Mechanisms of Integrin–Vascular Endothelial Growth Factor Receptor Cross-Activation in Angiogenesis

Ganapati H. Mahabeleshwar, Weiyi Feng, Kumar Reddy, Edward F. Plow, Tatiana V. Byzova

Abstract—The functional responses of endothelial cells are dependent on signaling from peptide growth factors and the cellular adhesion receptors, integrins. These include cell adhesion, migration, and proliferation, which, in turn, are essential for more complex processes such as formation of the endothelial tube network during angiogenesis. This study identifies the molecular requirements for the cross-activation between β3 integrin and tyrosine kinase receptor 2 for vascular endothelial growth factor (VEGF) receptor (VEGFR-2) on endothelium. The relationship between VEGFR-2 and β3 integrin appears to be synergistic, because VEGFR-2 activation induces β3 integrin tyrosine phosphorylation, which, in turn, is crucial for VEGF-induced tyrosine phosphorylation of VEGFR-2. We demonstrate here that adhesions and growth factor–induced β3 integrin tyrosine phosphorylation are directly mediated by c-Src. VEGF-stimulated recruitment and activation of c-Src and subsequent β3 integrin tyrosine phosphorylation are critical for interaction between VEGFR-2 and β3 integrin. Moreover, c-Src mediates growth factor–induced β3 integrin activation, ligand binding, β3 integrin-dependent cell adhesion, directional migration of endothelial cells, and initiation of angiogenic programming in endothelial cells. Thus, the present study determines the molecular mechanisms and consequences of the synergism between 2 cell surface receptor systems, growth factor receptor and integrins, and opens new avenues for the development of pro- and antiangiogenic strategies. (Circ Res. 2007;101:570-580.)

Key Words: angiogenesis • endothelial cell • β3 integrin signaling • vascular endothelial growth factor receptor • extracellular matrix proteins

Angiogenesis, the process of new blood vessel formation from preexisting vasculature, plays critical roles in tissue regeneration, postischemic tissue repair on myocardial infarction and stroke, and in the pathogenesis of cancer, rheumatoid arthritis, and diabetic microvascular disease. Angiogenesis is triggered by angiogenic growth factors and their receptors in coordination with extracellular matrix (ECM) receptors known as integrins. On integrin engagement, ECM triggers activation of intracellular signaling pathways essential for endothelial cell (EC) survival, proliferation, migration, morphogenesis, and organization of ECs into blood vessels. There are several manifestations of a tightly collaborative relationship between integrins and receptors for growth factors. On ECs, engagement of α5β3 integrin promotes phosphorylation and activation of vascular endothelial growth factor (VEGF) receptor (VEGFR)-2, thereby augmenting the mitogenic activity of VEGFs.

Among several integrins on ECs, α5β3 is the most abundant and influential receptor regulating angiogenesis. The upregulation of α5β3 during angiogenesis suggests that this integrin might play a crucial role during this process. Indeed, antagonists of α5β3, including blocking monoclonal antibody (LM609) and RGD cyclic peptides, were shown to be efficient inhibitors of neovascularization in several experimental animal models. Results of preclinical models have justified ongoing clinical trials of humanized monoclonal antibodies and cyclic peptides, which specifically target α5β3. That α5β3 regulates tumor-induced angiogenesis is clear, although its precise role remains disputed, indicating the need for conclusive mechanistic studies.

At the structural level, the function of this integrin is regulated by its cytoplasmic domain. Conserved regions present within the β3 integrin (β3) cytoplasmic domain include NPLY and NXXY motifs, the tyrosine residues of which can be phosphorylated to regulate interactions with signaling proteins containing SH3 and PTB domains. Phosphorylation of β3 modulates several intracellular events, including VAV-1/Rho GTPase activation, actin cytoskeleton reorganization and regulation of the phosphatidylinositol 3-kinase/AKT pathway, which is involved in the regulation of basic cellular functions such as cell spreading and survival. Thus, phosphorylation of β3 cytoplasmic domain is critical for α5β3 integrin–dependent functions because it regulates α5β3 affinity, avidity, and ligand-binding strength. Although the responses regulated by β3 phosphorylation are numerous and functionally important, this phenomenon has not been thoroughly studied, leaving critical gaps in our understanding of this integrin function.
understanding of the molecular mechanisms underlying the process of receptor cross-activation.

**Materials and Methods**

Reagents and plasmids used in this study, cell culture and transfection, isolation of primary mouse ECs, cell adhesion assays, cell migration assays, tube formation assays, time-lapse video microscopy, in vitro kinase assays, fibrinogen and WOW-1–binding assays, and immunoprecipitation and immunoblotting methods are described in the online data supplement at http://circres.ahajournals.org.

**Statistical Analysis**

Values were expressed as mean±SD. Probability values were based on the paired t test. Results were considered statistically significant with a probability value less than 0.05.

**Results**

β3 Integrin Tyrosine Phosphorylation Is Required for Maximum Tyrosine Phosphorylation of VEGFR-2

As a first step to examine the relationship between integrin ligation, β3 phosphorylation and VEGFR-2 activation, we monitored phosphorylation of β3 at Tyr747 and Tyr759 in ECs plated on the αvβ3 ligand vitronectin, the αvβ1 ligand collagen, or the αvβ6/αvβ1 ligand laminin. As a control, the ECs were maintained in suspension in the presence or absence of VEGF stimulation. As shown in Figure 1A, vitronectin, but not laminin or collagen, was able to induce β3 phosphorylation, which was augmented on VEGF treatment. At the same time, phosphorylation of β3 was minimal in cells in suspension or plated on laminin or collagen despite stimulation with VEGF (Figure 1A). Human umbilical vein ECs (HUVECs) also exhibited differential adhesion to various integrin ligands (Figure IIA and IIB in the online data supplement). Analysis of VEGF-2 tyrosine phosphorylation in the same set of samples revealed that basal level VEGF-2 activation can be triggered by αvβ3 ligation induced by vitronectin but does not occur in cells plated on collagen or laminin. Parallel analysis of β1 phosphorylation status showed no significant difference with VEGF stimulation (supplemental Figure IA). Thus, whereas VEGF stimulation promotes phosphorylation of αvβ3, ligation of αvβ6 also stimulates VEGF-2 phosphorylation, and activation, demonstrating a mutual relationship between VEGF-2 and αvβ3.

β3 Integrin Tyrosine Phosphorylation Is Complementary to VEGF-Induced Tyrosine Phosphorylation of VEGFR-2

αvβ3 is expressed on proliferating ECs during angiogenesis and vascular remodeling and the blockade of αvβ3 suppresses angiogenesis in several in vivo models.13–16 Therefore, we assessed whether the blockade of αvβ3 affected tyrosine phosphorylation of the β3 subunit. Accordingly, HUVECs grown on gelatin-coated plates were incubated with anti-αv, anti-β3, anti-βv, and anti-β6 blocking antibodies and induced with VEGF for 5 minutes at 37°C. Cell lysates were analyzed for phosphorylation of β3 at Tyr747 and Tyr759. Figure 1B shows that both anti-αv and anti-β3 blocking antibodies inhibited VEGF-induced phosphorylation of β3 at both residues. Simultaneously, control IgG, anti-βv, or anti-β6 blocking antibodies had no substantial effects on VEGF-induced phosphorylation of β3.

To further examine the consequences of integrin blocking antibodies, cell lysates were analyzed for tyrosine phosphorylation of VEGFR-2. Results indicated that only anti-αv and anti-β3 function blocking antibodies suppressed VEGF-induced phosphorylation of VEGFR-2, whereas anti-βv or anti-β6 blocking antibodies had no effect on VEGF-induced activation of VEGFR-2 (Figure 1C). Furthermore, treatment of HUVECs grown on gelatin-coated plates with VEGF-2 inhibitor significantly reduced VEGF-induced β3 phosphorylation (Figure 1D). Taken together, these results demonstrate the cross-activation between the 2 receptors: αvβ3 ligation controls not only tyrosine phosphorylation of β3 but also of VEGFR-2, and VEGF stimulation promotes not only VEGFR-2 but also β3 tyrosine phosphorylation.

As VEGF-2 activation and signaling seemed to be tightly associated with integrins, we extended our examination to the interaction of VEGFR-2 with other EC integrins. Results indicate that VEGF induced interaction of VEGFR-2 with β3 (Figure 1E). A small fraction of VEGF-2 was also found to interact with β1 but not with β3 (supplemental Figure IB). Thus, of the 3 classes of integrins expressed on ECs, only αvβ3 forms a complex with VEGFR-2 after VEGF treatment.

**VEGF Differentially Induces Interaction of Src Family Tyrosine Kinases With β3 Integrin in Endothelial Cells**

The presence of Src family tyrosine kinases (SFKs) is critical for the phosphorylation of several intracellular signaling molecules following integrin-mediated cell adhesion and cell spreading.22 Therefore, we sought to analyze the degree and range of SFKs interactions with β3 and VEGFR-2 on VEGF stimulation. In resting ECs, only small amounts of c-Src associated with both VEGFR-2 and β3. VEGF stimulation dramatically enhanced interaction of c-Src with VEGFR-2 and β3. No basal level or VEGF-induced interaction was found between Fyn and β3 or VEGF-2. A moderate amount of Yes was always associated with β3 and VEGFR-2, even after VEGF induction (Figure 2A and 2B). Therefore, we conclude that c-Src is the major tyrosine kinase associated with β3 following stimulation of cells with growth factor, possibly the kinase responsible for phosphorylation of β3 cytoplasmic tyrosines. To confirm these observations, kinetic analyses of β3 and c-Src tyrosine phosphorylation were performed. VEGF induced phosphorylation of β3 at 2.5 minutes, and it remained phosphorylated up to 30 minutes. Maximal β3 phosphorylation was observed between 5 and 15 minutes. VEGF also induced a similar activation phosphorylation of c-Src in ECs (Figure 2C), leading us to consider that activated c-Src might be responsible for β3 phosphorylation, which is crucial for activation of β3 integrin–dependent cellular signaling and EC functions.

**Adhesion and Growth Factor–Induced β3 Integrin Tyrosine Phosphorylation Is Mediated Through c-Src**

The cytoplasmic tyrosine motifs of β3 integrin known to regulate FAK phosphorylation, integrin-dependent actin cy-
Figure 1. Phosphorylation of β3 integrin cytoplasmic tyrosine is crucial for VEGFR-2 activation. Cells were induced with 20 ng/mL VEGF for 5 minutes. A, HUVECs were either kept in suspension or plated on vitronectin, laminin, or collagen and allowed to adhere and spread for 30 minutes and were then induced with VEGF. Cell lysates were analyzed for phosphorylation of β3 cytoplasmic tyrosines and phospho (P)-VEGFR-2 using specific antibodies. B and C, Effect of integrin-blocking antibodies on β3 and VEGFR-2 tyrosine phosphorylation. HUVECs were incubated with specific or control antibody for 1 hour and then induced with VEGF. Lysates were analyzed for phosphorylation of β3 (B, top images) and VEGFR-2 (C, top image) using specific antibody. D, Serum-starved HUVECs were treated with 400 and 800 nmol/L VEGFR-2 inhibitor SU1498 (lanes 3 and 4) and then induced with VEGF. Lysates were analyzed for β3 tyrosine phosphorylation using specific antibodies. E, β3 interacts with VEGFR-2 following VEGF stimulation. HUVECs were induced with VEGF; lysates were immunoprecipitated with anti-VEGFR-2 antibody and then separately immunoblotted with anti-β3, anti-β1, and anti-β5 antibodies. HUVEC whole-cell lysate served as control (lane 1).
toskeletal reorganization, the ability of integrins to localize to focal contacts, and cellular adhesion and spreading. There-fore, we examined the role of c-Src in adhesion- and growth factor–induced phosphorylation of β3, cytoplasmic tyrosine motifs. Accordingly, HUVECs were either kept in suspension (Figure 3A, lane 1) or plated on vitronectin (lane 2) and then treated with VEGF (lane 3) or SU6656, a specific c-Src inhibitor (lane 4). Cell lysates were analyzed for β3 integrin phosphorylation. As anticipated, in comparison with HUVECs kept in suspension, cells plated on vitronectin showed high levels of β3 phosphorylation at Tyr747 and Tyr759, which were further augmented by VEGF. Treatment of adherent ECs with SU6656 also reduced basal adhesion-induced β3 tyrosine phosphorylation in these cells (supplemental Figure VIIB). As an independent approach, HUVECs were transfected with wild-type (WT), dominant negative (DN), and catalytically active (CA) forms of Src. Cells transfected with DN-Src showed severe impairment in adhesion as well as VEGF-induced β3 phosphorylation (lanes 5 and 6). In contrast, expression of CA-Src dramatically enhanced adhesion as well as VEGF-induced phosphorylation of β3 on both Tyr747 and Tyr759 (lanes 7 and 8). Transfection of HUVECs with WT c-Src did not significantly alter adhesion or VEGF-induced β3 phosphorylation (lanes 7 and 8).

To further substantiate the specific role of c-Src in β3 phosphorylation, we used cellular systems in which c-Src expression is highly regulated. As shown in Figure 3B, no β3 phosphorylation was observed in any of these cell types.
Figure 3. c-Src directly phosphorylates \( \beta_3 \) integrin at cytoplasmic tyrosine motifs. A, HUVECs were detached and either kept in suspension or adhered in the presence of SU6656 (400 nmol/L) to vitronectin-coated plates. HUVECs were also transfected with WT Src, DN-Src, or CA-Src and were then induced with 20 ng/mL VEGF for 5 minutes. Cell lysates were resolved by SDS-PAGE and analyzed for phosphorylation of \( \beta_3 \) using specific antibodies. B, Src\(^{++}\), SYF, and SYF\(^{++}\)Src cells were either kept in suspension or plated on an uncoated plastic surface or on vitronectin-, laminin-, or collagen-coated surfaces and allowed to adhere and spread for 30 minutes. HUVECs plated on vitronectin-coated surface were used as a positive control (lane 1). Cell lysates were analyzed for phosphorylation of \( \beta_3 \) using specific antibody (B). C and D, Kinase assays were performed using recombinant \( \beta_3 \) cytoplasmic tail–glutathione S-transferase (GST) fusion protein (C). Kinase assays were also performed in the presence of c-Src inhibitor SU6656 (400 nmol/L) and recombinant protein tyrosine phosphatase (PTP) to ensure phosphorylation is tyrosine specific. Peptides derived from distinct cytoplasmic regions of \( \beta_3 \) were also used as substrates for kinase assays under similar conditions (D).

when either plated on uncoated plastic surfaces or kept in suspension (lanes 2 and 3). Attachment to vitronectin stimulated high levels of \( \beta_3 \) phosphorylation in Src\(^{++}\) and SYF\(^{++}\) c-Src cells but not in SYF cells (lane 4). Cells plated on laminin or collagen showed very low \( \beta_3 \) phosphorylation (lanes 5 and 6). On growth factor stimulation, \( \beta_3 \) phosphorylation was observed only in Src\(^{++}\) and SYF\(^{++}\) c-Src cells, not in SYF-cells (supplemental Figure IXA). Thus, c-Src controls
cell adhesion as well as growth factor-induced β3 tyrosine phosphorylation.

c-Src Directly Phosphorylates Cytoplasmic Tyrosine Motifs of β3 Integrin

To examine whether c-Src can directly mediate β3 tyrosine phosphorylation, c-Src was immunoprecipitated from VEGF-stimulated HUVECs. Immunocomplexes were incubated with purified full-length β3 cytoplasmic domain, and [γ-32P] ATP incorporation was monitored. As shown in Figure 3C, the immunoprecipitated c-Src can phosphorylate β3 cytoplasmic domain (lane 2). The inhibitor SU6656 blocked this process, confirming the specificity of the reaction (lane 3). Recombinant protein tyrosine phosphatase also prevented phosphorylation, indicating that it is a tyrosine substrate that is being phosphorylated (lane 4). Recombinant purified c-Src also phosphorylated β3 whereas none was observed without the substrate, demonstrating specificity (lanes 1 and 5). Together, these results clearly show that c-Src can directly phosphorylate β3 cytoplasmic tyrosines.

To further investigate phosphorylation of Tyr747 and/or Tyr759, kinase assays were performed using as substrate peptides derived from these 2 distinct β3 sites (Figure 3D). SU6656, which blocks c-Src autophosphorylation, was used to demonstrate specificity. In the absence of integrin substrate, a significant amount of c-Src autophosphorylation was observed, indicating that the immunoprecipitated c-Src complex is active. In the presence of Tyr747/Tyr759 β3 cytoplasmic peptide, a dramatic increase in [γ-32P] ATP incorporation was observed compared with control peptides devoid of tyrosine motifs. Similar results were observed using purified recombinant c-Src protein. As anticipated, [γ-32P] ATP incorporation was minimal in the presence of purified recombinant protein tyrosine phosphatase, demonstrating the role of c-Src in phosphorylation of tyrosine residues. Together, these results clearly indicate that c-Src directly phosphorylates both tyrosine motifs (Tyr747 and Tyr759) of the cytoplasmic tail of β3. Furthermore, VEGF-stimulated Akt phosphorylation was maximum in HUVECs grown on vitronectin compared...
with other integrin ligands (supplemental Figure VIA and VIB). However, the phosphatidylinositol 3-kinase/Akt pathway was not involved in VEGF-induced tyrosine phosphorylation (supplemental Figure VIC).

c-Src–Mediated β₃ Integrin Tyrosine Phosphorylation Is Critical for VEGF-Induced VEGFR-2–β₃ Integrin Macromolecular Complex Formation

Our previous results indicate that VEGF-induced VEGFR-2 tyrosine phosphorylation was maximal in cells plated on only α₅β₁ ligand vitronectin and that VEGFR-2 shows maximum interaction with β₃ in ECs. Therefore, we sought to examine the role of Src-mediated β₃ phosphorylation in β₃ interaction with VEGFR-2. Accordingly, c-Src activity was modified by overexpression of WT, DN, or CA forms of Src or by treatment of cells with Src and VEGFR-2 inhibitors; interaction between VEGFR-2 and β₃ was then assessed. In unstimulated cells, no interaction between β₃ and VEGFR-2 was observed (Figure 4A and 4B, lane 1), whereas VEGF induced strong interaction between VEGFR-2 and β₃ (lane 2). Treatment of cells with pharmacological inhibitors of c-Src and VEGFR-2 known to inhibit β₃ phosphorylation prevented VEGF-stimulated interaction between β₃ and VEGFR-2 (lanes 3 and 4). Likewise, transfection with DN-Src reduced, whereas CA-Src enhanced, interaction between the 2 receptors (lanes 6 and 7). Furthermore, c-Src also partially regulated VEGFR-2 phosphorylation in ECs (supplemental Figure VIIA). These results indicate that the c-Src–mediated phosphorylation of β₃ integrin essentially regulates interaction between β₃ integrin and VEGFR-2 in ECs.

To further analyze the roles of β₃ cytoplasmic tyrosine motifs in β₃ integrin/VEGFR-2 interaction, we used lung ECs derived from β₃ knock-in mice in which Tyr747 and Tyr759 were mutated to phenylalanine (DiYF). Supplemental Figure IIC shows that VEGF induced phosphorylation of β₃ at Tyr747 and Tyr759 in WT but not in DiYF EC. To further assess the role of c-Src–mediated phosphorylation of β₃ integrin essentially regulates interaction between β₃ integrin and VEGFR-2 in ECs.

Figure 5. c-Src–mediated phosphorylation of β₃ cytoplasmic tyrosine is required for α₅β₁ outside-in signaling. A and B, Src++, SYF, and SYFSrc cells were incubated on vitronectin-, collagen-, or laminin-coated plates. Attached cells per field were counted. The number of Src++ cells that adhered on vitronectin was assigned a value of 100%. C, HUVECs were either transfected with various forms of Src or treated with SU6656 (400 nmol/L) and then suspended on vitronectin-coated plates. The number of nontransfected HUVECs that adhered on vitronectin was assigned a value of 100%. D, WT and DiYF mouse lung microvascular ECs were suspended on vitronectin- or bone sialoprotein–coated plates. Attached cells per field were counted, and the number of WT cells that adhered on vitronectin was assigned a value of 100%. BSA-coated plates were used as control. Asterisks indicate significant difference over control (P<0.028).
Figure 6. β3 cytoplasmic tyrosine motifs are required for αvβ3-dependent directional migration of ECs and EC tube formation. A and B, HUVECs (A) or WT and DiYF mouse lung microvascular ECs (B) were transfected with various forms of Src and were then seeded on vitronectin-coated upper wells of Boyden-type migration chambers. Cells were allowed to migrate, and nonmigrated cells adherent to the top surface were removed. Migrated cells were stained, and cells per field were counted. Numerical values are represented as a bar diagram, and fold changes over control are indicated. C, WT and DiYF mouse lung microvascular ECs were grown on vitronectin-, laminin-, or collagen-coated plates. A wound was created across the cell monolayer by scraping away a swath of cells. Representative cell paths (n=6) are shown tracked by time-lapse video microscopy in the presence of 20 ng/mL VEGF over a period of 10 hours.
c-Src–mediated tyrosine phosphorylation of β3 integrin controls VEGF-induced β3 integrin and VEGFR-2 interaction in ECs.

**c-Src Is Critical for Growth Factor–Induced β3 Integrin Activation and Ligand Binding**

An intrinsic property of integrins is an increase in soluble ligand binding in response to stimulation, a process called integrin activation. Our results indicate that c-Src directly phosphorylates β3 on cytoplasmic tyrosines, which might affect integrin functional activity. To address this issue, HUVECs were transfected with WT, DN, and CA forms of c-Src and then stimulated with VEGF. Subsequently, αvβ3 activation was assessed by WOW-1 binding as described under Materials and Methods. As anticipated, VEGF induced a 6-fold increase in WOW-1 binding (Figure 4D). DN-Src reduced αvβ3 activity triggered by VEGF by at least 2-fold. In contrast, CA-Src promoted WOW-1 binding to unstimulated as well as to VEGF-stimulated cells (Figure 4D). Similar results were observed using fibronectin as a soluble ligand for αvβ3 (Figure 4E). Furthermore, activation and ligand binding were also assessed using Src+++, SYF, and SYF++Src cells. Basic fibroblast growth factor stimulation resulted in a dramatic increase in integrin activation in Src+++ and SYF++Src but not in SYF cells, as measured by WOW-1 or fibronectin binding (supplemental Figure IXB and IXD). No differences in β3 expression levels were found between these 3 cell lines (supplemental Figure IXC). Thus, c-Src and c-Src–dependent β3 cytoplasmic tyrosine phosphorylation are essential for VEGF-induced αvβ3 activation and ligand binding to activated integrin, both crucial steps in integrin signaling.

**c-Src Is Required for αvβ3 Integrin–Dependent Cellular Adhesion to Distinct Ligand**

To examine the role of c-Src in αvβ3-dependent cell adhesion to ECM ligands, we used Src+++ SYF, and SYF++Src cell systems. Src+++ and SYF++Src cells displayed the highest levels of adhesion to vitronectin, which is primarily recognized by αvβ3 SYF cells showed at least 3-fold lower adhesion on vitronectin compared with Src+++ or SYF++Src cells (Figure 5A and 5B), although no differences in cell adhesion to collagen or laminin were observed. Adhesion of HUVECs on vitronectin was assessed on expression of WT, DN, and CA forms of Src or on treatment with inhibitor SU6656 (Figure 5C). CA-Src promoted αvβ3-dependent cell adhesion, whereas DN-Src and Src inhibitor caused impairment of adhesion to vitronectin. These results indicate that c-Src plays a crucial role in αvβ3-dependent cell adhesion to ECM ligands.

Our results also indicated that Src phosphorylates β3 on cytoplasmic tyrosines, which may be required for αvβ3-dependent ECM reorganization. To evaluate this, WT and DiYF mouse lung microvascular ECs were incubated on vitronectin or bone sialoprotein, αvβ3-specific ligands. DiYF ECs showed significantly impaired adhesion to vitronectin as well as bone sialoprotein (Figure 5D). No significant differences in adhesion were found between WT and DiYF ECs incubated on BSA-coated plates. These results clearly show that β3 cytoplasmic tyrosines and c-Src–mediated phosphorylation of these residues are essential for αvβ3-dependent cellular adhesion to distinct ECM ligands.

**β3 Integrin Cytoplasmic Tyrosines Are Required for Directional Migration of Endothelial Cells**

EC motility is the defining feature of angiogenesis, required for the organization of proliferating ECs into vessel-like structures. To assess the role of Src-mediated β3 tyrosine phosphorylation in αvβ3-dependent EC migration, HUVECs transfected with various forms (DN and CA) of Src were evaluated in migration assays using VEGF as an agonist and vitronectin as a substrate. DN-Src significantly reduced EC migration in response to VEGF, whereas CA-Src dramatically increased both basal and VEGF-induced EC migration (Figure 6A). Pharmacological inhibitors of c-Src and VEGFR-2 also reduced EC migration triggered by VEGF (supplemental Figure VIIIB). To further evaluate the c-Src–mediated β3 phosphorylation in αvβ3-dependent EC migration, WT and DiYF mouse lung microvascular ECs were transfected with WT, DN, and CA forms of c-Src and stimulated with VEGF (Figure 6B). VEGF stimulation induced increases in WT EC migration by 2.5-fold in WT-Src and 3.5-fold in CA-Src cells. DN-Src significantly tempered the VEGF-induced increase in migration in only WT ECs. Surprisingly, c-Src activity modulation did not result in any significant differences in migration of DiYF ECs. These results clearly indicate that c-Src mediates signaling through tyrosine phosphorylation of β3 integrin. Lack of these tyrosine residues in the cytoplasmic domain of β3 severely impaired c-Src–mediated αvβ3 integrin–dependent EC migration.

To examine the role of β3 cytoplasmic tyrosine motifs in directional migration of ECs, WT and DiYF ECs were cultured on collagen-, laminin-, or vitronectin-coated plates. Wounds were created, and VEGF-A165-stimulated cell migration was monitored by time-lapse video microscopy. Cell paths were recorded and are presented in Figure 6C. WT and DiYF ECs plated on laminin exhibited similar migration speeds, 53.2±4.8 and 47.2±4.2 μm/h, respectively. On collagen, WT and DiYF ECs also showed similar migration rates, of 43.2±3.5 and 44.5±3.3 μm/h, respectively. On vitronectin, WT and DiYF ECs showed relatively slow but similar migration at 30.8±3.2 and 28.3±2.7 μm/h, respectively. However, when random movement was distinguished from directed migration, the results became quite different. Despite the high speed of migration, the average distance of directed migration from site of origin was relatively low for...
WT cells on laminin and collagen, 71 ± 6.2 and 92 ± 7.2 μm respectively (Figure 6C). No differences between WT and DiYF cells were found. In contrast, WT ECs showed maximum directed migration when plated on vitronectin (229 ± 6.6 μm). Importantly, directed migration by DiYF cells was dramatically impaired (77 ± 5.4 μm versus 229 ± 6.6 μm for WT). WT ECs plated on αβ ligand vitronectin following induction with VEGF-A165 resulted in higher directional persistence of cell migration (supplemental Figure IVA). Simultaneously, DiYF ECs plated on vitronectin and induced with VEGF showed random movement with no distinct pattern. Furthermore, ECs stimulated with VEGF grown on vitronectin also exhibited greater rates of proliferation without significant differences in rates of apoptosis (supplemental Figures IIIA, IIIB, IVB, VA, VB). These results clearly demonstrate that β3 integrin cytoplasmic tyrosine motifs are required for persistent and directional migration of ECs during the process of angiogenesis.

β3 Integrin Cytoplasmic Tyrosine Phosphorylation Is Crucial for Organization of the Angiogenic Program in Endothelial Cells

To further evaluate the functional significance of c-Src-dependent β3 phosphorylation, the ability of ECs to organize into precapillary tube-like structures was tested. Accordingly, WT and DiYF mouse microvascular ECs were transfected with the various activation forms of Src. These cells were seeded on Matrigel-coated plates and allowed to organize into precapillary tube-like structures. Overexpression of DN-Src significantly reduced, whereas the CA form of Src dramatically enhanced, VEGF-induced tube formation on Matrigel in only WT cells (Figure 6D and 6E). Both VEGF and the varied forms of Src failed to modify the degree of tube formation in DiYF ECs. From all of these results, we conclude that c-Src-dependent β3 integrin cytoplasmic tyrosine phosphorylation is essential for αβ-dependent EC migration as well as precapillary endothelial tube formation on extracellular matrix substrates. Thus, β3 cytoplasmic tyrosine motifs are crucial for initiation of the angiogenic program in ECs and ultimately regulate the processes of angiogenesis.

Discussion

This study focused on the mechanisms and molecular requirements for the crosstalk and cross-activation between 2 families of cell surface receptors on endothelium: integrins, receptors for extracellular matrix, and tyrosine kinase receptors, represented by VEGFR-2. The major findings in this manuscript are: (1) there is an intimate and coordinated relationship between VEGFR-2 and αβ integrin; (2) adhesion- and growth factor–induced β3 integrin tyrosine phosphorylation is directly mediated by c-Src; (3) c-Src-dependent β3 integrin tyrosine phosphorylation is critical for interaction between VEGFR-2 and β3 integrin; and (4) c-Src is required for growth factor–induced β3 integrin activation, ligand binding, and αβ integrin–dependent cellular adhesion. We have demonstrated that VEGF induces association of its receptor VEGFR-2 with the β3 subunit of αβ, but not with β3 or β3 integrins, on ECs. Blocking antibodies against either the αv or β3 subunits independently blocked VEGF-induced phosphorylation of β3 cytoplasmic tyrosines and VEGF-induced VEGFR-2 phosphorylation. We found that phosphorylation of tyrosine within the β3 cytoplasmic domain occurred in response to VEGF and, in turn, was essential for VEGFR-2–β3 integrin association and VEGFR-2 activation and subsequent signaling. Thus, crosstalk between the 2 receptors determines the cellular responses to VEGF as well as to integrin ligation, which, in turn, is regulated by tyrosine phosphorylation events.

We have identified c-Src as a molecule that directly phosphorylates the cytoplasmic tyrosines of β3 in response to VEGF stimulation, enabling it to directly control VEGF-induced and integrin-mediated cellular responses such as cell adhesion and migration. Src, Yes, and Fyn triple mutant cells (SYF) exhibited severely impaired β3 tyrosine phosphorylation in response to growth factors, which was corrected by reexpression of c-Src alone. Potentially, kinases other than c-Src may also contribute to VEGF-induced β3 phosphorylation in other cell lines. In ECs, Src, but neither Yes nor Fyn, was able to interact with VEGFR-2 and β3 in a VEGF-dependent manner. Recent observations have demonstrated that Src is required for VEGF-induced vascular permeability, a response triggered by VEGFR-2 activation. Moreover, c-Src is able to modulate blood vessel development in several experimental animal models. Here we have demonstrated that growth factor–stimulated ligand binding to αβ on ECs is c-Src dependent. In triple knockout cells (SYF), the lack of Src activity resulted in deficient β3 phosphorylation, which, in turn, led to severe impairment in growth factor–induced ligand binding, a prerequisite for growth factor–modulated cell adhesion, spreading, and migration. Thus, we conclude that c-Src is crucial for integrin activation in response to growth factors and have also demonstrated the intimate association of Src and integrin signaling. In fact, several features of the Src knockout phenotype, including osteopetrosis, show a close resemblance to the phenotype of β3-null mice.

The presence of the β3/Src complex at the leading edges of adherent cells further demonstrates their intimate association in basic cellular processes. Furthermore, mouse embryonic fibroblasts containing c-Src (Src+/+ and SYF’Src) showed a greater tendency to adhere to vitronectin-coated surfaces than to collagen- or laminin-coated surfaces. These results strengthen our conclusion that c-Src is required mainly for vitronectin receptor signaling (αβ) rather than collagen or laminin receptor signaling. Interestingly, only Src, not Fyn knockout, mice display impairment of VEGF-induced vascular responses and tumor burden, indicating that Src is the main SFK in the regulation of EC functions. Our study also demonstrates that c-Src–dependent β3 cytoplasmic tyrosine phosphorylation is essential for αβ integrin–dependent EC migration as well as precapillary endothelial tube formation on ECM substrates. Thus c-Src, via direct phosphorylation of β3 integrin cytoplasmic tyrosines, controls the functional association between αβ and VEGFR-2, which, in turn, regulates activation of both receptors on ECs. This functional interplay is crucial for EC adhesion, migration, and initiation of angiogenic programming in ECs.
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References
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Materials and methods

Reagents and plasmids

Rabbit polyclonal anti-VEGFR-2, anti-β3-integrin, anti-β5-integrin, anti-β1-integrin, anti-Src, anti-Fyn, anti-Yes, and mouse monoclonal anti-phospho-tyrosine (PY20 and PY99) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-Src (Tyr-416), anti-phospho-VEGFR-2, and purified, recombinant Src were from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-phospho-β3-integrin antibodies (Tyr 747 and Tyr 759) were from Biosource International, Inc. (Camarillo, CA). Mouse monoclonal anti-β3-integrin, anti-β5-integrin, anti-β1-integrin, anti-αv integrin blocking antibodies were from Chemicon International, Inc. (Temecula, CA). Purified collagen, laminin, vitronectin, bFGF, and VEGF were purchased from R&D Systems (Minneapolis, MN). Matrigel was obtained from BD Biosciences (San Jose, CA). Protein G agarose, Alexa-488 labeled fibrinogen, and [γ32P] ATP were from Invitrogen (Carlsbad, CA). ProFound Co-Immunoprecipitation Kit was purchased from Pierce Biotechnology, Inc. (Rockford, IL). Wild type, dominant negative (K296R/Y528F) and catalytically active (Y529F) forms of Src cDNA cloned in pUSEamp vector were obtained from Upstate Cell Signaling Solutions (Charlottesville, VA). SU6656, SU1498, and recombinant protein tyrosine phosphatase were obtained from Calbiochem (San Diego, CA). Nucleofector transfection kits for primary endothelial cells were obtained from Amaxa Biosystems (Cologne, Germany). Boyden type cell migration chambers were obtained from Corning (Corning, NY). All other chemicals were analytical grade.
Cell culture and transfection

Human umbilical cord vein endothelial cells (HUVEC) cells were grown in DMEM medium supplemented with 10% FBS, 90 μg/mL heparin sulphate, 90 μg/mL endothelial cell growth factor, 10,000 U/mL penicillin, 10 μg/mL streptomycin. Cells were used between second and fifth passages. HUVECs were transiently transfected using the HUVEC Nucleofector kit according to manufacturer instructions. Forty-eight hours after transfection, cells were serum starved and used for experiments.

Src++, SYF, and SYF+c-Src cells were obtained from American Type Culture Collection. Cells were maintained in Dulbecco-modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and antibiotics and were grown at 37 °C in 6% CO2.

Isolation of primary mouse endothelial cells

DiYF mice were generated in the laboratory of Dr. David R. Phillips and maintained on a C57/Bl6 background (seven generations of backcrossing). Six- to eight-week old wild-type (WT) and DiYF mice were used in this study. We performed all procedures according to protocols approved by Cleveland Clinic Foundation Institutional Animal Care and Use Committee. WT and DiYF mouse lungs were removed by surgical procedure minced and digested using collagenase-dispase reagent (3 mg/mL). Digests were strained and the resulting cell suspension was plated on flasks coated with 5 μg/mL fibronectin.
Cell adhesion assay
The cell adhesion assay was performed as described previously. HUVEC’s or mouse embryonic fibroblasts (SYF and SYF + Src) or mouse lung endothelial cells were detached from the tissue culture flasks using 20 mM EDTA. Cells were washed twice with sterile PBS and re-suspended in DMEM. The cell suspensions were added to ligand-coated wells and placed in humidified incubator for 45 min. The wells were gently washed three times with DMEM and photographs were taken. The numbers of attached and spread cells per field were counted.

Cell migration assay
Transwell tissue culture inserts were coated with αvβ3 integrin ligand vitronectin for 24 h at 4 °C. HUVECs cells either transfected with various forms of c-Src or treated with inhibitors were trypsinized and 1X10^5 cells were added into each well. Cells were allowed to migrate for 12 h and fixed with 70% methanol for 15 min and stained with Giemsa. The non-migrated cells adhered to the top surface were removed and three random 10X fields were photographed using an inverted phase contrast microscope.

Tube formation assay
The formations of vascular tube-like structures by endothelial cells were assessed on the basement membrane matrix preparation. Twelve well plates were coated with 0.5 mL of Matrigel according to the manufacturer’s instructions. Wild type and DiYF lung microvascular endothelial cells were transfected with various forms of c-Src. These cells were detached, washed twice with sterile PBS and seeded on Matrigel-coated plates. Medium with or without 20 ng/mL
VEGF was added and cells were further incubated at 37 °C for 8 h. The tube formation was observed using an inverted phase contrast microscope (Leica) and photographs were taken. Using ImagePro software, the degree of tube formation was quantified by measuring the length of tubes in three random fields.

**Time-lapse video microscopy**

Endothelial cell motility on various extracellular matrix protein-coated plates induced with VEGF-A$_{165}$ was analyzed by time-lapse videomicroscopy$^3$. Time-lapse imaging was performed using a Leica DM IRB microscope supported by the Metamorph program (Molecular Devices). Images were acquired every 10 min for 10 hr using a Photometric Cool Snap Camera (Roper Scientific) under 5% CO$_2$ and at 37 °C in a stage incubator. Cell paths were generated from centroid positions and migration parameters were computed with ImagePro plus software.

**In vitro kinase assay**

HUVECs were induced with 20 ng/mL VEGF and lysed in Src kinase lysis buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 2 mM orthovanadate, 10 mg/mL pepstatin, 2.5 mM EDTA). Cell lysates were immunoprecipitated with anti-c-Src antibody. Immunocomplex or purified full-length c-Src in kinase assay buffer (20 mM Hepes (pH 7.4), 10 mM MnCl$_2$) containing 10 µg of purified GST conjugated β$_3$ integrin cytoplasmic domain with 5 µCi of [γ-32P]ATP and 10 µM ATP was incubated at 30 °C for 30 min with or without SU6656 and recombinant protein tyrosine phosphatase. The samples were resolved by SDS-PAGE, transferred to
nitrocellulose membrane, and autoradiographed. Membranes were reprobed with anti-c-Src antibody to insure equal loading.

Purified $\beta_3$ integrin cytoplasmic peptides specific for tyrosine-747 (DTANNPLYKEATSTFT-COOH), tyrosine-759 (KEATSTFTNITYRGT-COOH), and control peptides (DTANNPLF KEATSTFT-COOH and KEATSTFTNIT FRGT-COOH) were mixed with c-Src immunoprecipitates or purified full-length c-Src and kinase assay buffer containing 5 $\mu$Ci of $[^{32}\text{P}]$ATP and 10 $\mu$m ATP and incubated at 30 °C for 30 min with or without SU6656 and recombinant protein tyrosine phosphatase (PTP). Fractions of reaction mixtures were transferred to phosphocellulose paper and washed with 150 mM of H$_3$PO$_4$. These membranes were rinsed briefly in ethanol, air dried, and transferred to scintillation vials. $\gamma$-ATP incorporation was counted using a Beckman LS600IC liquid scintillation counter (Beckman, Fullerton, CA).

**Fibrinogen and WOW-1 binding assay**

To assess fibrinogen binding, semiconfluent HUVECs were transfected with various forms of c-Src. Cells were serum starved for 4 h and further induced with 20 ng/mL VEGF-A165. Fluorescein isothiocyanate (FITC)-labeled fibrinogen was added at a final concentration of 200 nM and cell were incubated for 30 min. Cells were fixed with 3.7% formaldehyde/PBS for 15 min and washed twice with ice-cold PBS. Fluorescence-activated cell sorting (FACS) was performed using a FACS Calibur (Becton Dickinson, San Jose, CA) and data were analyzed using CellQuest software program.
The WOW-1 Fab binding assay was performed as described previously. Semiconfluent HUVECs were transfected with various forms of c-Src and were serum starved for 4 h and further stimulated with 20 ng/mL VEGF-A165. WOW-1 Fab was added at a final concentration of 30 μg/mL, followed by addition of FITC-conjugated goat anti-mouse IgG at 10 μg/mL. After 30 min cells were fixed with 3.7% formaldehyde/PBS for 15 min, washed twice with PBS, and FACS analysis was performed as described above.

**Immunoprecipitation and immunoblotting**

Cells were lysed in immunoprecipitation lysis buffer (1% Noniodet P-40, 150 mM NaCl, 50 mM Tris-HCL (pH 7.8), 2 mM EDTA, 10 mM NaF, 10 mM Na₂₃PO₄, 2 mM Na₃VO₄ 10 μg/mL leupeptin, 4 μg/mL pepstatin and 0.1 U/mL aprotinin). Cell lysates were centrifuged at 13,000xg for 10 min. Supernatants were collected and assayed for protein concentration using the Bio-Rad protein assay method. Cell lysates containing 700–800 μg of total protein were pre-cleared and were immunoprecipitated with the indicated antibody. Immunocomplexes were denatured using Laemmli sample buffer and proteins were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and probed with indicated antibody. When appropriate, nitrocellulose membranes were stripped and blotted according to manufacturer's instructions. Endothelial cell surface biotinylation and Western blot analysis were performed as described previously. Co-immunoprecipitation of Src family tyrosine kinases with VEGF receptor-2 and β3-integrin were performed with ProFound Co-Immunoprecipitation Kit according to manufacturer's instructions. Bands were
analyzed by densitomeric analysis using Kodak 1D software and fold changes were indicated.

**Supplemental Methods**

**Reagents and plasmid**

Rabbit polyclonal anti-VEGFR-2, anti-β3-integrin, anti-β1-integrin and mouse monoclonal anti-phospho-tyrosine (PY20 and PY99) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-Akt and anti-p-Akt antibodies were obtained from cell signaling technology. Rabbit polyclonal anti-phospho-β3-integrin antibodies (Tyr 747 and Tyr 759) were from Biosource International, Inc. (Camarillo, CA). Purified collagen, laminin, vitronectin, fibronectin, and VEGF were purchased from R&D Systems (Minneapolis, MN). Annexin V-FITC apoptosis detection kit-I was obtained from BD Biosciences. Protein G agarose and CyQUANT NF cell proliferation assay kit were from Invitrogen (Carlsbad, CA). Dominant negative and catalytically active forms of Src cDNA cloned in pUSEamp vector were obtained from Upstate Cell Signaling Solutions (Charlottesville, VA). SU6656, SU1498, SH5, LY294002, wortmannin and Akt-inhibitor-IV were obtained from Calbiochem (San Diego, CA). Dominant negative and the myristylated form of Akt-1 cloned in K179M vector were expressed in primary endothelial cells using a nucleofector transfection kit from Amaza Biosystems (Cologne, Germany). Boyden-type cell migration chambers were obtained from Corning (Corning, NY). All other chemicals were analytical grade.
Cells Culture

Human umbilical cord vein endothelial cells (HUVECs) were grown in DMEM medium supplemented with 10% FBS, 90 μg/mL heparin sulphate, 90 μg/mL endothelial cell growth factor, 10,000 U/mL penicillin, and 10 μg/mL streptomycin. WT and DiYF mouse lung endothelial cells were isolated as described previously\(^6\).

Src\(^{++}\), SYF, and SYF+c-Src cells were obtained from American Type Culture Collection\(^1\). Cells were maintained in Dulbecco-modified eagle medium (DMEM) as described above.

Cell adhesion assays

HUVECs were detached from the tissue culture flasks using 20 mM EDTA, washed twice with sterile 1xPBS and re-suspended in DMEM. The cell suspensions were added to vitronectin-, collagen- or laminin-coated wells and placed in a humidified incubator for 45 min. These wells were gently washed three times with DMEM and photographs were taken. The numbers of attached and spread cells per field were counted.

Endothelial cell apoptosis assays

Endothelial cell apoptosis assays were performed using the Annexin V-FITC Apoptosis Detection Kit-1 according to manufacturer's instructions. Briefly, HUVECs were grown on vitronectin-, collagen- or laminin-coated plates, in the presence or absence of VEGF, for 18 hr in reduced serum medium (2% FBS). The cells were then detached using cell dissociation buffer. Cells were washed
and incubated with annexin-V and propidium iodide for 30 min. The numbers of annexin-V positive cells were counted using a FACSCalibur (Becton Dickinson, San Jose, CA) and data were analyzed using CellQuest software.

**Endothelial cell proliferation assays**

Endothelial cell proliferation on various extracellular matrix proteins were performed using CyQUANT NF cell proliferation assay kits according to the manufacturer's instructions. Accordingly, semiconfluent HUVECs were detached and plated on vitronectin-, collagen-, or laminin-coated 96 well plates. These cells were allowed to adhere and grow for 12 hr then were further stimulated with 20 ng/ml VEGF for 24 hr. These cells were then washed and incubated with 0.1 ml of CyQUANT NF dye reagent for 1 hr at 37°C. Fluorescence intensity of each sample was measured at the excitation wavelength of 485nm and emission wavelength of 530nm. The mean value of unstimulated cells grown on vitronectin was designated as 100% and relative proliferation rates were indicated.

**Endothelial cell migration assays**

Endothelial cell migration assays were performed as described earlier. Transwell tissue culture inserts were coated with $\alpha_v\beta_3$ integrin ligand vitronectin for 24 hr at 4°C. HUVECs, either treated with Src inhibitor SU6656 or VEGFR-2 inhibitor SU1498, were trypsinized and $1\times10^5$ cells were added into each well. These cells were further stimulated with 20ng/ml VEGF. Cells were allowed to migrate for 12 hr, fixed with methanol and stained with Giemsa. The non-migrated cells adhered to the top surface were removed using cotton swabs and several random 10X fields were photographed using an inverted phase contrast microscope.
Untreated cells which migrated on vitronectin were assigned the value of 1 and fold changes were indicated.

**Fibrinogen and WOW-1 binding assay**

The WOW-1 Fab and fibrinogen binding assay was performed as described above using Src++, SYF, and SYF+c-Src cells.

**Immunoprecipitation and immunoblotting**

Wherever cell lysates were subjected to immunoprecipitation, cells were lysed using immunoprecipitation lysis buffer-1 (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 7.8), 2 mM EDTA, 10 mM NaF, 10 mM Na$_2$P$_2$O$_7$, 2 mM Na$_3$VO$_4$, 10 µg/mL leupeptin, 4 µg/mL pepstatin and 0.1 U/mL aprotinin) for 20 min on ice. Cell lysates were centrifuged at 13,000×g for 10 min. Cell lysates containing 500 µg of total protein were pre-cleared and were immunoprecipitated with the indicated antibody. Immunocomplexes were denatured using Laemmli sample buffer (1x) and proteins were resolved by SDS-PAGE and probed with the indicated antibody.

When cell lysates were subjected directly to immunoblotting analysis, cells were lysed using radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 2 mM EDTA, 25 mM NaF, 2 mM Na$_3$VO$_4$ and one complete protease Inhibitor tablet from Roche Diagnostics per 50 ml of lysis buffer). Equal protein from total cell lysates were subjected to SDS-PAGE and Western blot analysis was performed using indicated antibody. Bands were analyzed by densitomeric analysis using Kodak 1D software and fold changes were indicated.
References


Supplemental Figure 1. (A) Adhesion and growth factor induced β₁ integrin tyrosine phosphorylation. HUVECs were serum starved for 4 hr and these cells were detached and plated on laminin- (lanes 1 & 2), collagen- (lanes 3 & 4), and fibronectin- (lanes 5 & 6) coated tissue culture plates. These cells were further stimulated with 20 ng /ml VEGF for 5 mins. Cells were lysed and equal amounts of total proteins were immunoprecipitated with rabbit-anti-β₁ integrin antibody. Immunocomplexes were resolved by reducing SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (upper panel A). Blots were reprobed with anti-β₁ integrin antibody as loading control (lower panel A). (B) VEGFR-2 and β₁ integrin interaction. HUVECs were grown on laminin (lanes 1 & 2), collagen (lanes 3 & 4) and fibronectin (lanes 5 & 6) coated plates. These cells were serum starved for 4 hr then stimulated with 20 ng /ml VEGF for 5 min. Cells were lysed, equal amounts of total proteins from cell lysates were immunoprecipitated with rabbit anti-β₁ integrin antibody. Immunocomplexes were separated by reducing SDS-PAGE and immunoblotted with rabbit-anti-VEGFR-2 and anti-β₁ integrin antibody (panel B). Results indicated that there is minimum interaction between VEGFR-2 and β₁-integrin irrespective of ligand substrate.

Supplemental Figure 2. Endothelial cell adhesion to extracellular matrix proteins. (A, B) HUVECs were serum starved for 4 hr and cells were detached from tissue culture plates. These cells were washed twice with sterile 1xPBS. Equal numbers of cells in suspension were added to vitronectin-, collagen- or
laminin-coated plates. Cells were allowed to adhere and spread for 45 min at 37°C in a humidified incubator. After 45 min unattached cells were removed and the plates were washed twice with 1xPBS. These cells were fixed using ice-cold 4% paraformaldehyde and photographs were taken (panel A). The number of cells adhered and spread were counted and indicated by bar diagram (panel B). The number of cells adhered on vitronectin was assigned the value of 100% and relative adhesion was indicated. (C) Wild type (lanes 1 & 2) and DiYF (lanes 3 & 4) mouse lung microvascular endothelial cells were stimulated with VEGF. Cell lysates were analyzed for phosphorylation of β3 integrin using specific antibodies (panel C)

Supplemental Figure 3. Endothelial cell apoptosis on various extracellular matrix proteins. (A-B) HUVECs were serum starved for 4 hr then detached from tissue culture plates. These cells were washed twice with 1xPBS and kept in suspension for 30 min or allowed to adhere on vitronectin-, laminin- or collagen-coated plates for 30 min. These cells were induced with 20 ng/ml VEGF for 5 min at 37°C in a humidified incubator. Medium was aspirated and unattached cells were removed by washing gently with 1xPBS. Numbers of apoptotic cells were determined using annexin V-FITC and propidium iodide staining (panel A). The percentages of apoptotic cells in suspension or adhered to vitronectin, collagen, or laminin were estimated and indicated by bar diagram (panel B). No significance differences were observed in apoptotic populations of cells when kept in suspension or plated on laminin, collagen or vitronectin.
Supplemental Figure 4. Migration and proliferation of endothelial cells on extracellular matrix proteins. (A) Wild type and DiYF microvascular endothelial cells were grown on laminin-, collagen- or vitronectin-coated plates. Cells were allowed to spread and grow into monolayers, wounds were created using a pipette tip and dislodged cells were removed by washing twice with sterile 1xPBS. The adherent cells were induced with 20ng/ml VEGF and subjected to time lapse video microscopy. Averages of total path length and direct distance of cells from site of origin are indicated. (B) Proliferation of endothelial cells on various integrin ligands. HUVECs were detached and plated on vitronectin-, collagen- and laminin-coated 96 well plates. These cells were allow to grow for 12 hr then further stimulated with 20 ng/ml VEGF for 24 hr. At the end of the 24 hours the proliferative index of these cells were estimated. HUVECs grown on vitronectin were assigned the value of 100% and relative fold changes are indicated.

Supplemental Figure 5. Role of integrin ligands in regulation of endothelial cell apoptosis. HUVECs were grown on vitronectin-, collagen- and laminin-coated 6 well plates. The cells were further stimulated with 20 ng/ml VEGF for 18 hr in reduced serum medium (2% FBS). Numbers of apoptotic cells were counted using annexin V-FITC and propidium iodide staining (panel A). Percentages of apoptotic endothelial cells on each of the matrix proteins is indicated (panel B).
Supplemental Figure 6. VEGF induced Akt phosphorylation. (A, B) HUVECs were grown on laminin, collagen and vitronectin. These cells were serum starved for 4 hr and stimulated with 20 ng/ml VEGF for 5 min. Cells were lysed and cell lysates containing equal amounts of total protein were resolved by SDS-PAGE and analyzed by Western blot using anti-P-Akt antibody (upper panel A). The blot was reprobed with anti-Akt antibody as loading control (lower panel A). Densitometry analysis was performed and fold change over control were indicated (panel B). (C) Role of PI-3 kinase/Akt pathway in β3 integrin phosphorylation. HUVECs were transiently transfected with catalytically active Myr-Akt (lane 3), dominant negative Akt (DN-Akt) constructs (lane 4) or treated with PI-3 kinase inhibitors, 10 μM LY294002 (lane 6), 10 nM Wortmanin (lane 7) or pretreated with pharmacological inhibitors of Akt, 1 μM SH5 (lane 5), 700 nM Akt-inhibitor-IV (lane 8) for 1 hr and further stimulated with 20 ng/ml VEGF for 5 min. Equal amounts of total protein from cell lysates were separated on SDS-PAGE and analyzed for phosphorylation of β3 integrin by Western blot using specific antibodies (upper panels C). Portions of cell lysate were also analyzed for equal loading using anti-β3 integrin and the activation status of Akt was determined using anti-phospho-Akt antibody (lower panels C).

Supplemental Figure 7. Role of Src in adhesion-induced VEGFR-2 phosphorylation. (A) HUVECs were transfected with dominant negative (lanes 3 & 4) or the catalytically active form of Src (lanes 7 & 8), or pretreated with 400 nM Src inhibitor SU6656 (lanes 5 & 6). These cells were detached and plated on
vitronection-coated tissue culture plates and induced with 20 ng/ml VEGF for 5 min. Equal amounts of total proteins from cell lysates were separated by SDS-PAGE and analyzed for phosphorylation of VEGFR-2 using specific antibody (upper panel A). (B) SU6656 reduces adhesion induced β3 integrin tyrosine phosphorylation. HUVECs were serum starved in the presence and absence of the pharmacological inhibitor of Src SU6656 (400 nM) for 2 hr. These cells were detached and plated separately on vitronectin- (lanes 1 & 2) and fibronectin- (lanes 3 & 4) coated plates. Cells were allowed to adhere and spread for 30 min then adherent cells were lysed using RIPA lysis buffer. Cell lysates were analyzed for phosphorylation of β3 integrin using specific antibodies (upper panels B). The blot was reprobed with anti-β3 integrin antibody as loading control.

Supplemental Figure 8. Role of Src in β3 integrin and VEGFR-2 interaction. (A) HUVECs were transfected with the catalytically active form of Src. These cells were serum starved for 4 hr then stimulated with 20 ng/ml VEGF for 5 min. Cell lysates were individually immunoprecipitated with anti-β3 integrin antibody, immunocomplexes were resolved by SDS-PAGE and immunoblotted with anti-VEGFR-2 antibody (upper panel A). HUVEC whole cell lysate was used as positive control (lane 1). HUVECs stimulated with VEGF show significant increases in VEGFR-2 and β3 integrin interaction compared to unstimulated cells (lanes 2 & 3). Surprisingly, CA-Src trasfected cells even in the absence of VEGF also show moderate amounts of interaction between VEGFR-2 and β3 integrin in
endothelial cells (lane 4). (B) Role of Src in $\alpha_v\beta_3$ integrin-dependent endothelial cell migration. HUVECs were treated with 400 nM SU6656 or 800 nM SU1498 for 1 hr and applied to the upper chamber of Boyden-type migration chambers. These cells were stimulated with 20 ng/ml VEGF for 12 hr. The numbers of migrated cells were counted and fold changes in migration are indicated (panel B).

Supplemental Figure 9. Role of c-Src in growth factor induced $\beta_3$ integrin tyrosine phosphorylation and activation. (A) Src++, SYF, and SYF+Src cells plated on plastic surface were induced with 20 ng/ml bFGF for 5 min and levels of $\beta_3$ integrin phosphorylation were detected using a specific antibody. (B-D) Src++, SYF, and SYF+Src cells were stimulated with bFGF (20 ng /ml) and incubated with WOW-1 Fab fragments (B) or FITC-fibrinogen (D) for 30 min. Cells were fixed and analyzed by flow cytometry. Expression levels of $\beta_3$ integrin and Src in these cells were confirmed by Western blot analysis (C).
Supplemental Figure-1

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- P-Tyr-\(\beta_1\) Integrin
- \(\beta_1\) Integrin

VEGF - + - + - + - +

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- VEGFR-2
- \(\beta_1\) Integrin
Supplemental Figure-2

A. Images showing cell adhesion on different substrates: Vitronectin, Collagen, Laminin.

B. Bar graph showing relative adhesion (% control) for different substrates: Vitronectin, Collagen, Laminin.

C. Immunoblotting analysis showing phosphorylation of β3 integrin (747, 759) and total β3 integrin in Wild-type and DiYF cells with or without VEGF treatment.
Supplemental Figure 3

A

Suspension

Vitronectin

Collagen

Laminin

B

% of non-apoptotic cells

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- VEGF
- + VEGF

Relative cell proliferation(% Control)

Vitronectin | Collagen | Laminin

0 | 50 | 100

200 | 250

Relative cell proliferation(% Control)
Supplemental Figure-5

A

Vitronectin

- VEGF

+ VEGF

Collagen

Laminin

B

% of non-apoptotic cells

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Supplemental Figure-6

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B

Relative Phosphorylation (folds)

Control DN

Myr-Akt

DN-Akt

SH5

LY294002

Wortmannin

Akt-Inhibitor IV

VEGF        - +      +      +       +      +      +      +

Laminin                 Vitronectin                Collagen

C

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β₃-p-Tyr-747

β₃-p-Tyr-759

β₃-Integrin

P-Akt
Supplemental Figure-7

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**P-VEGFR-2**

**VEGFR-2**

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**β3-p-Tyr-747**

**β3-p-Tyr-759**

**β3-Integrin**
Supplemental Figure-8

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VEGF      - - +       -
SU6656 SU1498

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VEGFR-2

β3 Integrin

B

Folds increase in migration

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SU6656 SU1498
Supplemental Figure-9

A. Src ++ cells
   - P-β3 integrin-747
   - β3 integrin
   - SYF cells
     - P-β3 integrin-747
     - β3 integrin
   - SYF+Src cells
     - P-β3 integrin-747
     - β3 integrin
   - bFGF: -/+  

B. WOW1 binding, Mean of fluorescence
   - Control
   - 20ng/ml bFGF
   - Src ++  SYF  SYF+Src
   - Mean of fluorescence

C. Src++  SYF  SYF+Src
   - Src
   - β3 Integrin
   - Actin

D. Fibrinogen binding, Mean of fluorescence
   - Control
   - 20ng/ml bFGF
   - Src++  SYF  SYF+Src
   - Mean of fluorescence