

# CD47 Is Necessary for Inhibition of Nitric Oxide-stimulated Vascular Cell Responses by Thrombospondin-1\*

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Jeff S. Isenberg<sup>‡</sup>, Lisa A. Ridnour<sup>§</sup>, Julie Dimitry<sup>¶</sup>, William A. Frazier<sup>¶</sup>, David A. Wink<sup>§</sup>, and David D. Roberts<sup>‡1</sup>

From the <sup>‡</sup>Laboratory of Pathology and <sup>§</sup>Radiation Biology Branch, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, Maryland 20892 and the <sup>¶</sup>Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

CD36 is necessary for inhibition of some angiogenic responses by the matricellular glycoprotein thrombospondin-1 and is therefore assumed to be the receptor that mediates its anti-angiogenic activities. Although ligation of CD36 by antibodies, recombinant type 1 repeats of thrombospondin-1, or CD36-binding peptides was sufficient to inhibit nitric oxide (NO)-stimulated responses in both endothelial and vascular smooth muscle cells, picomolar concentrations of native thrombospondin-1 similarly inhibited NO signaling in vascular cells from wild-type and CD36-null mice. Ligation of the thrombospondin-1 receptor CD47 by recombinant C-terminal regions of thrombospondin-1, thrombospondin-1 peptides, or CD47 antibodies was also sufficient to inhibit NO-stimulated phenotypic responses and cGMP signaling in vascular cells. Thrombospondin-1 did not inhibit NO signaling in CD47-null vascular cells or NO-stimulated vascular outgrowth from CD47-null muscle explants in three-dimensional cultures. Furthermore, the CD36-binding domain of thrombospondin-1 and anti-angiogenic peptides derived from this domain failed to inhibit NO signaling in CD47-null cells. Therefore, ligation of either CD36 or CD47 is sufficient to inhibit NO-stimulated vascular cell responses and cGMP signaling, but only CD47 is necessary for this activity of thrombospondin-1 at physiological concentrations.

Thrombospondin-1 (TSP1)<sup>2</sup> is a secreted glycoprotein that plays a relatively minor role in development of the murine vascular system (1, 2), but its regulated appearance in the extracellular matrix plays important roles in responses to acute injury and in several chronic disease states in the adult. TSP1 is a

major component of platelet  $\alpha$ -granules and is released from platelets upon activation, where it modulates platelet adhesion and the properties of fibrin clots formed following acute vascular injury (3, 4). TSP1 released from platelets or produced locally in response to cytokines and growth factors also plays an important role in recruitment of mononuclear cells during the early phases of wound repair, and the absence of TSP1 delays excisional wound repair in mice (5).

TSP1 also accumulates in the neointima of atherosclerotic lesions (6), where it may stimulate vascular smooth muscle cell (VSMC) proliferation and migration by enhancing responsiveness to platelet-derived growth factor (7). Antibody blocking of TSP1 can reverse this response and enhance the re-endothelialization of an injured artery (8). An N700S coding sequence polymorphism in TSP1 that alters its conformation is associated with increased risk of premature familial myocardial infarction (reviewed in Ref. 9).

TSP1 is also a potent modulator of angiogenesis (10). The N-terminal domain of TSP1 stimulates angiogenesis through its interactions with  $\alpha_3\beta_1$  and  $\alpha_4\beta_1$  integrins (11, 12), but the central type 1 repeats contain sequences that potently inhibit angiogenesis via CD36 and/or heparan sulfate proteoglycan receptors (13, 14). Under most circumstances, the net activity of intact TSP1 is anti-angiogenic. Thus, the absence of TSP1 enhances experimental and tumor-induced angiogenic responses (15–17).

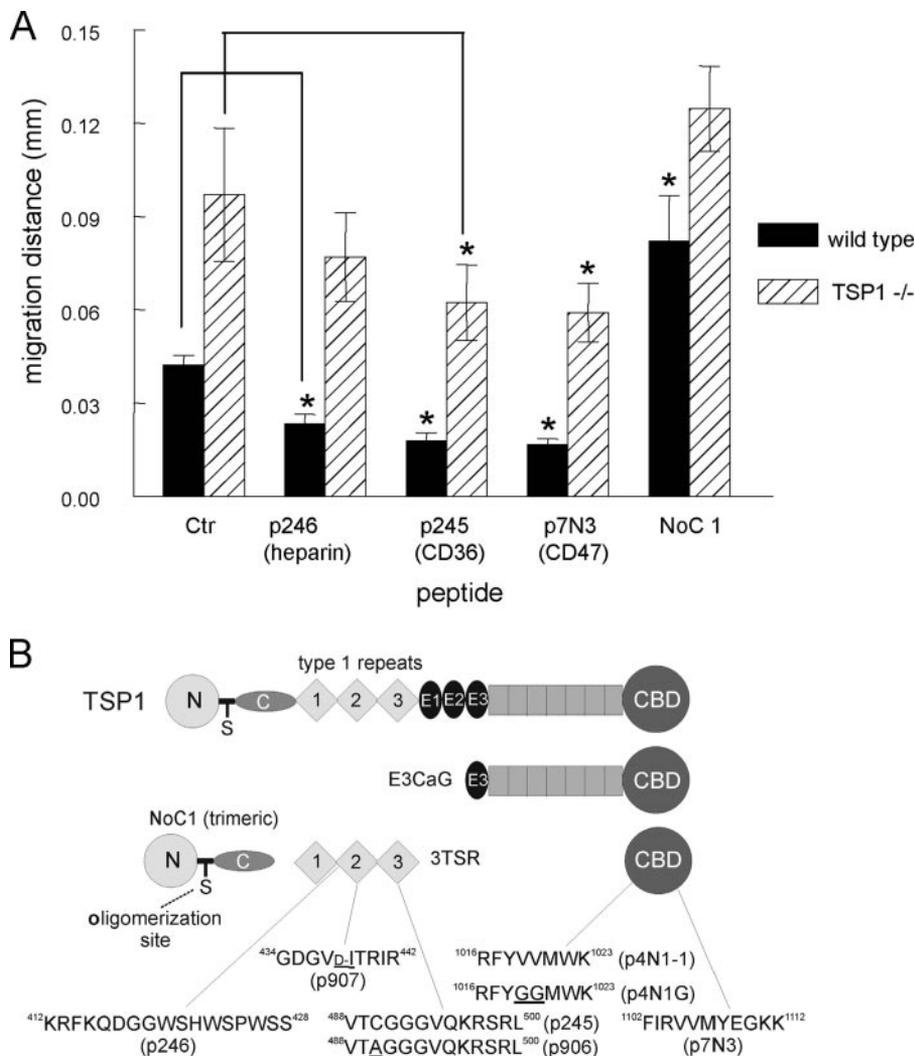
Vascular effects of TSP1 are mediated by its binding to receptors on both endothelial and VSMCs. Endothelial cells express at least eight TSP1 receptors, and several of these are shared on VSMCs. Based on the activity of CD36 antibody FA6-152 to block the inhibitory effect of TSP1 on FGF2-induced microvascular endothelial cell motility and the failure of TSP1 to inhibit corneal angiogenesis induced by FGF2 in CD36<sup>-/-</sup> mice, CD36 is considered to be the primary TSP1 receptor that mediates this anti-angiogenic activity (13, 18). Some evidence indicates that TSP1 also inhibits angiogenesis through  $\beta_1$  integrins, CD47, LRP/calreticulin, and heparan sulfate proteoglycans (14, 19–21), but the necessity of these receptors for TSP1 to inhibit angiogenesis has not been confirmed in the respective receptor-null mice. To develop effective angiogenesis inhibitors based on TSP1, it is important to establish whether additional signaling pathways may allow TSP1 to inhibit angiogenesis in a CD36-independent manner.

We recently found that the anti-angiogenic activity of TSP1 is dramatically potentiated in the presence of low concentrations of nitric oxide (NO) donors (22). This activity is mediated

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<sup>1</sup> To whom correspondence should be addressed: Laboratory of Pathology, Center for Cancer Research, NCI, NIH, Bldg. 10, Rm. 2A33, 10 Center Dr., MSC1500, Bethesda, MD 20892-1500. Tel.: 301-496-6264; Fax: 301-402-0043; E-mail: droberts@helix.nih.gov.

<sup>2</sup> The abbreviations used are: TSP1, thrombospondin-1; CBD, thrombospondin-1 C-terminal binding domain; DEA/NO, diethylamine NONOate; DETA/NO, diethyltriamine NONOate; HASMC, human aortic vascular smooth muscle cell; HUVEC, human umbilical vein endothelial cell; MASMC, murine aortic smooth muscle cell; NoC1, trimeric thrombospondin-1 residues 1–356; 3TSR, thrombospondin type 1 repeats; VSMC, vascular smooth muscle cell; FGF2, fibroblast growth factor 2; FCS, fetal calf serum; BSA, bovine serum albumin.



**FIGURE 1. NO-stimulated vascular cell outgrowth is modulated by ligation of several TSP1 receptors.** A, wild-type and TSP1<sup>-/-</sup> muscle biopsies were explanted in three-dimensional collagen matrices and incubated in growth medium in the presence of exogenous NO (10 μM) and cell migration measured at day 7. Explants were treated with TSP1-derived peptides specific for heparan sulfate proteoglycans (peptide 246, 10 μM), CD36 (peptide 245, 10 μM), CD47 (peptide 7N3, 10 μM), or NoC1 (0.1 μg/ml), which binds to several β<sub>1</sub> integrins. Results are representative of three experiments with those differing from the respective controls with *p* < 0.05 indicated by the asterisk. B, origins of the recombinant proteins and peptides. Amino acid residues differing from the native TSP1 sequence are underlined.

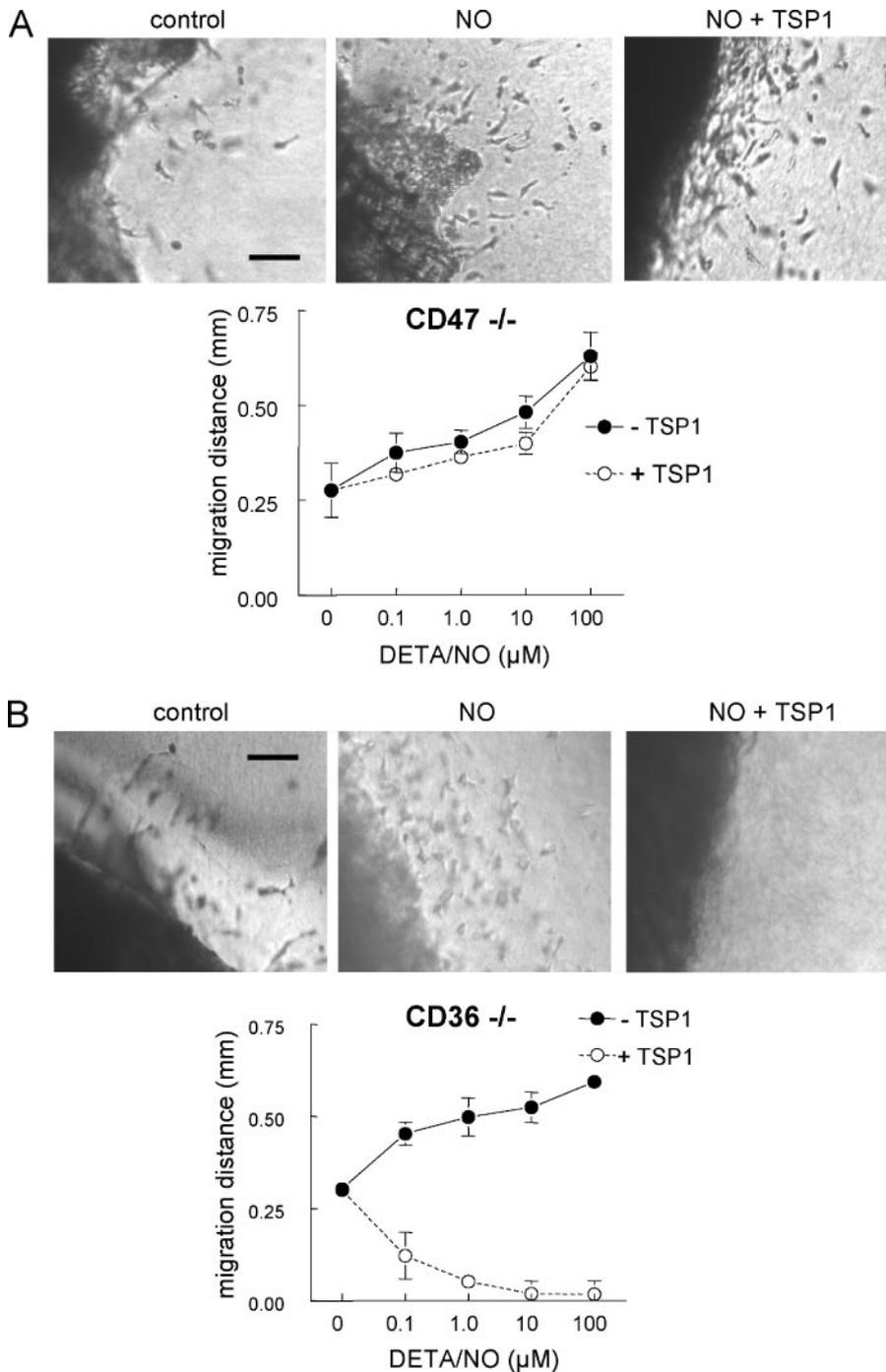
at least in part through inhibition by TSP1 of cGMP signaling via NO-mediated activation of soluble guanylyl cyclase in both endothelial cells (22) and VSMCs (23). Moreover, TSP1-null vascular cells exhibit elevated basal cGMP levels and enhanced cGMP and phenotypic responses to exogenous NO. The inhibitory activity of TSP1 on NO signaling was replicated by an agonist CD36 antibody and by a recombinant CD36-binding region of TSP1, suggesting that this activity is also mediated by CD36. However, our attempts to confirm this hypothesis led us to revise this model. We present here evidence that a second TSP1 receptor, CD47, plays the primary role in mediating the inhibitory activities of TSP1 for vascular cells. Remarkably, CD47 is also necessary for inhibition of NO signaling by CD36 ligands.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—Human umbilical vein endothelial cells (HUVECs, Cambrex, Walkersville, MD) were maintained in

endothelial cell growth medium (Cambrex) with 5% FCS in 5% CO<sub>2</sub> at 37 °C. Cells were utilized at passages 4–8. Purity of cultures was monitored by immunochemical staining with monoclonal human anti-CD31 antibody and monoclonal anti-α smooth muscle actin from Sigma. Human aortic smooth muscle cells (HASMCs, Cambrex) were maintained in smooth muscle cell growth medium with the manufacturer’s additives (SM-GM, Clontec) and 5% FCS in 5% CO<sub>2</sub> at 37 °C. Cells utilized were within passages 4–9. Purity of primary cultures was monitored by immunochemical staining with monoclonal human anti-CD31 antibody and α-smooth muscle actin (Sigma). DEA/NO and DETA/NO were kindly provided by Dr. Larry Keefer (NCI, National Institutes of Health (NIH), Frederick, MD). TSP1 was prepared from human platelets obtained from the NIH blood bank as previously described (24). Recombinant proteins expressed in insect cells containing the N-terminal domains (NoC1), type 1 repeats (3TSR), or C-terminal regions of TSP1 (E3CaG1) were generously provided by Dr. Deane Mosher (University of Wisconsin) and Dr. Jack Lawler, Harvard Medical School (25, 26). The recombinant C-terminal cell-binding domain (CBD) was prepared as previously described (27). Murine anti-human CD36 antibody (clone SMΦ) was purchased from Chemicon International (Temecula, CA). CD36 antibody clone FA6-152 was purchased from Immunotech (Beckman Coulter). CD36 antibody clone 185-1G2 was purchased from Neomarkers (Fremont, CA). Anti-cyclin D1 (IgG<sub>1</sub>) and estrogen receptor-α (IgG<sub>2a</sub>) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HSP-60 monoclonal antibody (IgM) was purchased from Affinity Bioreagents Inc. (Golden, CO). Anti-CD47 antibody (clone CIKm1) was from ICN (Costa Mesa, CA). Type I collagen (Vitrogen) was from Cohesion Technologies (Palo Alto, CA). Peptides 246, 245, 7N3, 4N1-1, and 761 were prepared as described (28). Peptides 906 and 907 were prepared by Peptides International (Louisville, KY). B6H12 (anti-CD47) was purified by protein G affinity chromatography (Pierce) from conditioned media of the respective hybridoma (American Type Culture Collection).

**Murine Cell Cultures**—Murine ASMCs were obtained from aortic segments harvested sterily from C57B1/6 CD47-null or



**FIGURE 2. CD47 but not CD36 is necessary for TSP1 inhibition of *ex vivo* angiogenesis.** *A*, CD47<sup>-/-</sup> muscle biopsies were explanted in three-dimensional collagen matrix and incubated in growth medium. Explants were treated with a slow release exogenous NO-donor (DETA/NO, 0.1–100  $\mu\text{M}$ ) with or without TSP1 (1  $\mu\text{g/ml}$ ). Cell migration was determined at 7 days as described. Results are representative of three experiments. Representative photomicrographs are shown of CD47<sup>-/-</sup> explants incubated in basal medium, with or without DETA/NO (10  $\mu\text{M}$ ) with or without TSP1 (1  $\mu\text{g/ml}$ ) (scale bars: 50  $\mu\text{m}$ ). *B*, CD36<sup>-/-</sup> muscle biopsies were explanted in three-dimensional collagen matrix and incubated in growth medium with or without TSP1 (1  $\mu\text{g/ml}$ ) and exogenous NO-donor (DETA/NO, 0.1–100  $\mu\text{M}$ ). Cell migration was determined at 7 days. Results are representative of three experiments. Representative photo micrographs are shown of CD36<sup>-/-</sup> explants incubated in basal medium, with or without DETA/NO (10  $\mu\text{M}$ ) with or without TSP1 (1  $\mu\text{g/ml}$ ) (scale bars: 50  $\mu\text{m}$ ).

CD36-null mice as described (29) and cultured in SM-GM (Cambrex) plus 20% FCS. In the case of CD36-null cells, culture flasks were pre-coated with 1% gelatin prior to cell plating. Wild-type C57B1/6 ASMC cultures were prepared as previously described (7). Cell culture purity was determined by

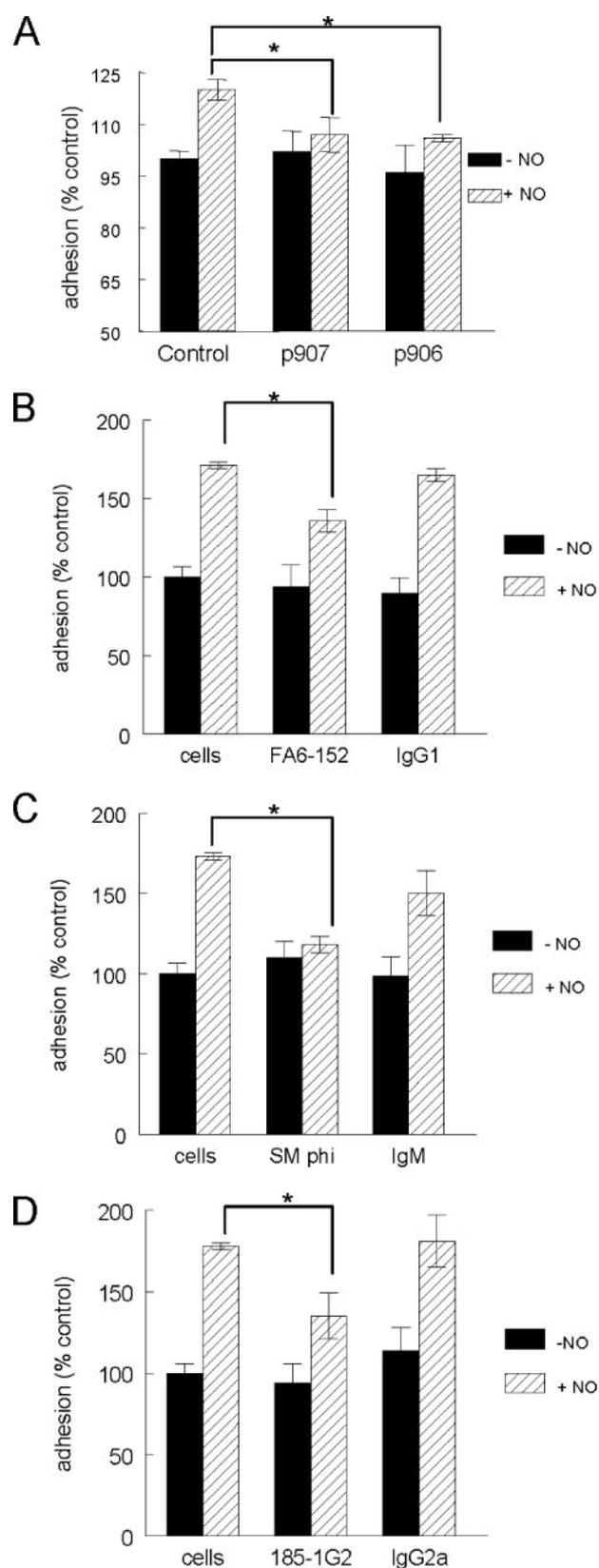
immunohistochemistry staining for  $\alpha$ -smooth muscle actin. Cells were used within passages 1–4 to minimize overgrowth of other cell types.

**Animals**—C57B1/6 WT and TSP1-null (1) CD47-null (30) and CD36-null mice (31) were all extensively backcrossed on the C57B1/6 background and were housed in a pathogen-free environment. Handling and care of animals was in compliance with the guidelines established by the Animal Care and Use Committees of the National Cancer Institute and of Washington University School of Medicine.

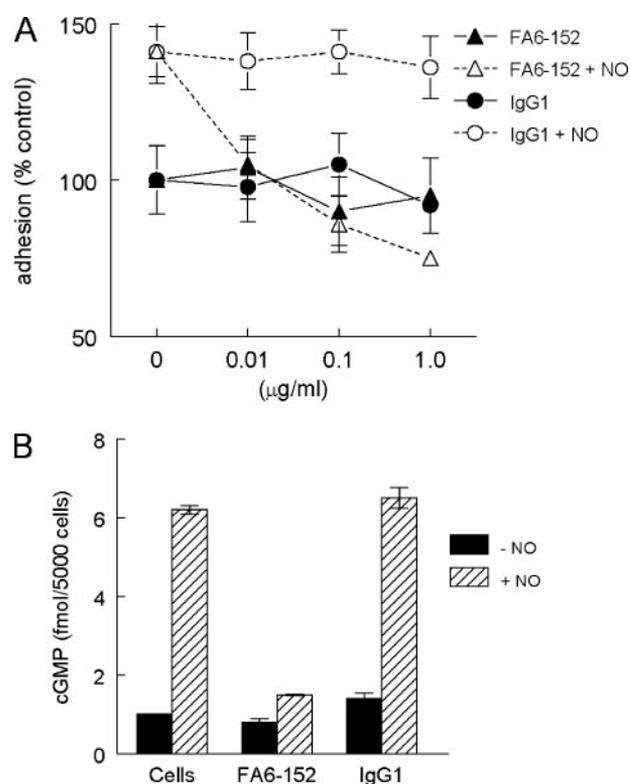
**Explant Invasion Assay**—Muscle biopsies from the pectoralis major muscle of 8- to 12-week-old wild-type or transgenic mice were harvested and explanted into 100  $\mu\text{l}$  of type I collagen gel in 96-well tissue culture plates as described (7). Following gelation, the embedded explants were overlaid with 75  $\mu\text{l}$  of EGM plus 2% FCS in the presence or absence of a dose range of DETA/NO (0.01–1000  $\mu\text{M}$ ) and other indicated treatments and then incubated at 37  $^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 7 days, at which time maximum vascular cell migration through the matrix was measured.

**Cell Proliferation**—Proliferation of vascular cells was measured with a non-radioactive colorimetric assay (CellTiter 96, Promega, Madison, WI). Briefly, to each well of a 96-well culture plate (Nunc, Denmark)  $5 \times 10^3$  cells were suspended in 100  $\mu\text{l}$  of culture medium with indicated treatments and incubated for 72 h at 37  $^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. Following incubation 20  $\mu\text{l}$  of tetrazolium compound/solubilization agent was added, and incubation continued for 4 h under the same conditions. The plate was then read on a MR580 Microelisa Auto Reader (Dynatech) at a wavelength of 490 nm. Appropriate zero time controls were run for all assays, and the optical density readings obtained were then subtracted from those obtained at 72 h.

**Cell Adhesion Assay**—Cell adhesion was carried out in 96-well culture plates. After pre-coating wells with type I collagen (3  $\mu\text{g/ml}$  in Dulbecco's phosphate-buffered saline) harvested cells were plated at a density of  $1 \times 10^4$  cells/well in



**FIGURE 3. Agonist or antagonist antibody ligation of CD36 inhibits NO-driven endothelial cell adhesion.** A, HUVECs ( $1 \times 10^4$  cell/well) were plated in 96-well culture dishes pre-coated with type I collagen ( $5 \mu\text{g/ml}$ ), preincubated with the CD36-binding peptides p906 or p907 ( $10 \mu\text{M}$ ) or CD36 antibodies FA6-152 ( $0.1 \mu\text{g/ml}$ ) (B), SM $\Phi$  ( $0.1 \mu\text{g/ml}$ ) (C), and 185-1G2 ( $0.1 \mu\text{g/ml}$ ) (D) and exposed to DEA/NO ( $10 \mu\text{M}$ ). Cells were also concurrently and under similar conditions treated with isotype-matched control antibodies (IgG<sub>1</sub>,

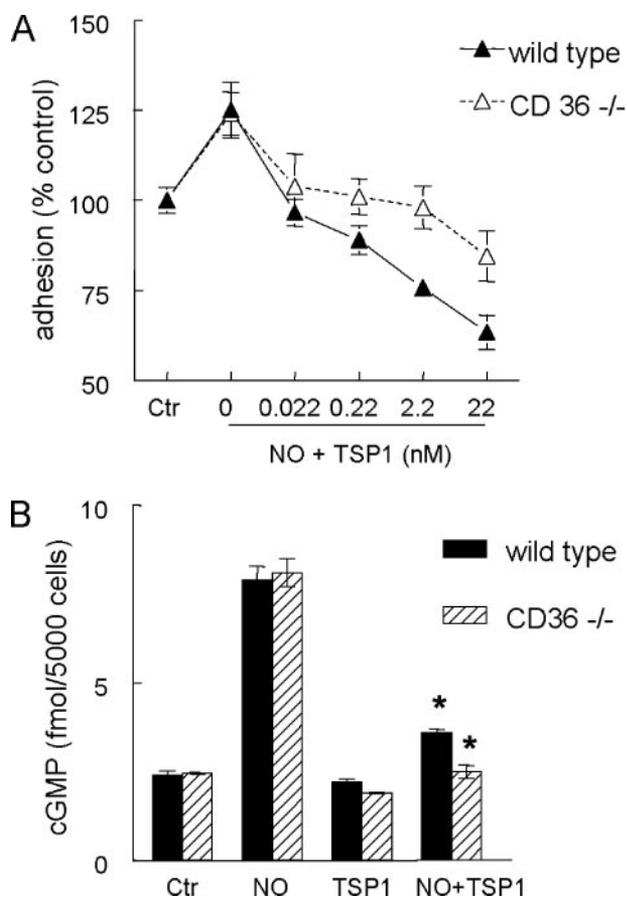


**FIGURE 4. Antibody ligation of CD36 inhibits HASMC NO signaling.** A, HASMCs ( $1 \times 10^4$  cell/well) were plated in 96-well culture dishes pre-coated with type I collagen ( $5 \mu\text{g/ml}$ ), preincubated with the CD36 monoclonal antibody FA6-152 ( $0.01$ – $1 \mu\text{g/ml}$ ), or IgG<sub>1</sub> control and exposed to DEA/NO ( $10 \mu\text{M}$ ). Following incubation for 1 h at  $37^\circ\text{C}$  plates were washed, and cells were fixed, stained, developed, and read at  $570 \text{ nm}$ . B, HASMC cells were plated ( $5 \times 10^3$  cells/well) on 96-well plates and weaned over 24 h from serum, then treated in serum-free medium with  $0.1\%$  BSA with DEA/NO ( $10 \mu\text{M}$ ) with or without FA6-152 ( $1 \mu\text{g/ml}$ ) or an isotype control antibody ( $1 \mu\text{g/ml}$ ) for 5 min. Cells were lysed, and cGMP levels were determined by enzyme-linked immunosorbent assay.

medium plus  $0.1\%$  BSA and treatment agents and incubated in  $5\% \text{ CO}_2$  for 1 h. Wells were washed with phosphate-buffered saline, and cells were fixed with  $1\%$  glutaraldehyde for 10 min, washed, and stained with  $1\%$  crystal violet for 20 min. Excess stain was rinsed away, adherent cells were treated with  $10\%$  acetic acid, and plates were read at  $570 \text{ nm}$ .

**Intracellular cGMP Measurement**—HUVECs ( $10^4$  cells/well) were grown overnight in 96-well culture plates containing full growth medium with  $2\%$  FCS and weaned in growth medium without additives and  $1\%$  FCS over 24 h before treatment with NO donors and other agents in SM-GM without additives plus  $0.1\%$  BSA. Intracellular cGMP levels were determined according to the manufacturer's instructions using an enzyme immunoassay kit (Amersham Biosciences). In other experiments ASMCs from wild-type and CD36 or CD47-null mice were plated onto 96-well culture plates and incubated overnight in full growth medium. They were then weaned off serum as described to SM-GM plus  $0.1\%$  BSA and treated with NO donor

IgM, and IgG<sub>2a</sub>, respectively) ( $0.1 \mu\text{g/ml}$ ). Following incubation for 1 h at  $37^\circ\text{C}$ , the plates were washed, and the cells were fixed, stained, developed, and read at  $570 \text{ nm}$ . Results are expressed as a percentage of the untreated control and represent the mean  $\pm$  S.D. of at least three separate experiments.



**FIGURE 5. CD36 is not necessary for TSP1 inhibition of NO-stimulated vascular cell adhesion and cGMP accumulation.** *A*, murine ASMCs from wild-type and CD36-null animals ( $1 \times 10^4$  cell/well) were plated in 96-well culture dishes pre-coated with type I collagen ( $5 \mu\text{g/ml}$ ) and incubated in SM-GM plus 0.1% BSA with DEA/NO ( $10 \mu\text{M}$ ) with or without exogenous TSP1 (0.022–22 nM). Following incubation for 1 h at  $37^\circ\text{C}$  plates were washed, and cells were fixed, stained, developed, and read at 570 nm. Results are expressed as percent control and represent the mean  $\pm$  S.D. of at least three separate experiments. *B*, wild-type and CD36-null ASMCs were plated ( $5 \times 10^3$  cells/well) on 96-well plates and weaned over 24 h from serum, then treated in serum-free medium with 0.1% BSA with DEA/NO ( $10 \mu\text{M}$ ) with or without TSP1 ( $1 \mu\text{g/ml}$ ) for 5 min. Cells were lysed, and cGMP levels were determined by enzyme-linked immunosorbent assay. Results presented are representative of those obtained in three independent experiments. \*,  $p < 0.05$  versus respective NO controls.

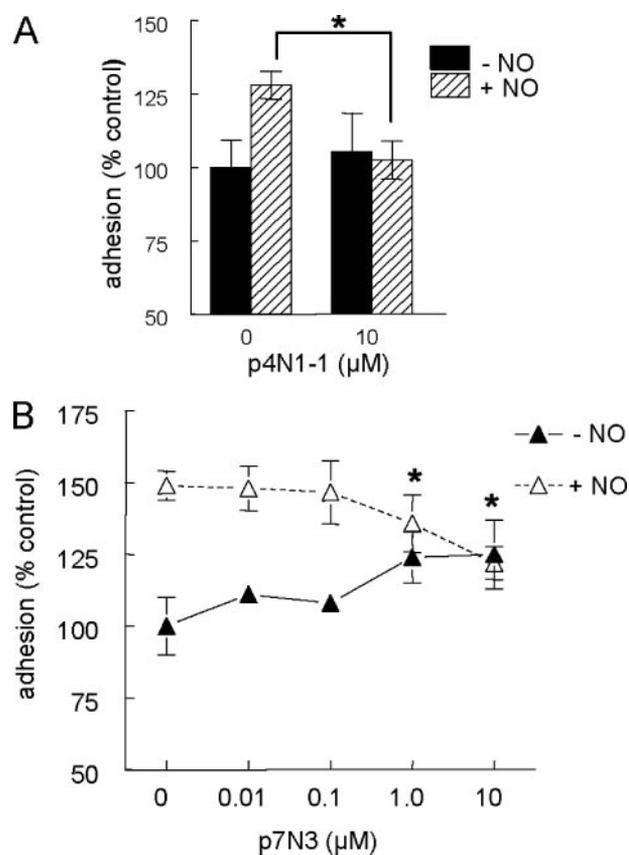
and other agents as indicated. Intracellular cyclic nucleotides were determined via immunoassay.

**Statistics**—All studies were repeated in triplicate, and results are presented as the mean  $\pm$  S.D., with analysis of significance done using the Student's *t* test and a  $p < 0.05$  taken as significant.

## RESULTS

**Peptide Ligands of Three TSP1 Receptors Inhibit Explant Angiogenic Responses**—As previously reported (22), sustained exposure to exogenous NO released by the donor DETA/NO stimulated vascular outgrowth from muscle explants in three-dimensional collagen cultures to a greater extent in those from TSP1-null when compared with WT mice (Fig. 1*A*, controls). Consistent with their reported affects on endothelial cells *in vitro* and angiogenesis *in vivo* (14, 20), a CD36-binding peptide from the third type 1 repeat of TSP1 (p245, VTCCGGVQKRSRL), a CD47-

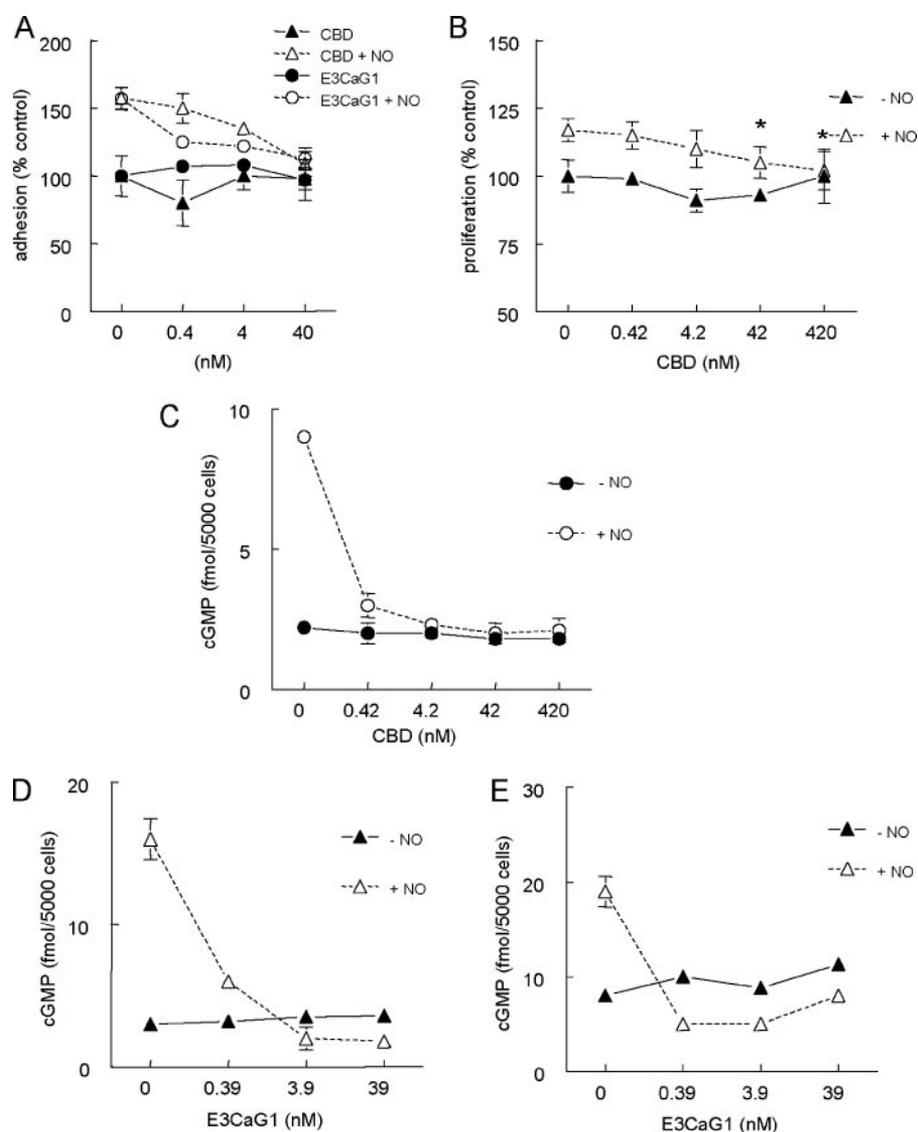
## TSP1 Inhibits NO Signaling via CD47



**FIGURE 6. Ligand of CD47 is sufficient to block NO-driven endothelial cell adhesion.** *A*, HUVECs ( $1 \times 10^4$  cell/well) were plated in 96-well culture dishes pre-coated with type I collagen ( $5 \mu\text{g/ml}$ ), and incubated in EGM plus 0.1% BSA with or without DEA/NO ( $10 \mu\text{M}$ ) and CD47 peptides p4N1-1 and p7N3 at the indicated concentrations (*B*). Following incubation for 1 h at  $37^\circ\text{C}$  plates were washed, and cells were fixed, stained, developed, and read at 570 nm. Results are expressed as percent control and represent the mean  $\pm$  S.D. of at least three separate experiments.

binding peptide from the C-terminal module of TSP1 (p7N3, FIRVVMYEGKK), and to a lesser extent a heparin- and transforming growth factor- $\beta$ -binding peptide from the second type 1 repeat (p246, KRFKQDGGWSHWSPWSS) inhibited vascular cell outgrowth from both wild-type and TSP1-null explants stimulated by NO (Fig. 1). Conversely, the described pro-angiogenic activities of the N-terminal region of TSP1 (11, 12) were reflected by enhanced vascular outgrowth from explants in the presence of recombinant NoC1. Assuming that these peptides act as agonists of their respective receptors, this indicates that ligating CD36, heparan sulfate proteoglycans, or CD47 is sufficient to inhibit NO-stimulated vascular outgrowth, whereas ligating  $\beta_1$  integrins or other TSP1 N-module receptors enhances vascular outgrowth under the same conditions.

**CD47 but Not CD36 Is Necessary for Inhibition by TSP1 of Explant Angiogenic Responses**—Although the former data show that ligating CD36 or CD47 is sufficient to inhibit explant angiogenesis stimulated by NO, they do not prove that the respective receptors are necessary for activities of the peptide ligands or of intact TSP1. Furthermore, although these peptides clearly bind to the indicated receptors, structural studies have raised concerns that VVM peptides may not represent the true CD47 binding site in the C-terminal domain of TSP1 (32). To directly address the roles of CD36 and CD47 in the inhibitory



**FIGURE 7. The C-terminal domain of TSP1 is sufficient to inhibit NO-stimulated responses in vascular smooth muscle cells.** *A*, HASMCs ( $1 \times 10^4$  cell/well) were plated in 96-well culture dishes pre-coated with type I collagen ( $5 \mu\text{g/ml}$ ) and incubated in EGM plus 0.1% BSA with or without DEA/NO ( $10 \mu\text{M}$ ) with or without the recombinant CBD or E3CaG1 (0.4–40 nM). Following incubation for 1 h at  $37^\circ\text{C}$  plates were washed and cells were fixed, stained, developed, and read at 570 nm. *B*, HASMCs ( $5 \times 10^3$ /well) were plated in 96-well culture plates, treated with CBD (0.42–420 nM) with or without DETA/NO ( $10 \mu\text{M}$ ) and incubated for 72 h at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere, then developed with MTS reagent and read at a wavelength of 490 nm. Results are expressed as percent control and represent the mean  $\pm$  S.D. of at least three separate experiments. *C*, HASMCs were plated ( $5 \times 10^3$  cells/well) in 96-well plates and weaned over 24 h from serum, then treated in serum-free medium with 0.1% BSA with DEA/NO ( $10 \mu\text{M}$ ) with or without CBD (0.42–420 nM) for 5 min. Cells were lysed, and cGMP levels were determined by enzyme-linked immunosorbent assay. Results presented are representative of those obtained in three independent experiments. In other experiments HASMCs (*D*) or HUVECs (*E*) were plated ( $5 \times 10^3$  cells/well) in 96-well plates, weaned of serum, and treated with E3CaG1 (0.39–39 nM) with or without DEA/NO ( $10 \mu\text{M}$ ), and cGMP levels were determined as described.

activity of TSP1, muscle explants from mice lacking the respective receptors were placed into three-dimensional collagen cultures (Fig. 2). Similar to wild-type explants, NO dose-dependently stimulated vascular outgrowth in CD36- or CD47-null explants. Remarkably, the ability of exogenous TSP1 to antagonize this response was preserved in CD36-null explants (Fig. 2*B*) but lost in CD47-null explants (Fig. 2*A*). Thus, CD47 is necessary for the anti-angiogenic activity of TSP1 in this assay, but CD36 is not. This result was unexpected given that a recombinant CD36-binding domain of TSP1 (3TSR) and a CD36 antibody (SM $\Phi$ ) described to be an agonist based on its ability to

mimic TSP1 (13) were shown previously to inhibit endothelial cell adhesion on type I collagen stimulated by acute NO exposure (22). This prompted us to re-examine the role of CD36 in this activity of TSP1.

*CD36 Ligation Is Sufficient but Not Necessary for Inhibition of NO Responses by TSP1*—A CD36-binding peptide derived from the third type 1 repeat (p906, VTAGGGVQKRSRL) and a derivative of the second type 1 repeat with enhanced CD36-binding and anti-angiogenic activity (p907, GDGV(D/I)TRIR (33)) similarly inhibited NO-stimulated endothelial cell adhesion (Fig. 3*A*). As previously reported, the CD36 agonist antibody SM $\Phi$  inhibited NO-stimulated cell adhesion, whereas a control IgM was inactive (Fig. 3*C*). However, two CD36 antibodies that were reported to antagonize inhibition by TSP1, FA6-152 (13) and 185-1G2 (19), also inhibited NO-stimulated adhesion (Fig. 3, *B* and *D*). Isotype-matched control antibodies did not inhibit NO-stimulated cell adhesion, demonstrating the specificity of these CD36 antibodies for blocking an NO response. Thus, various CD36 ligands are sufficient to inhibit NO-stimulated endothelial cell responses, but their mechanism of action may differ from the previously described TSP1 responses that were antagonized by the CD36 antibody FA6-152 (13).

Antagonism of NO signaling by TSP1 is conserved in VSMCs (23). Consistent with the data for endothelial cells in Fig. 3, the CD36 antagonist antibodies FA6-152 and 185-1G2 but not isotype-matched control antibodies were dose-dependent inhibitors of NO-stimulated HASMC adhesion (Fig. 4*A* and results not shown). The FA6-152 antibody also prevented NO-induced accumulation of cGMP in HASMCs (Fig. 4*B*), consistent with its effects on NO-stimulated adhesion but not with its reported activity as a TSP1 antagonist (13, 18). Therefore, inhibition of NO signaling by CD36 ligation is conserved in both types of vascular cells but is independent of the ability of CD36 ligands to mimic or inhibit TSP1 activity in other angiogenesis assays.

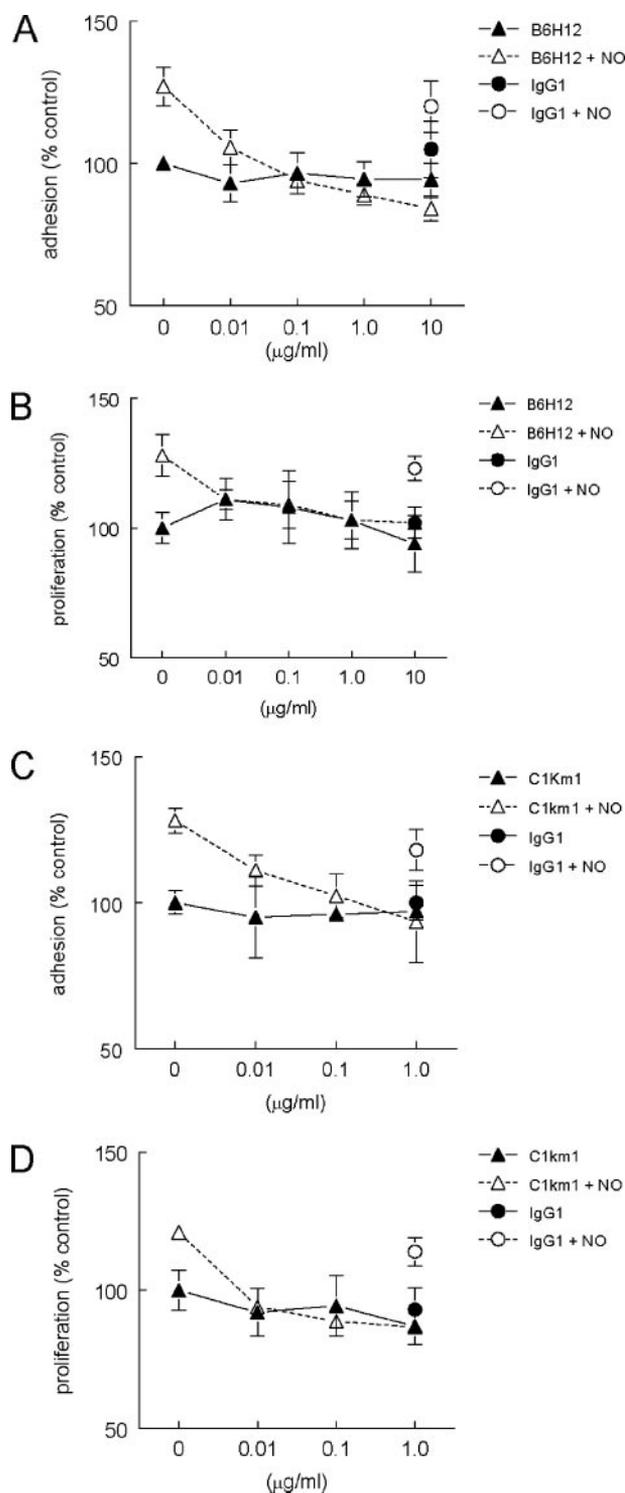
To clarify the role of CD36 in inhibition by TSP1 of NO-stimulated responses, we used MAMCs derived from WT and CD36-null mice (Fig. 5). Low dose NO significantly stimulated

adhesion of WT MAMCs on type I collagen, and TSP1 at 22 pM inhibited this response to control levels (Fig. 5A). Higher doses of TSP1 further inhibited adhesion below baseline. Remarkably, 22 pM TSP1 inhibited NO-stimulated adhesion of CD36-null MAMC to the same extent as in WT cells. Although higher concentrations of TSP1 further suppressed NO-induced adhesion of WT cells to levels below the basal level of the untreated controls, in the CD36-null cells further inhibition by TSP1 was only seen at 22 nM.

These data established that CD36 is not necessary for picomolar concentrations of TSP1 to inhibit an NO-stimulated response, although a secondary inhibitory response at nanomolar concentrations of TSP1 does require CD36. We have shown that picomolar concentrations of TSP1 inhibit NO signaling at the level of cGMP (22, 23). To establish whether inhibition by TSP1 of NO signaling through cGMP requires CD36, cGMP levels were analyzed in the WT and CD36-null MAMCs (Fig. 5B). As shown previously (23), NO-stimulated cGMP levels in WT cells were inhibited by exogenous TSP1. This activity of TSP1 does not require CD36, however, because the cGMP response was also completely inhibited by TSP1 in CD36-null MAMC (Fig. 5B). Notably, basal cGMP levels were similar in WT and CD36-null cells, suggesting that the previously reported effects of endogenous TSP1 on basal cGMP levels in vascular cells (22, 23) do not require CD36.

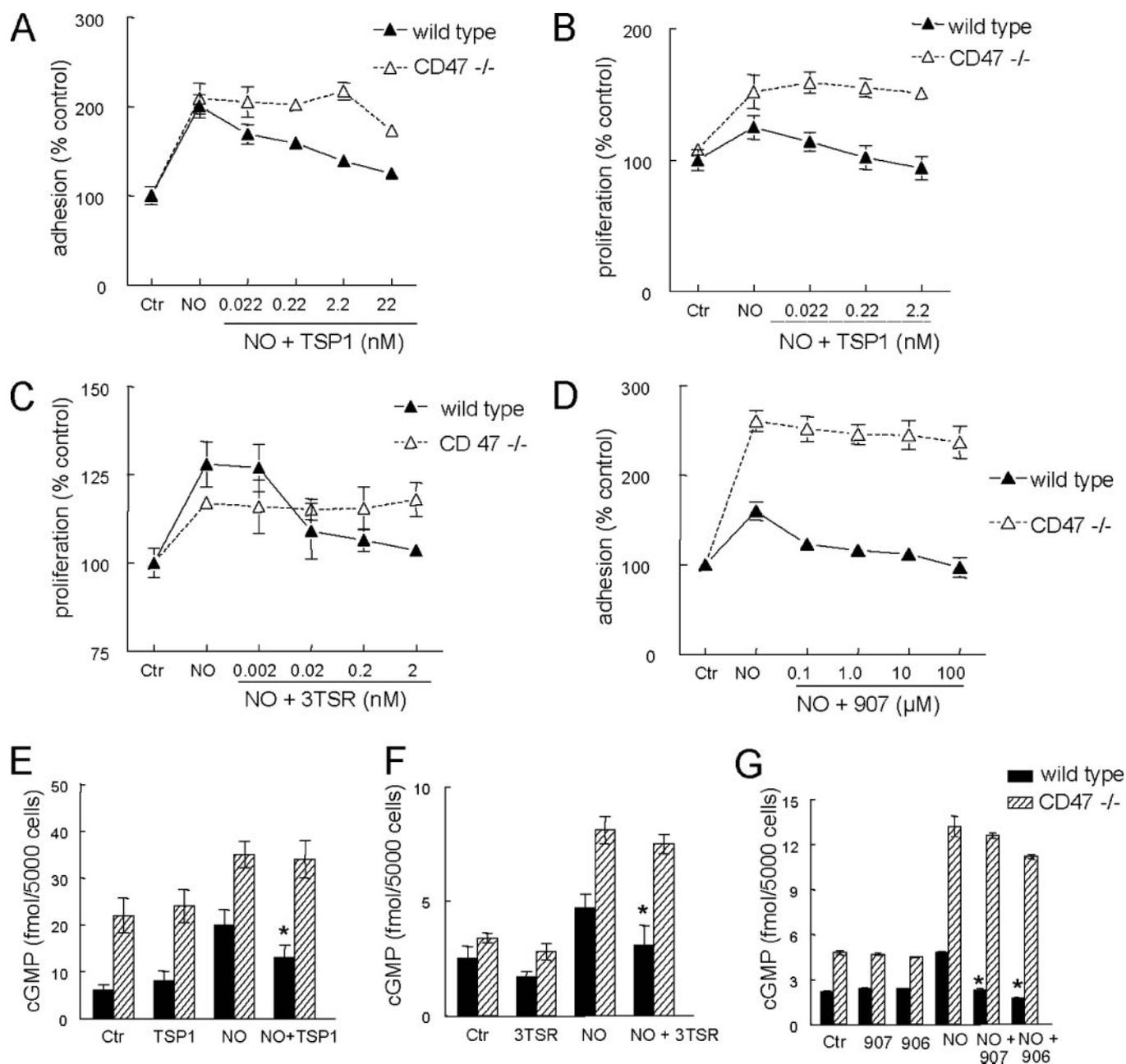
**CD47 Ligation Inhibits NO Responses**—The explant data in Fig. 2 suggested that CD47 could mediate the CD36-independent regulation of NO signaling by TSP1. To further examine the role of CD47, we tested two CD47-binding sequences identified in the CBD of TSP1 (34). Peptides containing the first (4N1-1, Fig. 6A) or second VVM motifs from this domain (7N3, Fig. 6B) inhibited NO-stimulated endothelial cell adhesion on type I collagen. Inhibition by peptide 7N3 was dose-dependent and maximal at 10  $\mu$ M. Specificity was confirmed using a control peptide in which the first VVM motif was substituted by GGM (p4N1G, RFYGGMWK), which at 10  $\mu$ M did not significantly inhibit NO-stimulated adhesion (data not shown).

Although the VVM peptides clearly bind to CD47 (34, 35), crystal structures of recombinant C-terminal regions of TSP1 and TSP2 have raised doubts about the exposure of the VVM motifs in native TSP1 (32, 36). Based on a crystal structure for this domain of the paralog TSP2 (36), the third type 2 repeat, the Ca-binding repeats, and the G module fold together to form the C-terminal globular domain of TSP1. A recombinant construct containing these elements of TSP1 (E3CaG1, Fig. 1B) at  $\geq 0.4$  nM inhibited NO-driven but not basal HASMC adhesion to collagen (Fig. 7A). Recombinant G module (CBD), which is also documented to interact with CD47 and to signal through that receptor (27), was slightly less active but also dose-dependently inhibited NO-stimulated HASMC adhesion on type I collagen but not basal adhesion on the same substrate (Fig. 7A). CBD also inhibited NO-stimulated HASMC proliferation but required much higher concentrations to reach basal levels (Fig. 7B). Consistent with their effects on NO-stimulated adhesion, CBD and E3CaG1 were equipotent dose-dependent inhibitors of NO-stimulated cGMP levels in HASMCs (Fig. 7, C and D). E3CaG1 also potentially inhibited NO-driven cGMP production



**FIGURE 8. Antibody ligation of CD47 inhibits NO-stimulated endothelial cell adhesion and proliferation.** HUVECs ( $1 \times 10^4$  cell/well) were plated in 96-well culture dishes pre-coated with type I collagen (5  $\mu$ g/ml) and incubated in EGM plus 0.1% BSA with or without B6H12 (0.01–10  $\mu$ g/ml) (A) or C1K1m1 (0.01–1  $\mu$ g/ml) (C). An isotype-matched control antibody (IgG<sub>1</sub>) was also tested at comparable doses. Following incubation for 1 h at 37 °C plates were washed and cells were fixed, stained, developed, and read at 570 nm. HUVECs ( $5 \times 10^3$  cells/well) were plated on 96-well culture plates and incubated for 72 h in EGM plus 1% FCS with or without DETA/NO (10  $\mu$ M) and antibodies B6H12 (0.01–10  $\mu$ g/ml) (B) or C1K1m1 (0.01–1  $\mu$ g/ml) (D). An isotype-matched control antibody (IgG<sub>1</sub>) was also tested at comparable doses. Cell proliferation was assayed via the colorimetric change obtained after incubation with MTS reagent at 490 nm. Results are expressed as percent control and represent the mean  $\pm$  S.D. of at least three separate experiments.

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**FIGURE 9. CD47 is necessary for TSP1 inhibition of NO-driven vascular cell responses.** A, murine wild-type and CD47-null ASMCS ( $1 \times 10^4$  cell/well) were plated in 96-well culture dishes pre-coated with type I collagen ( $5 \mu\text{g/ml}$ ) and incubated in SM-GM plus 0.1% BSA with DETA/NO ( $10 \mu\text{M}$ ) with or without TSP1 (0.022–22 nM) for 1 h, plates were washed, and cells were fixed, stained, developed, and read on a microplate reader at 570 nm. In other experiments, wild-type and CD47-null ASMCS ( $5 \times 10^3$  cell/well) were plated on 96-well culture plates and incubated for 72 h in SM-GM plus 1% FCS with DETA/NO ( $10 \mu\text{M}$ ) with or without TSP1 (0.022–2.2 nM) (B) or 3TSR (0.002–2 nM) (C), and proliferation was determined as described. In other experiments wild-type and null cells were plated in 96-well culture dishes pre-coated with type I collagen ( $5 \mu\text{g/ml}$ ) and incubated in SM-GM plus 0.1% BSA with DETA/NO ( $10 \mu\text{M}$ ) with or without peptide 907 (0.1–100  $\mu\text{M}$ ) for 1 h, plates were washed, and cells were fixed, stained, developed, and read at 570 nm (D). Wild-type and CD47-null ASMCS were plated ( $5 \times 10^3$  cells/well) in 96-well plates and weaned over 24 h from serum, then treated in serum-free medium with 0.1% BSA with DEA/NO ( $10 \mu\text{M}$ ) with or without TSP1 (1  $\mu\text{g/ml}$ ) (E), 3TSR (0.2 nM) (F), or CD36-binding peptides derived from the second (p907, 1  $\mu\text{M}$ ) or third type 1 repeats (p906, 1  $\mu\text{M}$ ) (G) for 5 min. The cells were lysed, and intracellular cGMP levels were determined. \*,  $p < 0.05$  versus NO alone (E–G).

in HUVECs, demonstrating that this function of CD47 is conserved in both endothelial and VSMC (Fig. 7E).

Because E3CaG1 also contains an integrin binding site, we used two CD47 antibodies to independently verify that CD47 ligation is sufficient to inhibit NO-stimulated responses (Fig. 8). B6H12 inhibits CD47-dependent endothelial and T-cell chemotaxis,  $\alpha_v\beta_3$  integrin activation, and calcium mobilization (34, 37–39) but stimulates CD47-dependent activation of  $\alpha_4\beta_1$

integrin (28). B6H12 was a dose-dependent inhibitor of NO-stimulated HUVEC adhesion on type I collagen (Fig. 8A). B6H12 also prevented stimulation of HUVEC proliferation by sustained exposure to NO (Fig. 8B). CIK1 is a nonblocking CD47 antibody for integrin function (28) and an activating antibody for T-cell receptor signaling (40). CIK1 treatment also inhibited HUVEC adhesion and proliferation (Fig. 8, C and D). Nonspecific isotype-matched antibodies did not block the NO-

driven increase in cell adhesion or proliferation (Fig. 8, A–D). The activities of these antibodies confirm that CD47 ligation is sufficient to inhibit NO signaling.

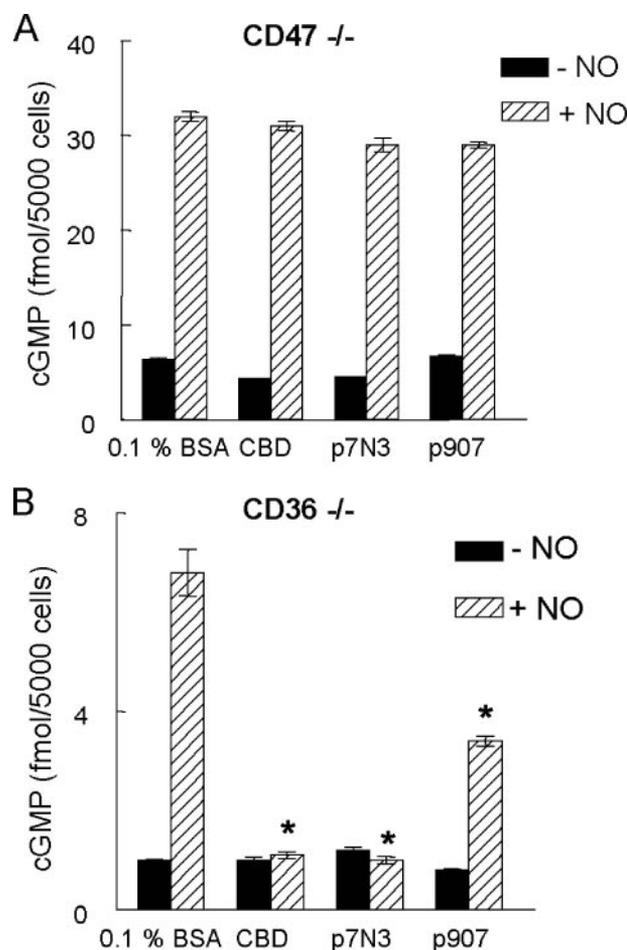
**CD47 Is Necessary for Inhibition of NO Responses by TSP1 in Vascular Cells**—In contrast to CD36, CD47 is required for inhibition of NO-stimulated MASMCM responses by exogenous TSP1 (Fig. 9). Exogenous TSP1 had no effect on NO-stimulated adhesion of CD47-null MASMCM at concentrations up to 2.2 nM and only moderately inhibited the response at 22 nM (Fig. 9A). Similarly, TSP1 did not inhibit NO-stimulated proliferation in CD47-null MASMCM (Fig. 9B). Remarkably, recombinant type 1 repeats of TSP1 that bind to CD36 also failed to inhibit NO-stimulated proliferation in CD47-null cells (Fig. 9C), and a CD36-binding peptide analog derived from the second type 1 repeat failed to inhibit NO-stimulated adhesion in CD47-null cells (Fig. 9D).

The inability of CD47-null cells to respond to exogenous TSP1 extended to NO-stimulated cGMP signaling. As reported previously for TSP1-null endothelial cells and MASMCMs (22, 23), basal cGMP levels were significantly higher in CD47-null MASMCMs (Fig. 9E). Addition of NO elevated these levels, but exogenous TSP1 did not significantly inhibit the basal or NO-stimulated cGMP levels in the CD47-null MASMCMs (Fig. 9E). Similar results were obtained using recombinant type 1 repeats and peptide 907, which bind to CD36 and inhibited NO-stimulated cGMP responses in WT but not in CD47-null MASMCMs (Fig. 9, F and G). NO-stimulated cGMP was slightly inhibited by a CD36-binding peptide from the third type 1 repeat (p907, Fig. 9G).

To further define the roles of CD36 and CD47, we compared cGMP responses of VSMCMs from the respective null mice to peptide and recombinant protein ligands (Fig. 10). Two CD47 ligands (CBD and peptide 7N3) and a CD36 ligand (peptide 907) did not significantly inhibit NO-stimulated cGMP accumulation in CD47-null MASMCMs (Fig. 10A). In contrast, CBD and its derived peptide 7N3 completely inhibited NO-stimulated cGMP in CD36-null MASMCM (Fig. 10B). Therefore, CD47 is necessary for inhibition of cGMP signaling and downstream VSMCM responses to NO by either CD36 or CD47 ligands, but CD36 is not necessary for inhibition by a CD47 ligand. Remarkably, peptide 907, a reported CD36 ligand (33), partially inhibited NO-stimulated cGMP in CD36-null cells (Fig. 10B). The mechanism by which peptide 907 inhibits NO-induced cGMP formation in the CD36-null cells is unknown, but possibilities include binding to another member of the class B scavenger receptor family expressed on these cells (41) or interaction of peptide 907 with CD47.

## DISCUSSION

Although the inability of TSP1 to inhibit angiogenesis in corneas of CD36-null mice is strong evidence that CD36 is a necessary TSP1 receptor in the context of FGF2-driven corneal angiogenesis (18), ligation of several other TSP1 receptors is sufficient to inhibit angiogenic responses *in vitro* and *in vivo*. TSP1-derived peptides or recombinant regions of TSP1 that bind to heparan sulfate proteoglycans (14, 42) or CD47 (20) each have such activities. However, these previous studies did not determine whether the respective receptors are necessary

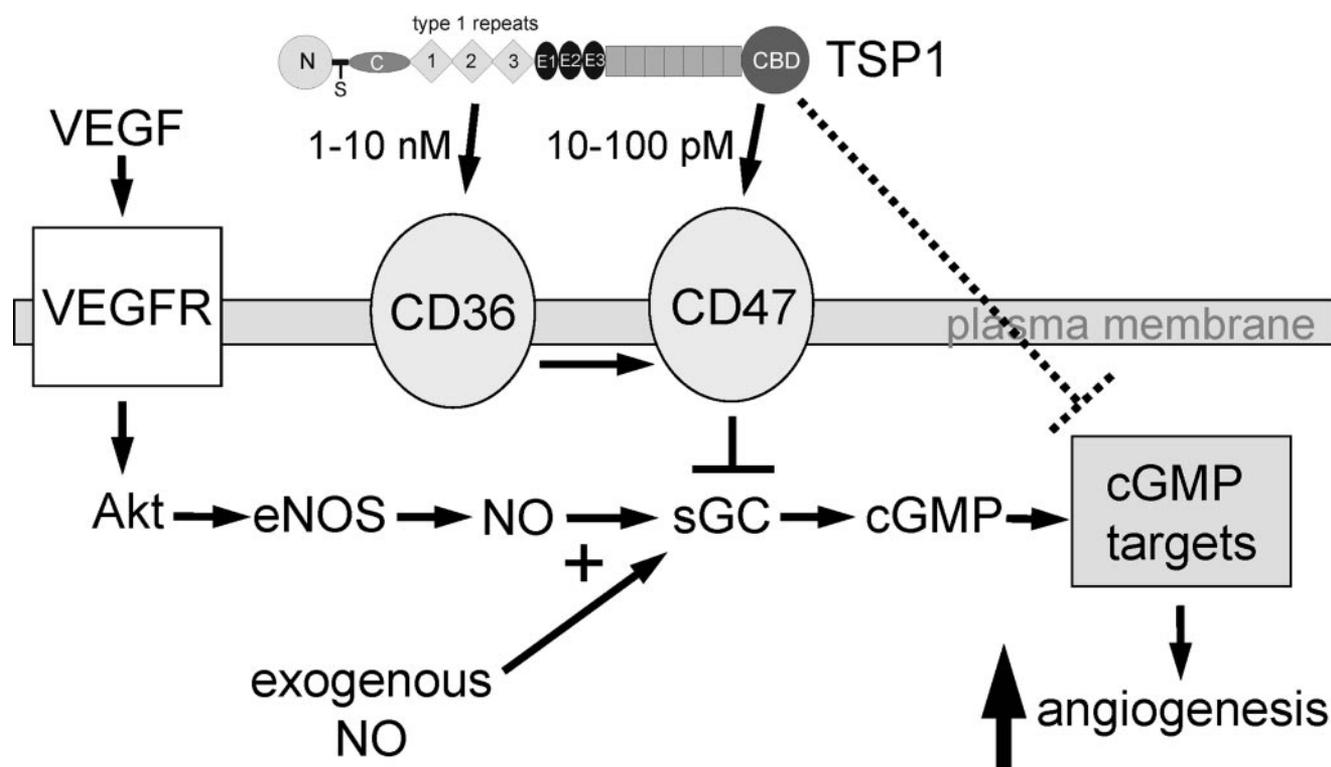


**FIGURE 10. CD47 is necessary for inhibition of NO signaling by both CD36- and CD47-binding sequences of TSP1.** Murine CD47-null ASMCMs (A) or CD36-null ASMCMs (B) were plated ( $5 \times 10^3$  cells/well) in 96-well plates, weaned from serum over 24 h, and treated in serum-free medium with 0.1% BSA with DEA/NO (10  $\mu$ M) with or without CBD (1  $\mu$ g/ml), peptide 7N3 (1  $\mu$ M), or peptide 907 (1  $\mu$ M) for 5 min, the cells were lysed, and cGMP levels were determined. Results are expressed as percent control and represent the mean  $\pm$  S.D. of at least three separate experiments. \*,  $p < 0.05$  versus -NO.

for the activity of native TSP1. The present data provide genetic evidence that CD47 but not CD36 is essential to the anti-angiogenic activity of TSP1 in the context of NO, a key mediator of signaling for several major angiogenic factors (43–46). CD47-binding recombinant domains of TSP1 lacking its CD36 binding sites are sufficient to inhibit cGMP signaling downstream of NO. Although CD36 is not necessary and, therefore, does not mediate this activity of TSP1, we found that various CD36 ligands are sufficient to inhibit the same NO-stimulated responses, provided that CD47 is expressed. This activity, however, is independent of the ability of a given CD36 ligand to block TSP1 interaction with CD36.

The CD36-binding type 1 repeats of TSP1 were previously shown to inhibit NO signaling, but we now demonstrate that this activity also requires CD47. This should not be interpreted to show that CD47 is a receptor for the type 1 repeat sequences, although that cannot be excluded. Our data indicate that, in the context of NO stimulation, CD47 is the essential inhibitory receptor for TSP1, and CD36 ligation is sufficient to mediate a secondary inhibitory response that also requires CD47 (Fig. 11). Basal cGMP levels are elevated in CD47-null and in TSP1-null

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**FIGURE 11. Regulation of NO signaling by TSP1 via CD36 and CD47.** Endogenous NO synthesis is stimulated via Akt-mediated phosphorylation of endothelial nitric-oxide synthase (*eNOS*) downstream of vascular endothelial growth factor (*VEGF*) receptor (57). Ligand of soluble guanylyl cyclase (*sGC*) mediated by endogenous or exogenous NO. CD47 is downstream of CD36, because ligation on CD36 cannot inhibit signaling in the absence of CD47. The requirement for CD47 is consistent with lateral interactions with CD36 or with CD36 signaling requiring a convergent CD47 signal at some point upstream of *sGC*. The mechanism by which CD47 ligation regulates *sGC* is not known. TSP1 also inhibits NO signaling downstream of cGMP (22), but the receptors mediating this second signal have not been defined.

vascular cells, but this is not observed in CD36-null MASM. Therefore, the ability of endogenous TSP1 to suppress basal cGMP levels also requires CD47 but not CD36.

*In vitro* studies have shown that CD36 mediates only a subset of endothelial cell interactions with TSP1 (47, 48), and CD36 now appears to be necessary only for a subset of the anti-angiogenic activities of TSP1. Antibody blockade of CD36 prevented the inhibitory effects of TSP1 and peptides derived from its type 1 repeats on FGF2-driven endothelial cell migration (13), and exogenous TSP1 could not inhibit FGF2-driven angiogenesis in the mouse cornea in the absence of CD36 (18). However, we now show that CD36 antibodies that block TSP1 inhibition in the cornea can themselves inhibit angiogenic responses stimulated by NO. The basis for a differential requirement of CD36 in FGF2- versus NO-dependent angiogenic responses remains to be determined. However, it is notable that NO signaling is required for angiogenic responses to vascular epidermal growth factor but not to FGF2 (44, 49, 50).

Low to moderate concentrations of NO (<1–30 nM) elicit pro-survival and pro-angiogenic responses from vascular cells (22, 23, 46). Bi-directional cross-talk with TSP1 potently regulates this angiogenic activity of NO (22, 51). Consistent with previous publications, we find that CD36 ligation is sufficient to block pro-angiogenic responses, but only if CD47 is also expressed. As with exogenous TSP1, CD36 antibodies effectively block several NO-stimulated vascular cell responses, including cell proliferation, adhesion, migration, and cGMP accumulation. However, agonist (SMΦ) and antagonist anti-

body (FA6-152 and 185-1G2) ligation of CD36 equally inhibited NO signaling. These results contrast with previous reports that have classified these CD36 antibodies on the basis of their abilities to mimic or block the inhibitory activity of TSP1. Therefore, we propose that both agonist and antagonist antibodies elicit signaling through CD36 that is independent of TSP1.

Activation of CD47 has been described to inhibit (52) or stimulate extracellular signal-regulated kinase phosphorylation (53). We found that NO-stimulated extracellular signal-regulated kinase phosphorylation in endothelial cells was inhibited by exogenous TSP1 (51), consistent with the evidence that CD47 mediates inhibition of angiogenic responses by TSP1. This was supported by the ability of CD47 antibodies, peptide ligands, and two forms of the C-terminal CD47-binding domain of TSP1 to inhibit NO-stimulated vascular cell responses. Moreover, neither NO-stimulated VSMC adhesion on collagen nor NO-stimulated cGMP levels could be blocked by pretreatment with TSP1 in CD47-null cells. Taken together these results demonstrate that either CD36 or CD47 ligation is sufficient to mimic TSP1 inhibition of NO-driven pro-angiogenic cell responses, but only CD47 expression is absolutely required for TSP1 inhibition of these angiogenic responses.

Given that CD47 is necessary for some responses to both CD36 and CD47 ligands, we must reconsider the roles of these two receptors in vascular cell responses to TSP1. It has been generally assumed that if TSP1 binds to a receptor and genetic evidence shows that receptor to be necessary for a specific response, one can infer that TSP1 binds to the same receptor to

mediate that response. This may not be valid logic for a protein such as TSP1 that engages multiple receptors on vascular cells. Convergent signaling downstream of CD36 and CD47 may mediate a single response even if TSP1 binds only to CD47. Alternatively, the cross-talk between CD36 and CD47 may occur at the plasma membrane (Fig. 11). CD36 and CD47 both associate with lipid rafts and with certain integrins that themselves bind TSP1 (28, 54, 55). The physical proximity of CD47, its associated integrins, or one of its cytoplasmic binding partners may be necessary for signal transduction through CD36. Such cooperative signaling by a CD36- $\beta_1$  integrin-CD47 complex has been proposed for another CD36 ligand,  $\beta$ -amyloid (56), and may explain the recent evidence that  $\beta_1$  integrins are also necessary for some inhibitory responses of vascular cells to TSP1 (19).

Together, these considerations suggest that multiprotein complexes containing two or more TSP1 receptors could mediate its actions on vascular cells. Further, because other TSP family members bind to different subsets of these receptors, the potential exists for finely tuned spatial or temporal regulation during development, wound healing, and pathological states.

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