Molecular Basis of Antiangiogenic Thrombospondin-1 Type 1 Repeat Domain Interactions With CD36

Philip A. Klenotic,* Richard C. Page,* Wei Li, Joseph Amick, Saurav Misra, Roy L. Silverstein

Objective—Antiangiogenic activity of thrombospondin-1 and related proteins is mediated by interactions between thrombospondin type 1 repeat (TSR) domains and the CD36, LIMP-2, Emp sequence homology (CLESH) domain of the endothelial cell receptor CD36. We sought to characterize key molecular determinants of the interaction between thrombospondin-1 TSR domains and the CD36 CLESH domain.

Approach and Results—Recombinant thrombospondin-1 TSR2 and TSR(2,3) constructs inhibited microvascular endothelial cell migration, microvascular endothelial cell tube formation, and vessel sprouting in aortic ring assays. Interaction with CD36 CLESH decoy peptides negated these effects. Mutational analyses identified a cluster of residues that confer positive charge to the TSR2 surface and mediate interaction with CD36 CLESH. Antiangiogenic activity was significantly reduced by charge-neutralizing mutations of the Arg-Trp ladder in TSR2, but not TSR3. Additionally, I438 and K464 of TSR2 were shown to be required for CD36 CLESH binding to TSR2. A complementary acidic cluster within CD36 CLESH is also required for antiangiogenic activity.

Conclusions—Thrombospondin-1 interacts with CD36 CLESH through electrostatic interactions mediated by a positively charged TSR2 surface and multiple negatively charged CD36 CLESH residues. Two key residues serve as specificity determinants that identify other TSR domains that interact with CD36 CLESH. (Arterioscler Thromb Vasc Biol. 2013;33:1655-1662.)

Key Words: angiogenesis ■ CD36 ■ thrombospondin-1

The matricellular protein thrombospondin-1 (TSP-1) was the first endogenous protein inhibitor of angiogenesis to be identified.1 TSP-1, first isolated from human platelets,2 inhibits endothelial cell proliferation3 and migration and promotes apoptosis in the context of proangiogenic signals.4,5 At early stages of dermal wound healing, TSP-1 is released from platelet alpha granules into thrombi and the extracellular matrix to delay vascular remodeling.6 Within the tumor microenvironment, downregulation of TSP-1 is critical for many tumor cell types to continue unregulated cellular growth, metastasis, and seeding to secondary sites.7 Among the 5 members of the thrombospondin family, only TSP-1 and TSP-2 have been shown to have antiangiogenic activity to date. The functions of TSP-3 and TSP-4 are currently unclear. TSP-5, also called cartilage oligomeric matrix protein, regulates attachment and survival of chondrocytes and potentially of other musculoskeletal cells,8-11 but no angiogenic functions for this protein have been reported.

TSP-1 and TSP-2 each contain 3 highly homologous thrombospondin type 1 repeat (TSR) domains. The other members of the thrombospondin family do not contain TSRs, emphasizing that TSR domains are important for antiangiogenic function. Furthermore, several other antiangiogenic proteins, such as brain angiogenesis inhibitor-1 (BAI1), also contain TSR domains. The best-studied TSRs, the second and third TSR domains of TSP-1 (called TSR[2,3]), bind and activate transforming growth factor-β,12-14 bind heparan sulfate proteoglycans and fibromectin,7 and promote endothelial cell apoptosis through the cell surface receptor CD36.17,18 Recombinant TSR domains and TSR-derived synthetic peptides exhibit potent antiangiogenic activity within tumors19 and attenuate tumor growth in mice.20-22 Although TSR-derived peptides have been tested in phase II clinical trials, results are mixed.23 A more detailed understanding of the structural mechanisms used by TSR domains to regulate angiogenesis may inform the design of future therapies against pathological angiogenesis.

Two available crystal structures of TSP-1–TSR(2,3)11,24 showcase a positively charged surface ridge within each TSR. These ridges have been proposed to serve as a recognition surface for TSP-1 and TSP-2 binding partners,25 most notably CD36. A ≈30-residue-long region within CD36 called the CLESH (CD36, LIMP-2, Emp sequence homology) domain is sufficient for TSR binding.26 We and others have suggested that the negatively charged CLESH domain interacts with TSR domains via the positively charged surface ridge.11,27 Moreover, we reported that phosphorylation of a CD36

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threonine adjacent to the CLESH domain negatively regulates TSP-1 binding. The basis and extent of TSR/CD36 CLESH interactions, however, remain poorly defined.

In the present study, we characterize the molecular basis of interaction between the TSR domains of TSP-1 and the CD36 CLESH domain. We show that only the TSR2 of TSP-1 binds to the CD36 CLESH domain to inhibit microvascular endothelial cell (MVEC) angiogenic functions. We propose and validate key specificity determinants that mediate the interaction of TSR2 with CD36 CLESH and allow for the identification of other TSR domains that interact with CD36 CLESH. This study provides a framework to better understand the CD36 CLESH/TSR binding interface and the molecular determinants that mediate TSP-1 binding to CD36 at the endothelial cell surface.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

TSR2 Charge-Neutralizing Mutations Abolish the TSP-1/CD36 CLESH Interaction

The TSR2 and TSR3 of TSP-1 were previously identified as the domains responsible for antiangiogenic inhibition of MVEC-d migration. Sequence analyses have also identified TSR domains within the antiangiogenic proteins TSP-2, BA11, and a disintegrin and metalloproteinase with thrombospondin motifs 1 and 4 (ADAMTS-1 and ADAMTS-4). Two conserved arginine residues (Figure 1A) participate in cation-π stacking with neighboring tryptophan residues to form the Cysteine, Tryptophan, Arginine (CWR)-layered core of each TSR domain. These arginine residues confer a positive charge to the surface ridge of the CWR-layered core (Figure 1B). On the basis of electrostatic surface calculations and molecular modeling, we hypothesized that novel Arg-to-Met mutations could reduce the positive charge of the CWR-layered core surface ridge (Figure 1B; Figure I in the online-only Data Supplement) without compromising the structural integrity of the TSRs.

We analyzed the effect of individual Arg-to-Met mutations of TSR(2,3) on the inhibition of MVEC-d migration in a modified Boyden chamber assay. Few cells migrated when incubated against a low growth factor media chemoattractant containing 20% of normal growth factors (Figure 1C). Incubation against a normal growth factor media chemoattractant containing 100% of normal growth factors resulted in a 3-fold increase in migration. Addition of recombinant TSR(2,3) to normal growth factor media reduced MVEC-d migration to a level similar to that observed with low growth factor media. Arg-to-Met mutations in the TSR2 domain (R440M and R442M) fully abrogated the inhibitory activity of TSR(2,3). In contrast, the equivalent Arg-to-Met mutations in the TSR3 domain (R497M or R499M) had no significant effect on the inhibitory activity of TSR(2,3).

Because the effect of Arg-to-Met mutations could be ascribed to disruptions in the structure of TSR2, we acquired solution nuclear magnetic resonance heteronuclear single quantum coherence (NMR HSQC) spectra for uniformly N-labeled wild-type, R440M- and R442M-TSR2. HSQC spectra for R440M- and R442M-TSR2 are well dispersed and very similar to the HSQC spectrum of wild-type TSR2 (Figure II in the online-only Data Supplement), verifying that the R440M...
R442M mutations do not substantially alter the tertiary structure of TSR2.

**TSR2 Is the Critical TSR Required for CD36 CLESH Binding**

To confirm that TSR2 is the minimal domain responsible for the CD36 CLESH-dependent antimigratory activity of TSP-1, we conducted MVEC-d migration assays with TSR(2,3), TSR2, and TSR3 domain constructs. TSR2 attenuated MVEC-d migration to the same extent as the TSR(2,3) construct (Figure 2A). Attenuation of MVEC-d migration by TSR2 was reversed on addition of a competing GST-CD36 CLESH fusion protein, whereas GST alone had no effect (Figure 2A). In comparison, only TSR3 mildly attenuated MVEC-d migration and this attenuation was insensitive to CD36 CLESH (Figure 2A).

We next performed solid-phase binding assays to directly measure binding of CD36 CLESH to TSR(2,3), TSR2, or TSR3. In a binding assay using CD36 CLESH-ubiquitin fusion protein (CLESH-Ub) probed with an anti-ubiquitin antibody, the CLESH-Ub fusion bound similarly to TSR2 and TSR(2,3) (Figure 2B). We observed minimal binding of CLESH-Ub to TSR3 or in a control experiment measuring binding of ubiquitin to TSR2.

**Figure 2.** The CLESH domain of CD36 binds and reverses the antimigratory activity of thrombospondin type I repeat 2 (TSR2), but not TSR3. A, Microvascular endothelial cell (MVEC)-d migration in a modified Boyden chamber toward low growth factor media (low GF) or media containing a full complement of growth factors (normal GF) compared with MVEC-d migration toward media containing a full complement of growth factors in the presence of recombinant TSR(2,3), TSR2, TSR2 and GST-CD36 CLESH (TSR2+CLESH), TSR2 and GST (TSR2+GST), TSR3 or TSR3 and GST-CD36 CLESH (TSR3+CLESH). Migrated cells were detected by DAPI staining. B, CLESH-ubiquitin or Ub alone was incubated in microtiter plate wells that were blocked (blocked well) or pretreated with TSR(2,3), TSR2, or TSR3. After incubation and extensive washing, bound CD36 CLESH-Ub or Ub alone was detected by sequential incubations with anti-ubiquitin primary antibody, horseradish peroxidase–conjugated secondary antibody, and TMB-ELISA substrate. Data represent mean±SD of 3 independent experiments (**P<0.001). N.S. indicates no significant difference.

CLES
TSR2, but Not TSR3, Inhibits Angiogenesis in a CD36 CLESH-Dependent Manner

Solid-phase binding assays and MVEC-d migration assays suggested that TSR2 exhibits CD36 CLESH-dependent anti-angiogenic activity, whereas TSR3 does not. To evaluate this hypothesis, we performed ex vivo angiogenesis assays that examined branching in MVEC-b tube formation and assessed vessel sprouting of aortic rings. MVEC-b tube formation assays with TSR2 reduced tube formation to levels similar to that observed in low growth factor-containing media (Figure 3A; Figure III in the online-only Data Supplement).

Tube formation was fully restored by the addition of CD36 CLESH (Figure 3A). In comparison, TSR3 slightly inhibited MVEC-b tube formation; however, this inhibition was insensitive to CD36 CLESH (Figure 3A). TSR2 also significantly impaired vessel sprouting in an aortic ring assay (Figure 3B; Figure IV in the online-only Data Supplement). This inhibitory activity was reversed on addition of CD36 CLESH (Figure 3B). TSR3 had no significant effect on aortic ring vessel sprouting and exhibited no CD36 CLESH dependence (Figure 3B).

Figure 4. Thrombospondin type I repeat 3 (TSR3) lacks key determinants required for CD36 CLESH binding. A, Chemical shift perturbation (CSP, Δδ) histogram for TSR2 titrated with a 10× molar excess of CLESH-ubiquitin (green) or Ub (black). Dashed lines indicate the cutoff values for residues exhibiting CSPs >3σ or 6σ above the noise. B, Significant CSPs mapped to the structure of TSR2 (white) shown in cartoon representation. Residues are colored corresponding to CSPs >3σ (cyan) or 6σ (blue) above the noise. C, Sequence alignment of the second and third TSR domains of TSR(2,3). Residues I438 and K464, which confer a positive charge to TSR2 and corresponding residues in TSR3, are highlighted (black). D, APBS-calculated electrostatic surface representations of wild-type TSR2, I438Q/K464Q mutant TSR2, wild-type TSR3, and Q495I/Q521K mutant TSR3 colored according to the solvent accessible surface potential from +5 kT (blue) to –5 kT (red). E, MVECs-d were allowed to migrate toward low growth factor media (low GF), media containing a full complement of growth factors (normal GF), or full media containing recombinant wild-type TSR(2,3), I438Q/K464Q-TSR(2,3), Q495I/Q521K-TSR3, or BA1-TSR3 with or without CD36 CLESH. F, MVECs-b plated onto Matrigel were incubated for 6 hours against low growth factor media (low GF), media containing a full complement of growth factors (normal GF), or full growth factor media in the presence of Q495I/Q521K-TSR3 (TSR3 Q495I/Q521K) or Q495I/Q521K-TSR3 and CD36 CLESH (TSR3 Q495I/Q521K+CLESH). Tube formation was assessed by counting the number of branch points per 10× field as detected by phase contrast microscopy. G, Aortic rings were cultured in Matrigel with full growth factor media (normal GF), or full growth factor media with TSR2, Q495I/Q521K-TSR3 (TSR3 Q495I/Q521K) or Q495I/Q521K-TSR3 and CD36 CLESH (TSR3 Q495I/Q521K+CLESH). Total vessel formation per aortic ring after 6 days was assessed by phase contrast microscopy. Data represent mean±SD of 3 independent experiments (**P<0.001). N.S. indicates no significant difference.
Conversion of TSR2 to TSR3 Eliminates CD36 CLESH Effects

We performed solution NMR HSQC titrations to identify TSR2 residues that interact with CD36 CLESH or are located near the CLESH-binding site (Figure 4A). Such residues are predicted to exhibit chemical shift perturbations on addition of CLESH-Ub. As a control, we observed no significant chemical shift perturbations induced by ubiquitin alone (Figure 4A). Residues that undergo significant chemical shift perturbations (>3σ above noise) cluster along the surface ridge of the CWR-layered core and a nearby pocket (Figure 4B). The NMR data, in combination with migration assay results for wild-type and Arg-to-Met mutant TSR(2,3) constructs, confirm that the positively charged surface ridge of the TSR2 CWR-layered core is a key determinant for CD36 CLESH binding.

We compared putative CLESH-interacting TSR2 residues that were identified in our NMR HSQC experiments with the sequence of TSR3. We identified 2 nonconserved residues, I438 and K464 (Figure 4C). These 2 amino acids contribute to the overall positive character of the CLESH-binding surface of TSR2 (Figure 4D). The corresponding residues in TSR3, Q495, and Q521 result in a more neutral surface (Figure 4D). We therefore introduced dual I438Q/K464Q mutations in TSR(2,3) to neutralize the CLESH-binding surface of TSR2 (Figure 4D). We also introduced dual Q495I/Q521K mutations in TSR3 to increase the overall positive character of TSR3 and produce a surface with similar charge characteristics as TSR2 (Figure 4D).

Although wild-type TSR(2,3) effectively inhibited MVEC-d migration, TSR(2,3)-I438Q/K464Q was only partly effective (Figure 4E). Furthermore, although CD36 CLESH blocked the inhibition of MVEC-d migration by wild-type TSR(2,3), it had no effect on TSR(2,3)-I438Q/K464Q (Figure 4E). In contrast, TSR3-Q495I/Q521K effectively inhibited migration, and this inhibitory activity was sensitive to CD36 CLESH (Figure 4E). These results suggest that Q495I/Q521K mutations within TSR3 were sufficient to confer CD36 CLESH sensitivity to a TSR domain. We tested whether this converted TSR domain also exhibits antiangiogenic activity similar to wild-type TSR2 (Figure 3). MVEC-b tube formation assays (Figure 4F) and aortic ring assays (Figure 4G) confirmed the C36 CLESH-dependent antiangiogenic activity of TSR3-Q495I/Q521K. These results suggest that the I438/K464 and Q495/Q521 pockets contribute significantly to the surface charge characteristics of the TSR domains, and that these residues represent key molecular determinants for CD36 CLESH-dependent antiangiogenic activity.

Sequence alignments of TSR domains from proteins with antiangiogenic activity (Figure 1A; Figure V in the online-only Data Supplement) identify TSP-2 TSR2 and BAIII1 TSR2 as positively charged TSR domains similar to TSP-1 TSR2. We tested whether BAIII1 TSR3 exhibits antiangiogenic activity in a CD36 CLESH-dependent manner (Figure 4E). BAIII1 TSR3 effectively inhibited MVEC-d migration in a manner similar to TSP-1 TSR(2,3). Additionally, CD36 CLESH significantly reduced BAIII1 TSR3 inhibition of MVEC-d migration.

Acidic Residues in CD36 CLESH Are Critical for Interaction With TSR2

The CD36 CLESH domain has 4 acidic residues, E101, D106, E108, and D109 (Figure 5A), that potentially interact with the positively charged CLESH-binding surface of TSR2. We therefore tested individual and multiple alanine mutations of these amino acids in MVEC-d migration assays. Although wild-type CD36 CLESH blocked the inhibition of MVEC-d migration by TSR2 (Figure 5A; TSR2+CLESH), the E101A-, D106A-, E108A-, and D109A-CD36 CLESH mutants only partly rescued MVEC-d migration (Figure 5B). Furthermore, the triple mutant D106A/E108A/D109A- and quadruple mutant E101A/D106A/E108A/D109A-CD36 CLESH did not rescue MVEC migration at all (Figure 5B). In combination with NMR binding studies (Figure 4), these data show that the CD36 CLESH acidic side chains mediate the interaction between CD36 CLESH and the positively charged surface of TSR2.

Position 92 of the Extended CD36 CLESH Domain Regulates Interaction With TSR2

Our laboratory previously demonstrated that phosphorylation of the extended CD36 CLESH domain at T92 (Figure 5A) significantly blocked TSP-1 binding in an in vitro ELISA assay.27 Mutagenesis of CD36 suggested a steric component to the inhibition of CD36/TSP-1 interactions by T92 phosphorylation.27 To further explore this possibility, we performed MVEC-d migration assays in the presence of TSR2 and CD36 CLESH in which T92 is substituted by tryptophan, glutamate or arginine, amino acids with bulky side chains and a range of charge states. In comparison with wild-type CD36 CLESH, T92W-, T92E-, or T92R-CD36 CLESH only partly restored MVEC-d migration blocked by TSR2 (Figure 5B). The similar effect of neutral, negatively charged, and positively charged bulky side-chain substitutions at T92 suggest that phosphorylation of T92 may sterically rather than electrostatically interfere with CD36 CLESH/TSP-1 interactions.

Molecular Model of the TSR2/CD36 CLESH Complex

Our MVEC-d migration assays and in vitro ELISA and NMR binding studies provide residue-specific restraints that can be used to model the interaction between TSR2 and CD36 CLESH. We prepared a list of ambiguous restraints in which each TSR2 residue found by migration assays, ELISA or NMR, contributes to CD36 CLESH binding was restrained to a distance of ≤3.5 Å from E101, D106, E108, or D109 of CD36 CLESH. Complementary restraints required these CD36 CLESH residues to be positioned at ≤3.5 Å from TSR2 residues found to contribute to CD36 CLESH binding. We used these ambiguous restraints as an input for a rigid body/surface angle dynamics simulated annealing protocol to generate a model of the TSR2/CD36 CLESH complex. Consistent with the results of MVEC migration assays for the R440M, R442M, I438Q, and K464Q TSR(2,3) mutants, the model suggests that the interaction between CD36 and TSP-1 is mediated by extensive electrostatic contacts between negatively...
charged CD36 CLESH carboxylate side chains and residues that confer a positive charge to TSR2 (Figure 6).

**Discussion**

Although the interactions between TSP-1 TSR domains and the CD36 CLESH domain are central to the CD36-dependent antiangiogenic and antimigratory activities of TSP-1, the molecular basis for the TSP-1/CD36 CLESH interaction has been poorly defined to date. In this study, we combined structural and sequence data, mutagenesis, protein–protein interaction experiments, and in vitro functional assays to characterize key determinants that regulate TSR interactions with CD36 CLESH.

Our NMR experiments identified a cluster of TSR2 surface residues that were perturbed on addition of CD36 CLESH, suggesting a direct contact with the CD36 CLESH domain. Corneal pocket and endothelial cell migration assays previously implicated these residues in TSP-1 antiangiogenic activity. Using molecular modeling, we identified an allowed side-chain rotamer for methionine that places the methionine δ-sulfur atom in a position for S–π interactions and preserves the π-stacking arrangement with neighboring tryptophan indole rings. The arginine to methionine substitutions within the CWR-layered cores of TSR2 and TSR3 likely neutralize the charged WR-stacking motif surface ridges. We indeed observed that these mutations disrupt TSR2 interactions with CD36 CLESH.

In contrast to the highly conserved WR-stacking motif, an I-K positively charged patch on TSR2 formed by I438 and K464 serves as a differentiating factor between TSR2 and TSR3. The I-K patch is a key determinant that can be used to identify CD36 CLESH-interacting TSR domains on the basis of amino acid sequence (Figure V in the online-only Data Supplement). We hypothesize that among the 5 TSR-containing proteins with known antiangiogenic activity, only TSP-1 TSR2, TSP-2 TSR2, and BAI1 TSR3 contain the necessary determinants for interacting with CD36 CLESH. These 3 TSR domains contain residues with side chains that would confer a positive charge to the I-K patch, whereas other TSR domains within these proteins contain neutral patches that are likely unsuitable for CD36 CLESH binding. The TSR-containing fragment of BAI1 was previously identified as an inhibitor of intracranial glioma growth and progression through a CD36-dependent mechanism. Our I-K patch sequence-based categorizations predict that BAI1 TSR3 is responsible for BAI1 antiangiogenic activity, consistent with previous reports from our laboratory and others. Indeed, we found that BAI1 TSR3 suppressed endothelial cell migration, and this inhibition was partly reversed by CD36 CLESH (Figure 4E). In contrast, the TSR domains of ADAMTS-1 and ADAMTS-4 contain sequence differences that neutralize both the I-K patch and the WR-stacking motif, and most likely do not interact with CD36 CLESH.

Our modeling suggests that the CD36 CLESH sequence binds in an extended conformation along the positively charged TSR surface. Our NMR and mutagenesis data show that both electrostatic and steric determinants (eg, the phosphorylation state of T92) regulate TSR/CLESH binding. Interestingly, NMR titrations of CD36 CLESH with TSR2 suggest that CD36 CLESH remains unstructured and dynamic in the presence of TSR2 (data not shown). We speculate that CD36 CLESH may interact with TSR2 as an ensemble of conformations that satisfy overall electrostatic complementarity without enforcing rigid pairwise interactions between specific CD36 CLESH and TSR2 residues. The distributed electrostatic nature of the TSR2/CD36 CLESH interaction is consistent with the relatively small chemical shift perturbations observed in our NMR experiments. Such a fuzzy interaction hypothesis requires further investigation. However, it is noteworthy that the circulating protein histidine-rich glycoprotein also contains a CLESH-like domain that interacts with...
The identification of TSR2 as the source of CD36 CLESH-mediated antiangiogenic activity is relevant for the development of thrombospondin-based therapeutics. A recombinant TSP-1 construct containing the TSR1, TSR2, and TSR3 domains\(^{19,40}\) inhibited growth of human pancreatic cancer cells in an orthotopic mouse model.\(^{40}\) However, this construct contains multiple epitopes that interact with distinct signaling pathways, including activation of transforming growth factor-\(\beta\), via the Arginine, Phenylalanine, Lysine (RFK) motif located between TSR1 and TSR2.\(^{12–16}\) Peptidomimetics modeled on sequences within TSP-1 TSR2 had limited success as a result of rapid clearance.\(^{22}\) Although these peptide therapeutics were based on putative CD36-interacting epitopes, they most likely act through an entirely different pathway by activating transforming growth factor-\(\beta\).\(^{22}\) We hypothesize that these differences are attributable to the unusual structure of the TSR domains and the inability of small peptides to fully recapitulate the positively charged surface ridge required for binding to CD36 CLESH.

The results of the present study identify molecular determinants that regulate the CD36 CLESH-dependent antiangiogenic activity of TSP-1. The identification and characterization of the antiangiogenic properties of recombinant, bacterially expressed TSR domains like TSP1-TSR2 and BAI1 TSR3 may contribute further to the development of TSR-based therapies in malignant gliomas and other cancers.}

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**Disclosures**

None.

**References**


**Figure 6.** Molecular model of the thrombospondin type I repeat 2 (TSR2)/CD36 CLESH complex. **A**, Cartoon representation of the model TSR2/CD36 CLESH complex. **B**, Lowest energy model of the TSR2/CD36 CLESH complex with TSR2 shown as a surface representation. **C**, Ensemble of the 10 lowest energy models for the TSR2/CD36 CLESH complex with TSR2 shown as a surface representation. Modified CPK coloring is used throughout **A**, **B**, and **C** for nitrogen (blue), oxygen (red), sulfur (yellow), and carbon atoms of CD36 CLESH (pink) and TSR2 (white). Carbon atoms of TSR2 residues that participate in CLESH binding are colored according to identification by NMR (cyan), mutagenesis (green), or both (orange).

TSP-1 TSR domains. Although the histidine-rich glycoprotein CLESH contains a similar number of acidic residues as the CD36 CLESH, its acidic residues are located in a different arrangement than in CD36.\(^{39}\)

Interestingly, TSP1-TSR3 exhibited mild but reproducible antiangiogenic activity that is independent of CD36 CLESH. The mechanism by which TSR3 exerts this effect is not known. TSR3 may interact with a distinct region of CD36, with a distinct endothelial receptor or with an extracellular signaling factor. The characterization of the mode of action of TSR3 will require further study.

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**Table 1:** Summary of Studies Investigating Thrombospondin-1 and CD36 Interactions

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**Table 2:** Summary of References


**Significance**

By interacting with the cell surface receptor CD36, the matricellular protein thrombospondin-1 (TSP-1) exhibits potent antiangiogenic activity. Downregulation of TSP-1 within tumor microenvironments promotes unregulated cellular growth and metastasis. Previous studies identified an interaction between the TSP-1 type 1 repeat domains and CD36. Here, we identify the specific molecular determinants within the TSP-1 type 1 repeat domains and the CLESH domain of CD36 that mediate the TSP-1/CD36 interaction. Our results provide a framework for identifying which TSP-1 type 1 repeat domains within other proteins, such as TSP-2 and brain angiogenesis inhibitor-1, also regulate angiogenesis by interacting with CD36. These findings give molecular insights into the antiangiogenic effects of TSP-1 and present a basis for engineering antiangiogenic or antitumor therapeutics based on TSP-1 type 1 repeat domains.
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Molecular basis of anti-angiogenic thrombospondin-1 type 1 repeat domain interactions with CD36

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Supplemental Figure I – Molecular models for mutation of WR stacking motif Arginine residues. A, Structure of the WR-stacking motif with wild-type TSR(2,3)(PDB ID 3r6b). B, Structure in (A) with a R440M mutation demonstrates that a preferred methionine side-chain backbone-dependent rotamer places the δ-sulfur of methionine in position to participate in S-π interactions. C, Structure in (A) with a R440W mutation shows that no backbone-dependent preferred side-chain rotamers for tryptophan are allowed due to steric clashes. D, Structure in (A) with a R440H mutation shows that no backbone-dependent preferred side-chain rotamers for histidine are allowed due to steric clashes. E, Structure in (A) with a R440F mutation shows that no backbone-dependent preferred side-chain rotamers for phenylalanine are allowed due to steric clashes. F, Structure in (A) with a R440Y mutation shows that no backbone-dependent preferred side-chain rotamers for tyrosine are allowed due to steric clashes.
**Supplemental Figure II** – Solution NMR $^1$H/$^{15}$N-HSQC spectra for wild-type and Arg-to-Met mutant TSR2 constructs are similar and indicate that Arg-to-Met mutations do not significantly alter the structure of the TSR domain. **A**, HSQC of wild-type TSR2. Residues within the WR-stacking motif of wild-type TSR2 are labeled. **B**, HSQC of R440M-TSR2. **C**, HSQC of R442M-TSR2.
Supplemental Figure III – Tube formation Pics Representative phase contrast microscopy images of MVEC-b tube formation assays. Aortic rings were cultured in Matrigel with media containing a low level of growth factors (“EGM-2 Low Growth Factor”), a full complement of growth factors (“EGM-2”), or full media containing recombinant wild-type TSR2 (“EGM-2 +TSR2”), TSR2 and CD36 CLESH (“EGM-2 +TSR2 + CLESH”), TSR3 (“EGM-2 +TSR3”), TSR3 and CD36 CLESH (“EGM-2 +TSR3 + CLESH”), Q495I/Q521K-TSR3 (“EGM-2 +TSR3(Q495I/Q521K)”) or Q495I/Q521K-TSR3 and CD36 CLESH (“EGM-2 +TSR3(Q495I/Q521K)”).
Supplemental Figure IV—Representative phase contrast microscopy images of aortic ring assays. Aortic rings were cultured in Matrigel with media containing a full complement of growth factors (“EGM-2 media”), or full media containing recombinant wild-type TSR2 (“EGM-2 +TSR2”), TSR2 and CD36 CLESH (“EGM-2 +TSR2 + CLESH”), TSR3 (“EGM-2 +TSR3”), TSR3 and CD36 CLESH (“EGM-2 +TSR3 + CLESH”), Q495I/Q521K-TSR3 (“EGM-2 +TSR3(Q495I/Q521K)”) or Q495I/Q521K-TSR3 and CD36 CLESH (“EGM-2 +TSR3(Q495I/Q521K)”).
Supplemental Figure V – Determinants proposed to modulate CD36 CLESH binding are highlighted within sequence alignments of TSR domains for proteins with anti-angiogenic activity. The CD36 CLESH-binding TSR2 domain of TSP-1 (denoted as **) contains two arginine residues (gray highlights) and an “I-K” patch comprised of an isoleucine (I438) and lysine (K464) (cyan highlights). The TSR3 domain of TSP-1, which does not bind CD36 CLESH, harbors two glutamine residues (red highlights) in place of the “I-K” patch. Within the TSR domains of TSP-2, BAI1, ADAMTS-1 and ADAMTS-4, only TSP-2 TSR2 and BAI1 TSR-3 (domains denoted as **) contain both necessary arginine residues (gray highlights) and at least one residue that would confer a positive charge to the “I-K” patch (a lysine or arginine residue, cyan highlights) while containing no residues that would neutralize the “I-K” patch (glutamine or asparagine, red highlights).
Supplemental Methods:

Molecular basis of anti-angiogenic thrombospondin-1 type 1 repeat domain interactions with CD36

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Supplemental Materials and Methods

Materials
Monoclonal mouse anti-GST antibody MAB3117 was purchased from Chemicon International, monoclonal mouse anti-ubiquitin antibody P4D1 was purchased from Santa Cruz Biotechnology and HRP-linked sheep anti-mouse IgG was purchased from GE Healthcare. One Step Ultra TMB-ELISA chemiluminescent detection reagent was purchased from Thermo Scientific and Vectashield mounting medium for fluorescence with DAPI from Vector Laboratories. BD Matrigel Matrix Basement Membrane from BD Biosciences was used for all tube formation and aortic ring assays.

Cells
Human dermal microvascular endothelial cells (MVEC-d) and human brain microvascular endothelial cells (MVEC-b) were purchased from Lonza and grown in microvascular endothelial cell growth medium, EGM-2 (Lonza), supplemented with an EGM-2 bullet kit (Lonza) (5% fetal bovine serum, 0.4% human fibroblast growth factor-2, 0.1% VEGF, 0.1% human endothelial growth factor, 0.1% R3-IGF-1, 0.04% hydrocortisone, 0.1% ascorbic acid, and 0.1% GA-100). Cells were maintained at 37°C, 5% CO2 and used between passages 4 and 8.

Recombinant Proteins
The second and third TSR domains of thrombospondin-1 were cloned into the pGST-parallel-1 expression vector1 to produce the TSR(2,3) plasmid as previously described.2 A TSR2 expression construct was generated from pGST-parallel-1-TSR(2,3) by insertion of a stop codon between residues P472 and I473. The TSR3 expression construct was made by inserting an EcoRI site 5' of residue I473 and an Xhol site 3' of residue P529 by standard PCR methods; the restriction fragment was cloned into the pGST-parallel-1 vector. The BAI1 TSR3 construct was cloned using the same two restriction sites. The extended CLESH domain of CD36 (amino acids 81-117) was cloned into both the pGST-parallel-21 and pGST-parallel-1-ubiquitin (pGST-par1Ub) expression vectors to generate GST-CD36 CLESH and CD36 CLESH-Ub fusion proteins. Mutant TSR(2,3), TSR2, GST-CD36 CLESH or CD36 CLESH-Ub constructs were generated by QuikChange mutagenesis (Stratagene). TSP-1 TSR2, TSR3 and TSR(2,3) constructs and BAI1 TSR3 constructs were transformed into RosettaGami (EMD/Millipore) E. coli cells for protein expression, while GST-CD36 or CD36-Ub plasmids were expressed in Rosetta2(DE3) cells (EMD/Millipore). Proteins were expressed in LB media or 15NH4Cl supplemented minimal media3 and purified using GSTrap and HiLoad 16/60 Superdex 75 gel filtration columns (GE Healthcare) as previously described.2

Statistical Analyses
Data are represented as mean±SD. Multiple comparisons were analyzed by 1-way ANOVA using Prism (GraphPad Software, Inc.). Pairwise comparisons were analyzed post hoc using Tukey’s test. Statistically significant differences are denoted as ** for P<0.001 and * for P<0.01. Comparisons for which P>0.05 are denoted as N.S. for no significant difference.

Endothelial Cell Migration Assays
Microvascular endothelial cell (MVEC-d) migration studies followed a modified Boyden chamber procedure. Low passage MVEC-ds were washed with PBS, then removed from 10 cm dishes upon addition of 0.05% Trypsin/EDTA and incubation at 37°C, 5% CO2 for 5 min. The
cells were counted using a Coulter Counter and normalized to 300,000 cells per ml in EGM-2 with 10% of normal growth factors (low GF). Five hundred microliters of either EGM-2 (low GF) or EGM-2 (normal GF) were dispensed into a 24-well tissue culture treated plate. A 0.8 µm filter chamber was placed into each well and 300 µl of suspended cells were placed inside each chamber with or without TSR and/or CD36 CLESH-containing proteins. The plate was incubated at 37°C, 5% CO₂ for four hours, and washed with PBS, followed by removal of excess non-migrated cells with a cotton swab. The membrane with the migrated cells attached was removed from the chamber with a razor blade, placed onto a Superfrost Plus microscope slide with Vectashield mounting media containing DAPI, and sealed with clear nail polish. Migrated cells were visualized and counted using a Leica DMIRB inverted microscope with a 10X objective lens (Leica Microsystems). Each experiment was performed in triplicate with five fields of stained nuclei counted per membrane.

**Endothelial Cell Tube Formation Assays**
Microvascular endothelial cells (MVEC-b) at low passage were used for all tube formation experiments. Three hundred microliters of Matrigel added to the bottom of a 24-well tissue culture treated plate and allowed to polymerize for 30 min at 37°C. To each matrigel-covered well, 75,000 MVEC-b in 500 µl of EGM-2 were added with or without TSR and CD36 CLESH proteins and placed at 37°C, 5% CO₂ for 6 hours. Tube formation was assessed by quantification of the number of branch points per viewed field using phase contrast microscopy. Tube formation experiments were performed in triplicate.

**Aortic Ring Assays**
Aorta from wild type C57/Bl6 mice 12 wks of age were harvested, cut into 1 mm sections and incubated overnight at 37°C, 5% CO₂ in low GF EGM-2. Individual sections were placed onto 100 µl of polymerized Matrigel in a 48-well tissue culture-treated plate then covered with an extra 150 µl of liquid Matrigel and polymerized for 30 min at 37°C. One ml of EGM-2 with full growth factors was added to each well with or without TSR and CD36 CLESH containing proteins and incubated at 37°C, 5% CO₂ for 6 days. New media and tested proteins were added on days 1, 3 and 5. On day 6, the aortic sections were washed twice with PBS and visualized by phase contrast microscopy. Angiogenesis was measured by counting the individual vessel sprouts per aorta. Aortas from three separate mice were used and multiple 1 mm sections for each condition were tested.

**Solid Phase Binding Assays**
The wells of a 96-well polystyrene plate (Fisher Scientific) were coated with 1 µg purified recombinant TSR2 or TSR(2,3) in coating buffer (0.5 M NaHCO₃) overnight at 4°C. Coated wells were washed twice with PBS. Non-specific binding sites were blocked with 1.0% BSA in PBS+0.1% Tween 20 (PBST) at 22°C for 1 hour. After three washes with PBST, purified recombinant GST or GST-CD36 CLESH in PBS were added to a final concentration of 10 µg/µl and incubated for 3 hours at 22°C. Wells were washed twice with PBST and anti-GST primary antibody (1:2,000) was added to each well and incubated at 22°C for 1 hour. Unbound primary antibody was removed with five washes of PBST followed by addition of HRP-conjugated anti mouse IgG (1:2,000) to each well for 1 hour at 22°C. Unbound secondary antibody was removed with five washes of PBST. Bound antibody was detected by TMB-ELISA / H₂SO₄ reagent quantified at 450 nm using a SpectraMax 190 Microplate Reader (Molecular Devices).
Nuclear Magnetic Resonance Spectroscopy Data Collection and Analysis

Nuclear magnetic resonance (NMR) spectroscopy data for TSR2 constructs were acquired at 25°C on a Bruker 900 MHz Avance spectrometer equipped with a cryoprobe. NMR samples for titration analyses contained 200 µM uniformly $^1$H/$^{15}$N-labeled TSR2. NMR samples for resonance assignments contained 1 mM uniformly $^1$H/$^{15}$N/$^{13}$C-labeled TSR2. All NMR datasets were processed with in-house scripts using NMRPipe and visualized with Sparky. Backbone chemical shift assignments for TSR2 were determined using three-dimensional $^1$H/$^{15}$N/$^{13}$C-HNCA,-HNCOCA, -HNCO, -HNCACO and –HNCACB experiments. Chemical shift assignments were made for 98.3% of all backbone $^1$H and $^{15}$N atoms and 90.5% of all backbone $^{13}$Cα, $^{13}$Cβ and $^{13}$C' atoms.

Two-dimensional $^1$H/$^{15}$N-heteronuclear single quantum coherence (HSQC) spectra for titration analyses of 200 µM uniformly $^1$H/$^{15}$N-labeled TSR2 with and without a 10x molar excess of unlabeled CD36 CLESH-Ub were acquired utilizing a spin-state selective gradient-enhanced HSQC pulse sequence. $^1$H/$^{15}$N-HSQC resonances were peak-picked using Sparky and normalized chemical shift perturbations ($\Delta\delta$) were calculated from the differences in proton ($\Delta^1$H) and nitrogen ($\Delta^{15}$N) chemical shifts and plotted with matplotlib using the following equation:

$$\Delta\delta = \sqrt{\Delta^1H + \left(\frac{\Delta^{15}N}{5}\right)^2}$$

Electrostatic Surface Calculations

Electrostatic surface calculations for TSR2 and TSR3 constructs were carried out using the Adaptive Poisson-Boltzmann Solver (APBS, version 1.3). Calculations included explicit hydrogens for all protein residues and simulations included 150 mM monovalent ions at 37°C. Molecular-structure figures colored by APBS-calculated solvent accessible surface charge were generated in PyMOL.

Computational Modeling of the TSR2/CD36 CLESH Complex

A CD36 CLESH peptide was docked to TSR2 using ambiguous intermolecular distance restraints, derived from $^1$H/$^{15}$N chemical shift perturbation (CSP) maps and mutagenesis data, as input into a combined rigid body/torsion angle dynamics simulated annealing protocol in Xplor-NIH. The crystal structure of TSR(2,3) (PDB ID 3r6b) was used as the starting structure of TSR2 for docking calculations. An extended polypeptide chain was used as the initial model of CD36 CLESH for docking calculations. The combined rigid body/torsion angle dynamics simulated annealing protocol began with rigid body minimization guided by ambiguous distance restraints and van der Waals repulsion terms, followed by combined rigid body/torsion angle dynamics. Side-chains defined as interfacial residues by the $^1$H/$^{15}$N CSP maps and mutagenesis data were allowed to freely sample torsional space while favoring preferred rotamer conformations. The best TSR2/CD36 CLESH complex model was selected from a family of 100 models as the solution with the lowest total energy and no ambiguous distance restraint violations greater than 0.5Å. Molecular-structure figures of the best TSR2/CD36 CLESH model were generated using PyMOL.
References


5. Goddard T, Kneller D. SPARKY 3. 2006


