Recognition of the N-terminal Modules of Thrombospondin-1 and Thrombospondin-2 by α6β1 Integrin*

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In addition to its recognition by αβ1 and αβ1 integrins, the N-terminal pentraxin module of thrombospondin-1 is a ligand for αβ1 integrin. αβ1 integrin mediates adhesion of human microvascular endothelial and HUVE cells to immobilized thrombospondin-1 and recombinant N-terminal regions of thrombospondin-1 and thrombospondin-2. αβ1 also mediates chemotaxis of microvascular cells to thrombospondin-1 and thrombospondin-2. Using synthetic peptides, LALERKDHSG was identified as an αβ1-bind- ing sequence in thrombospondin-1. This peptide inhibited αβ1-dependent cell adhesion to thrombospondin-1, thrombospondin-2, and the E8 fragment of murine laminin-1. The Glu residue in this peptide was required for activity, and the corresponding residue (Glu90) in the N-terminal module of thrombospondin-1 was required for its recognition by αβ1, but not by αβ1. αβ1 was also expressed in human umbilical vein endothelial cells; but in these cells, only certain agonists could activate the integrin to recognize thrombospondin-1. Selective activation of αβ1 integrin in microvascular endothelial cells by the anti-β1 antibody TS2/16 therefore accounts for their adhesion responses to thrombospondins and explains the distinct functions of αβ1 and αβ1 integrins as thrombospondin receptors in microvascular and large vessel endothelial cells.

Integrins play a major role in mediating interactions between cells and their extracellular matrix environment. In addition to providing physical anchoring of cells, engagement of integrins transmits signals into cells that regulate their survival and behavior (reviewed in Ref. 1). Conversely, cells can modulate their interactions with the extracellular matrix through regulating the expression or ligand-binding activities of specific integrins (1). Although some extracellular matrix ligands have been identified for most known integrins (reviewed in Ref. 1), the role of additional integrins expressed on endothelial cells as TSP antagonists remains incomplete.

Thrombospondins (TSPs) are a family of five extracellular matrix proteins (3, 4). TSP1 modulates cell behavior by altering cell adhesion, motility, proliferation, survival, gene expression, and differentiation. Some cellular responses to TSP1 are mediated by non-integrin receptors (5–8), but integrins also play important roles in mediating activities of TSP1 in several cell types. To date, interactions of TSP1 with αβ3, αβ1, αβ1, and αβ1 have been demonstrated (9–12). αβ1 also serves as a receptor for thrombospondin-2 (TSP2) (12), but the recognition site identified for αβ1 is not conserved in TSP2 (13).

One of the best characterized biological activities of TSP1 and TSP2 is to modulate angiogenesis. Inhibition of endothelial cell chemotaxis is mediated by the TSP1 receptor CD36 (14). Heparan sulfate proteoglycans and CD47 may also contribute to the anti-angiogenic activities of TSP1 (15–17). In certain contexts, however, TSP1 and recombinant or proteolytic fragments of TSP1 exhibit pro-angiogenic activities (18, 19). We previously reported that αβ1 mediates a pro-angiogenic activity of the N-terminal pentraxin module of TSP1 (18). This integrin is constitutively expressed on venous and microvascular endothelial cells, but its ability to bind TSP1 is regulated by cell-cell signaling involving VE-cadherin (18). Because β1-dependent interactions of TSP1 or TSP2 with endothelial cells could not be completely inhibited by αβ1 antagonists, we examined the role of additional β1 integrins expressed on endothelial cells as TSP receptors. This effort revealed that a second β1 integrin, αβ1, selectively functions as a TSP1 and TSP2 receptor in large vessel endothelial cells. However, microvascular cells are refractory to αβ1 antagonists. We have therefore examined the role of additional β1 integrins as TSP receptors in microvascular cells and report here that αβ1 is a major integrin receptor for TSP1 and TSP2 that mediates adhesion and chemotaxis of microvascular endothelial cells. Furthermore, we identify a specific sequence in the N-terminal module of TSP1 that mediates this interaction and demonstrate that this sequence antagonizes interactions of αβ1 with its well characterized ligand laminin-1.

EXPERIMENTAL PROCEDURES

Cell Culture—Human dermal microvascular endothelial (HDMVE) cells and human lung microvascular endothelial (HMVE-L) cells (Cambrex Bio Science Inc., Walkersville, MD) were grown under the conditions specified by the manufacturer. Adult iliac vein endothelial cells (AG10773A; NIA Repository, Coriell Institute for Medical Research, Camden, NJ) were grown on flasks coated with 0.1% gelatin in medium 199 containing 10% fetal bovine serum (FBS), 2 mM glutamine, 30 μg/ml heparin. Human umbilical vein endothelial (HUVE) cells (Clonetics BioWhittaker Inc.) were maintained in medium 199 containing 10% fetal bovine serum (FBS), 2 mM glutamine, 30 μg/ml heparin. Human umbilical vein endothelial (HUVE) cells (Clonetics BioWhittaker Inc.) were maintained in medium 199 containing

2 M. J. Calzada, L. Zhou, J. M. Sipes, J. Zhang, H. C. Krutzsch, M. L. Irueña-Arispe, D. S. Annis, D. F. Mosher, and D. D. Roberts, submitted for publication.
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20% FBS, 2 mM glutamine, 80 μg/ml endothelial cell mitogen, 10 μg/ml heparin, and penicillin/streptomycin. The human fibrosarcoma cell line HT-1080 (American Type Culture Collection, Manassas, VA) was grown in Dulbecco’s modified Eagle’s medium containing 10% FBS. Jurkat T cells were maintained in RPMI 1640 medium (BIO SOURCE International, Camarillo, CA) supplemented with 10% FBS, 2 mM glutamine, and penicillin/streptomycin. The breast carcinoma cell line MDA-MB-231 (American Type Culture Collection) was propagated weekly in RPMI 1640 medium containing 10% fetal calf serum.

Proteins and Peptides—TSP1 was purified from human platelets obtained from the National Institutes of Health Blood Bank (20). The protein was >95% intact based on analysis by SDS gel electrophoresis. The recombinant trimeric proteins consisting of the N-terminal module (N) and C-terminal module (C) of TSP1 (residues 1–140680) were expressed in E. coli and purified as described previously (21). A recombinant portion of TSP1 (residues 1–175 of the mature protein) was prepared as described previously (15). Site-directed mutagenesis of Glu90 in TSP1-(1–175) to Ala was performed as described previously (12). Residue numbers refer to the mature sequence of secreted TSP1. The forward and reverse mutation-inducing primer sequences were 5′-GAGGTTGCTTTCGCCGCCAGGACGACGCT-3′ and 5′-GAGGTTGCTTTCGCCGCCAGGACGACGCTT-3′, respectively. After growth on LB broth/ampicillin plates at 30 °C, the mutated plasmid was transformed into Escherichia coli XL-1 Blue cells for isolation, transformed into Rosetta cells (Novagen) for protein expression and concentration, and purified by a phenol/chloroform extraction. The recombinant TSP1-(1–175) to Ala was performed as described previously (12). Murine laminin-1 was provided by Dr. Lance Liotta (Laboratory of Pathobiology, NCI). The E8 fragment of murine laminin-1 was prepared as described (22).

Antibodies and Reagents—The β_1-activating antibody TS2/16 (23) was produced by the hybridoma cell line obtained from the American Type Culture Collection. The monoclonal antibody GoH3 was from Chemicon International, Inc. (Temecula, CA). The αβ_1-blocking antibody PI1B5 was from Sigma. The fluorescein-conjugated anti-mouse antibody used in flow cytometry was purchased from ICN Biomedicals, Inc. (Aurora, OH). The αβ_1 integrin function inhibitor (4-(2-methylphenyl)aminocarbonylamino phenyl)acetyl-L-DVP (24) was obtained from Bachem (Torrance, CA). Peroxidase-labeled goat anti-mouse IgG was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD).

Cell Adhesion Assay—Laminin-1 (5 μg/ml), TSP1 (40 μg/ml), STD-VCAM-1 (5 μg/ml), and NoC1 and NoC2 (30 μg/ml) in Dulbecco’s phosphate-buffered saline (DPBS) and TSP1-(1–175) (5 μg/ml) in 20 μg/ml NoC1 were used. The function-blocking antibodies, W6/32 and 3E10, were conjugated at 30 μg/ml to a 2% bovine serum albumin (BSA) for 2 h. After washing, 5 × 10^5 cells were added to the plates in 100 μl of DPBS and were incubated for 1 h at 37 °C. Cells were washed three times with DPBS and fixed with 1% glutaraldehyde in DPBS for 1 h. After washing, cells were incubated for 1 h with rabbit antibody against integrin β_1, then with FITC conjugated goat anti-rabbit IgG. After washing, cells were then incubated with rat anti-mouse IgG followed by FITC conjugated goat anti-rabbit IgG. The plates were washed and counterstained with propidium iodide. The results were analyzed by FACScan or LSR.

Results—αβ_1 Integrin Mediates Microvascular Endothelial Cell Adhesion to TSP1—Residues 175–242 of the N-terminal module of TSP1 contain a recognition site for αβ_1 integrin (13) that is partially responsible for adhesion of microvascular endothelial cells to TSP1 (18). However, TSP1-(1–175) was only severalfold less active than intact TSP1 in mediating adhesion of antibody TS2/16-activated HDMVE cells (Fig. 1A). This region contains an αβ_1-binding site that mediates adhesion of large vessel endothelial cells, but not an αβ_1 antagonist did not significantly inhibit adhesion of HDMVE cells to TSP1-(1–175) or intact TSP1 (Fig. 1B). Because adhesion to this region was inhibited by a β_1-blocking antibody (data not shown), we used function-blocking antibodies specific for other α subunits to define its specificity. An antibody against α5β_1 integrin was inactive (data not shown), but an α4-blocking antibody reproducibly decreased adhesion of HDMVE cells to TSP1 and TSP1-(1–175) (Fig. 1B). Inhibition of adhesion to TSP1-(1–175) by antibody GoH3 was comparable to that obtained using the known α6β_1 ligand laminin-1 (Fig. 1B).

Flow Cytometry Analysis—HUE and HDMVE cells were washed with DPBS containing 0.2% BSA and incubated with Puck’s saline containing 0.2% EDTA and 10% FBS at 37 °C for 6 min. Cells were dislodged and resuspended in a large volume of Puck’s saline/EDTA solution, centrifuged, resuspended in DPBS containing 0.2% BSA at a density of 6 × 10^6 cells/ml, and stored on ice. Cells (1 × 10^6/labeled reaction) were incubated with 2 μg of antibody TS2/16 as a control or with 2 μg of antibody GoH3 for 1 h. Cells were washed twice with DPBS and 0.2% EDTA and incubated with fluorescein isothiocyanate-conjugated anti-mouse antibody for 1 h. Labeled cells were washed again and fixed with 300 μl of 1% formaldehyde in DPBS. Flow cytometry analysis was performed using a BD Biosciences flow cytometer.

Immunoprecipitation Analysis—HUE or HDMVE cells grown in 10-cm dishes were dislodged with 2 mM EDTA, centrifuged, and resuspended in DPBS containing 0.2% EDTA and 0.5% deoxycholate. Cells were lysed with radioimmunoprecipitation assay buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EGTA, and 1 mM NaF supplemented with 10 μg/ml each antipain, pepstatin A, chymostatin, leupeptin, aprotinin, and soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride), and the lysate was preclarified by centrifugation. Equal volumes with equal protein concentrations were immunoprecipitated using the anti-α6 antibody GoH3 prebound to anti-mouse IgG agarose (Sigma). The immune complexes were washed six times with Tris-buffered saline, diluted with sample buffer containing 10% β-mercaptoethanol, heated, and fractionated on SDS gels (Bio-Rad). After transfer to a polyvinylidene fluoride membrane, the proteins were detected using horse-radish peroxidase-conjugated streptavidin and visualized using chemiluminescent substrate (Pierce).

Chemotaxis—HDMVE cells at passages 5–8 were used 2–3 days after passage. Modified Boyden chambers and 8-μm pore polycarbonate membranes (Nouvorare, Gaithersburg, MD) coated with 100 μg/ml gelatin in 0.1% aqueous acetic acid were used. Cells were dislodged with 2 mM EDTA and allowed to recover for 30 min suspended in complete medium. After centrifugation, the cells were resuspended in medium 199 containing 0.1% BSA and added (56 μl) to the upper chamber in the absence or presence of TSP1 (30 μg/ml), NoC1 or NoC2 (20 μg/ml), or both, in the lower chamber for 24 h at 37 °C. Cells on the lower surface of the membranes were fixed and stained. Migrated cells were counted microscopically.

Results—αβ_1 Integrin Mediates Microvascular Endothelial Cell Adhesion to TSP1—Residues 175–242 of the N-terminal module of TSP1 contain a recognition site for αβ_1 integrin (13) that is partially responsible for adhesion of microvascular endothelial cells to TSP1 (18). However, TSP1-(1–175) was only severalfold less active than intact TSP1 in mediating adhesion of antibody TS2/16-activated HDMVE cells (Fig. 1A). This region contains an αβ_1-binding site that mediates adhesion of large vessel endothelial cells, but an αβ_1 antagonist did not significantly inhibit adhesion of HDMVE cells to TSP1-(1–175) or intact TSP1 (Fig. 1B). Because adhesion to this region was inhibited by a β_1-blocking antibody (data not shown), we used function-blocking antibodies specific for other α subunits to define its specificity. An antibody against α5β_1 integrin was inactive (data not shown), but an α4-blocking antibody reproducibly decreased adhesion of HDMVE cells to TSP1 and TSP1-(1–175) (Fig. 1B). Inhibition of adhesion to TSP1-(1–175) by antibody GoH3 was comparable to that obtained using the known α6β_1 ligand laminin-1 (Fig. 1B).

αβ_1 integrin expression was measured using several independent isolates of HDMVE cells, but
not using HUVE cells (Fig. 2A). An α6β1 antagonist reproducibly inhibited adhesion of antibody TS2/16-activated HUVE cells to TSP1 (1–175), but not to laminin-1 (Fig. 2A). HUVE cell adhesion to this known α6β1 integrin ligand was only slightly inhibited by the anti-α6β1 antibody (Fig. 2A), suggesting that α6β1 integrin is either absent or not functional in these cells. To determine whether the distinct behavior of HDMVE and HUVE cells was unique to endothelial cells from these anatomical sites, we tested microvascular and large vessel cells from different organs. Adhesion of iliac vein endothelial (AG10773A) cells was also insensitive to the anti-α6β1 antibody (Fig. 2B), whereas HMVE-L cell adhesion was inhibited by this antibody (Fig. 2C).

Therefore, the selective expression or function of α6β1 as a TSP1 receptor may be a general characteristic of microvascular endothelial cells.

**α6β1 Integrin Also Recognizes TSP2**—Adhesion of HDMVE cells to the trimeric N-terminal regions of both TSP1 (NoC1) and TSP2 (NoC2) was β1-dependent (Fig. 3A), but the anti-α6 antibody was a more effective inhibitor of HDMVE cell adhesion to NoC2 than to NoC1 (Fig. 3B). The lesser sensitivity for NoC1 may be explained by the presence of an α6β1-binding site in NoC1, but not in NoC2. However, α6β1 appears to be the...
Fig. 3. N-terminal regions of TSP1 and TSP2 support αβ1 integrin binding. Adhesion of antibody TS2/16-activated HDMVE cells to NoC1 and NoC2 (30 μg/ml) was assessed in the presence of the β1-blocking monoclonal antibody 13 (5 μg/ml) (A) or antibody GoH3 (5 μg/ml) (B). Cell attachment was quantified, and results are expressed as the number of cells/mm² (±S.D.) from at least three different experiments.

Fig. 4. Expression of αβ1 integrin in large vessel and small vessel endothelial cells. A, α6 mRNA levels were quantified by reverse transcription-PCR. Products were analyzed after 30 cycles of amplification using a 1.5% agarose gel stained with ethidium bromide. B, α6 and β1 integrin-specific antibodies were used to compare protein expression of this TSP1 receptor by flow cytometry on the surface of HUVE and HDMVE cells. C, HUVE and HDMVE cells were surface-biotinylated and immunoprecipitated using the anti-α6 antibody GoH3. Precipitates were analyzed by SDS-7.5% polyacrylamide gel electrophoresis under reducing conditions, transferred to a polyvinylidene fluoride membrane, and visualized by chemiluminescence. D, α6 expression levels in HMVE-L and AG10773A cells were quantified in cell lysates prepared as described for C, immunoprecipitated with the anti-α6 antibody GoH3, and visualized by chemiluminescence.

major β1 integrin in HDMVE cells that recognizes TSP2.

α6β1 Integrin Expression Levels Are Similar in Large and Small Vessel Cells—The levels of α6β1 expression were measured to examine why microvascular and large vessel endothelial cells differed in their utilization of α6β1 as a TSP receptor. α6 mRNA was expressed at similar levels in HUVE and HDMVE cells based on reverse transcription-PCR (Fig. 4A). Cell-surface expression of α6 and β1 subunits assessed by flow cytometry did not differ between HUVE and HDMVE cells (Fig. 4B). However, analysis of the total expression levels by immunoprecipitation using the same anti-α6 antibody revealed some differences in the α6 subunit (Fig. 4C). On an underexposed blot (Fig. 4C, upper panel), more intact 120-kDa α6 subunit was reproducibly detected in HDMVE cells than in HUVE cells. By densitometry, this represented a 1.6-fold difference in α6 subunit between HDMVE and HUVE cells. After a longer exposure (Fig. 4C, lower panel), we could also detect differences in the pattern of processed α6 subunits or integrin-associated proteins. In addition to the intact α6 subunit, HUVE cell immunoprecipitates contained 70- and 43-kDa proteins. The apparent masses of these proteins are consistent with the unreduced and reduced molecular masses reported for a structural variant of the α6 integrin called α6 parvus (26). Although the gel was run under reducing conditions, it is possible that the 70-kDa band corresponds to partially unreduced α6 parvus. In the α6 immunoprecipitate from HDMVE cells, these bands were less abundant relative to the intact 120-kDa α6 subunit, but an additional unknown protein was also detected. Likewise, a slight difference in the expression levels of this integrin was also found in HMVE-L and AG10773A cells (Fig. 4D). These minor differences may not be sufficient to account for the differential function of α6β1 in these cells.

Activation States of α6β1 Integrin Differ in HUVE and HDMVE Cells—The differences in expression of α6β1 integrin in HUVE and HDMVE cells are probably insufficient to account for the differences in its function. Alternatively, the activating antibody TS2/16 may not stimulate α6β1 integrin equally in microvascular and large vessel endothelial cells. To test this hypothesis, we compared the activity of other integrin activation agonists in these cells (Fig. 5). In contrast to antibody TS2/16, MnCl2 and lipopolysaccharide stimulated anti-α6β1 antibody-inhibitable adhesion of HUVE cells to TSP1 and lami-


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**Fig. 5. α₆β₁ integrin is differently activated in HUVE cells.** Adhesion of HUVE cells to TSP1 and laminin-1 was assayed in the presence of different activation agonists. HUVE cells grown for 2 days in HDMVE cell-conditioned medium were activated with antibody TS2/16 (5 μg/ml) in the absence or presence of the α₂β₁-blocking antibody (5 μg/ml). HUVE cells grown in their growth medium were activated with lipopolysaccharide (LPS; 100 ng/ml) or MnCl₂ (0.4 mM) in the absence or presence of the α₂β₁-blocking antibody. Cell attachment was quantified after 1 h. Results are expressed as the number of cells/mm² (± S.D.) from three different experiments.

**Fig. 6. Identification of an α₆β₁ inhibitory sequence in TSP1.** Adhesion of HDMVE cells to NoC2 (30 μg/ml), TSP1-(1–175) (30 μg/ml), or the E8 fragment of laminin-1 was assayed in the absence (Control) or presence of TSP1 synthetic peptides containing the underlined conserved acidic residues at positions 35, 111, 126, 127, and 145. HDMVE cells were seeded on dishes coated overnight with the proteins. Cells were incubated for 1 h in the presence of the β₁-activating antibody TS2/16 (10 μg/ml) and the indicated peptides at 200 μM. Cell attachment was quantified after 1 h by fixation with 1% glutaraldehyde and staining with Diff-Quick solution. Results are expressed as the number of cells attached per mm² (± S.D.).

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**Identification of an α₆β₁-Binding Sequence in the N-terminal Module of TSP1**—The smallest portions of TSP1 and TSP2 that supported α₆β₁-dependent adhesion contain amino acids 1–175 of mature TSP1 and amino acids 1–859 of TSP2, suggesting that the α₆β₁-binding site is localized in the N-terminal modules of both proteins. The N-terminal modules of TSP1 and TSP2 are evolutionarily related to the G modules of laminin α subunits. Given that the laminin G modules contain an α₆β₁-binding site (28, 29), we considered that the position of the α₆β₁-binding sequence may be conserved in TSP1. Therefore, two synthetic peptides were synthesized that aligned with a proposed α₆β₁-binding sequence from laminin-1, peptide AG-32 (30). However, neither LFVQEDRALYI (TSP1-(140–151)) nor ATGGKWSITLF (TSP1-(131–141)) inhibited HDMVE cell adhesion to TSP1 or NoC2 (Fig. 6) (data not shown).

Because integrin recognition sites often require an Asp or Glu residue (2), our second approach to identify a sequence recognized by α₆β₁ integrin in TSP1 and TSP2 was to search for conserved Asp or Glu residues in the N-terminal modules of the known TSP1 and TSP2 sequences. Such conserved acidic residues are present at positions 14, 35, 90, 111, 126, 127, 145, and 162 of TSP1. We tested synthetic peptides containing six of the conserved acidic residues that were identified on this basis. Control peptides for each had Ala substituted for the conserved Asp or Glu residues. The conserved Asp/Glu residue at position 162 was previously identified as an α₆β₁-binding site in TSP1 and TSP2 (12) and did not affect α₆β₁-mediated adhesion. Synthetic peptides containing acidic residues 35 (ELT-

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**α₆β₁ Integrin Mediates Cell Spreading on TSP1**—Loss of cell-cell contact induces endothelial cell spreading on TSP1 mediated by α₆β₁ integrin (18). α₆β₁ integrin also mediates endothelial cell adhesion and spreading on TSP1 and fragments from the N-terminal heparin-binding domain of TSP1 and TSP2. On the other hand, α₆β₁ integrin mediates spreading on several cell types (32, 33). Our results show that HDMVE cells spread on TSP1 and TSP1-(1–175) in an α₆β₁-dependent manner based on inhibition by peptide LALERKDHS.

α₆β₁ integrin is known to participate in HDMVE adhesion to TSP1 (18), and heparan sulfate proteoglycan or low density lipoprotein receptor-related
protein may contribute to adhesion to TSP1-(1–175).

Glu\textsuperscript{90} of TSP1 Is Required for α\textsubscript{6}β\textsubscript{1} Integrin Recognition—Several residues in the inhibitory sequence LALERKDHSG are highly conserved among TSP1 and TSP2 sequences from different species (Fig. 9A). Notably, the Glu residue is completely conserved in all known TSP1 and TSP2 sequences. To test the role of this residue, we mutated Glu\textsuperscript{90} in TSP1-(1–175). Mutation of Glu\textsuperscript{90} to Ala completely inhibited HDMVE cell adhesion to this portion of TSP1 (Fig. 9B). This loss of activity was not due to a global alteration in folding of the protein because the same mutation did not affect adhesion of Jurkat T cells to TSP1-(1–175) mediated by α\textsubscript{6}β\textsubscript{1} integrin (Fig. 9C) (12). Furthermore, the mutant protein retained the heparin-binding activity of the native sequence (34) (data not shown). These results indicate that Glu\textsuperscript{90} plays an important role in α\textsubscript{6}β\textsubscript{1}-mediated adhesion to this portion of TSP1. The high degree of homology for this sequence among TSPs from different species and the total inactivation following mutation of the conserved Glu\textsuperscript{90} residue strongly suggest that this constitutes the α\textsubscript{6}β\textsubscript{1}-binding site.

Role of α\textsubscript{6}β\textsubscript{1} Integrin in Endothelial Cell Chemotaxis—TSP1 inhibits microvascular endothelial cell motility induced by fi-
broblast growth factor-2 through binding to its receptor CD36 (14), but TSP1 also stimulates chemotaxis of murine lung capillary cells (LE-II) and bovine aortic endothelial cells (35). The stimulatory activity of TSP1 was inhibited by an antibody that recognizes its N-terminal module (35), suggesting that this domain of TSP1 stimulates endothelial cell chemotaxis. Consistent with the function of \( \alpha_6 \beta_1 \) integrin in aortic endothelial wound repair (18), the anti-\( \alpha_6 \beta_1 \) antibody P1B5 partially blocked HDMVE cell chemotaxis stimulated by TSP1 or NoC1 (Fig. 10, A and B). The anti-\( \alpha_6 \beta_1 \) antibody GoH3 partially blocked migration stimulated by TSP1, NoC1, or NoC2 (Fig. 10, A–C), demonstrating that \( \alpha_6 \beta_1 \) integrin also mediates chemotaxis of endothelial cells to TSP1 and TSP2. The effects of the \( \alpha_6 \) - and \( \alpha_6 \) -blocking antibodies on TSP1- or NoC1-stimulated endothelial cell chemotaxis were additive (Fig. 10, A and B), indicating that both \( \alpha_6 \beta_1 \) and \( \alpha_6 \beta_1 \) integrins are necessary for migration to TSP1. Only the \( \alpha_6 \)-blocking antibody inhibited NoC2-stimulated migration, and no additivity was observed (Fig. 10C). Given that NoC2 lacks the binding site for \( \alpha_6 \beta_1 \) integrin, \( \alpha_6 \beta_1 \) integrin may be the primary receptor mediating endothelial cell chemotaxis to TSP2.

Motility responses of endothelial cells to TSP1 involve both random (chemokinesis) and directional (chemotaxis) components (35). To define the contribution of each to HDMVE cell migration induced by the \( \alpha_6 \beta_1 \)-binding domains of TSP1 and TSP2, we exposed the cells to each with or without a gradient (Table I). TSP1, NoC1, or NoC2 was added to the upper chamber, the lower chamber, or both. Our results show that TSP1, NoC1, or NoC2 in the lower chamber gave the strongest stimulation of migration (Table I). However, in the absence of gradient, when the proteins were added to the upper and lower chambers, we also observed endothelial cell motility. These
to the lower chamber. Cells (2,406,864/H9251) peptides derived from this region of the laminin located in the C-terminal proteolytic E8 fragment (28, 29). Two to the laminin peptide AG-32.

Furthermore, we have identified a peptide sequence from that TSP1 and TSP2 are two additional ligands for this inte-
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results indicate that as reported for native TSP1, the N-terminal domains of TSP1 and TSP2 have both directional and random effects on cell migration.

**DISCUSSION**

Several extracellular proteins have been demonstrated to be ligands for α6β1 integrin, including laminin-1 (α1β1γ1), laminin-8 (α4β4γ1) (36), laminin-10/11 (α5) (37), invasin (38), fertinil-β/ADAM-2 (39), CytR1 (40), meltrin-γ/ADAM-9 (41), and human papilloma virus-16 (42). We have now demonstrated that TSP1 and TSP2 are two additional ligands for this integ.

Furthermore, we have identified a peptide sequence from TSP1 that is specifically recognized by α6β1 and demonstrated that a specific Glu residue is essential for the activity of this peptide and that of a recombinant N-terminal module of TSP1. Microvascular endothelial cell α6β1 mediates both adhesion and motility responses to TSP1 and TSP2.

Although linear peptides are sufficient for recognition by many integrins, previous attempts to define recognition sites for α6β1 in its protein ligands have yielded conflicting results. The mechanism by which laminins bind α6β1 has been the most extensively studied. An α6β1-binding site in laminin-1 is clearly located in the C-terminal proteolytic E8 fragment (28, 29). Two peptides derived from this region of the laminin α1 subunit, NRWHISYITRFAG (AG-10) and TWYKIAFQNRK (AG-32), show some specificity for antagonizing α6β1 (30), but other studies using recombinant fragments containing the same sequences argue against this hypothesis (43, 44). Consistent with the latter, we detected no activity in TSP1 peptides paralogous to the laminin peptide AG-32.

Phage display screening for α6β1 integrin ligands also failed to identify any known laminin sequences, but identified three peptides that inhibit laminin-1 binding to α6β1: VSWSFSRHRYSPPFAVS, HRWMHPHVFAVRQGAS, and FGRIPSPLAY-

**Fig. 10. α6β1-dependent endothelial cell chemotaxis to TSP1, NoC1, and NoC2.** Chemotaxis was assessed in modified Boyden chambers. HDMVE cell chemotaxis was induced by 30 μg/ml TSP1 (A) or 20 μg/ml recombinant NoC1 (B) or NoC2 (C) in medium 199 with 0.1% BSA added to the lower chamber. Cells (2–3 × 10^6/well) added to the upper chamber were allowed to migrate for 6 h at 37 °C in 5% CO2. HDMVE cell migration induced by the indicated attractants was assayed in the absence or presence of the α6β1-blocking antibody (2 μg/ml), the α6β1-blocking antibody (10 μg/ml), or both combined. Migrated cells were counted microscopically after fixation. Results are presented as the number of cells migrated per field (±S.D.).

|                 | Upper chamber | Lower chamber | Upper and lower chambers |
|-----------------|---------------|---------------|--------------------------|
| BSA             | 17.6 ± 3      | 77.3 ± 5.7    | 66.4 ± 4.6               |
| TSP1            | 15.6 ± 4.6    | 77.3 ± 5.7    | 66.6 ± 5.7               |
| NoC1            | 15 ± 2.6      | 66.3 ± 4.6    | 66 ± 6.5                 |
| NoC2            | 0             | 66.4 ± 13.7   | 66.5 ± 4.9               |

**Fig. 11. Alignment of laminin G module sequences with the α6β1-binding sequences of thrombospondins.** Alignment of laminin α3–5 sequences and strand assignments are according to Ref. 44. Only the N-terminal portion of the extended D–E loop of laminin α1G3 is shown. The underlined sequence indicates the α6β1-binding peptide identified in human (h) TSP1.

**TABLE I**

**HDMVE cell motility responses to TSP1, NoC1, and NoC2**

Migration induced by TSP1, NoC1, and NoC2 was analyzed in a modified Boyden chamber. Different gradient conditions were created by adding TSP1 (20 μg/ml) or NoC1 or NoC2 (15 μg/ml) in solution to the upper or lower chamber. Chemotactic activity is expressed as the number of cells migrated per field (±S.D.) from triplicate values.

**TYSFR** (45). These sequences also bear no relationship to our TSP1 peptide. Finally, an α6β1-binding consensus sequence was identified in ADAM-2: XD/E/ECD (46). The active ADAM-2 sequences resemble our TSP1 peptide in that they contain one or two Glu residues, the first having the same spacing from a conserved Asp residue as seen in the TSP1 sequences (Fig. 9A). However, based on differential regulation by phorbol esters and divalent cations, the activation states of α6β1 that recognize laminin and ADAM-2 may be different (47), implying that the recognition mechanisms also differ. Therefore, it remains to be determined whether TSPs and ADAM-2 share a common binding mechanism for α6β1.

Based on sequence alignment of TSP N-terminal modules with laminin G modules (27), the α6β1-binding sequence in TSP1 should span the C-terminal end of strand D and the loop between strands D and E of its predicted secondary structure (Fig. 11). Therefore, we predict that α6β1-binding sites in laminins may also reside in the D–E loops of their G modules. Based on a previous alignment of G modules (44), Glu residues are common in the D–E loops (Fig. 11). The positions of the Glu residues are variable, however; so further work is needed to determine whether any of these are part of the α6β1-binding sites in laminins. The D–E loop is surface-exposed on laminin G modules (44), suggesting that the inhibitory sequence we identified may also be exposed on the TSP N-terminal modules.

Does α6β1 play a role in angiogenesis? *In vivo* expression studies demonstrated that α6 integrins are highly expressed in capillary endothelial cells (48), although α6β1 and α6β4 could not be distinguished by this method. The α9 null mouse is not informative for this issue because α9β4 plays a critical role in
epithelial integrity that results in perinatal lethality (49). αβ4 also recognizes laminin-8 and laminin-1 E8 fragments, but we do not know whether αβ4 can recognize TSP1 or TSP2. However, an αβ4-blocking antibody prevents cord formation by endothelial cells plated on Matrigel, implicating αβ4 integrin in capillary morphogenesis (50). The αβ6 ligand Cyr61 is also a stimulator of angiogenesis (51) and promotes vascular smooth muscle chemotaxis mediated by αβ6 integrin (52). Deletion of Cyr61 disrupts developmental angiogenesis (53). Our results demonstrate that αβ6 integrin also mediates TSP1- and TSP2-stimulated chemotaxis in microvascular endothelial cells. Thus, the αβ6 ligands TSP1 and Cyr61 share at least two target cell types and some biological activities. However, a report published during the review of this manuscript identified a recognition sequence in Cyr61 for αβ6 that bears no similarity to the sequence we identified in TSP1 (25).

With the addition of αβ6, microvascular and large vessel endothelial cells are now known to differ in their expression or regulation of three TSP1 receptors. CD36 is expressed selectively in capillary endothelium (55) and is clearly required for some activities of TSP1 (14). Both αβ6 and αβ1 are generally expressed in endothelial cells in vitro, but we have shown that αβ6 functions selectively in large vessel endothelial cells, whereas αβ1 is preferentially activated in microvascular cells. We are currently investigating the mechanism for this differential integrin activation and its functional role in mediating the effects of thrombospondins on angiogenesis.

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