Inhibition of endothelial cell migration by thrombospondin-1 type-1 repeats is mediated by β_1 integrins

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The anti-angiogenic effect of thrombospondin-1 has been shown to be mediated through binding of the type-1 repeat (TSR) domain to the CD36 transmembrane receptor. We now report that the TSR domain can inhibit VEGF-induced migration in human umbilical vein endothelial cells (HUVEC), cells that lack CD36. Moreover, we identified β_1 integrins as a critical receptor in TSR-mediated inhibition of migration in HUVEC. Using pharmacological inhibitors of downstream VEGF receptor effectors, we found that phosphoinositide 3-kinase (PI3k) was essential for TSR-mediated inhibition of HUVEC migration, but that neither PLCγ nor Akt was necessary for this response. Furthermore, β_1 integrins were critical for TSR-mediated inhibition of microvascular endothelial cells, cells that express CD36. Together, our results indicate that β_1 integrins mediate the anti-migratory effects of TSR through a PI3k-dependent mechanism.

Introduction

Thrombospondin-1 (TSP1) was first identified as a major component of platelet α-granules and as a cell adhesion molecule found in the matrix (Bornstein, 2001; Lawler, 2002). Since then, TSP1 has emerged as a complex protein with domain-specific and cell type–specific effects in cell adhesion, cell signaling, wound healing, and angiogenesis (Adams and Lawler, 2004). TSP1 is a large homotrimeric glycoprotein consisting of multiple domains that bind to cell surface receptors such as integrins, the integrin-associated protein (IAP/CD47), or CD36, and to extracellular molecules including heparin sulfate proteoglycans and sulfatides (Chen et al., 2000). The binding sites for these receptors on TSP1 are dispersed throughout the molecule, with many domains binding multiple receptors. Soluble TSP1 is a specific inhibitor of angiogenesis and tumor growth in mice that mediates its effects via modulation of endothelial cell adhesion, proliferation, and motility (Good et al., 1990; Volpert et al., 1998; Iruela-Arispe et al., 1999). Although TSP1 can bind to numerous receptors, the primary anti-angiogenic activity of TSP1 has been localized to the procollagen domain and type-1 repeat (TSR) sequence (Tolsma et al., 1993b; Vogel et al., 1993; Iruela-Arispe et al., 1999). The TSR domain has been studied extensively and the crystal structure described (Tan et al., 2002).

The three TSRs of TSP1 comprise an 18-kD peptide with part of its biological activity mapped to the CSVTCG sequence (Tolsma et al., 1993a; Dawson et al., 1997), which recognizes CD36 (Asch et al., 1992). CD36 has been shown to be an important receptor for TSP1 signaling under many experimental conditions (Jimenez et al., 2000). There have also been several reports of TSP1 mediating an anti-angiogenic effect through sequence motifs other than CSVTCG in the TSR domain (Dawson et al., 1999). The carboxyl terminus, containing the IAP/CD47-binding sites, and the amino terminus, containing $\alpha_3\beta_1$-binding sites, may also have a role in TSP1's anti-angiogenic effects (Kanda et al., 1999; Chandrasekaran et al., 2000). The multiplicity of domains and receptors has made it difficult to determine the precise mechanisms by which TSP1 regulates cellular functions, including its anti-angiogenic effects.

Integrins are required for cell proliferation, survival, and migration (Howe et al., 1998; Hynes et al., 2002), and are important to the growth of new blood vessels because endothelial cells proliferate in an anchorage-dependent manner (Ingber, 1990; Meredith et al., 1993). Integrin antagonists that prevent binding

of $\alpha_v\beta_3$ (Brooks et al., 1994), $\alpha_v\beta_5$ (Friedlander et al., 1995; Kumar and Li, 2001), and $\alpha_5\beta_1$ (Kim et al., 2000) to ECMs suppress tumor growth via angiogenesis inhibition. Interestingly, certain endogenous angiogenesis inhibitors such as endostatin (Wickstrom et al., 2002; Sudhakar et al., 2003) and tumstatin (Maeshima et al., 2001; Wickstrom et al., 2002; Sudhakar et al., 2003) have been shown to bind directly to integrins.

Migration and reorganization of endothelial cells is induced both in vivo and in vitro by members of the VEGF-platelet–derived growth factor supergene family and to a lesser extent by FGF growth factor families (Nguyen et al., 1994; Ribatti et al., 2000; Ferrara, 2004). Overexpression of VEGF by tumor cells has been associated with tumor growth, metastasis, and greater risks of tumor recurrence (Ferrara, 2004). Inhibition of VEGF secretion as well as the inhibition of endothelial cell migration and proliferation increases the apoptotic rate of tumor cells and blocks capillary sprout formation (Bjorndahl et al., 2004). In addition, several reports indicate that growth factor signaling pathways can be regulated by integrin clustering and occupation (Ingber, 1990; Miyamoto et al., 1996; Tsou and Isik, 2001), suggesting crosstalk between integrin and growth factor signaling pathways.

In the tumor microenvironment, TSP1 is a potent angiogenesis inhibitor, and the loss of TSP1 expression by tumor cells contributes to the angiogenic phenotype. Endothelial cell migration is a critical component of angiogenesis (Zetter, 1980), and inhibition of endothelial migration contributes to TSP1 suppression of angiogenesis. A better understanding of the mechanism of TSP1 inhibition of endothelial cell migration could provide insight for therapeutic strategies. The purpose of the current study was to investigate the mechanism through which the TSR domain inhibits cell migration induced by VEGF. We used human umbilical vein endothelial cells (HUVEC), cells that lack the CD36 receptor, along with human microvascular endothelial cells (HMVEC) that express CD36, to investigate this mechanism. Our results are consistent with a role for β_1 integrins as mediators of TSR inhibition of endothelial cell migration.

Results

TSR inhibits VEGF-induced HUVEC migration

It has been reported that the TSRs mediate an anti-angiogenic effect through the CD36 receptor on endothelial cells (Jimenez et al., 2000). Here, we show that TSR inhibited VEGF-induced HUVEC migration in a dose-responsive manner (Fig. 1 A). In fact, TSR inhibited migration as efficiently as TSP1 (Fig. 1 A). The inhibitory effect of TSP1 and TSR on cell migration was not the result of decreased adhesion, as assessed by measuring cell attachment to fibronectin in the presence or absence of TSP1 or TSR (Fig. 1 B).

Although it has been reported that HUVEC do not express the CD36 receptor (Swerlick et al., 1992), we considered the possibility that the HUVEC used in this study might express CD36. In the presence of a known blocking antibody against CD36, TSR was still able to inhibit VEGF-induced mi-
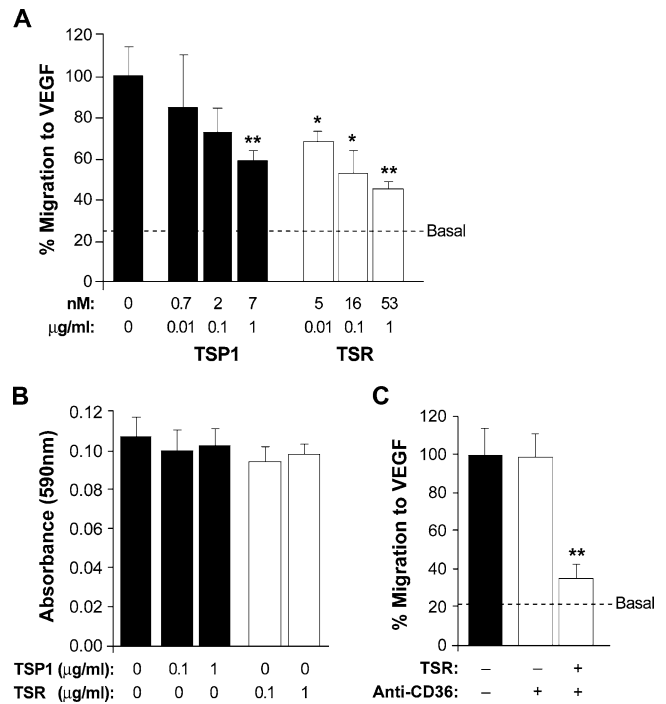

Figure 1. **TSP1 and TSR inhibit VEGF-induced HUVEC migration.** (A) A modified Boyden chamber assay was used to characterize VEGF-induced (3–5 ng/ml) migration of HUVEC. Cell migration is expressed as percentage of the maximal migration induced by VEGF. Dashed lines indicate basal migration levels, in the absence of VEGF. TSP1 and TSR concentrations are presented as both nM and μg/ml. Where indicated, suspended cells were treated with TSP1 (solid bars) or TSR (open bars) for 30 min, placed in migration chambers, and allowed to migrate to VEGF for 4 h. (B) The effect of cell adhesion in TSP1/TSR-treated cells was measured. HUVEC were pretreated with either TSP1 (solid bars) or TSR (open bars) for 30 min before plating on fibronectin-coated wells for 1.5 h. Adhesion was determined by measuring the absorbance at 590 nm of crystal violet–stained cells. (C) HUVEC were treated with an antibody against CD36 (5 μg/ml) for 10 min, followed by the addition of TSR (16 nM) for 30 min and were allowed to migrate to VEGF for 4 h. Error bars indicate SDs. *, $P < 0.05$, **, $P < 0.01$ compared with VEGF alone as determined using t test for unpaired data.

gration in HUVEC (Fig. 1 C). FACS analysis also confirmed the absence of CD36 in HUVEC compared with HMVEC, which express significant levels of this protein (unpublished data). These findings demonstrate that TSR can repress VEGF-stimulated HUVEC migration and suggests that this effect is mediated in the absence of CD36.

TSR inhibits HUVEC migration through β_1 integrins

Because TSP1 binds to β_1 and β_3 integrins via various domains across TSP1, we considered the possibility of TSR associating with β integrins in HUVEC. FACS analysis showed abundant levels of β_1 on these cells (Table I). Antibodies against β_1 and β_3 integrin subunits were used to examine the role of these receptors in TSR inhibition of VEGF-induced migration. A β_1 integrin antibody (clone PC410) was able to inhibit cell migration in a dose-dependent manner (Fig. 2 A). The PC410 β_1 antibody is also able to activate the β_1 integrin signaling pathway (Miller et al., 1999). When used at 2 μg/ml, a concentration that has only a slight effect on VEGF-induced migration,

Table I. **Alpha integrin subunit expression in HUVEC**

Integrin subunit	% maximal mean expression
β_1	1.000
α_1	0.014
α_2	1.050
α_3	0.870
α_4	0.003
α_5	0.930
α_6	0.620
α_v	0.980

Cells (0.5×10^6) were incubated with primary antibodies, washed, incubated with IgG conjugated to R-phycoerythrin, fixed, and analyzed for fluorescence using flow cytometry. Relative fluorescence intensity is expressed as a percentage of β_1 fluorescence.

the PC410 antibody was able to prevent the inhibition of migration mediated by TSR (Fig. 2 B). On the contrary, a nonactivating β_1 antibody (MAB2000) had no protective effect (Fig. 2 B). Similarly, a β_3 integrin antibody could not rescue TSR-

inhibited migration (Fig. 2 C). Collectively, these data suggest that the inhibitory effect of TSR on cell migration is overcome by the activation of β_1 integrins.

β integrin small interfering RNA (siRNA) was used to confirm the role of β_1 integrins in the TSR-inhibited migration. Fig. 2 D shows that β_1 and β_3 integrin levels were significantly reduced in cells transfected with β_1- or β_3-siRNA compared with cells transfected with a control siRNA. Silencing of β_1 or β_3 integrins did not prevent VEGF-induced cell migration (Fig. 2 E, solid and striped bars, respectively). We hypothesized that in HUVEC, β_3 integrins mediated adhesion and migration when β_1 levels were reduced by siRNA. It has previously been shown that overexpression of nonfunctional β_1 integrin stabilized β_3 integrin mRNA (Retta et al., 2001). Moreover, in microvascular endothelial cells (Cheng et al., 1991) and smooth muscle cells (Clyman et al., 1992), β_3 integrins were shown to mediate cell adhesion and migration on fibronectin. In cells with reduced β_1 levels, TSR was ineffective in inhibiting VEGF-induced migration (Fig. 2 E, solid bars). In contrast, reducing the level of β_3
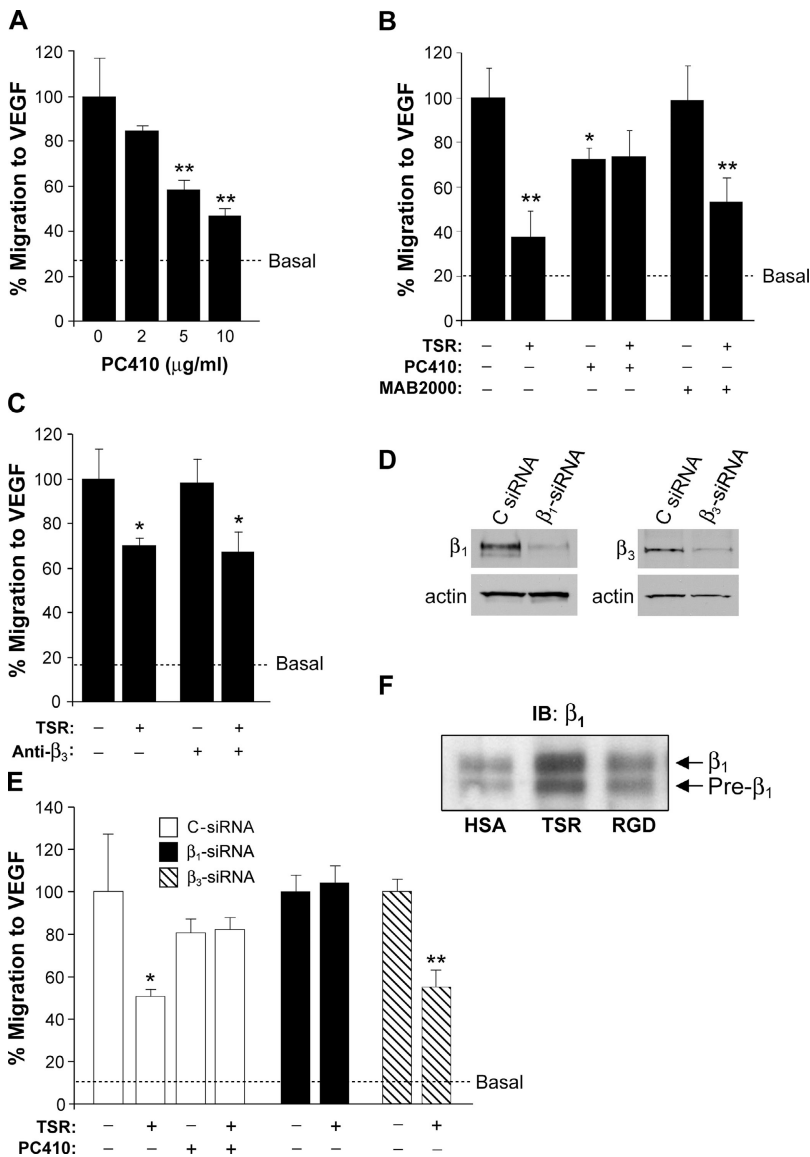

Figure 2. **TSR inhibits migration through β_1 integrins.** (A) Cells were treated with increasing concentrations of an antibody (clone PC410) against β_1 and were allowed to migrate to VEGF for 4 h. (B) Cells were treated with antibodies against β_1 (2 μg/ml) followed by addition of TSR (16 nM) before migration to VEGF for 4 h. Treatment with the PC410 β_1 antibody prevented TSR from inhibiting migration, whereas MAB2000 β_1 antibody had no effect. (C) Treatment with the β_3 antibody had no effect on TSR-mediated inhibition of migration. Cells were treated with an antibody against β_3 (2 μg/ml) followed by addition of TSR (16 nM) before migration to VEGF for 4 h. (D) HUVEC were transfected with either control siRNA (C-siRNA) or β_1-or β_3-siRNA. 72 h after transfection, cells were lysed and integrin β_1 and β_3 levels were analyzed by Western blotting. Membranes were stripped and probed for actin to show equal protein loading. (E) siRNA-transfected cells were used in the migration assay 72 h after transfection. Where indicated, control siRNA cells were treated with the PC410 β_1-activating antibody followed by treatment with TSR (16 nM) for 30 min and were then allowed to migrate to VEGF for 4 h. (F) Ligand-coated Spherotech beads were incubated with suspended HUVEC, beads were isolated, and protein resolved by SDS-PAGE. Beads coated with TSR and RGD (but not HSA) show specific binding to β_1 integrins. *, $P < 0.05$, **, $P < 0.01$ compared with relevant control (VEGF alone or antibody alone, respectively) as determined using a t test for unpaired data.

Figure 3. **TSR complexes with and functions through $\alpha_5\beta_1$ integrins.** (A) Cells were treated with α subunit antibodies (2 μg/ml) for 15 min, treated with TSR (16 nM) for 30 min, and allowed to migrate toward VEGF for 4 h. (B) Spherotech magnetic substrate-coated beads were used to prepare supramolecular complexes from suspended HUVEC. Western blotting (WB) analysis using an $\alpha_5\beta_1$ integrin antibody shows major bands in fractions prepared from beads coated with TSR, RGD, and β_1 antibodies BD15 and MAB2000 (β_1), but not for HSA (top). The blot was stripped and probed for β_1 (bottom). (C) Spherotech magnetic substrate-coated beads were used to prepare supramolecular complexes from HUVEC lysate using 1% NP-40 lysis buffer. Membranes were probed with an $\alpha5\beta1$ antibody. (D) Fibronectin-adherent HUVEC treated with Spherotech magnetic substrate-coated beads were immunostained for $\alpha_5\beta_1$ (bottom). Arrows show a representative bead by phase contrast (top). The arrowhead denotes a cell margin (top). (E) Quantification of the percent positive beads presented in D. *, $P < 0.05$, **, $P < 0.01$ compared with VEGF alone as determined using t test for unpaired data.
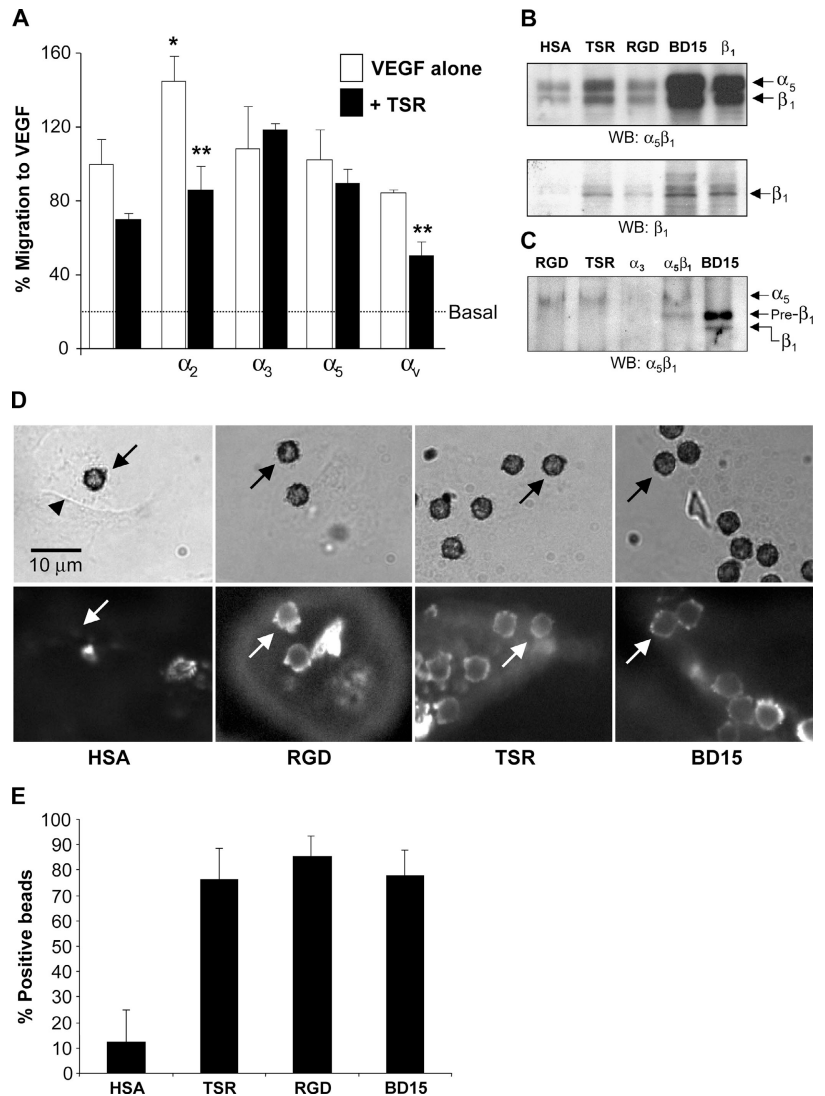
had no effect on TSR's ability to inhibit cell migration (Fig. 2 E, striped bars). In cells that were transfected with control siRNA, TSR was able to inhibit VEGF-induced migration, and this inhibition was diminished by the presence of PC410 (Fig. 2 E, open bars). Together, these results indicate that inhibition of VEGF-induced HUVEC migration by TSR is strictly dependent on levels of β_1 integrin expression and activation.

TSR complexes with β_1 integrins

Next, we examined whether there was a direct interaction between TSR and β_1 integrins in HUVEC using protein-coated beads. Beads coated with TSR, an adhesion tripeptide Arg-Gly-Asp (RGD), or horse serum albumin (HSA) were incubated with suspended HUVEC and bead fractions were separated on SDS-PAGE and analyzed by Western blotting. The immature ($\sim$105 kD) and the mature ($\sim$125 kD) forms (Salicioni et al., 2004) of β_1 integrins were detected in fractions prepared from beads coated with either RGD or TSR peptides (Fig. 2 F), but not from beads coated with HSA, indicating that TSR has the ability to bind β_1 integrins with the same efficiency as RGD peptide, the canonical RGD β_1 ligand.

TSR functions through β_1 integrins

Next, we sought to identify the α subunit that β_1 partners with for TSR-mediated inhibition of VEGF-induced migration. FACS analysis showed the expression levels of various α subunits in HUVEC (Table I). Abundant levels of α_2, α_3, α_5, and α_v, lower levels of α_6, and minimal levels of α_1 were detected. Although a recent report shows α_4 expressed in HUVEC (Calzada et al., 2004b), barely detectable levels of α_4 were observed here. Several antibodies against α subunits were examined in the migration assay. Blocking either α_3 or α_5 prevented TSR from inhibiting VEGF-induced cell migration, whereas antibodies to the α_2 and α_v subunits had no significant effect (Fig. 3 A).

To examine this in further detail, suspended HUVEC were incubated with protein-coated beads, and bead fractions were prepared and analyzed by Western blotting. Both α_5 and β_1 integrins were detected in fractions prepared from beads coated with either RGD or TSR peptides as well as beads coated with specific β_1 integrin antibodies, whereas barely detectable levels were present in fractions prepared from beads coated with HSA (Fig. 3 B, top). β_1 integrins were detected in

peptide- and antibody-coated beads, but not with HSA-coated beads (Fig. 3 B, bottom). Interestingly, $\alpha_5\beta_1$ integrins were the only dimers detected when β_1 antibody-coated beads were used with biotinylated HUVEC (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200407060/DC1), indicating that in our experimental conditions, $\alpha_5\beta_1$ integrins were the predominant β_1 dimers expressed.

To determine whether α_5 or β_1 integrins were the preferential receptor for TSR, immunoprecipitation experiments were performed with HUVEC lysate. Fig. 3 C shows that only the α_5 subunit was detected in RGD and TSR peptide-coated bead immunoprecipitates, whereas BD15 antibody-coated beads specifically recognize β_1 integrins. As expected, the $\alpha_5\beta_1$-coated beads recognize both α_5 and β_1. No specific signal was detected with α_3 integrin antibody-coated beads, even when the membrane was stripped and reprobed with an α_3-specific antibody (unpublished results). These data suggest that in native conditions, both α_5 and β_1 integrins are potential receptors for TSR.

We also examined whether TSR-coated beads were able to bind and cluster with $\alpha_5\beta_1$ integrins in HUVEC. Adherent cells were incubated with beads coated with HSA, BD15, TSR, or RGD and immunostained for $\alpha_5\beta_1$. Beads coated with TSR showed a distinct staining for $\alpha_5\beta_1$ (Fig. 3 D, bottom). Beads adherent to HUVEC are evident by phase contrast (Fig. 3 D, top). Quantification of these results using computerized image analysis confirmed that >75% of the beads coated with RGD, TSR, or BD15 displayed $\alpha_5\beta_1$ integrin staining, whereas <15% of the HSA-beads were positive (Fig. 3 E). Together, our results suggest that TSR inhibition of HUVEC migration is mediated by binding to the $\alpha_5\beta_1$ integrin, a major fibronectin receptor in HUVEC.

TSR inhibition of VEGF-induced migration depends on PI3k

Phosphoinositide 3-kinase (PI3k) and PLCγ were examined as potential downstream targets of TSR function in VEGF-induced HUVEC migration. As expected, treatment of cells with a PLCγ inhibitor, U73122, resulted in a dose-responsive inhibition of migration (Fig. 4 A). In the presence of TSR, addition of U73122 appeared to have an additive effect on inhibiting migration (Fig. 4 B). Because PLCγ inhibition did not interfere with the inhibitory effects of TSR, it is unlikely that PLCγ function is a rate-determining component of TSR-mediated inhibition.

In the presence of the PI3k inhibitor, LY294002, there was a dose-responsive inhibition of cell migration (Fig. 4 C). Similar results were shown using wortmannin (unpublished data). Addition of TSR did not further reduce migration in the presence of LY294002 (Fig. 4 D), suggesting PI3k as a candidate for mediation of TSR effects.

PI3k functions through β_1 integrins in VEGF-induced migration

Considering our results indicating that TSRs inhibitory effects on migration depend on PI3k and β_1, we next examined whether β_1 integrins mediate TSR's inhibitory effects via PI3k. Inhibition of cell migration induced by treatment with
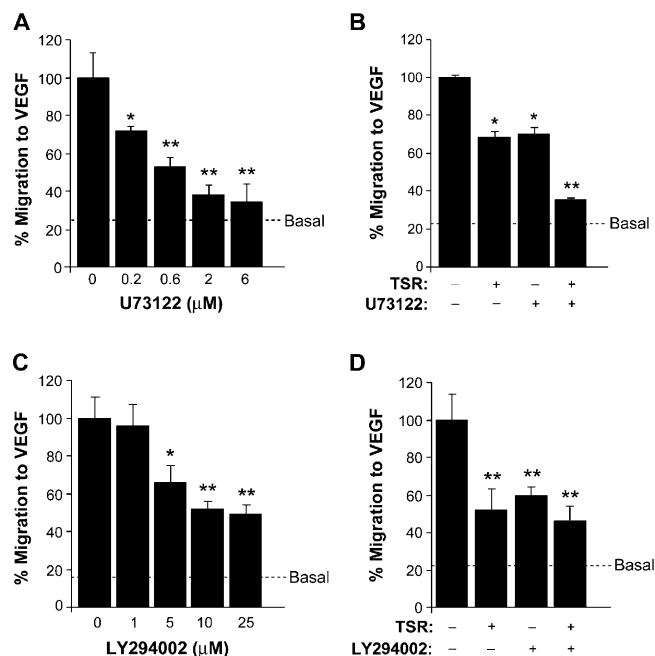
Figure 4. **TSR inhibition of migration is dependent on PI3k, but independent of PLCγ.** (A) HUVEC were treated with the indicated concentrations of the PLCγ inhibitor U73122 and were allowed to migrate to VEGF (5 ng/ml) for 4 h. (B) HUVEC were treated with U73122 (2 μM) for 10 min followed by treatment with TSR (16 nM) for 30 min and allowed to migrate to VEGF for 4 h. (C) Cells were pretreated with increasing amounts of a PI3k inhibitor, LY294002, and allowed to migrate to VEGF (5 ng/ml) for 4 h. (D) Cells were treated with LY294002 (5 μM) for 10 min followed by treatment with TSR (16 nM) for 30 min, and allowed to migrate to VEGF for 4 h. *, P < 0.05, **, P < 0.01 compared with VEGF alone as determined using t test for unpaired data.

LY294002 was blocked by preincubation of cells with PC410, whereas MAB2000 had no protective effect (Fig. 5 A). These results suggest that the role of PI3k on cell migration is at least in part dependent on the level of activation of β_1 integrins at the time TSR was added. Interestingly, we found that the p85 subunit of PI3k coimmunoprecipitated with β_1 integrins (Fig. 5 B), indicating that a pool of PI3k was directly associated with β_1 integrins in HUVEC. Using β_1 siRNA, we next examined whether the PI3k associated with β_1 integrins was responsible for LY294002 mediated inhibition of cell migration. In cells with diminished levels of β_1 integrins, pretreatment with LY294002 alone or in association with TSR was not able to inhibit VEGF-induced migration (Fig. 5 C, solid bars) compared with control siRNA-treated cells (Fig. 5 C, open bars). Conversely, overexpression of the myristoylated p110 subunit of PI3k, a constitutively active form of PI3k associated with the cell membrane, prevented both LY294002 and TSR from inhibiting migration (Fig. 5 D, solid bars). The functionality of the overexpressed p110 was confirmed by increased Akt phosphorylation levels in the transfected cells (Fig. 5 D, inset). These data further support the hypothesis that TSR inhibits HUVEC migration via regulation of the PI3k activity associated with β_1 integrins.

Contrary to what was observed with the PI3k inhibitor, inhibition of VEGF-induced cell migration using U73122 could not be rescued using PC410, indicating that although

Figure 5. TSR requires PI3k and β_1 integrin for inhibiting migration. (A) Where indicated, cells were treated with antibodies against β_1 (PC410 or MAB2000; 2 μg/ml) for 10 min, followed by treatment with either LY294002 (5 μM) or U73122 (2 nM) for 15 min and were allowed to migrate to VEGF for 4 h. (B) The p85 subunit of PI3k coimmunoprecipitates with β_1 integrins. Near-confluent HUVEC were lysed and 200 μg of protein was immunoprecipitated with either anti-β_1 antibody (MAB2000) or a mouse IgG control antibody. Immunocomplexes were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was probed with an mAb against the p85 subunit of PI3k, stripped, and reprobed with an mAb against β_1. (C) TSR requires β_1 integrins and PI3k to inhibit migration. Cells were transfected with either control- or β_1-siRNA. Cells were treated with LY294002 (5 μM) for 10 min, then treated with TSR (16 nM) for 30 min and allowed to migrate to VEGF for 4 h. (D) Cells were infected with pLNCX retrovirus, either empty (vec) or containing myristoylated p110 (m110). Cells were split 24 h after infection, and 48 h later subjected to a migration assay or lysed for Western analysis. For the migration assay, cells were treated with either LY294002 (10 μM) or TSR (16 nM) and allowed to migrate to VEGF for 4 h. *, $P < 0.05$, **, $P < 0.01$ compared with relevant control (VEGF alone or antibody alone, respectively) as determined using a t test for unpaired data.
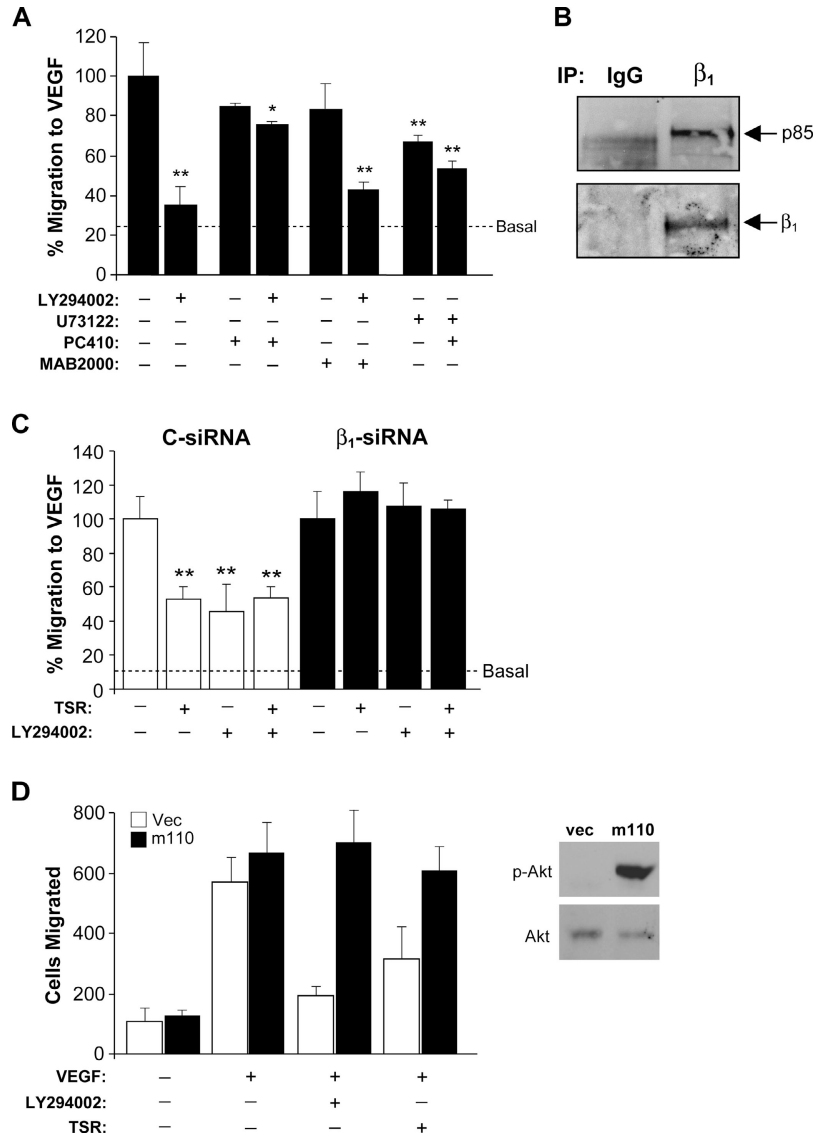
PLCγ functions downstream from the VEGF receptor in migration, it does not require β_1 integrins for activity (Fig. 5 A).

Active Akt is not required for VEGF-induced migration

Because Akt is a downstream target of PI3k, we examined whether it is part of the TSR-β_1-PI3k pathway. TSR had no effect on VEGF-induced Akt phosphorylation, whereas treatment with LY294002 inhibited VEGF-induced Akt phosphorylation (Fig. 6 A, top). Furthermore, TSR caused no additional decrease in Akt phosphorylation in combination with LY294002. MAPK phosphorylation was then examined by reprobing the blot. As expected, neither TSR nor LY294002 treatments had any effect on MAPK phosphorylation induced by VEGF (Fig. 6 A, bottom). We also examined the effect of down-regulation of Akt expression by siRNA on cell migration. The dramatic decrease of Akt protein levels (Fig. 6 B, inset) had no significant effect on VEGF-induced migration. Interestingly, treatment with TSR and LY294002 was able to significantly inhibit migration in both control siRNA cells and Akt-siRNA cells

(Fig. 6 B). These results suggest that although PI3k is necessary for VEGF-induced migration, activation of its downstream effector, Akt, is not required.

TSR inhibits migration through β_1 integrins in cells expressing CD36

Thus far, our data show a dependence on β_1 integrins for TSR's inhibitory effect on HUVEC migration, cells that lack CD36. We also examined the role of β_1 integrins in HMVEC, cells that endogenously express CD36. In the presence of a CD36 antibody, we show that TSR is no longer able to inhibit VEGF-induced HMVEC migration, as anticipated (Fig. 7 A). Surprisingly, however, in the presence of this antibody, the full length TSP1 could still inhibit VEGF-induced migration (Fig. 7 A). These data suggest the presence of an additional receptor mediating the anti-migratory effects. Interestingly, addition of the PC410 antibody was sufficient to prevent both TSP1 and TSR from inhibiting VEGF-induced migration (Fig. 7 B). Similar to HUVEC, an antibody against the β_3 subunit had no effect on either TSP1 or TSR inhibition of HMVEC migration (Fig. 7 B).
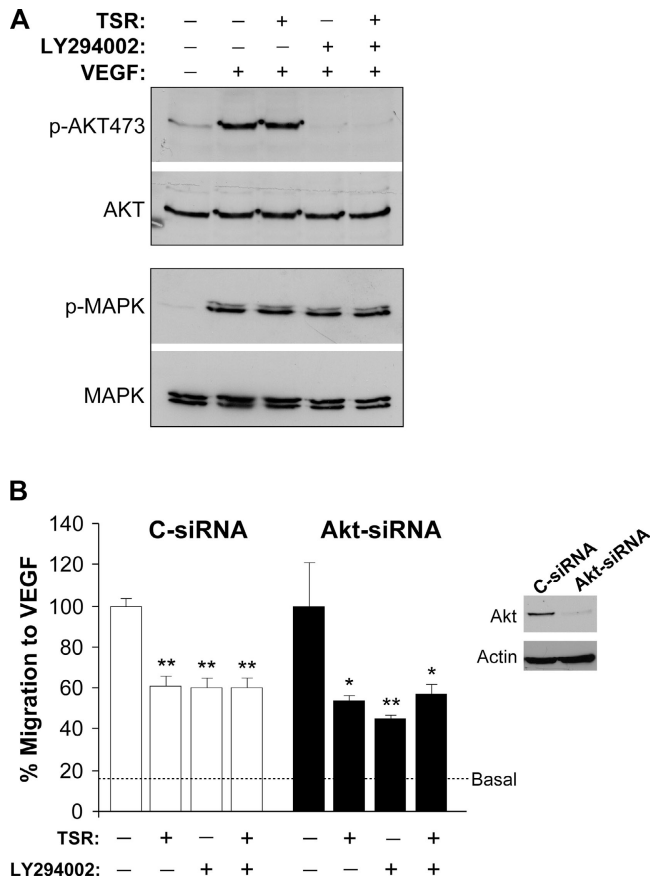
Figure 6. **TSR does not require Akt to inhibit migration.** (A) Where indicated, near-confluent and serum-starved HUVEC were treated with LY294002 (5 μM) for 10 min, followed by treatment with TSR (16 nM) for 2 h and then with VEGF (5 ng/ml) for 5 min before cell lysis. Lysates were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed for either phospho-ser473Akt or phospho-MAPK, stripped, and probed for total Akt or MAPK. (B) Cells were transfected with Akt siRNA as described in Materials and methods. Where indicated, cells were treated with LY294002 (5 μM) for 10 min, then treated with TSR (16 nM) for 30 min and allowed to migrate to VEGF (5 ng/ml) for 4 h. The inset is a Western blot showing Akt-siRNA treatment greatly reduces total Akt protein levels. *, P < 0.05; **, P < 0.01 compared with VEGF alone as determined using a *t* test for unpaired data.

Figure 7. **TSP1 and TSR inhibit migration through β_1 integrins in cells expressing CD36.** (A) HMVEC-d were treated with a blocking antibody against CD36 (5 μg/ml) for 10 min, followed by the addition of either TSP1 (7 nM) or TSR (5 nM) for 30 min. Cells were then placed in Transwell chambers and allowed to migrate to VEGF for 4 h. (B) HMVEC-d were treated with antibodies (2 μg/ml) against either β_1 (PC410) or β_3 integrins for 10 min followed by the addition of either TSP1 (7 nM) or TSR (5 nM) for 30 min. Cells were allowed to migrate to VEGF (5 ng/ml) for 4 h. Error bars indicate SDs. *, P < 0.05, **, P < 0.01 compared with relevant control (VEGF alone or antibody alone, respectively) as determined using a *t* test for unpaired data.

These data support a potentially important role for β_1 integrins in facilitating the anti-migratory effect of TSP1 on endothelial cells, even when CD36 is expressed.

Discussion

Soluble TSP1 inhibits angiogenesis by interacting directly with endothelial cells. Several recent studies indicate that down-regulation of TSP1 may have a critical role in the switch to an angiogenic phenotype (Campbell et al., 1998; Laderoute et al., 2000; Doll et al., 2001; Watnick et al., 2003). It is well documented that endothelial cell migration and proliferation are critical components of angiogenesis (Folkman, 1995), yet neither the mechanism of VEGF-mediated endothelial cell migration nor the inhibitory effects of TSP1 are fully understood. Several studies have reported that the anti-angiogenic effects of TSP1 are mediated at least in part by CD36, which triggers a signaling
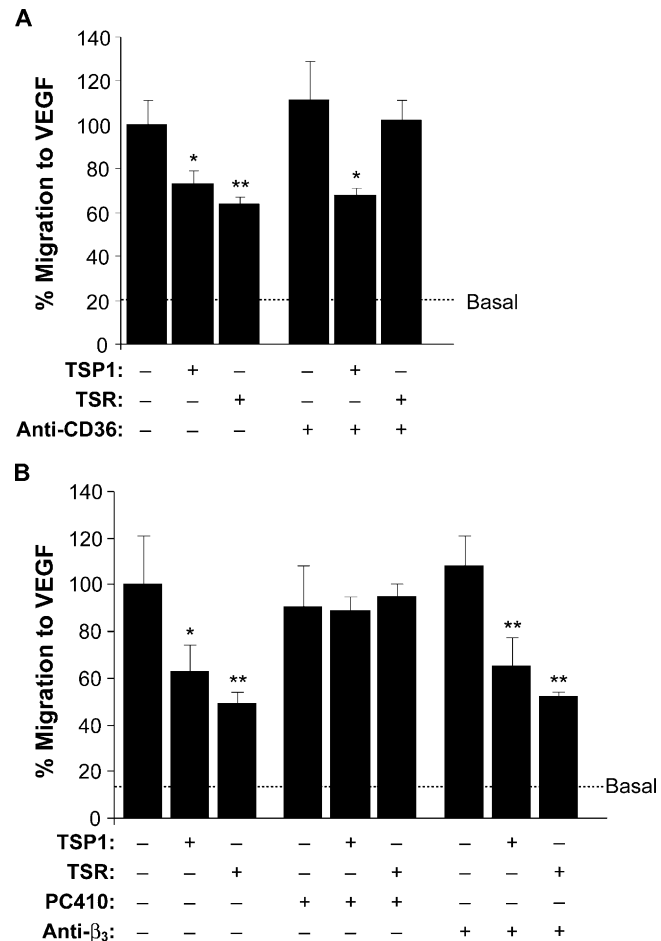
cascade leading to endothelial cell apoptosis and collapse of tumor vessels (Dawson et al., 1997; Jimenez et al., 2000).

Our study has focused on exploring the mechanism by which the TSR domain of TSP1 inhibits VEGF-induced migration of HUVEC. VEGF was used as the chemoattractant to induce migration, as we have previously shown VEGF to be a more potent chemotactic factor for HUVEC migration than bFGF (Yoshida et al., 1996). In fact, VEGF has recently been shown to be the principal effector of the angiogenesis pathway in endothelial cells, whereas FGF acts primarily by increasing VEGF expression (Ferrara, 2004). Consistent with a recent report (Calzada et al., 2004b), we find that TSP1 inhibits VEGF-mediated HUVEC migration. Therefore, we investigated whether TSR, the previously identified anti-angiogenic domain of TSP1, mediated inhibition of HUVEC migration. We found that TSR inhibits VEGF-induced HUVEC migration via a CD36-independent pathway.

TSP1 recognizes multiple receptors, several of which are integrins. In particular, various domains of TSP1 can bind β integrins. Furthermore, several reports indicate that growth factor signaling pathways can be regulated by integrin clustering and occupation (Ingber, 1990; Miyamoto et al., 1996; Tsou and Isik, 2001), suggesting crosstalk between integrin and growth factor signaling pathways (Zachary and Gliki, 2001). We hypothesized that TSR may function through β_1 integrins as an alternative pathway to CD36. We found, by using either a β_1-activating antibody or by suppressing β_1 integrins, that TSR was unable to inhibit VEGF-induced migration, indicating that β_1 is necessary for TSR inhibition of VEGF-induced migration.

We also found a direct interaction of TSR with β_1 integrins by coimmunoprecipitation and colocalization. Because TSR does not contain an RGD sequence, a sequence present in several ECM proteins that is recognized by integrins, our data further indicate that TSR binds β_1 through an RGD-independent sequence. This is consistent with a recent report showing involvement of β_1 in TSR inhibition of cell adhesion (Calzada et al., 2004a). Endostatin, for example, has been shown to bind to $\alpha_5\beta_1$ (Wickstrom et al., 2002; Sudhakar et al., 2003) through a non-RGD sequence that promotes endothelial cell adhesion (Wickstrom et al., 2004). Our data indicate that TSR also binds through $\alpha_5\beta_1$. α5 integrins were highly expressed in our cells, and blocking antibodies to α_5 subunits were able to suppress TSR inhibition of cell migration. We found that $\alpha_5\beta_1$ binds directly to TSR, and is the most likely target for TSR binding. However, α_3 subunits were also highly expressed in our cells, and blocking antibodies to α_3 were able to suppress the TSR inhibition of cell migration, despite the lack of any direct evidence for binding of TSR to α_3. A potential alternate explanation could be activation of the $\alpha_3\beta_1$ integrins downstream of TSR binding to $\alpha_5\beta_1$.

We also investigated the role of downstream signaling molecules as effectors of TSR binding to β_1 integrins. Thus, we examined two signaling proteins, PLCγ and PI3k, that have been shown to be downstream of VEGF-induced migration in endothelial cells (Shuster and Herman, 1998; Qi and Claesson-Welsh, 2001). Consistent with earlier reports (Zeng et al., 2001), we found that inhibition of PLCγ activation was able to block VEGF-mediated HUVEC migration. Because simultaneous pretreatment of HUVEC with TSR plus a PLCγ inhibitor had an additive effect on inhibiting migration, we concluded that PLCγ is not a rate-limiting effector of the TSR-signaling pathway.

There are numerous reports of β_1 integrins functioning through PI3k in other cell types (Berrier et al., 2000; Reyes-Reyes et al., 2001; Woods et al., 2001; Armulik et al., 2004). Our results indicate that PI3k is critical for the β_1-dependent inhibition of HUVEC migration by TSR. These data are consistent with a published report (Dimmeler et al., 2000) showing that inhibition of PI3k alone was sufficient to reduce VEGF-induced cell migration. Treatment with TSR was able to inhibit cell migration as efficiently as the PI3k inhibitors. Simultaneous treatment with TSR and LY294002 had no additive effect beyond that of either LY294002 or TSR alone, implying that these two agents operate via a common pathway. We also found that PI3k complexes with β_1 integrins. In addition, the PI3k inhibitor LY294002 did not inhibit migration in the presence of the β_1-activating antibody or in cells where β_1 integrin levels were decreased. These results indicate that inhibition of the pool of PI3k available to associate with β_1 integrins is sufficient to inhibit VEGF-induced migration. In support of this hypothesis, a recent report showed that blockage of α_5 integrins in colon cancer was able to decrease PI3k activation leading to inhibition of cell attachment and induction of apoptosis (Murillo et al., 2004). Another study has identified the membrane-proximal segment domain of β_1 integrins as an important regulator of PI3k-dependent signaling (Armulik et al., 2004).

Akt is a downstream target of PI3k for VEGF-induced proliferation (Gerber et al., 1998; Thakker et al., 1999). Moreover, a recent report showed that migration is increased by activating Akt (Dimmeler et al., 2000). Here, we found that active Akt is not required for VEGF-induced migration, as migration was not decreased when Akt levels were silenced. Furthermore, decreasing Akt expression had no effect on TSR inhibition of migration. Altogether, our data raise the possibility that β_1 integrins can regulate cell migration via a PI3k-dependent, Akt-independent pathway. This is not entirely unexpected because the time frame of these effects is not consistent with the cell survival role of Akt.

Together, our data support a pathway for VEGF-mediated HUVEC migration that requires activation of both β_1 integrins and PI3k, as VEGF-stimulated HUVEC migration is blocked by either β_1 silencing or by inhibition of PI3k activity. TSR binds to $\alpha_5\beta_1$ integrins outside of their RGD-binding site. We propose that this binding allows adhesion of the heterodimer to the ECM but prevents integrin conformational changes proximal to the membrane, which then blocks the activation of PI3k and results in the inhibition of cell migration. In the absence of β_1, the residual HUVEC migration is probably mediated via β_3 integrins. Under these conditions, TSR no longer inhibits migration because it does not bind β_3. This alternate pathway for VEGF-mediated migration does not proceed via PI3k because the PI3k inhibitor was unable to block migration in the absence of β_1 integrins.

Our study describes a novel pathway for TSR inhibition of VEGF-induced endothelial migration through β_1 integrins. We also report that β_1 integrins contribute to TSR's inhibitory effect on migration in HMVEC, cells that endogenously express CD36. In HMVEC, a cell type that expresses both CD36 and β_1 integrins, each receptor may differentially contribute to TSP1-mediated migration inhibition depending upon microenvironmental cues and on the nature of the migration stimulus. Because fragments of TSP1 and analogs of TSP1 domains are currently being tested in the clinical setting as anti-cancer drugs, it will become increasingly important to understand the nature of the receptors by which TSP1-derived reagents exert their anti-endothelial cell effects.

Materials and methods

Materials

mAbs (from CHEMICON International) against integrin subunits included: activating anti-β_1 (clone P4C10), used for FACS and migration assays; a nonactivating anti-β_1, MAB2000, used in migration assays and immunoprecipitation; anti-β_1 (clone B3B11), used for immunoblotting; anti-β_3 (clone 25E11) used in migration assays; the β integrin partners kit for α

subunits; and $\alpha_5\beta_1$ (AB1950) for immunoblotting. β_1 mAbs K20 (Beckman Coulter) and BD15 (Biosource) were used to coat beads, and β_1 monoclonal clone W1B10 (Sigma-Aldrich) was used to detect β_1 from bead supramolecular complexes. β_3 mAb (Biosource International) was used for immunoblotting. pAbs recognizing MAPK (C-16), His (G-18), and β-actin were purchased from Santa Cruz Biotechnology, Inc. Phosphorylated Akt (ser473) antibody was from Cell Signaling and antibody against total Akt was from Upstate Biotechnology. Anti-active MAPK was from Promega. The antibody to CD36 was from NeoMarker (Clone 185-1G2). The anti-p85 mAb was from BD Biosciences. Recombinant VEGF was provided by Genentech, Inc. Fibronectin was from BD Biosciences. All other reagents and chemicals were from Sigma-Aldrich unless otherwise indicated.

Proteins
TSP1 and the thrombospondin peptide, TSR, were isolated or prepared as described previously (Miao et al., 2001). The TSR peptide contained all three TSRs.

Cell culture
Primary HUVEC and dermal HMVEC (Cambrex/Biowhittaker) were maintained according to the supplier's directions. For experiments, cells were grown to subconfluence and used between passages 3–7 for HUVEC and passages 4–8 for HMVEC.

Adhesion assay
96-well plates (Falcon) coated with 10 μg/ml fibronectin were washed with PBS and blocked with endothelial basal medium (EBM) containing 2% BSA. Where indicated, cells were pretreated with TSP1 or TSR for 30 min. 4×10^4 cells/well in EBM/2% BSA were plated in quadruplicate and incubated for 1.5 h at 37°C. Cells were washed with PBS, fixed, and stained in 0.5% crystal violet in 20% MeOH/PBS. Cells were washed extensively with H_2O, solubilized with 1% SDS, and absorbance read at 590 nm.

Migration assay
Cell migration assays were performed using modified Boyden chambers (Transwell-Costar Corp.) coated with 10 μg/ml fibronectin. Subconfluent cells were trypsinized (0.01% trypsin/5 mM EDTA; Cambrex), neutralized (Cascade Biologics, Inc.), washed with EBM/0.1% BSA, and resuspended. Cells were pretreated with inhibitors for 30 min in suspension. Typically, 5×10^5 cells were added to the top of each migration chamber and were allowed to migrate to the underside of the chamber for 4 h in the presence or absence of VEGF (3–5 ng/ml) in the lower chamber. Cells were fixed and stained (Hema 3 Stain System; Fisher Diagnostics). The number of migrated cells per membrane was captured using bright-field microscopy connected to a Spot digital camera (Diagnostic Instruments). Migrated cells from the captured image were counted using NIH Image software. Each determination represents the average of three individual wells, and error bars represent the SD. Migration was normalized to percent migration, with migration to VEGF alone representing 100% migration. Due to the variability inherent in endothelial cell migration assays, a threefold increase in VEGF-induced migration over basal levels was considered the minimum criteria for an experiment to be considered valid. Each experiment was repeated a minimum of three times to ensure data consistency. Data were analyzed with a two-sided unpaired t test. For all statistical comparisons, cell treatments were compared with VEGF alone, except in experiments where antibodies were used, in which case cell treatments were compared with antibodies alone.

siRNA transfection
β_1 and β_3 integrin siRNAs were purchased as four oligonucleotide SmartPools (Dharmacon Research, Inc.), containing an approximate 50% guanidine-cytosine content (Dharmacon Research, Inc.). The control sequence used was fluorescein siRNA with an approximate guanidine-cytosine content of 50% (QIAGEN). The Akt siRNA was commercially available as a SmartPool (Cell Signaling). HUVEC were trypsinized, washed with HBSS, and resuspended (10^6 cells) in HUVEC solution (Amaxa Biosystems) containing 1 nM siRNA duplex, and were transfected using a Nucleoporator (Amaxa Biosystems) following the manufacturer's instructions. After transfection, cells were immediately plated in dishes containing complete media. The following day cells were split, and 48 h later either lysed or subjected to migration assays, as described.

Cell treatment, immunoprecipitation, and immunoblotting
For conditions where lysates were examined by Western analysis, subconfluent HUVEC were serum starved for 4 h before treatment with pharmacological inhibitors, TSR, or growth factors. Pharmacological inhibitors were

added for 15 min. TSR was typically added for 2–4 h. VEGF was added for 5 min before cell harvest. Lysate preparation and Western analysis were performed as described previously (Short et al., 1998). For immunoprecipitations, cell lysates were incubated with primary antibody overnight at 4°C, followed by incubation with protein G–Sepharose for 1 h at 4°C. Bead complexes were washed with cold radioimmunoprecipitation assay buffer and boiled with SDS-PAGE sample buffer. Immunoreactivity was detected on Hyperfilm using ECL (Amersham Biosciences). Images from ECL autoradiograms were captured using Adobe software.

Bead coating and isolation of supramolecular complexes
Aliquots of Sphero carboxyl Ferro magnetic beads (4.5 μm; Spherotechnic) were coated as recommended by the manufacturer. In brief, beads were coated for 2 h in 0.01 M sodium acetate, pH 5.0, containing 1.25 mg/ml EDAC (Sigma-Aldrich) and 0.3 mg ligand/ml beads. Beads were washed with HBSS/1% HSA and kept in the same solution until added to cells. HUVEC were incubated for 2 h in HBSS/1% HSA, briefly trypsinized, washed, and held in suspension for 30 min at RT. Cells (0.15 $\times$ 10^6 per condition) were resuspended in HBSS/0.1% HSA containing 0.1 mM $MnCl_2$. Isolation of supramolecular complexes was performed as described previously (Plopper and Ingber, 1993; Green et al., 1999). SDS-PAGE sample buffer was added, and samples were heated for 10 min at 65°C and analyzed by SDS-PAGE and Western blotting.

For immunoprecipitation experiments, suspension cells were pelleted and lysed with 1% digitonin buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 25 mM NaF, 5 mM EDTA, 1 mM Na_3VO_4, and 1 mM PMSF. 900 μg of total cell lysate was incubated overnight at 4°C with 10 μl of coated beads. Beads were washed three times with the same buffer containing 0.2% digitonin. For more stringent conditions, cells were lysed in 1.5% CHAPS buffer containing 50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, and 2 mg/ml aprotinin. Bead fractions were washed the next day using 1% NP-40 buffer containing 137 NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 20 mM Tris-HCl, pH 7.4, 50 mM NaF, 1 mM Na_3VO_4, and 1 mM PMSF.

Immunofluorescence
HUVEC were plated on fibronectin-coated coverslips (10 μg/ml) for 2 h in serum-free EBM. Magnetic beads coated with integrin-specific antibodies, HSA, RGD, or TSR were added and allowed to adhere for 30 min. Cells were gently washed with PBS, fixed in 3.7% formaldehyde in PBS, and permeabilized with 0.5% Triton X-100/PBS. Nonspecific staining was blocked with 2% BSA and coverslips were incubated with primary antibodies against $\alpha_5\beta_1$ in 2% BSA/PBS. Coverslips were washed extensively with PBS and stained with TRITC-conjugated goat anti–mouse IgG in 2% BSA/PBS for 30 min. Coverslips were washed in PBS, rinsed in deionized water, mounted in Permafluor, and viewed on a microscope (TE-2000 E; Nikon).

Preparation of myristoylated p110 retrovirus
10 μg of vector or myristoylated p110 constructs (pLNCX) was transfected in the Phoenix-Ampho packaging cell line (Dal Canto et al., 1999). Viral supernatant was collected after 24 or 48 h and passed through a 0.45-μm filter. Subconfluent cells were incubated with viral supernatant in the presence of 8 μg/ml polybrine in serum containing media and incubated overnight. Cells were then split and 48 h later used in either the migration assay or lysed for protein expression analysis.

Flow cytometry
Flow cytometric measurements of integrin subtypes expressed on the surface of endothelial cells was performed as described previously (Short et al., 1998), using a FACS Vantage SE instrument (Becton Dickinson). Data were analyzed using CellQuest software (Becton Dickinson), and relative fluorescence intensity was expressed as a percentage of β_1 fluorescence.

Online supplemental material
A supplemental figure (Fig. S1) has been made available at http://www.jcb.org/cgi/content/full/jcb.200407060/DC1).

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