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Src-mediated coupling of focal adhesion kinase to integrin αvβ5 in vascular endothelial growth factor signaling

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Vascular endothelial growth factor (VEGF) promotes vascular permeability (VP) and neovascularization, and is required for development. We find that VEGF-stimulated Src activity in chick embryo blood vessels induces the coupling of focal adhesion kinase (FAK) to integrin αvβ5, a critical event in VEGF-mediated signaling and biological responsiveness. In contrast, FAK is constitutively associated with β1 and β3 integrins in the presence or absence of growth factors. In cultured endothelial cells, VEGF, but not basic fibroblast growth factor, promotes the Src-mediated phosphorylation of FAK on tyrosine 861, which contributes to the formation of a FAK/αvβ5 signaling complex. Moreover, formation of this FAK/αvβ5 complex is significantly reduced in $pp60^{c\text{-}src}$-deficient mice. Supporting these results, mice deficient in either $pp60^{c\text{-}src}$ or integrin β5, but not integrin β3, have a reduced VP response to VEGF. This FAK/αvβ5 complex was also detected in epidermal growth factor-stimulated epithelial cells, suggesting a function for this complex outside the endothelium. Our findings indicate that Src can coordinate specific growth factor and extracellular matrix inputs by recruiting integrin αvβ5 into a FAK-containing signaling complex during growth factor–mediated biological responses.

Introduction

Vascular endothelial growth factor (VEGF)* was originally described as a vascular permeability (VP) factor secreted by tumor cells, expressed in hypoxic tissues (Senger et al., 1983; Connolly et al., 1989; Marti et al., 2000), mitogenic for endothelial cells (Ferrara and Davis-Smyth, 1997), and essential for development (Carmeliet et al., 1996; Ferrara et al., 1996). Recent evidence demonstrates that mice lacking the nonreceptor tyrosine kinase, $pp60^{c\text{-}src}$, have defects in VEGF-mediated vascular responses (Eliceiri et al., 1999; Paul et al., 2001). Specifically, in $pp60^{c\text{-}src}$- or $pp62^{c\text{-}yes}$-deficient mice, the VP-promoting functions of VEGF were distinguished from the mitogenic functions of VEGF because their blood vessels were resistant to VEGF-mediated VP, even though these animals showed normal VEGF-mediated angiogenesis (Eliceiri et al., 1999). However, chick embryos or mice transduced with kinase-deleted Src, which suppresses multiple Src family kinases (SFKs), fail to undergo VEGF-mediated angiogenesis (Eliceiri et al., 1999). Together these findings demonstrate that, in general, SFKs are compensatory during embryogenesis and angiogenesis, but that VEGF-induced VP is dependent on a subset of SFKs, such as Src or Yes (Eliceiri et al., 1999).

Angiogenesis requires the coordination of growth factor receptors and integrins (Brooks et al., 1994; Friedlander et al., 1995), leading to the activation of downstream signals in endothelial cells (Eliceiri et al., 1998; Short et al., 1998). Two pathways of growth factor–induced angiogenesis have been identified in which basic FGF (bFGF) induces angiogenesis dependent on integrin αvβ3 ligation, whereas VEGF induces angiogenesis dependent on the ligation of integrin αvβ5 (Friedlander et al., 1995). The mechanisms underlying the selective coordination of inputs from growth factors and the extracellular matrix (Plopper et al., 1995; Miyamoto et al., 1996; Giancotti and Ruoslahti, 1999), such as the

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*Abbreviations used in this paper: aa, amino acid(s); bFGF, basic FGF; CAM, chorioallantoic membrane; EB, Evan's Blue; FAK, focal adhesion kinase; GST, glutathione S-transferase; HA, hemagglutinin; HUVEC, human umbilical vein endothelial cell; MAP, mitogen-activated protein; SFK, Src family kinase; VEGF, vascular endothelial growth factor; VP, vascular permeability.

Key words: VEGF; vascular permeability; Src; tyrosine kinase; integrin

VEGF pathway with integrin αvβ5, remains poorly understood. For example, whereas αvβ5-deficient mice develop normally (Huang et al., 2000), the ligation state of integrin αvβ5 and Src kinase activity in normal animals are critical during VEGF-induced angiogenesis in vivo (Friedlander et al., 1995; Eliceiri et al., 1999).

Recent work from several laboratories indicates that Src and focal adhesion kinase (FAK) are activated by growth factor receptors and/or after integrin-mediated cell adhesion (Parsons and Parsons, 1997; Schlaepfer and Hunter, 1998). Src and FAK also associate with the cytoplasmic domain of growth factor receptors (Ralston and Bishop, 1985; Gould and Hunter, 1988; Kypta et al., 1990; Sieg et al., 2000), and after integrin-mediated cell adhesion, FAK can recruit Src to focal adhesions leading to Erk activation (Courtneidge et al., 1993; Aplin et al., 1998; Schlaepfer and Hunter, 1998; Wary et al., 1998). In addition to Src, several adapter and signaling molecules can associate with FAK (Cobb et al., 1994; Schlaepfer et al., 1994), including $p130^{Cas}$ (Polte and Hanks, 1995), paxillin (Turner and Miller, 1994), PI 3-kinase

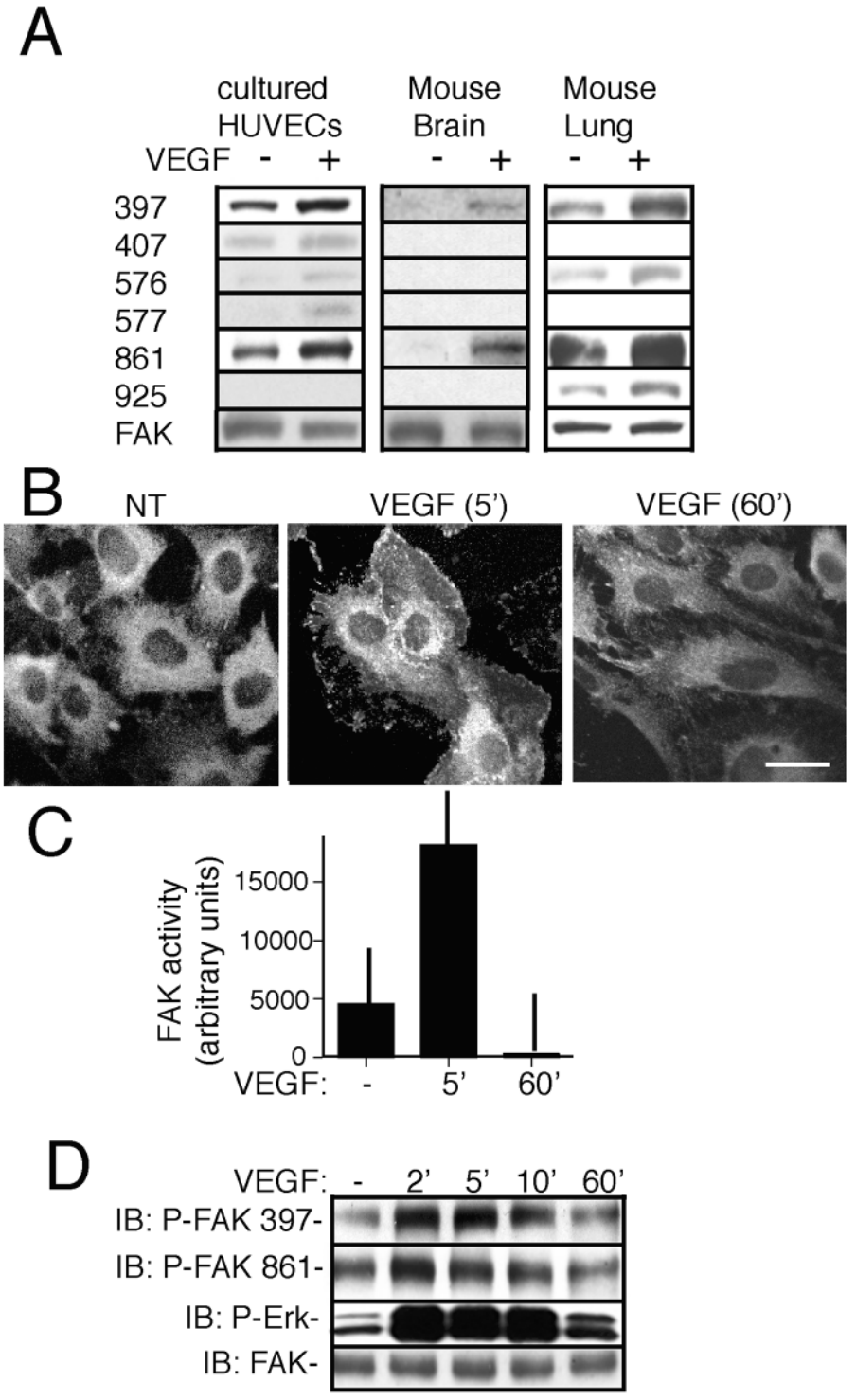

Figure 1. **VEGF promotes FAK phosphorylation and translocation in endothelial cells.** (A) Lysates of VEGF-stimulated primary HUVECs (20 ng/ml; 5 min), mouse brain and lung brain (2 μg/animal, 5 min) were prepared as described in Materials and methods and subjected to immunoblotting with anti-phosphotyrosine antibodies specific for aa 397, 407, 576, 577, 861, or 925 within FAK. The sensitivity and specificity of the phosphospecific antibodies were characterized in various tissues as described in the Materials and methods. These immunoblots are representative of three different experiments. (B) Translocation of endogenous FAK in VEGF-stimulated HUVECs (20 ng/ml; 5–60 min) to focal adhesions was determined by indirect immunofluorescence with an anti-FAK antibody in representative micrographs, as described in Materials and methods. Bar, 5 μm. (C) Representative FAK activity in lysates of VEGF-stimulated HUVECs (20 ng/ml; 5–60 min) was measured by immune complex in triplicate in vitro kinase assays as described in Materials and methods ($P < 0.05$). (D) Lysates of VEGF-stimulated HUVECs (20 ng/ml; 2–60 min) were subjected to immunoblotting with an anti-phosphotyrosine antibody specific for aa 397, 861, an anti-phospho Erk antibody, or an anti-FAK antibody. Each of these panels are representative of triplicate experiments.

(Chen and Guan, 1994), and Grb2 (Schlaepfer et al., 1994). However, the coordination of inputs from growth factor receptors leading to the selective recruitment or activation of specific integrins in vivo remains poorly understood. To investigate the mechanism by which the VEGF pathway coordinates with integrin αvβ5 and Src kinase, an in vivo angiogenesis model was used with a defined growth factor input, (i.e., VEGF), and a known requirement for a specific integrin, i.e., αvβ5. Although we have previously shown an Src-requirement for VEGF-mediated vascular responses (Eliceiri et al., 1999; Paul et al., 2001), experiments were designed to determine whether Src and its substrate, FAK, could functionally regulate αvβ5 during the VEGF-mediated response in intact blood vessels.

Evidence is provided that VEGF and other growth factors activate Src kinase, which induces the phosphorylation of tyrosine 861 (Y861) within the FAK COOH terminus, facilitating the association of FAK with integrin αvβ5 both in vivo and in vitro. Src deficiency or blockade of Src activity inhibits the formation of a VEGF-induced FAK/αvβ5 complex. In contrast, both β1 and β3 integrins were found to couple to FAK in the absence of growth factor stimulation. The physiological relevance of this pathway is underscored by the finding that mice lacking the integrin β5 subunit, or mice deficient in Src, have reduced VEGF-induced VP, suggesting a critical role for integrin αvβ5, together with Src kinase activity, in regulating VEGF-induced vascular responses in vivo.

Results

VEGF promotes FAK phosphorylation and translocation in endothelial cells

Previous studies from our laboratory demonstrated that Src kinase activity (Eliceiri et al., 1999) and integrin αvβ5 ligation (Friedlander et al., 1995) contribute to VEGF-mediated angiogenesis and/or VP. Based on these findings, we considered the role of FAK in VEGF-mediated vascular responses, as integrins as well as Src kinase(s) influence FAK phosphorylation and activation, leading to downstream signaling (for review see Aplin et al., 1998). To gain a molecular understanding of this phenomenon, experiments were designed to determine which Src phosphorylation sites within FAK were phosphorylated after VEGF stimulation. As an initial approach, lysates of VEGF-stimulated primary human endothelial cells (HUVECs) or VEGF-treated mouse tissues were immunoblotted with a panel of phosphotyrosine-specific antibodies. These antibodies were directed to the tyrosine-phosphorylated state of amino acids (aa) 397, 407, 576, 577, 861, or 925 within FAK, to detect the known substrate sites for Src. The profile of VEGF-induced tyrosine phosphorylation in cultured HUVECs was compared with lysates of mouse lung and brain tissues exposed to VEGF (5 min) (Fig. 1 A). VEGF-induced robust tyrosine phosphorylation of aa 397 and 861 on FAK within cultured endothelial cells as well as in intact mouse tissues (Fig. 1 A). Other tyrosines within FAK were phosphorylated to a minimal degree or below the detection limit.

FAK is found in focal contacts where it promotes downstream integrin-mediated signals (Parsons and Parsons, 1997; Schlaepfer and Hunter, 1998). To assess the role of VEGF in the recruitment of FAK to focal contacts, we examined the localization of FAK in quiescent or VEGF stimulated endothelial cells. Serum-starved HUVEC monolayers were treated for various times with VEGF, which induced the subcellular translocation of a fraction of the endogenous pool of FAK from a diffuse cytoplasmic distribution to focal adhesions within 5 min, consistent with previous observations (Takahashi et al., 1999). This subcellular translocation response was transient, as there was a complete loss of FAK in focal adhesions within 60 min (Fig. 1 B). The kinetics of the subcellular translocation correlated with a transient increase in FAK activity (3.5-fold increase within 5 min), followed by a decrease in FAK activity by 60 min in lysates of these cells (Fig. 1 C). Based on the prominent VEGF-induced tyrosine phosphorylation of aa 861 in endothelial cells (Fig. 1 A) (Abu-Ghazaleh et al., 2001), lysates of VEGF-stimulated HUVECs were immunoblotted with phosphotyrosine-specific anti-FAKY397, FAK Y861, phosphospecific anti–mitogen-activated protein (MAP) kinase (Erk), or anti-FAK antibodies (Fig. 1 D). Tyrosine phosphorylation of aa 861 within FAK was increased within 2–5 min, and returned to baseline levels within 60 min. The VEGF-induced Erk phosphorylation completely paralleled the kinetics of FAK phosphorylation, FAK activity and its subcellular translocation. These findings reveal that VEGF promotes a rapid but transient redistribution of FAK to focal contacts which parallels its activation kinetics, and the induction of downstream signaling to ERK.

VEGF induces FAK phosphorylation and formation of a FAK/αvβ5 complex in cultured endothelial cells

Ligation of integrin αvβ5 has been shown to be essential for VEGF-induced angiogenesis (Friedlander et al., 1995), although the mechanisms underlying the recruitment of intracellular signaling proteins to integrins in vivo remains poorly understood. For example, an autonomously expressed form of FAK lacking kinase activity, FAK-related non-kinase (Schaller et al., 1993), suppresses VEGF-induced angiogenesis (unpublished data), suggesting that FAK may have an essential role in VEGF-mediated vascular responses. Whereas data in Fig. 1 demonstrates that VEGF stimulation leads to the phosphorylation of FAK on aa 397 and 861 (Fig. 1 A) and its localization in focal contacts (Fig. 1 B), the capacity for phosphorylated FAK to coordinate with integrins in blood vessels is unknown. Therefore, lysates of starved or VEGF-stimulated HUVECs were subjected to immunoprecipitation with anti-integrin antibodies. These immunoprecipitates were then probed for the presence of FAK. VEGF induced a FAK/αvβ5 complex in endothelial cells (Fig. 2 A) that was associated with increased FAK phosphorylation (Fig. 1) and kinase activity (Fig. 1 C). Unlike that seen with αvβ5, αvβ3 showed a constitutive association with FAK that did not increase in response to VEGF (Fig. 2 A). Other angiogenic growth factors such as bFGF do not appear to promote FAK/αvβ5 coupling (Fig. 2 A, bottom). The specificity of the FAK/αvβ5 complex was supported by blotting for other candidate focal adhesion proteins. For example, these αvβ5 immunoprecipitates were probed for paxillin, $p130^{Cas}$, or PKC, which can bind FAK/integrin complexes (Fig. 2 B). Immunoblotting with an anti-phosphotyrosine

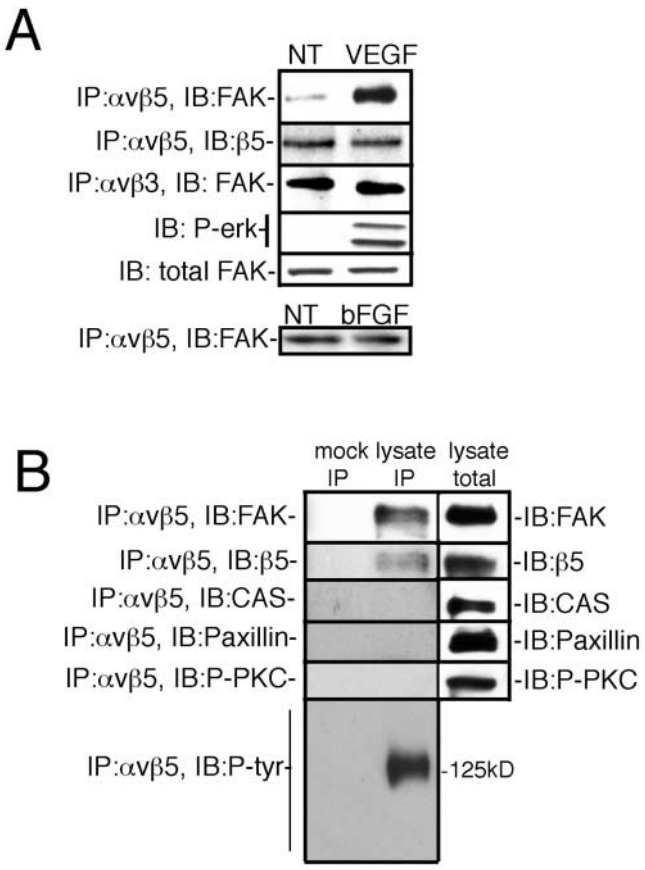

Figure 2. **VEGF-induced assembly of FAK with integrin αvβ5 in endothelial cells.** (A) FAK association with integrin αvβ5 or αvβ3 in HUVECs with or without VEGF (20 ng/ml; 5 min) was measured by immunoprecipitation from whole cell lysates with anti-αvβ5 or anti-αvβ3 monoclonal antibodies, respectively. These immune complexes were immunoblotted and detected with an anti-FAK antibody. Immunoblotting with an anti-phospho Erk antibody reveals the activation of the MAP kinase pathway in these VEGF-stimulated endothelial cells. The bottom panel shows the association of FAK with αvβ5 after stimulation with basic fibroblast growth factor. (B) The capacity for cytosolic proteins other than FAK to associate with αvβ5 was determined by immunoblotting αvβ5 immunoprecipitates from VEGF-stimulated HUVEC lysates with anti-p130^{Cas}, paxillin, phosphorylated pan PKC, or phosphotyrosine antibodies. No background bands in the molecular weight range of FAK were detected by FAK immunoblotting of mock immunoprecipitations performed with anti-αvβ5 in the absence of cell lysate (shown above) or with control antibodies (i.e., anti-VEGFR2) in the presence of lysate (unpublished data).

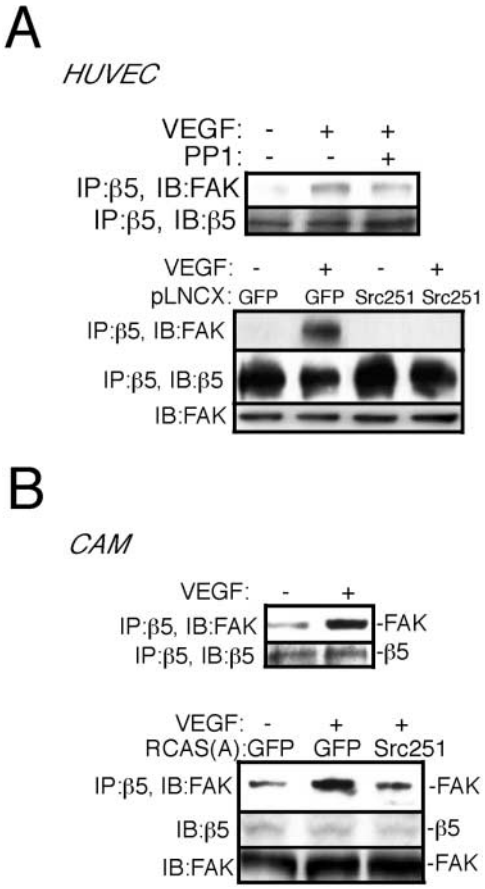

Figure 3. **Src kinase regulates VEGF-induced assembly of a FAK/αvβ5 complex in cultured endothelial cells and in the chick embryo during angiogenesis.** (A) The Src kinase family inhibitor, PP1 (Hanke et al., 1996) was used to inhibit (10 μM; 2 min pretreatment) VEGF-induced assembly of the FAK/αvβ5 complex in HUVECs (20 ng/ml; 5 min). Retroviral gene delivery of GFP or a dominant negative Src, kinase-deleted Src (Src 251) was used to suppress Src kinase activity in VEGF-stimulated HUVECs as described in Materials and methods. (B) Induction of a FAK/αvβ5 complex in the CAM was measured by stimulation of 10-d-old CAMs with VEGF (1 μg/ml; 5 min) as previously described (Eliceiri et al., 1999). Retroviral gene delivery of Src 251 or control GFP, was used to suppress Src kinase activity in angiogenic blood vessels as previously described (Eliceiri et al., 1999). Lysates of these CAMs were subjected to immunoprecipitation with an anti-αvβ5 antibody and the immunoprecipitates immunoblotted with an anti-FAK antibody. These immunoblots were representative of at least three separate experiments.

antibody did not reveal a significant population of additional tyrosine-phosphorylated proteins other than a 125-kD protein, most likely FAK, in the αvβ5 immunoprecipitations. Although we did not detect other proteins associated with FAK/αvβ5 complexes, this may be due to the brief VEGF stimulation (5 min) used in this experiment.

Src kinase regulates the formation of a FAK/αvβ5 complex after VEGF stimulation in cultured endothelial cells or on the chorioallantoic membrane of 10 1-d-old chick embryos during angiogenesis

Blockade of Src kinase activity (Eliceiri et al., 1999) or ligation of integrin αvβ5 disrupts VEGF-mediated signaling and angiogenesis, yet it fails to influence bFGF-mediated angiogenesis (Friedlander et al., 1995). However, inhibition of αvβ5 has no effect on VEGF-stimulated Src kinase activity (unpublished data). These findings suggest that Src functions upstream of integrin αvβ5. Therefore, we considered whether Src might be required for assembly of the FAK/αvβ5 complex in VEGF-stimulated endothelial cells or tissues. To test this possibility, we employed pharmacological or genetic approaches to suppress Src kinase activity in cultured endothelial cells in vitro or intact blood vessels in vivo. Pharmacological inhibition of Src kinase with PP1 (Hanke et al., 1996) or retroviral delivery of kinase-deleted Src (aa 1–251, Src 251) suppressed VEGF-induced levels of the FAK/αvβ5 complex in HUVECs (Fig. 3 A). To determine whether VEGF could induce a FAK/αvβ5 complex in blood

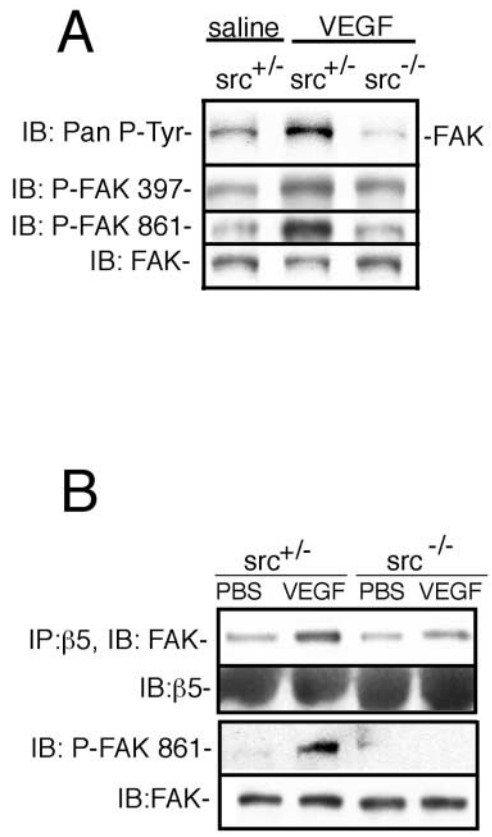

Figure 4. **VEGF-induced FAK phosphorylation and formation of FAK/αvβ5 complex is reduced in src$^{-/-}$ mice.** (A) Lysates of VEGF-stimulated mouse lungs (2 μg i.v./animal; 5 min) from src$^{-/-}$ or control mice were subjected to immunoblotting with a generic anti-phosphotyrosine antibody, or anti-phosphotyrosine antibodies specific for aa 397 or 861 within FAK, as described in Materials and methods. (B) The dermis of the ears of src$^{+/-}$ or src$^{-/-}$ mice were injected intradermally with VEGF (500 ng) or PBS, and after 5 min, whole tissue lysates were prepared for immunoprecipitation with an anti-β5 antibody, followed by immunoblotting for FAK, an anti-phosphotyrosine antibody specific for tyrosine 861 within FAK. Parallel lysates were subjected to immunoblotting with anti-FAK or β5 antibodies as loading controls. Laser scanning densitometry of the FAK/αvβ5 complex in the top row revealed a threefold increase in FAK/αvβ5 complex after VEGF stimulation in src$^{+/-}$ compared with only a 1.1-fold increase in src$^{-/-}$mice. These immunoblots were representative of at least three separate experiments.

vessels in vivo, chick chorioallantoic membranes (CAMs) were stimulated with VEGF and analyzed for the presence of a FAK/αvβ5 complex. Lysates from VEGF-stimulated contained elevated levels of the FAK/αvβ5 complex, compared with unstimulated controls (Fig. 3 B, top). The formation of the VEGF-induced FAK/αvβ5 complex was disrupted by exposing these CAMs to an avian-specific retrovirus (RCAS) expressing Src 251 (Fig. 3 B, bottom), providing genetic evidence for a Src requirement for the VEGF-induced assembly of the FAK/αvβ5 complex in vivo.

VEGF-induced FAK phosphorylation and formation of a FAK/αvβ5 complex is suppressed in src$^{-/-}$ mice

Although disruption of multiple SFKs with Src 251 blocks VEGF-mediated angiogenesis, mice lacking a single SFK showed a selective loss of VEGF-mediated VP, suggesting that multiple Src kinases can contribute to VEGF-dependent angiogenesis, yet selective SFKs are important for VP (Eliceiri et al., 1999; Paul et al., 2001). To substantiate the VEGF-mediated Src requirement for the FAK/αvβ5 association, mice lacking pp60$^{c\text{-}src}$ were injected with VEGF, and the injected tissues analyzed for FAK phosphorylation and assembly of the FAK/αvβ5 complex. Lysates were prepared from src$^{-/-}$ or control mouse tissues (dermis) stimulated with VEGF or saline and subjected to immunoblotting with phospho-specific antibodies directed to aa 397 or 861. VEGF induced an increase in FAK 397 and 861 phosphorylation in control mice, whereas only a minimal level of FAK phosphorylation was detected in src$^{-/-}$ mice (Fig. 4 A), suggesting that FAK is an important substrate for Src after VEGF stimulation in vivo. Although VEGF treatment increased the level of FAK associated with integrin β5 in src$^{+/-}$ control animals (threefold), the formation of this complex was significantly suppressed in src$^{-/-}$ mice (Fig. 4 B) (1.1-fold). These results provide genetic evidence in mice to corroborate the finding that the VEGF-induced phosphorylation of FAK and the association of phosphorylated FAK with αvβ5 depend on VEGF-mediated Src kinase activity.

A role for the growth factor–induced tyrosine phosphorylation of the COOH-terminal FAK aa 861 for assembly with integrin β5 in vivo

VEGF stimulation induces FAK/αvβ5 complex formation in vivo and in cultured endothelial cells (Figs. 2–4). Although both tyrosines 397 and 861 within FAK are prominently phosphorylated after VEGF stimulation, it remains unclear whether these sites are involved in the formation of the FAK/αvβ5 complex by phosphorylation of either of these sites. In addition, it is important to determine whether FAK/αvβ5 complexes can form in other cell types in response to other growth factors. To address these questions and to determine the functional requirement for tyrosines 397 and 861 in the assembly of the FAK/αvβ5 complex, epitope-tagged (hemagglutinin [HA]) full-length FAK constructs were expressed at similar levels in human epithelial cells (HEK-293) (Fig. 5 A). HA-tagged wild-type FAK (HA-FAK), or mutants of aa 397 (HA-FAK Y397F) or 861 (HA-FAK Y861F) were examined for their capacity to associate with endogenous αvβ5 in these cells. Like the endothelial cell response to VEGF, epithelial cells such as HEK-293 formed a FAK/αvβ5 complex in response to EGF, which was blocked by the Src inhibitor, PP1 (unpublished data). Lysates of EGF-stimulated HEK-293 cells expressing FAK constructs were subjected to immunoprecipitation with anti-αvβ5, and the immunoprecipitates were blotted with anti-HA to detect FAK. Wild-type FAK or the Y397F FAK mutant were readily detected in a complex with αvβ5, however the Y861F FAK mutant failed to form a complex with αvβ5 (Fig. 5 B). Immunoblotting of whole cell lysates with an anti-HA antibody revealed equivalent expression levels of each of these tagged FAK constructs (Fig. 5 B). These findings reveal that the tyrosine at position 861 is critical for the formation of FAK/αvβ5 complex, and that this complex may form in other growth factor–stimulated cell types.

To confirm the role of tyrosine phosphorylation of aa 861 in the formation of FAK/αvβ5 complexes in VEGF-treated endothelial cells, HA-tagged wild-type FAK or aY861F mu-

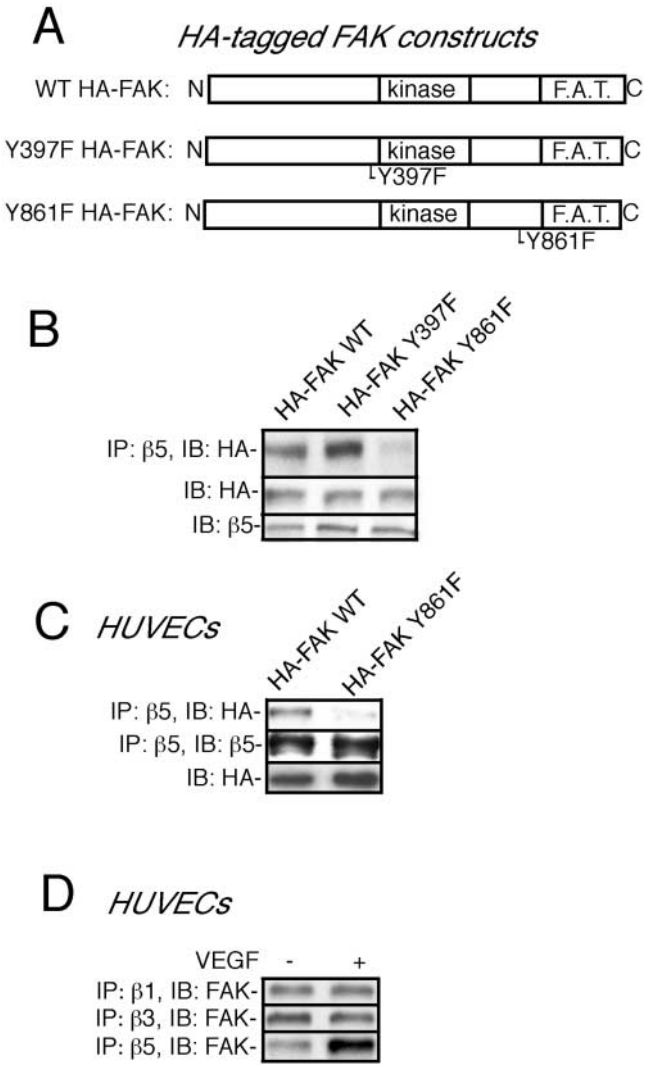

Figure 5. **A role for the growth factor–induced tyrosine phosphorylation of the COOH-terminal FAK aa 861 for assembly with integrin β5 in vivo.** (A) Various epitope-tagged (HA) FAK constructs (HA wild-type, Y397F, or Y861F) were expressed in HEK-293 cells, as previously described (Sieg et al., 2000). (B) Lysates of EGF-stimulated cells (20 ng/ml; 5 min) expressing these various FAK constructs were subjected to immunoprecipitation with an anti-αvβ5 antibody and immunoblotted with an anti-HA antibody to detect FAK/αvβ5 complexes. Expression levels of each HA-tagged construct was confirmed by HA immunoblotting. (C) Association of HA-WT FAK or HA-Y861 mutant FAK with integrin αvβ5 in VEGF-stimulated HUVECs. Parallel blotting of HUVEC lysates with anti-HA antibody reveals transient expression levels of HA-tagged FAK constructs. (D) Lysates of VEGF or mock-treated HUVECs were analyzed for the VEGF-induced association of FAK with αvβ5, αvβ3, or β1 integrins by immunoprecipitation with anti-αvβ5, αvβ3, or β1 antibodies and immunoblotting with an anti-FAK antibody. These immunoblots were representative from at least three different experiments.

tant FAK were expressed in HUVECs. After VEGF stimulation, theY861F FAK mutant failed to associate with αvβ5, whereas the WT FAK construct formed a complex with αvβ5 (Fig. 5 C), consistent with the VEGF-induced coupling of αvβ5 with endogenous FAK in these cells (Fig. 2 A). These results suggest that the VEGF-induced tyrosine phosphorylation of aa 861 is important in the formation of the FAK/αvβ5 complex in the endothelium.

Phosphorylation of the COOH-terminal FAK tyrosine 861 regulates assembly with integrin β5 in vitro

Previous findings have shown that the membrane proximal region of the β integrin cytoplasmic tail can bind FAK in vitro (Schaller et al., 1995), a region that is conserved between β1, β3, and β5 integrins. In support of this, we show that integrins αvβ3 and β1 (Fig. 5 D) have a constitutive baseline association with FAK, whereas only integrin αvβ5 supports increased assembly of a FAK/integrin complex in response to VEGF and other growth factors (Figs. 2 A, 3 B, and 5). Furthermore, the co-immunoprecipitation analysis of HA-FAK/αvβ5 in cultured cells suggests that the tyrosine phosphorylation of a specific aa, Y861 in the FAK COOH terminus, is important for the FAK/αvβ5 complex (Fig. 5). Therefore, to further characterize the mechanism of the Src-mediated FAK/αvβ5 interaction, in vitro binding studies were performed using NH_2- or COOH-terminal domains of FAK and various full-length or truncated fusion proteins of β5 and β3 cytoplasmic tails. NH_2-terminal (FAK NT; aa 1–410) and COOH-terminal (FAK CT; aa 852—1052) fragments of FAK were subjected to in vitro phosphorylation with active Src and allowed to bind to fusion proteins derived from integrin β5 or β3 cytoplasmic tails. Src failed to phosphorylate FAK NT in vitro (unpublished data), and therefore was not used in subsequent in vitro binding assays. However, Src induced tyrosine phosphorylation of the FAK CT in vitro as detected with phosphotyrosine antibodies to aa 861 and 925 (Fig. 6 C). Mock-treated or phosphorylated FAK CT protein was incubated with the full-length cytoplasmic tails of integrin β5 (glutathione S-transferase [GST]: aa 716–772) or β3 (GST: aa 716–762) (Fig. 6 A). Integrin-bound FAK was captured with glutathione-Sepharose and analyzed by immunoblotting with an anti-FAK antibody. As expected from our previous results (Fig. 2), FAK was constitutively associated with the full-length β3 cytoplasmic tail. Unexpectedly, some level of constitutive association was detected in complex with full-length β5. However, this may be anticipated, as β1, β3, and β5 integrin cytoplasmic tails share considerable sequence homology, particularly at the membrane-proximal domain, including the sequence (KLL[V/I]TIHDR[R/K]EFAKF] (Fig. 6 A, ●). Therefore, to determine the contribution of the sequences unique to the cytoplasmic tails of the β3 and β5 subunits, fusion proteins were prepared lacking the common membrane proximal sequence. Binding assays of mock-treated or phosphorylated FAK CT with these truncated β3 or β5 cytoplasmic tails revealed that Src-phosphorylated FAK CT bound selectively to the β5 tail compared with the β3 cytoplasmic tail, whereas nonphosphorylated FAK CT failed to bind either β3 or β5 cytoplasmic tails. To determine whether the phosphorylation of tyrosine 861 within the FAK CT by Src was required for the interaction of FAK with β5, a point mutant of the FAK CT (Y861F) was evaluated. Although the Src-phosphorylated FAK CT bound integrin β5, the mutant FAK CT (Y861) failed to bind integrin β5 (Fig. 6 C) even though it was phosphorylated on aa 925 as detected by immunoblot analysis (Fig. 6 C). These in vitro binding data with integrin tails lacking the membrane proximal domain are con-

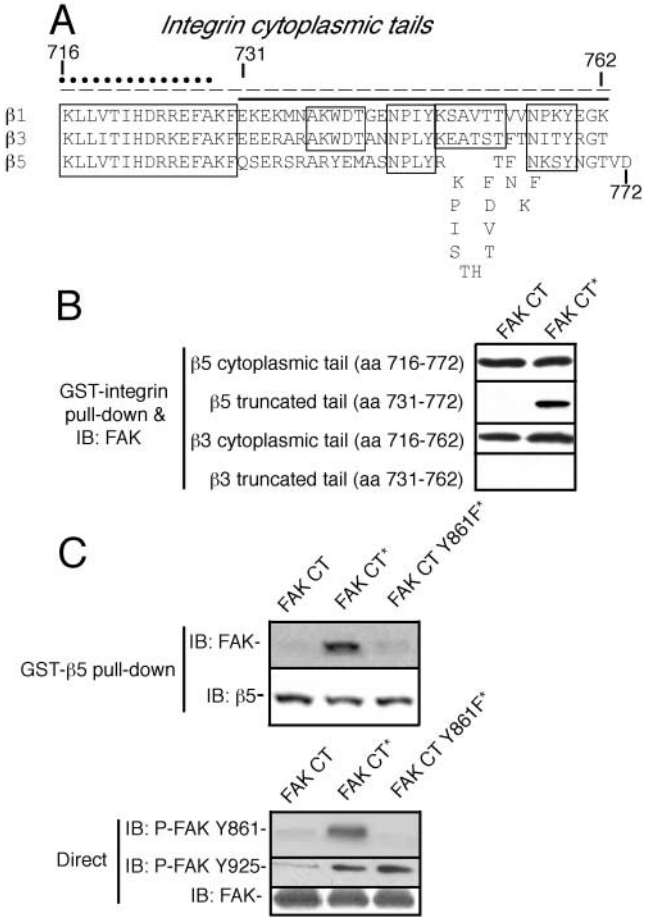

Figure 6. **Phosphorylation of the COOH-terminal FAK tyrosine 861 regulates assembly with integrin β5 in vitro.** (A) Alignment of the cytoplasmic tails of β integrin subunit for integrins β1, β3, and β5. Full-length fusion proteins of β3 and β5 cytoplasmic tails (dashed line), truncated β3 and β5 cytoplasmic tails lacking the membrane-proximal domain (solid line) used in this study, and previously mapped conserved peptide region of β1 integrin (Schaller et al., 1995; circles). Boxed regions represent conserved domains. (B) Associations between Src-phosphorylated FAK COOH terminus (FAK CT*) or mock-phosphorylated FAK CT (FAK CT) with GST fusions of full length β3 or β5 integrin cytoplasmic tails, or truncated β3 or β5 integrin cytoplasmic tails (5 min) were identified by immunoblotting glutathione-Sepharose pulldowns with an anti-FAK antibody, as described in Materials and methods. These blots were representative of at least three different experiments. (C) (upper) Phosphorylated FAK CT or a mutant of tyrosine 861 within the FAK CT (FAK CT Y861F) was incubated with the truncated β5 integrin cytoplasmic tail. (lower) Src-mediated phosphorylation of the FAK CT was determined by immunoblotting with phosphotyrosine specific anti-FAK antibodies recognizing aa 861 or 925.

sistent with the data from intact cells in which VEGF-induced an increase in FAK/αvβ5 but not FAK/αvβ3 complexes. Although the molecular basis of the interactions of many proteins which associate with integrin tails remains poorly understood, our findings suggest that the membrane proximal domain of integrin tails may contribute to the formation of a baseline of the FAK/integrin complex in vivo and in vitro.

VEGF induced VP defect in integrin β5–deficient mice

Although the in vitro findings provide an important insight into the molecular basis of Src-mediated FAK/αvβ5 interactions in the VEGF pathway, the functional requirement for integrin αvβ5 in a VEGF-mediated vascular response remained unknown. Previous studies have demonstrated that VEGF-mediated endothelial responses depend on Src kinase activity (Eliceiri et al., 1999) and integrin αvβ5, but not αvβ3 (Friedlander et al., 1995). In support of this, mice lacking a single Src family member such as $pp60^{c\text{-}src}$ fail to undergo VEGF-induced VP, whereas general suppression of Src with kinase-deleted Src blocks VEGF- but not bFGF-mediated angiogenesis (Eliceiri et al., 1999). Therefore, we reasoned that if Src and αvβ5 were both downstream of VEGF and on a common signaling pathway, one might predict that mice lacking αvβ5 would have a phenotype similar to that of $src^{-/-}$ mice. Control mice or those lacking integrin β5 or β3 were intradermally injected with VEGF and evaluated for VEGF-mediated VP. The β5-deficient mice had a significant decrease in VEGF-induced VP compared with control littermates (Fig. 7 A) ($P < 0.05$), which paralleled the loss of VP observed in $src^{-/-}$ mice (Eliceiri et al., 1999; Paul et al., 2001). Importantly, mice lacking β3 (Hodivala-Dilke et al., 1999) showed control levels of VP (Fig. 7 A), which is consistent with our previous findings that VEGF-dependent vascular responses depend primarily on αvβ5 (Friedlander et al., 1995). To corroborate these findings, control mice or mice lacking β5 were subjected to a stereotactic brain injection of saline or VEGF into the brain which is known to compromise the blood brain barrier (Fig. 7 B) (Eliceiri et al., 1999). The decrease in Evan's blue extravasation in cerebral blood vessels of $\beta5^{-/-}$ mice after VEGF administration suggests that there is a requirement for integrin β5 in the VEGF-mediated breakdown of the blood–brain barrier. Furthermore, the decrease in VEGF-induced VP in these β5-deficient mice was concomitant with a decrease in brain damage after cerebral ischemia (Fig. 7 C). Together, these results demonstrate an important role for integrin αvβ5 in VEGF-mediated endothelial responses in vivo that appears identical to that seen in mice lacking $pp60^{c\text{-}src}$.

Discussion

VEGF is unique among angiogenic growth factors, as it functions as both a mitogen/chemoattractant and as well as an inducer of VP in blood vessels (Senger et al., 1983; Ferrara and Davis-Smyth, 1997). Recent studies indicate that VEGF promotes integrin-dependent cell biological responses in vivo and in vitro (Friedlander et al., 1995; Soldi et al., 1999; Borges et al., 2000; Byzova et al., 2000), suggesting that the coordination of inputs from the extracellular matrix and growth factors are physiologically important. Although growth factors and integrin-mediated cell adhesion are known to activate nonreceptor tyrosine kinases such as FAK and Src, the mechanisms by which growth factor–induced biological processes in primary cells and tissues are regulated by integrins remains poorly understood. In this report, evidence is provided for a novel molecular mechanism to explain how Src can regulate integrin and growth factor–dependent signaling within blood vessels stimulated with VEGF, a process which may be applicable to other cell types.

An important finding of this study is that VEGF via Src induces the site-specific tyrosine phosphorylation of FAK on

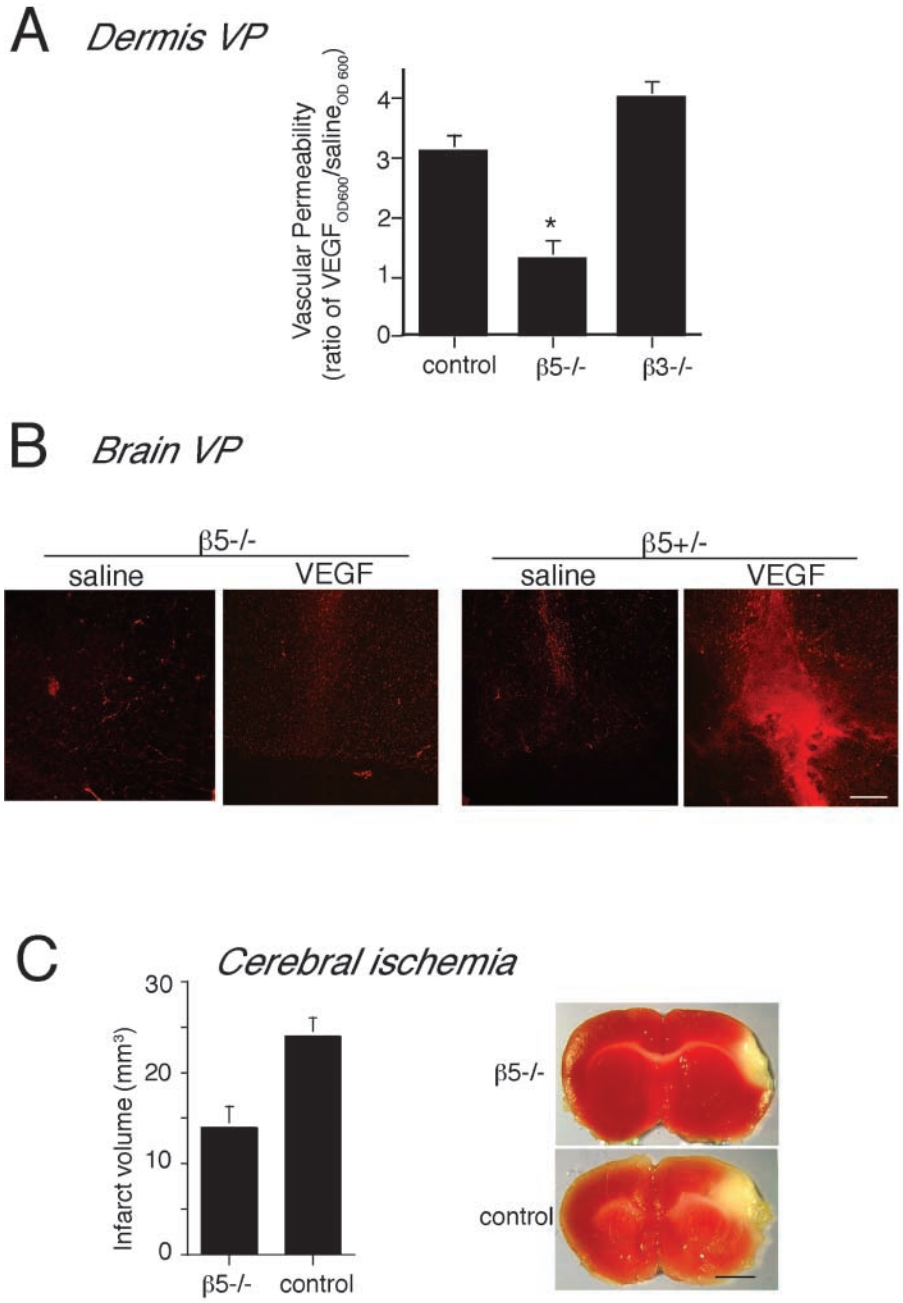

Figure 7. **VEGF-induced VP defect in integrin β5–deficient mice.** (A) The VEGF-induced VP response in the dermis of $\beta5^{-/-}$, $\beta3^{-/-}$, or control mice was determined by intradermal injection of VEGF (400 ng) into mice that had been previously injected with EB, a fluorescent VP indicator. The extravasation of EB from the blood vessels was quantitated by fluorimetry ($O.D._{600}$), as previously described (Eliceiri et al., 1999). ($n = 7$) (*, $P < 0.05$) (B) The VEGF-induced VP response in cerebral blood vessels was identified by the extravasation of EB from $\beta5^{-/-}$ or control mice that had been stereotactically injected with VEGF or saline, as previously described (Eliceiri et al., 1999; Paul et al., 2001). Laser confocal scanning microscopy was used to visualize the fluorescence of the extravasated EB in representative brain cross sections. Bar, 100 μm. (C) Infarct volumes following cerebral ischemia were determined in TTC-stained coronal sections of $\beta5^{-/-}$ or control mice, as previously described (Paul et al., 2001). Quantitation of the infarct volumes was measured as previously described (Paul et al., 2001) ($P < 0.02$) (left). Representative micrographs of TTC-stained brain sections reveal the zone of VEGF-mediated neuronal damage (right). Bar, 2 mm.

Y861, leading to the formation of a complex between FAK and αvβ5 in both cultured endothelial cells in vitro and blood vessels in vivo, and in EGF-stimulated epithelial cells. These findings are consistent with the emerging role of aa 861 in mediating cell migration in tumor (Slack et al., 2001) and endothelial cells (Abu-Ghazaleh et al., 2001). In this study we have shown that Src deficiency or blockade of Src activity suppresses FAK phosphorylation at aa 861, and thereby reduces VEGF-induced FAK/αvβ5 complex formation. These findings indicate that VEGF-induced Src activity and the phosphorylation of Y861 in FAK contribute to the formation of a FAK/αvβ5 complex. Although baseline levels of FAK associate with integrins β1, β3, and β5, only the β5 integrin supports increased levels of FAK/integrin complexes after VEGF stimulation. Our data suggests that this interaction depends on a region within the COOH-terminal half of the β5 cytoplasmic tail that contains an aa sequence distinct from that of β1 or β3. Direct genetic evidence for a role for integrin αvβ5 in the VEGF pathway is demonstrated in mice lacking integrin β5, which, like $src^{-/-}$ mice, have a defective VEGF-mediated VP response. In contrast, mice lacking integrin β3 have a normal VEGF-induced VP response. In combination with the biochemistry from endothelial cell immunoprecipitations and the in vitro binding assays, the lack of VEGF-mediated VP from Src or β5 knockout mice suggests that the VEGF-induced formation of the FAK/αvβ5 complex may be an important mechanism for coordinating growth factor–dependent integrin signaling during VEGF-mediated VP.

Previous studies from our laboratory demonstrate that SFKs (Eliceiri et al., 1999) and integrin αvβ5 (Friedlander et al., 1995) are required for VEGF-induced angiogenesis

and VP. In contrast, bFGF-induced angiogenesis depends on the ligation of integrin αvβ3 (Friedlander et al., 1995), and is independent of Src kinase activity (Eliceiri et al., 1999). Several other signaling molecules, such as PKC or eNOS, selectively contribute to the VEGF pathway (Friedlander et al., 1995; Ziche et al., 1997), suggesting that at least some of the upstream components of the VEGF and bFGF signaling pathways are distinct.

In addition to the role of VEGF as a mitogen and a VP factor, a functional role for VEGF in inducing edema and tissue damage has been identified after cerebral ischemia (van Bruggen et al., 1999). Direct genetic evidence for the pathophysiological relevance of integrin αvβ5 in the VEGF pathway is provided by the observation of a reduction in neuronal damage in β5-deficient mice after cerebral ischemia (Fig. 7 C). We have previously shown that Src deficiency or blockade of Src activity prevents VEGF-mediated VP, thereby reducing neuronal damage after stroke (Paul et al., 2001). In combination with the reduction in VEGF-induced VP (Fig. 7) and neuronal damage in $\beta5^{-/-}$ mice, these results suggest a link between integrin αvβ5 and the Src-dependent VEGF vascular response in vivo.

Evidence from several cell models indicates that integrin αvβ5 mediates cell biological processes that require costimulation with growth factors. For example, αvβ5-mediated cell adhesion, migration/invasion requires prestimulation with growth factors (Klemke et al., 1994; Brooks et al., 1997; Doerr and Jones, 1996; Lewis et al., 1996). In contrast, αvβ3-mediated cell migration/invasion in these cells are independent of growth factor stimulation. These studies suggest that in contrast to αvβ3, integrin αvβ5 may require an upstream priming signal from an activated growth factor receptor leading to Src kinase activation for biological function of the integrin αvβ5 and downstream signaling. The capacity for HEK-293 epithelial cells to form an Src-dependent FAK/αvβ5 complex in response to EGF and our results with VEGF-stimulated endothelial cells suggests that this pathway may have a general significance for a wide range of cell types in response to specific growth factors.

Data presented here indicate that a FAK/integrin complex can form in an integrin-specific manner depending on the stimulus. Although FAK can bind the membrane distal region of the β1 integrin tail (Lewis and Schwartz, 1995; Klingbeil et al., 2001), the FAK NH_2 terminus binds a conserved membrane proximal β1 integrin cytoplasmic tail sequence (Schaller et al., 1995). The molecular basis of this constitutive baseline association of FAK with the membrane proximal region of β integrins remains unknown; however, it is possible that the Src-mediated association of the FAK CT with the truncated β5 cytoplasmic tail may depend on a β5-specific distal sequence(s). There are no obvious motifs within the integrin β5 cytoplasmic tail, such as a phosphotyrosine binding domain that might account for such an interaction, but evidence presented here suggests that Src-mediated tyrosine phosphorylation of the FAK CT at aa 861 can contribute to the FAK/αvβ5 association. It is conceivable that phosphorylation of aa 861 influences the structure of FAK through intramolecular rearrangement, enabling it to bind the cytoplasmic tail of integrin β5. This may involve more than one interaction, such that the FAK NT might associate with the membrane proximal region of the β integrin cytoplasmic tail, as suggested by previous workers (Schaller et al., 1995), whereas the FAK CT associates selectively with the COOH terminus of the β5 integrin cytoplasmic tail. Recent findings indicate that the FAK NT may be important for coordinating with growth factors receptors (Sieg et al., 2000), whereas tyrosine phosphorylation of aa 861 in the FAK CT is increased during integrin-mediated cell migration (Abu-Ghazaleh et al., 2001; Slack et al., 2001). Furthermore, our data with different FAK mutants suggest that wild-type FAK interacts with αvβ5 through mechanism(s) distinct from Y397F/αvβ5 interactions. Not surprisingly, the Y397F mutation of aa 397 influences a wide range of other phosphorylation events, which may complicate the interpretation of this mutant in these assays. Indeed, phosphorylation of Y397 (Wennerberg et al., 2000), Y925 and other sites within FAK may influence the complexity of integrin-associated proteins in vivo, and mediate baseline levels of FAK/integrin interactions. We believe that the design of the in vitro binding assays with the FAK COOH terminus lacking aa 397, facilitates the analysis of the potential role of aa 861 in mediating growth factor–dependent interactions with the distal portion of the integrin tail. Although our in vitro binding data suggests that the FAK/αvβ5 complex forms in the absence of other proteins, it is possible that other focal-adhesion associated proteins (for review see Aplin et al., 1998) can associate with this FAK/αvβ5 complex in cells.

Evidence is provided that endothelial cells can coordinate VEGF-induced vascular responses through a specific integrin-mediated signaling mechanism. Although both Src kinase and integrin αvβ5 are necessary for these VEGF responses in blood vessels, we propose that the Src-mediated association of FAK with αvβ5 represents a novel mechanism for the coordination of different integrin and growth factor–dependent biological processes and may be applicable to various cell types in vivo.

Materials and methods

Antibodies and reagents

A rabbit polyclonal antibody raised against the COOH terminus of human FAK (C-20; Santa Cruz Biotechnology) was used for immunoprecipitations for in vitro kinase assays and immunoblotting. The anti-HA antibody was from Covance Research Products. Monoclonal antibodies directed to αvβ3 (LM609) or αvβ5 (P1F6) used for integrin immunoprecipitations from human or chick tissues. Rabbit polyclonal anti-β5 antibodies used to immunoprecipitate mouse integrin αvβ5 were from either Dr. M. Hemler (Harvard University, Boston, MA) (Ramaswamy and Hemler, 1990) or Chemicon International. The phospho-specific MAP kinase antibody was from New England Biolabs, and the phospho-FAK antibodies directed to tyrosines 397, 407, 576, 577, 861, or 925 were from Biosource. The specificity of these site-specific anti-phosphotyrosine antibodies targeting FAK was confirmed by immunoblotting various FAK mutants expressed in vitro, in cultured cells, and/or based on previous findings with these reagents (Sieg et al., 2000). RCAS (A)-GFP, and Src 251 were gifts of Dr. P. Schwartzberg (National Institutes of Health, Bethesda, MD) and H. Varmus (Sloan-Kettering, New York, NY). pLNCX–FAK-related non-kinase constructs were a gift of Dr. T. Parsons (University of Virginia, Charlottesville, VA). HUVECs were obtained from Biowhittaker. VEGF was from Peprotech, and bFGF was a gift of Dr. J. Abraham (Scios, Mountain View, CA). Protein A/G was from Pierce Chemical Co., and glutathione-Sepharose was from Amersham Pharmacia Biotech. All other reagents and media were from Sigma-Aldrich.

HUVEC, HEK-293, chick embryo, and mouse treatments

Low-passage (P2-P5) HUVEC were serum starved for 16 h in serum-free media before stimulation with growth factors. Gene delivery of various

constructs into HUVECs was performed by retroviral infection using the replication-defective murine Moloney retrovirus pLNCX and amphotropic packaging cells (ϕNX-Ampho, a gift of G. Nolan, Stanford University, Stanford, CA) as described previously (Eliceiri et al., 1999). HEK-293 cells expressing various FAK constructs (HA-tagged full-length wildtype FAK, or mutants Y397F and Y861F) were pooled populations of cells expressing HA-tagged FAK proteins. Both HUVECs and 293 cells were serum starved for 16 h in serum-free media before VEGF or EGF stimulation, respectively. Fertilized chick embryos (McIntyre Farms) were stimulated with growth factors or infected with retroviruses as previously described (Eliceiri et al., 1999). High-titer avian-specific retroviruses used for the transduction of CAM tissue with mutant constructs were prepared as previously described (Eliceiri et al., 1999). 48 h after infection with the retroviruses expressing GFP or mutant Src 251, chick CAMs were stimulated with VEGF for 5 min, and lysates were prepared for analysis.

$\beta5^{-/-}$ and control $\beta5^{+/-}$ mice were generated as previously described (Huang et al., 2000). $\beta3^{-/-}$ mice were generated as previously described (Hodivala-Dilke et al., 1999). $Src^{+/-}$ and $src^{-/-}$ mice were generated as previously described (Soriano et al., 1991), and were a gift of Drs. P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA), P. Stein (University of Pennsylvania, Philadelphia, PA), and P. Schwartzberg. Systemic intravenous VEGF injections (2 μg/animal in 100 μl), stereotactic brain injections and intradermal ear injections of anesthetized mice was performed with VEGF (500 ng in 5 μl) as previously described (Eliceiri et al., 1999). Statistical analysis of the quantitation of mouse matrigel angiogenesis, VP, and ischemia assays was performed with the Student *t* test.

Immunoprecipitation, immunoblotting, kinase assays, and immunostaining

For coimmunoprecipitation of FAK with integrin αvβ5 in HUVECs, lysis was performed in a buffer (HNG) containing 1% Brij (HNG buffer: 50 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol) (Berditchevski et al., 1997), and the lysates diluted with one volume of PBS for immunoprecipitation. For the HUVEC in vitro kinase assays and immunoprecipitation of FAK/αvβ5 complexes from mouse tissues, lysates were prepared in modified RIPA buffer as described previously (Eliceiri et al., 1999) and diluted with PBS for immunoprecipitation. To detect FAK/αvβ5 complexes in CAM tissue and HEK-293 cells, lysates were prepared in HNG buffer with 1.0% TX100 using a motorized grinder as necessary. SDS-PAGE and immunoblotting were performed as previously described (Eliceiri et al., 1999). FAK activity was measured by the ability of immunoprecipitated FAK to phosphorylate poly-Glu-Tyr (4:1) in an in vitro kinase assay. FAK was immunoprecipitated from equivalent amounts of protein from whole cell lysates as described above, subjected to the kinase assay, and the samples were analyzed by 16% SDS-PAGE as previously described (Eliceiri et al., 1998). Immunostaining of serum-starved HUVEC in the presence or absence of VEGF was performed with an anti-FAK antibody (Abedi and Zachary, 1997) and fixed in acetone as previously described (Takahashi et al., 1999).

In vitro binding assay

GST fusion proteins of NH_2- and COOH-terminal fragments of FAK and various β3 and β5 integrin cytoplasmic tails were prepared in *Escherichia coli* (BL21[DE3]). The FAK constructs were phosphorylated in vitro with active Src kinase (UBI), and the GST domain removed from the FAK constructs by Factor Xa (Amersham Pharmacia Biotech) cleavage. The integrin tail constructs retained the GST domain to facilitate the pulldown of FAK/integrin complexes after incubation with glutathione-Sepharose after 5–10 min in PBS on ice. Complexes were resolved by 16% SDS-PAGE and immunoblotted with anti-FAK or phosphospecific Y861 and Y925 antibodies.

In vivo VP models

Extravasation of Evan's Blue (EB) in the dermis after intradermal injection of VEGF was quantitated by extraction with formamide and spectrophotometry of eluted EB dye (Eliceiri et al., 1999). Laser scanning confocal microscopy was used to visualize the VP of cerebral blood vessels by detection of the fluorescence of the EB dye in brain cross sections (Eliceiri et al., 1999; Paul et al., 2001). Cerebral ischemia experiments were performed as previously described (Paul et al., 2001). In brief, permanent occlusion of the middle cerebral artery was performed in anesthetized mice by coagulation using a heating filament (Nawashiro et al., 1997). The brains were removed after 24 h and the infarcts determined by staining 1-mm coronal brain sections with 2% TTC. The infarct was measured from digital images of the sections and the volume calculated by summing the infarcted nonstained areas multiplied by their thickness (Eliasson et al., 1997).

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