated Adhesive Activities of Thrombospondin-1*

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The recognition of extracellular matrix components can be regulated by conformational changes that alter the activity of cell surface integrins. We now demonstrate that conformational regulation of the matrix glycoprotein thrombospondin-1 (TSP1) can also modulate its binding to an integrin receptor. F18 1G8 is a conformation-sensitive TSP1 antibody that binds weakly to soluble TSP1 in the presence of divalent cations. However, binding of the antibody to melanoma cells was strongly stimulated by adding exogenous TSP1 in the presence of calcium, suggesting that TSP1 undergoes a conformational change following its binding to the cell surface. This conformation was not induced by known cell surface TSP1 receptors, whereas binding of F18 was stimulated when TSP1 bound to fibronectin but not to heparin or fibrinogen. Conversely, binding of F18 to TSP1 enhanced TSP1 binding to fibronectin. Exogenous fibronectin also stimulated TSP1-dependent binding of F18 to melanoma cells. Binding of the fibronectin-TSP1 complex to melanoma cells was mediated by α3β1 and α5β1 integrins. Furthermore, binding to F18 or fibronectin strongly enhanced the adhesive activity of immobilized TSP1 for some cell types. This enhancement of adhesion was mediated by α3β1 integrin and required that the α5β1 integrin be in an active state. Fibronectin also enhanced TSP1 binding to purified α3β1 integrin. Therefore, both fibronectin and the F18 antibody induce conformational changes in TSP1 that enhance the ability of TSP1 to be recognized by α3β1 integrin. The conformational and functional regulation of TSP1 activity by fibronectin represents a novel mechanism for extracellular signal transduction.

Thrombospondin-1 (TSP1) is an extracellular matrix protein that displays a complex variety of biological activities (reviewed in Refs. 1 and 2). TSP1 can both promote and inhibit cell adhesion, motility, and proliferation. TSP1 also contains sequences that can both activate latent transforming growth factor-β1 and inhibit its activation (3). In various animal models, TSP1 expression has been both positively and negatively correlated with tumor growth and angiogenesis (4–10). Some of these disparities in the cellular responses to TSP1 may arise from the presence of several functional sites on TSP1, which, when coupled with differences in the expression or activation state of the corresponding cell surface receptors between cell types, could result in opposing responses to this protein in different cell types. Yet, a growing list of examples are known in which a single cell type can respond to TSP1 as both an activator and an inhibitor of a specific response (11–13). Therefore, additional mechanisms must exist either to regulate cellular responsiveness to TSP1 or specific ligand binding activities of TSP1 itself.

The latter mechanism for regulating TSP1 activity is consistent with several previous reports that soluble TSP1 has activities distinct from immobilized TSP1. Soluble TSP1 generally inhibits angiogenic responses (14, 15), but immobilized TSP1 can stimulate angiogenic responses in several contexts (13, 16, 17). Such distinct responses could arise from differences in TSP1 receptor signaling because of the increased valency of immobilized versus soluble TSP1 or from alterations in signal transduction resulting from physical immobilization of a specific TSP1 receptor. Both of these models have ample precedent from investigation of other cell-matrix interactions (reviewed in Refs. 18 and 19). However, a third possibility is suggested by conformational studies of TSP1. Based on electron microscopic and spectroscopic analyses, the conformation of TSP1 is modulated by calcium binding (20–22). Characterization of the epitopes recognized by several monoclonal antibodies to TSP1 has identified calcium-dependent epitopes on the molecule (21). Thus, TSP1 exists in at least two major conformational states, one of which is induced by calcium binding. Remarkably, some antibodies that recognize only the calcium-free state in vitro readily bind to TSP1 in a calcium-rich environment in some tissues (23, 24). Therefore, binding to some TSP1 receptors may induce conformational changes that resemble those induced by removing calcium, and both states may exist in vivo. Other extracellular matrix components may regulate the conformation of TSP1. A recent study of protease sensitivity suggested that binding of TSP1 to fibronectin reversibly alters its conformation to resemble the calcium-depleted state (25).

In addition to the reversible conformational changes induced by binding of calcium or specific TSP1 ligands, TSP1 is a substrate for protein-disulfide isomerase (26, 27). Several disulfide isomer forms of TSP1 have been described and can also be differentiated by specific TSP1 antibodies (26, 28). These may also have different functional activities relating to inactivation of specific proteases or binding to the integrin α3β1 (29). Therefore, TSP1 can exist in multiple conformational states,
some of which can interconvert reversibly, whereas others are restricted by covalent disulfide bonds.

While characterizing a new conformation-specific antibody for TSP1, we found that the TSP1 conformer recognized by this antibody is specifically induced following binding of TSP1 to the surface of some cells. We subsequently found that this conformation is induced by binding to fibronectin in vitro or by association with cell-surface-associated fibronectin on cells. We further report that the conformation induced by binding of this antibody and fibronectin enhance the recognition of TSP1 by one of its cell surface receptors, αβ1 integrin. This reversible conformational change therefore modulates the ability of TSP1 to differentially engage specific cell surface TSP1 receptors and to thereby trigger specific biological responses.

**EXPERIMENTAL PROCEDURES**

**Proteins and Peptides—**TSP1 and plasma fibronectin were purified from human platelets or plasma, respectively, obtained from the National Institutes of Health Blood Bank (30,31). Fibrinogen and heparin-BSA were obtained from Sigma. Human fibroblast and lung fibrocytes were provided by Dr. Ralph Simmons, Fibrogenex, Inc., Chicago, IL. Purified αβ1 integrin was obtained from Chemicon. MBP-invasive 497 fusion protein was purified as described (32). Synthetic peptides derived from TSP1 that are recognized by the αβ1 integrin or other TSP1 receptors were synthesized as previously described (33). Integrin antagonists GRGDNP (αβ1 (34)) and (4-((2-methylphenyl)aminocar-bonyl)aminophenyl)acetyl-LDVP (αβ1 (35)) were obtained from Bachem (Torrance, CA). A non-peptide antagonist of α-containing integrins (SB223245) was provided by Dr. William H. Miller (SmithKline Beecham Pharmaceuticals, King of Prussia, PA) (36). Proteins were labeled with 125I using Iodogen (Pierce) as described previously (37).

**Antibodies—**F18 1G8 is an IgG1 secreted by a hybridoma derived from a BALB/c mouse immunized with formalin-fixed, thrombin-activated human platelets. Fab fragments were prepared from intact immunoglobulin using immobilized papain (Pierce) and radiolabeled using IODO-Beads according to the manufacturer's instructions (Pierce). TSP1 antibodies A6.1, D4.6, and C6.7 were provided by Dr. William Frazier (Washington University, St. Louis, MO). TSP1 antibody ab-TSP1 (HHB432) and the β1 integrin-activating antibody TS2/16 (38) were prepared from the respective hybridomas obtained from the American Type Culture Collection. A β1 integrin function-blocking antibody (αBo, αBo, αβ1, αβ2) and radiolabeled antibody TS2/16 (38) were obtained from Bachem (Torrance, CA). A non-peptide antagonist of α-containing integrins (SB223245) was provided by Dr. William H. Miller (SmithKline Beecham Pharmaceuticals, King of Prussia, PA) (36). Proteins were labeled with 125I using Iodogen (Pierce) as described previously (37).

**Platelets—**Platelets were obtained by venipuncture using a two-syringe technique and purified where indicated using a discontinuous arabinogalactan gradient as described previously (42). Platelets were diluted to 200,000/μl in 0.145 mM NaCl, 2.7 mM KCl, 3.8 mM Hepes, 0.1% glucose, 0.35% bovine serum albumin, pH 7.4. Divalent cations were added before use as indicated for each experiment. 125I-Labeled antibody binding was measured as previously described (42).

**Results**

**F8 Recognizes a Calcium-dependent Epitope on Platelets and TSP1—**F18 is secreted by a hybridoma derived from a mouse immunized with activated human platelets. The binding sites on platelets for the antibody were increased 10-fold following activation with thrombin and induced in binding purified TSP1 on platelets in plasma. (Table I) Fab fragments were used for these studies to preclude interactions with Fc receptors on the platelets, but a similar induction of binding was observed using the intact antibody (data not shown). Calcium was more active than magnesium for inducing the F8 epitope, and a combination of both cations was somewhat less effective than calcium alone. The antibody bound to purified platelet TSP1 Instrument). In some experiments, binding of TSP1 antibodies to TSP1 bound to immobilized proteins as described above was detected by an enzyme-linked immunoassay. After incubating with the indicated antibodies for 1 h, the wells were washed three times and incubated for 1 h in 1% BSA/Tris with 1% BSA/Tris for 30 min. The wells were washed again, and binding was detected using o-phenylenediamine substrate (Sigma).

**Functional Regulation of Thrombospondin-1 by Fibronectin**

In some experiments, binding of TSP1 antibodies to TSP1 bound to immobilized proteins as described above was detected by an enzyme-linked immunoassay. After incubating with the indicated antibodies for 1 h, the wells were washed three times and incubated for 1 h in 1% BSA/Tris with 1% BSA/Tris for 30 min. The wells were washed again, and binding was detected using o-phenylenediamine substrate (Sigma).
Functional Regulation of Thrombospondin-1 by Fibronectin

Table I

| Conditions                  | Binding sites/cell | K_d (nM) |
|-----------------------------|-------------------|----------|
| Experiment 1                |                   |          |
| Platelets + 5 mM EGTA       | 2,700             | 4.4 x 10^{-8}  |
| + 5 mM EGTA + thrombin      | 6,200             | 1.4 x 10^{-9}  |
| +Ca^{2+} + thrombin         | 26,800            | 2.4 x 10^{-8}  |
| Experiment 2                |                   |          |
| Platelets + thrombin + 5 mM EGTA | 2,400   | 5.8 x 10^{-9}  |
| + thrombin + 2 mM Mg^{2+}   | 10,000            | 1.8 x 10^{-9}  |
| + thrombin + 2 mM Ca^{2+}   | 15,500            | 2.7 x 10^{-9}  |
| + thrombin + 2 mM Ca^{2+} + 2 mM Mg^{2+} | 7,200 | 1.3 x 10^{-8}  |

Figure 1. Antibody F18 1G8 recognizes TSP1. A, wells in a 96-well plate were coated by incubating with TSP1 (1 μg/well) diluted in PBS with 1 mM calcium or 2.5 mM EDTA. After removing unbound TSP1 and blocking nonspecific sites with 1% BSA, binding of F18 to the respective wells was measured in buffer containing 1 mM CaCl_2 or 5 mM EDTA as indicated. Wells were incubated with 15 μg/ml F18 for 2 h followed by washing and incubation with 125I-anti-mouse IgG (84,000 cpm/well) for 1 h. Bound radioactivity was corrected for nonspecific binding to wells without TSP1 (225 cpm for EDTA and 174 cpm for calcium) and is presented as mean ± S.D., n = 2. B, wells were coated with F18 (2 μg/well) diluted in 0.1 M sodium carbonate, pH 9. Binding of 125I-TSP1 (10 μCi/μg, 105,000 cpm/well) was determined in the presence of calcium or EDTA as indicated. Nonspecific binding of 125I-TSP1 to wells without antibody (576 cpm in EDTA and 311 cpm in Ca) was subtracted, and net binding is presented.

2B). The latter result confirmed that similar amounts of TSP1 bound to each immobilized protein and that the increased binding of F18 to TSP1 bound to fibronectin was not an artifact of an increased TSP1 binding capacity on fibronectin compared with fibrinogen. The response for immobilized plasma fibronectin was dose-dependent, and cellular fibronectins from two sources produced similar enhancements of F18 binding to the TSP1-fibronectin complex (Fig. 2C).

Although some binding of F18 could be detected to TSP1 complexed with high concentrations of immobilized heparin-BSA (results not shown), when heparin and fibronectin concentrations that supported comparable levels of TSP1 binding were compared, as verified by quantifying direct 125I-TSP1 binding and binding of two other TSP1 antibodies, antibody F18 demonstrated enhanced binding to TSP1 complexed with fibronectin but much weaker binding to TSP1 complexed with heparin-BSA (Fig. 2D). Therefore, binding to fibronectin specifically induces the F18 epitope on TSP1.

Binding of F18 to TSP1 Enhances Fibronectin Binding—If (Fig. 1) formed a stable complex with TSP1 by gel filtration and specifically immunoprecipitated TSP1 from surface-labeled activated platelets (data not shown).

The divalent cation dependence for binding to platelets could indicate either that the antibody recognizes an epitope specific to a calcium-replete conformation of TSP1 or that the TSP1 receptor mediating its binding to platelets is calcium-dependent. Based on solid phase binding assays, however, F18 bound to TSP1 immobilized on plastic independent of divalent cations (Fig. 1A). In a reverse assay, soluble TSP1 bound preferentially to immobilized F18 when calcium was absent (Fig. 1B). This divalent cation dependence is similar to that of the previously reported TSP1 antibody A6.1 (21), but the two antibodies did not cross-compete for binding to TSP1 (data not shown). Therefore, this antibody defines a distinct calcium-dependent epitope on TSP1 that is preferentially exposed when calcium is removed, but this divalent cation dependence could not account for the inverse calcium-dependence for F18 binding to activated platelets.

Induction of the F18 Epitope by TSP1 Binding to Fibronectin—The above data suggested that binding of TSP1, either to a cell surface TSP1 receptor or to another TSP1 ligand bound to its respective cell surface receptor, induces a TSP1 conformational epitope that is recognized by F18. Preliminary studies using the blocking antibody 10E5 excluded a role for the platelet TSP1 receptor CD36 (11) and expressed in the ability of labeled F18 to recognize TSP1 bound to immobilized fibronectin, fibrinogen, or type I collagen substrates (Fig. 2).
the enhancement of antibody F18 binding induced by fibronectin resulted from a conformational change in TSP1, then binding of these two ligands to TSP1 may be thermodynamically linked. To test this hypothesis, we compared the binding of radiolabeled fibronectin to TSP1 and to TSP1 complexed with F18 (Fig. 3). Fibronectin binding was 3-fold higher to the immobilized TSP1-F18 complex than to immobilized TSP1 itself. This enhancement was specific, because immobilized F18 alone had no binding activity for fibronectin and complexing TSP1 with a control TSP1 antibody, ah-TSP1, did not enhance fibronectin binding relative to immobilized TSP1 without antibodies (Fig. 3).

Based on the dose-dependence for binding of 125I-fibronectin to immobilized TSP1 alone or complexed to F18, we determined that F18 increased the number of binding sites for fibronectin (10$^{22}$ pmol/well, respectively) but did not significantly alter the apparent affinity of fibronectin for immobilized TSP1.

F18 Binding to Melanoma Cells Is Mediated by a TSP1-Fibronectin Complex—F18 bound at low levels to a suspension of A2058 melanoma cells, but its binding was reproducibly and dose-dependently enhanced by adding exogenous TSP1 (Fig. 4A). In contrast, binding of labeled TSP1 to the same cells was not significantly increased by TSP1.

### FIG. 2. Binding to fibronectin specifically induces the TSP1 epitope recognized by F18. A, binding of 125I-F18 to TSP1 immobilized on various matrix proteins. The indicated proteins (2.5 µg/well in PBS) were coated on Immulon 2 polystyrene wells. After blocking with PBS containing 1% BSA, TSP1 at the indicated concentrations was added and incubated for 1 h at 37 °C. The wells were washed three times and incubated with 125I-F18 (15 µCi/µg) for 1 h at 37°C. The wells were washed, and bound radioactivity was quantified and presented as mean ± S.D., n = 2. F18 binding to TSP1 was significantly enhanced in a dose-dependent fashion when TSP1 was coated on fibronectin (FN).

### FIG. 3. Effects of TSP1 antibodies on 125I-fibronectin binding to TSP1. TSP1 (20 µg/ml) was coated on Immulon-2 wells alone or after mixing with the indicated TSP1 antibody (4 µg/ml). Control wells were coated with BSA. The wells were incubated with 125I-fibronectin (FN) for 1 h, washed, and counted. Fibronectin binding is presented as mean ± S.D. F18 (4 µg/ml) complexed with TSP1 significantly increased fibronectin binding (p = 0.04), but the control antibody ah-TSP1 did not (p > 0.6).

(●), but no significant effect on binding occurred when TSP1 was bound to fibrinogen (FG) (△) or the BSA control (○). Binding of 125I-A6.1 (14 µCi/µg) to TSP1 immobilized on various matrix proteins. A6.1 binding was determined to TSP1 incubated with immobilized fibronectin (●), fibrinogen (○), type I collagen (ColI, △), or BSA control (○). C, both plasma and cellular fibronectins induce the F18 epitope on TSP1. Immuno 2 wells were coated using the indicated concentrations of human plasma fibronectin (●), foreskin fibroblast fibronectin (○), or lung fibronectin (Δ). The wells were incubated with 25 µg/ml TSP1 followed by 125I-F18 (3 µCi/µg). Net binding, corrected for nonspecific binding to wells without fibronectin and preincubated with TSP1 (198 cpm), is presented as mean ± S.D., n = 2. D, fibronectin but not heparin binding induces the F18 epitope. Wells coated with 1 µg/well plasma fibronectin, 1 ng of heparin-BSA, or BSA alone were incubated with 0.5 µg/ml TSP1 followed by the indicated TSP1 antibodies, horseradish peroxidase-conjugated anti-mouse antibody, and o-phenylenediamine. A duplicate plate with removable wells was incubated with 0.5 µg/ml 125I-TSP1 to directly quantify the bound TSP1 under each condition. The immobilized ligand concentrations were chosen based on this assay to obtain equivalent levels of bound TSP1. Antibody binding quantified by the enzyme-linked immunoassay was normalized to the actual TSP1 bound and is presented as mean ± S.D., n = 2.
not significantly induced by the addition of F18 (Fig. 4B). This result indicates either that these two TSP1 binding interactions are not thermodynamically linked or that TSP1 bound to its major binding site on these cells is not recognized by the F18 antibody. Because high affinity binding of TSP1 to A2058 cells was previously shown to be mediated by the heparin-binding domain of TSP1 (37), this result also suggests that cell surface heparan sulfate proteoglycans are not the TSP1 receptor that induces the epitope recognized by F18 and is consistent with the failure of heparin binding to induce the F18 epitope on TSP1 in vitro.

TSP1-dependent binding of 125I-F18 to A2058 cells was further enhanced by the addition of plasma fibronectin with the TSP1 (Fig. 4C). As observed for F18 binding to platelets, the enhancement of F18 binding was at least partially calcium-dependent, because addition of EDTA significantly inhibited both basal binding of F18 and the TSP1-fibronectin-stimulated binding of F18 (Fig. 4C).

β1 Integrin-dependent Binding of TSP1 Antibody F18 to Melanoma Cells—Of the known cell surface TSP1 receptors, only integrins require divalent cations for binding to TSP1, suggesting that binding of TSP1 to an integrin induced the epitope recognized by F18 on platelets and melanoma cells. We considered both integrins that have been reported to bind directly to TSP1 (α5β1, α4β1, ανβ1, αIIbβ3, and α6β3) and integrins that could bind fibronectin to serve as a bridging ligand for TSP1 (ανβ1, αβ1, and α5β1). Using A2058 melanoma cells, we could not inhibit TSP1-enhanced F18 binding using an αν integrin antagonist (Fig. 5), but a β1 integrin-blocking antibody inhibited the fibronectin-enhanced binding of F18 to melanoma cells (Fig. 4C, p < 0.0001).

The β1 integrin dependence was specific to cells where exogenous TSP1 enhanced F18 binding (Fig. 4D). Basal binding of F18 to MDA-MB-435 breast carcinoma cells was only slightly stimulated by exogenous TSP1, and the β1 integrin antibody had no significant effect on F18 binding to MDA-MB-435 cells (Fig. 4B). Both of these negative results suggested that direct binding of TSP1 to the ανβ1 integrin, which is the primary adhesive receptor for TSP1 in these cells (46), does not induce the F18 epitope.

Inhibition of TSP1/fibronectin-dependent F18 binding to melanoma cells by several integrin subunit antagonists further suggested that the TSP1/fibronectin complex is bound through integrins that recognize fibronectin (Fig. 5A). Function-blocking antibodies specific for ανβ1 and α6β3 integrins both inhibited F18 binding to or below basal levels (p < 0.002). Specific peptide antagonists of α5β1 (35) and α8β1 integrins (34) yielded similar inhibition, whereas an αν-specific antagonist
Adhesion—Because we noted previously that spreading of breast carcinoma cells on a TSP1 substrate, which is mediated by α5β1 integrin, was enhanced when the protein was adsorbed in the absence of divalent cations (46), we examined the effect of F18 binding on the adhesive activity of immobilized TSP1 for this integrin. In preliminary experiments, we found that adding soluble F18 moderately stimulated spreading of MDA-MB-435 breast carcinoma cells on immobilized TSP1 (results not shown). Because TSP1 immobilized on a plastic substrate may have limited ability to undergo conformational changes in response to antibody binding, we repeated this experiment by pre-incubating TSP1 in solution with TSP1 antibodies prior to adsorbing the complex on plastic (Fig. 6). Remarkably, F18 strongly enhanced the spreading of MDA-MB-435 and MDA-MB-231 breast carcinoma cells on substrates coated with a low concentration of TSP1. Formation of a complex of TSP1 with F18 dramatically increased the outgrowth of both lamellar and filopodial processes on MDA-MB-231 cells attaching on TSP1 (Fig. 6A). This enhancement of cell spreading was specific in that all of the other TSP1 antibodies examined either had no effect or partially inhibited spreading of MDA-MB-231 cells under the same conditions (Fig. 6B). The spreading response in MDA-MB-231 cells, which have a largely inactive α5β1 integrin (46), was dependent on activation of β1 integrins using antibody TS2/16 (Fig. 6C), indicating that β1 integrin activation was required for the response to the F18-TSP1 complex. The α5β1 integrin in MDA-MB-435 cells is normally partially active (46), and enhanced spreading on TSP1 complexed with F18 was stimulated only moderately by the β1 integrin-activating antibody (results not shown).

Integrin antagonists were used to verify that the enhanced adhesion response of breast carcinoma cells to immobilized TSP1 was mediated by α5β1 integrin (Fig. 6D). In this experiment, α5β1 integrin was activated using IGF1, and complexing the TSP1 with F18 further stimulated the IGF1-dependent spreading. Both α5β1- and β1-specific function blocking antibodies reversed the F18-dependent spreading of IGF1-activated cells on TSP1, whereas the α5β1- and α5β1-blocking antibodies that prevented TSP1-fibronectin-mediated F18 binding to melanoma cells had no effect on F18-enhanced adhesion (Fig. 6D). Therefore, the F18-enhanced spreading is not mediated by fibronectin on the surface of breast carcinoma cells.

Complexing TSP1 with F18 similarly enhanced α5β1 integrin-mediated responses in two other cell types. F18 enhanced spreading of human umbilical vein endothelial cells on TSP1 (Fig. 7A). As expected, based on the known regulation of α5β1 integrin activation by cell contact in endothelial cells (13), cells from sparse cultures showed greater stimulation of spreading on the immobilized F18-TSP1 complex than did cells from confluent cultures. Similarly, F18 stimulated outgrowth of neurite-like processes in OH-1 small cell lung carcinoma cells on a TSP1 substrate (Fig. 7B). A 2.3-fold stimulation of process formation was observed in cells without EGF, whereas cells treated with EGF did not show a significant response. In the presence of EGF, the outgrowth response to immobilized TSP1 is presumably maximal and could not be further stimulated.

Complex Formation with Fibronectin Enhances α5β1 Integrin-dependent Adhesive Activity of Immobilized TSP1—Most cell types that interact with TSP1 through the α5β1 integrin also express integrins that recognize fibronectin, precluding an analysis of the effect of fibronectin binding on the interaction of TSP1 with α5β1 integrin. However, some small cell lung carcinoma cells lack any functional fibronectin receptors but exhibit α5β1 integrin-dependent adhesion and outgrowth of neurite-like processes on TSP1 (11). We therefore used these cells to

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**Fig. 5.** TSP1-fibronectin-dependent binding of 125I-F18 to melanoma cells is dependent on α5β1 and α6β1 integrins. A, a suspension of A2058 melanoma cells was incubated with 125I-F18 (3 μCi/μg) alone (Control) or in the presence of 5 μg/ml TSP1 (+TSP1). For all other conditions, A2058 cells were pre-incubated with fibronectin for 30 min, centrifuged, and resuspended in binding buffer. Binding of 125I-F18 was measured to these cells (+TSP1+FN) alone or in the presence of function blocking antibodies for α5β1 (5 μg/ml) or α6β1 (5 μg/ml), 1 μM α5β1 integrin antagonist (4-A-(2-methylphenyl)aminomethylcarbonylamino-phenylacetyl-LDVP (4aLDVP), 0.4 μM α6β1 peptide antagonist GRGDNP, or 1 μM α1 integrin antagonist SB223245. Significant inhibition relative to the control containing both TSP1 and fibronectin is indicated by an asterisk for: α5β1-blocking antibody; p = 0.0014; α5β1-blocking antibody, p = 0.0016; α6β1 integrin antagonist 4aLDVP, p < 0.0001; and the α6β1 peptide antagonist GRGDNP, p < 0.0001. B, TSP1-fibronectin-mediated binding of F18 does not require an intact cytoskeleton. Melanoma cells were resuspended in complete cell culture medium, treated with cytochalasin D (1 μM), nocodazole (10 μM), or an equivalent concentration of dimethyl sulfoxide (DMSO) for 30 min at room temperature in the presence or absence of fibronectin, and then centrifuged and resuspended with 125I-F18 (6.2 μCi/μg) alone (control) or with 5 μg/ml TSP1 and 10 μg/ml fibronectin (TSP1+FN). Binding was determined after 1 h with orbital mixing at 4°C. (36) was inactive. Antibody and peptide antagonists of several other known TSP1 receptors, including CD36, CD47, and α6β1, were tested but were also negative (results not shown).

The preceding data show that fibronectin adsorbed on plastic (Fig. 2) or bound to β1 integrins on the cell surface (Figs. 4 and 5) induces a conformation change in TSP1 detected by the F18 antibody. Because binding of fibronectin to cell surface integrins induces conformation changes that initiate fibronectin matrix assembly (reviewed in Ref. 47), which may also be induced by adsorption of fibronectin to plastic, the cytoskeleton could be required as a scaffold for cell surface fibronectin to induce this epitope on TSP1. However, neither disruption of the actin cytoskeleton using cytochalasin D nor disruption of microtubules using nocodazole significantly inhibited the TSP1-fibronectin-mediated binding of F18 to melanoma cells (Fig. 5B). Therefore, the observed response does not require an intact cytoskeleton.

**Binding of TSP1 to F18 Augments α5β1 Integrin-dependent**
examine the effect of complexing TSP1 with fibronectin on its recognition by \( \alpha_3 \beta_1 \) integrin. Complexes of TSP1 with fibronectin or with antibody F18 produced similar enhancements of OH-1 process outgrowth relative to cells plated on TSP1 alone (Fig. 8). Neither F18 nor fibronectin-coated substrates alone induced neurite formation. Therefore, conformational changes induced by binding of fibronectin to TSP1 also enhance its ability to interact with the \( \alpha_3 \beta_1 \) integrin.

**Fibronectin Enhances TSP1 Binding to Purified \( \alpha_3 \beta_1 \) Integrin—**

The above data demonstrated that changing the conformation of TSP1 modulates \( \alpha_3 \beta_1 \) integrin-mediated responses to TSP1 in three cell types but did not exclude the possibility that fibronectin modulated the function of this integrin indirectly. To directly examine the effect of fibronectin on binding of TSP1 to \( \alpha_3 \beta_1 \) integrin, we established a competitive assay to measure the affinity of TSP1 for \( \alpha_3 \beta_1 \) integrin (Fig. 9). We used an \( ^{125}\text{I} \)-labeled invasin fragment as a tracer, because preliminary experiments indicated that TSP1 binding to \( \alpha_3 \beta_1 \) integrin was of too low an affinity to quantify in a direct assay, consistent with a previous report that recombinant \( \alpha_3 \beta_1 \) integrin binding to immobilized TSP1 could not be detected using a direct binding assay (32). In contrast, TSP1 was an effective competitive inhibitor of invasin binding to purified \( \alpha_3 \beta_1 \) integrin (Fig. 9A). Using the LIGAND program (48), the apparent \( K_d \) for this interaction was determined to be \( 2.6 \pm 0.3 \times 10^6 \text{ M}^{-1} \). Inhibition by TSP1 was specific, because fibronectin did not inhibit invasin binding to the same integrin (Fig. 9B and results not...
shown). Complexing TSP1 with fibronectin, however, increased the activity of TSP1 to inhibit invasin binding (Fig. 9B, \( p = 0.012 \) compared with TSP1 alone using a 2-sided \( t \) test). Based on the dose dependence for inhibition by the TSP1-fibronectin complex (Fig. 9A), complexing with fibronectin significantly increased the affinity of TSP1 for \( \alpha_3\beta_1 \) integrin (4.8 \( \pm \) 1.5 \( \times \) 10\(^6\) M\(^{-1} \), \( p = 0.004 \)). Therefore, the stimulation of TSP1 binding to \( \alpha_3\beta_1 \) integrin by fibronectin could be verified using purified proteins.

**DISCUSSION**

Much progress has been made toward defining both ligand-and receptor-binding specificities of extracellular matrix proteins. However, to understand the diverse biological activities that have been reported for these proteins we must also consider the possibility that some of their binding activities are differentially regulated. Previously, the regulation of cellular responses to integrin ligands has been studied primarily in terms of the activation state of integrin receptors (“inside out signaling,” reviewed in Ref. 18) and intracellular cross-talk between the signals from integrins and other cell surface receptors (reviewed in Ref. 49). Activation of the \( \alpha_3\beta_1 \) integrin to recognize TSP1 is regulated by the former pathway in several cell types (11, 13, 46). However, we now present evidence for an additional mechanism to modulate the same integrin-ligand interaction based on allosteric regulation of integrin ligand conformation. The active conformation of TSP1 is preferentially recognized and can be induced or stabilized by the TSP1 antibody F18. We further identified fibronectin as an extracellular matrix and cell surface-associated protein that specifically induces the same conformational and functional state of TSP1. F18 is a TSP1 antibody that recognizes a conformation that is not favored when soluble platelet TSP1 is in physiological media containing divalent cations but is induced when TSP1 is bound on the surface of platelets and melanoma cells or ad-
sorbed on plastic. Fibronectin specifically induces this conformational change and can serve as a bridging ligand to present TSP1 in this conformation on the surface of cells (see Fig. 10A). Exposure of this TSP1 epitope on melanoma cells requires the $\alpha_\beta_1$ and $\alpha_\beta_3$ integrins, presumably to bind fibronectin. Although most high affinity binding of TSP1 to melanoma and other cell types is mediated by sulfated glycoconjugates (37, 46), F18 does not recognize TSP1 bound to this ligand but instead binds to a minor fraction of the TSP1 that binds to the cell through fibronectin and its $\beta_1$ integrin receptors.

In addition to mediating the binding of TSP1 to the melanoma cell surface, fibronectin binding alters the biological activity of TSP1 for processes mediated by one of its receptors, $\alpha_\beta_1$ integrin (Fig. 10B). Because most cells also express fibronectin receptors, we could only quantify this activity in small cell lung carcinoma cells that do not recognize fibronectin. However, fibronectin presumably modulates interactions of TSP1 with $\alpha_\beta_1$ integrin in other cell types as well. $\alpha_\beta_1$ integrin participates in the interactions of several cell types with TSP1 (11, 13, 46, 50, 51), and therefore this regulation may be important for understanding the effect of TSP1 on many cell types. In addition, the apparently unfavorable equilibrium between the active conformation and the less active calcium-replete form of TSP1 in physiological media may explain the failure to detect direct binding of TSP1 to recombinant $\alpha_\beta_1$ integrin (32).

Although both $\alpha_\beta_1$ and $\alpha_\beta_3$ integrins have been reported to be TSP1 receptors in several cell types (46, 51, 52), the present results suggest that the role of these integrins in adhesion to TSP1 may be indirect rather than direct. We have observed that medium containing serum provides sufficient fibronectin to promote TSP1 binding via this mechanism. Therefore, fibronectin derived from the growth media or secreted by the cell binds to a minor fraction of the TSP1 that binds to the cell through fibronectin and its $\beta_1$ integrin receptors.

The binding of TSP1 to fibronectin is mediated by at least two domains in each protein (45, 53–56), an interaction that was recently shown to modify the sensitivity of TSP1 to limited proteolysis (25). Although the proteolysis data implied that binding to fibronectin alters the conformation of TSP1, its functional relevance to the biological activities of TSP1 were unknown. Cell-adhesive activities of TSP1/fibronectin mixtures (57). Additional reports of cross-talk between integrin-ligand binding activities of fibronectin or TSP1 versus cross-talk among the intercellular signals induced by each protein. Additional reports of cross-talk between fibronectin and TSP1 to regulate cell behavior include the suppression of TSP1-induced endothelial cell apoptosis by fibronectin (58) and disruption by TSP1 of focal adhesions in endothelial cells attached on fibronectin (59). Using a cell line that lacks functional fibronectin-binding integrins, we have now demonstrated that fibronectin binding can alter the ability of TSP1 to be recognized by $\alpha_\beta_1$ integrin. This modulation of receptor binding appears to be specific in that heparin and fibrinogen binding activities of TSP1 were not modulated by the same conformational change. Conversely, fibronectin binding specifically modulates signal transduction from TSP1 in the matrix by regulating the ability of TSP1 to interact with a specific integrin receptor (Fig. 9). Thus, both intracellular and extracellular signaling pathways may mediate the combined effects of these two proteins on cell function.

The interaction of TSP1 with fibronectin on the surface of activated platelets was previously demonstrated by cross-linking (60). Because TSP1 was not required for fibronectin binding to platelets (61), TSP1 may also bind to the surface of activated platelets through integrin-associated fibronectin, as demonstrated here for its binding to melanoma cells. Both $\alpha_\beta_1$ and $\alpha_\beta_3$ integrins have been implicated as TSP1 receptors on platelets (62, 63). Although an $\alpha_\beta_1$ antibody partially inhibited F18 binding to activated platelets, previous work has questioned the relevance of this antibody inhibition to the proposed function of $\alpha_\beta_1$ as a TSP1 receptor (64). Our results in melanoma cells further suggest that the contribution of $\alpha_\beta_1$ integrin in F18 binding to platelets may also be to bind fibronectin as a bridging molecule rather than acting as a direct receptor for TSP1. The role of $\beta_1$ integrins that bind fibronectin as indirect receptors for TSP1 on platelets should therefore be further examined.

Although conformational regulation of integrin function has been widely observed, only a few examples of conformational regulation of extracellular matrix protein function have been reported. Binding of fibronectin to $\alpha_\beta_1$ integrin induces exposure of a self-association site that mediates fibronectin fiber assembly (reviewed in Ref. 47). The exposure of an anti-adhesive site in fibronectin may also be conformationally regulated and is induced following binding to glycosaminoglycans (65). The binding of fibronectin to the platelet surface also induces a change in its conformation and exposes a neoepitope recognized by a function-blocking antibody (42). In TSP1, conformational changes are now known to regulate its binding to two integrins, $\alpha_\beta_1$ (43) and $\alpha_\beta_3$. The regulation of $\alpha_\beta_3$ integrin binding requires covalent modification of disulfide bonds in TSP1 (43), whereas regulation of $\alpha_\beta_1$ integrin binding appears to be reversible. It is likely that further examples of such functional regulation will be found in other matrix proteins.

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Conformational Regulation of the Fibronectin Binding and α3β1 Integrin-mediated Adhesive Activities of Thrombospondin-1

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