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Differential αv integrin–mediated Ras-ERK signaling during two pathways of angiogenesis

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Antagonists of αvβ3 and αvβ5 disrupt angiogenesis in response to bFGF and VEGF, respectively. Here, we show that these αv integrins differentially contribute to sustained Ras-extracellular signal–related kinase (Ras-ERK) signaling in blood vessels, a requirement for endothelial cell survival and angiogenesis. Inhibition of FAK or αvβ5 disrupted VEGF-mediated Ras and c-Raf activity on the chick chorioallantoic membrane, whereas blockade of FAK or integrin αvβ3 had no effect on bFGF-mediated Ras activity, but did suppress c-Raf activation. Furthermore, retroviral delivery of active Ras or c-Raf promoted ERK activity and angiogenesis, which anti-αvβ5 blocked upstream of Ras, whereas anti-αvβ3 blocked downstream of Ras, but upstream of c-Raf. The activation of c-Raf by bFGF/αvβ3 not only depended on FAK, but also required p21-activated kinase-dependent phosphorylation of serine 338 on c-Raf, whereas VEGF-mediated c-Raf phosphorylation/activation depended on Src, but not Pak. Thus, integrins αvβ3 and αvβ5 differentially regulate the Ras-ERK pathway, accounting for distinct vascular responses during two pathways of angiogenesis.

Introduction

Angiogenesis is regulated by signals derived from receptors for both growth factors and ECM molecules (Eliceiri and Cheresh, 2000). For example, inhibition of integrin αvβ3 during bFGF stimulation suppresses the sustained phase of extracellular signal–related kinase (ERK) signaling (Eliceiri et al., 1998) leading to endothelial apoptosis and inhibition of angiogenesis (Brooks et al., 1994a,b; Eliceiri et al., 1998). Although anti-αvβ3 blocks bFGF-mediated angiogenesis, VEGF-induced angiogenesis is disrupted by anti-αvβ5 (Friedlander et al., 1995), providing evidence that distinct signaling pathways regulate angiogenesis.

It is well recognized that cell proliferation requires both growth factor stimulation and integrin-mediated adhesion to the ECM (Aplin et al., 1999). A key mechanism by which adhesion events influence cellular behavior is based on integrin-mediated activation of the Ras-ERK cascade (Renshaw et al., 1997), which regulates gene expression, cell proliferation, survival, differentiation, and migration (Klemke et al., 1997; Hood et al., 2002). A number of reports have characterized the intracellular pathways leading to ERK activation in vitro (Kolch, 2000). Previous reports reveal that growth factor receptors or integrins recruit adaptor proteins and nucleotide exchange factors that convert Ras to its GTP-bound form leading to recruitment of c-Raf to the cytoplasmic membrane where it can be activated (Feig and Cooper, 1988). Although the mechanisms of c-Raf regulation are incompletely understood, they are thought to involve membrane recruitment and subsequent phosphorylation by protein kinases such as p21-activated kinase (PAK) and Src, which are activated in convergent cascades by growth factor receptors together with integrins (Fabian et al., 1993; King et al., 1998; del Pozo et al., 2000; Schlessinger, 2000). Recent reports indicate that the differential phosphorylation of c-Raf by these kinases not only regulates its activation in an on/off binary way, but also controls its subcellular localization and subsequent distinct cellular responses. Specifically, bFGF-induced phosphorylation of c-Raf on serine 338 by PAK leads to c-Raf translocation to the mitochondria and endothelial cell (EC) survival in the face of apoptotic stress, whereas VEGF-induced phosphorylation of c-Raf on tyrosines 340/341 by Src leads to EC survival in response to receptor-mediated apoptosis (Alavi et al., 2003).

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Key words: integrin alphaV; FGF receptor; VEGF receptor; c-Raf protein; MAP-ERK kinase

Abbreviations used in this paper: CAM, chick chorioallantoic membrane; EC, endothelial cell; ERK, extracellular signal–related kinase; FRNK, FAK-related nonkinase; PAK, p21-activated kinase; PAK_{83-149}, PAK-1 auto-inhibitory domain.

Although integrins and growth factor receptors promote coordinated signaling activities, there appear to be multiple mechanisms that govern how signals derived from these receptors converge. In vitro analyses have implicated Ras (Schlaepfer et al., 1994), c-Raf (Lin et al., 1997), or MEK (Renshaw et al., 1997) as a regulatory destination for integrin signals in the ERK cascade. Based on previous reports indicating that angiogenesis requires both an early, αv integrin–independent, and a sustained αv-dependent activation of ERK (Eliceiri et al., 1998), we sought to establish the mechanisms by which αv integrins influence the ERK pathway during bFGF- versus VEGF-dependent neovascularization within intact blood vessels. In this report, we present the unexpected finding that αvβ3 and αvβ5, together with FAK, play very distinct roles in the activation of the Ras-ERK cascade leading to EC survival during angiogenesis in response to bFGF and VEGF, respectively.

Results

αv integrins contribute to sustained Ras, Raf-1, and ERK activation during angiogenesis in vivo

Antagonists of integrins αvβ3 and αvβ5 disrupt angiogenesis induced by bFGF and VEGF, respectively, in chick embryos, mice, and rabbits (Friedlander et al., 1995, 1996). These integrins potentiate growth factor–mediated, sustained ERK signaling during angiogenesis, which is critical for neovascularization in these tissues (Eliceiri et al., 1998).

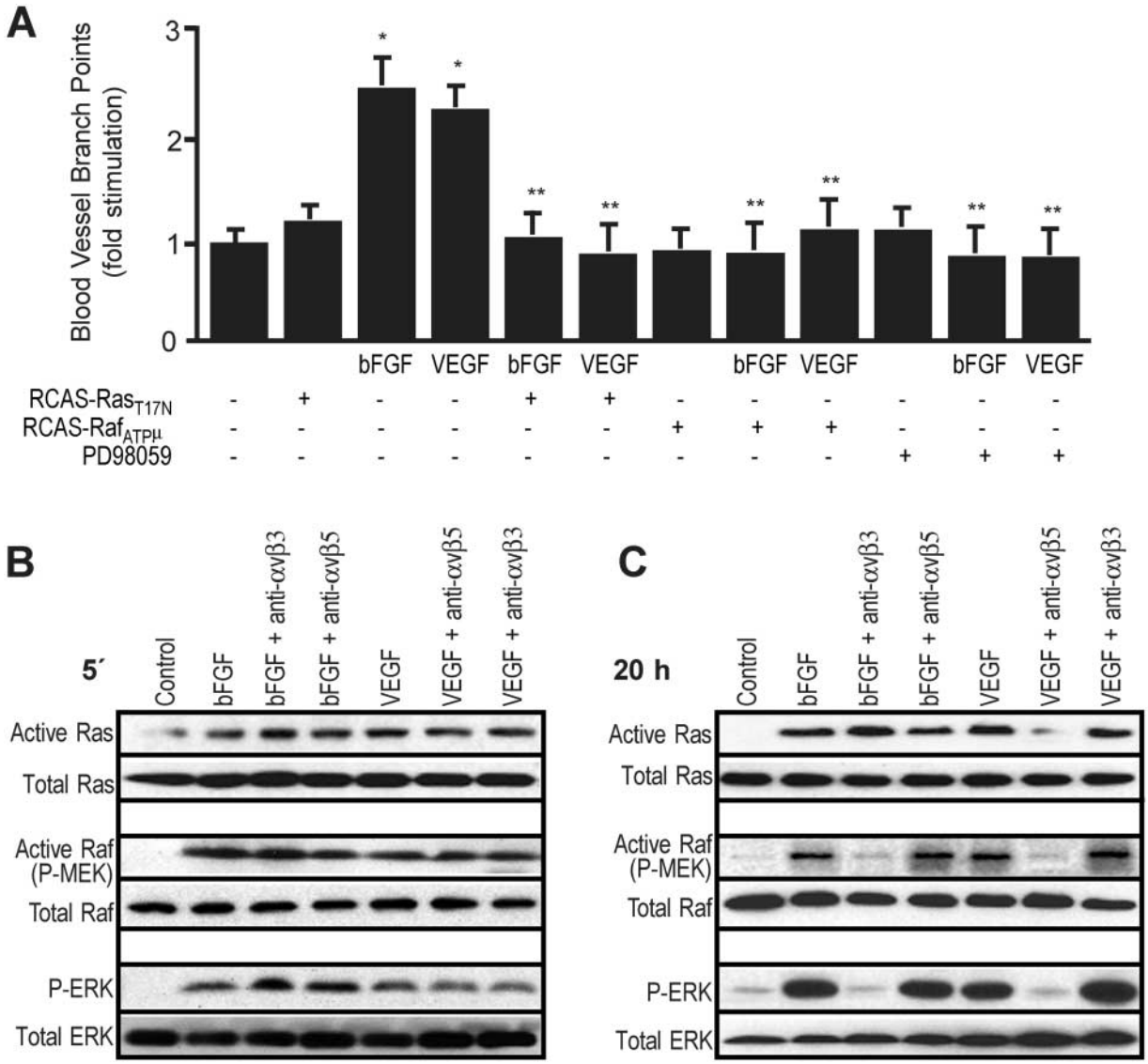

Figure 1. **FGF and VEGF require and stimulate Ras, Raf, and ERK activity during angiogenesis in cooperation with integrins αvβ3 and αvβ3.** (A) 10-d-old chick CAMs were exposed to filter paper disks saturated with RCAS-Ras T_{17N} (inactive Ras), RCAS-$Raf_{ATPμ}$ (inactive c-Raf), or PD98059 (MEK inhibitor) followed by stimulation with either 2 μg/ml bFGF or VEGF for 72 h. Blood vessels were enumerated by counting vessel branch points in a double-blinded manner. Each bar represents the mean ± SEM of 24 replicates. *, $P < 0.05$ relative to control; **, $P < 0.05$ relative to treatment. (B) 10-d-old chick CAMs were exposed to filter paper disks saturated with either bFGF or VEGF for 5 min, followed by excision and detergent extraction of the tissues. 1 h before excision, the embryos were i.v. injected with 30 μg function-blocking antibodies selective for either integrin αvβ3 (LM609) or αvβ5 (P1F6). Relative Ras, c-Raf, and ERK was determined as described in Materials and methods. (C) Chick CAMs were treated as above with the exception that CAM tissue was excised 20 h after initial exposure to bFGF and VEGF.

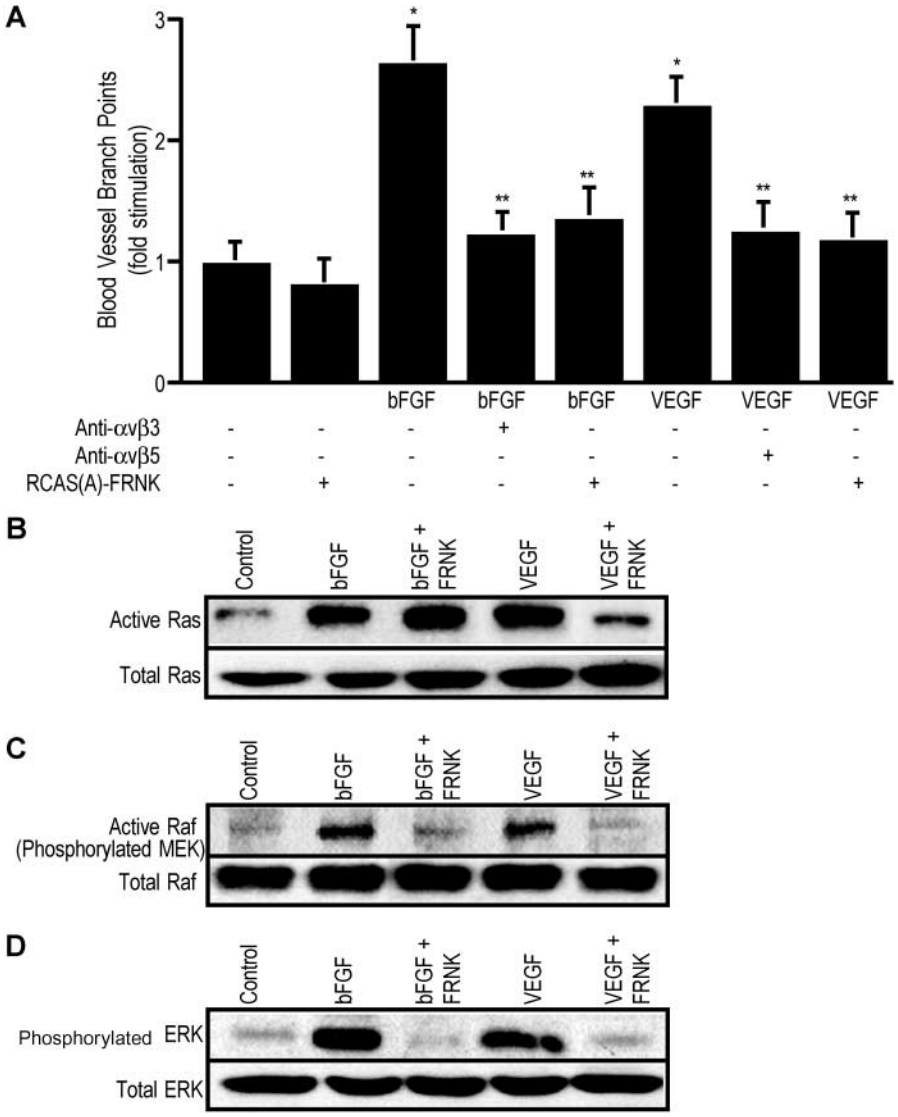

Figure 2. **FAK is required for angiogenesis and signaling during bFGF- and VEGF-induced angiogenesis.** (A) 10-d-old chick CAMs were exposed to filter paper disks saturated with RCAS(A)-FRNK (inactive FAK) or i.v. injected with anti-αvβ3 or -αvβ5 followed by stimulation with either 2 μg/ml bFGF or VEGF for 72 h. Blood vessels were enumerated by counting vessel branch points in a double-blinded manner. Each bar represents the mean ± SEM of 20 replicates. *, $P < 0.05$ relative to control; **, $P < 0.05$ relative to treatment. (B) 10-d-old chick CAMs were exposed to filter paper disks saturated with RCAS-FRNK (inactive FAK) followed by stimulation with either 2 μg/ml bFGF or VEGF for 20 h. Tissues were then excised and subjected to detergent extraction. Relative Ras activity was determined using a pulldown assay with the Ras-binding domain of c-Raf followed by SDS-PAGE and immunoblotting for Ras as described in Materials and methods. (C) Chick CAMs were treated as above with the exception that c-Raf was immunoprecipitated from the tissue extracts and subjected to an in vitro kinase assay using kinase-dead MEK as a substrate as described in Materials and methods. The above blot was probed with an anti-c-Raf antibody as a loading control. (D) Chick CAMs were treated as above with the exception that total CAM lysates were electrophoresed and probed with antibodies directed against the active phosphorylated form of ERK or an anti-ERK antibody as a loading control.

Therefore, experiments were designed to establish how αvβ3 and αvβ5 contribute to the Ras-ERK pathway during angiogenesis in response to bFGF versus VEGF. For this purpose, bFGF or VEGF was used to stimulate the growth of new blood vessels on chick chorioallantoic membranes (CAMs) of 10-d-old embryos. CAMs treated with growth factor for either 5 min (early) or 20 h (late/sustained) were exposed to function-blocking antibodies selective for either αvβ3 or αvβ5. Alternatively, tissues were transduced with a retroviral vector encoding mutationally inactive forms of Ras or c-Raf, or treated with PD98059, a pharmacological inhibitor of MEK.

As expected, disruption of Ras, c-Raf, or MEK activity in these tissues suppressed angiogenesis induced with either growth factor (Fig. 1). In the absence of integrin antagonists, the activity of Ras, c-Raf, and ERK was induced within 5 min and remained active for at least 20 h after either bFGF or VEGF stimulation (Fig. 1, B and C). Intravenous administration of anti-αvβ3, either 1 h before growth factor addition, or 1 h before harvest at the 20-h time point, showed no diminution of bFGF-induced early or late Ras activity in CAM tissues (Fig. 1, B and C). However, anti-αvβ3 did prevent the bFGF-induced sustained c-Raf and ERK activity (Fig. 1, B and C). In contrast, i.v. injection of anti-αvβ5 completely blocked VEGF-mediated late Ras and c-Raf, as well as ERK activity (Fig. 1, B and C). Importantly, anti-αvβ3 did not suppress VEGF-induced, nor did anti-αvβ5 suppress bFGF-induced Ras, c-Raf, or ERK signaling (Fig. 1, B and C). These findings suggest that ligation of specific αv integrins differentially regulates bFGF- and VEGF-mediated activation of the Ras-ERK signaling pathway in angiogenic tissue in vivo. In this case, VEGF-mediated (but not bFGF-mediated) activation of Ras depends on the coordinated ligation of a specific αv integrin. Our findings can likely be attributed to a direct effect on blood vessels because VEGF preferentially activates ECs, and αvβ3 is exclusively expressed by ECs within these tissues.

FAK is required for Ras-ERK activity and angiogenesis in vivo

FAK is stimulated on integrin ligation (Schaller, 2001) and plays a critical role in growth factor signaling (Sieg et al., 2000). To further evaluate the role of integrin signaling in angiogenesis, we asked whether FAK activity was required

for angiogenesis and if so, how it might contribute to bFGF- or VEGF-mediated Ras-ERK activation. For this purpose, CAMs stimulated with either bFGF or VEGF were transduced with a retrovirus encoding FAK-related nonkinase (FRNK), an autonomously expressed form of FAK containing the COOH-terminal region of FAK, but lacking its kinase domain. Consistent with analyses using integrin antagonists (Brooks et al., 1994a; Friedlander et al., 1995) and reports examining FAK's role in growth factor–induced ERK activity and migration (Renshaw et al., 1999; Sieg et al., 2000), blockade of FAK activity in these tissues disrupted angiogenesis induced with either growth factor (Fig. 2 A). Although FAK was required for c-Raf and ERK activity in response to either growth factor, only Ras activity downstream of VEGF was FAK dependent (Fig. 2, B–D). These results are consistent with those obtained when anti-αvβ3 and anti-αvβ5 were applied to these tissues. Specifically, VEGF-mediated Ras activity requires FAK and αvβ5, whereas bFGF activation of Ras is both FAK- and αvβ3-independent. However, both growth factors require their respective αv integrin and FAK for activation of c-Raf and ERK leading to angiogenesis.

Integrin antagonists differentially suppress angiogenesis in response to active Ras or Raf

To further establish the role αv integrins play in Ras-Erk signaling and angiogenesis, unstimulated CAMs were transduced with active forms of Ras (G12V-Ras) or c-Raf (Raf-

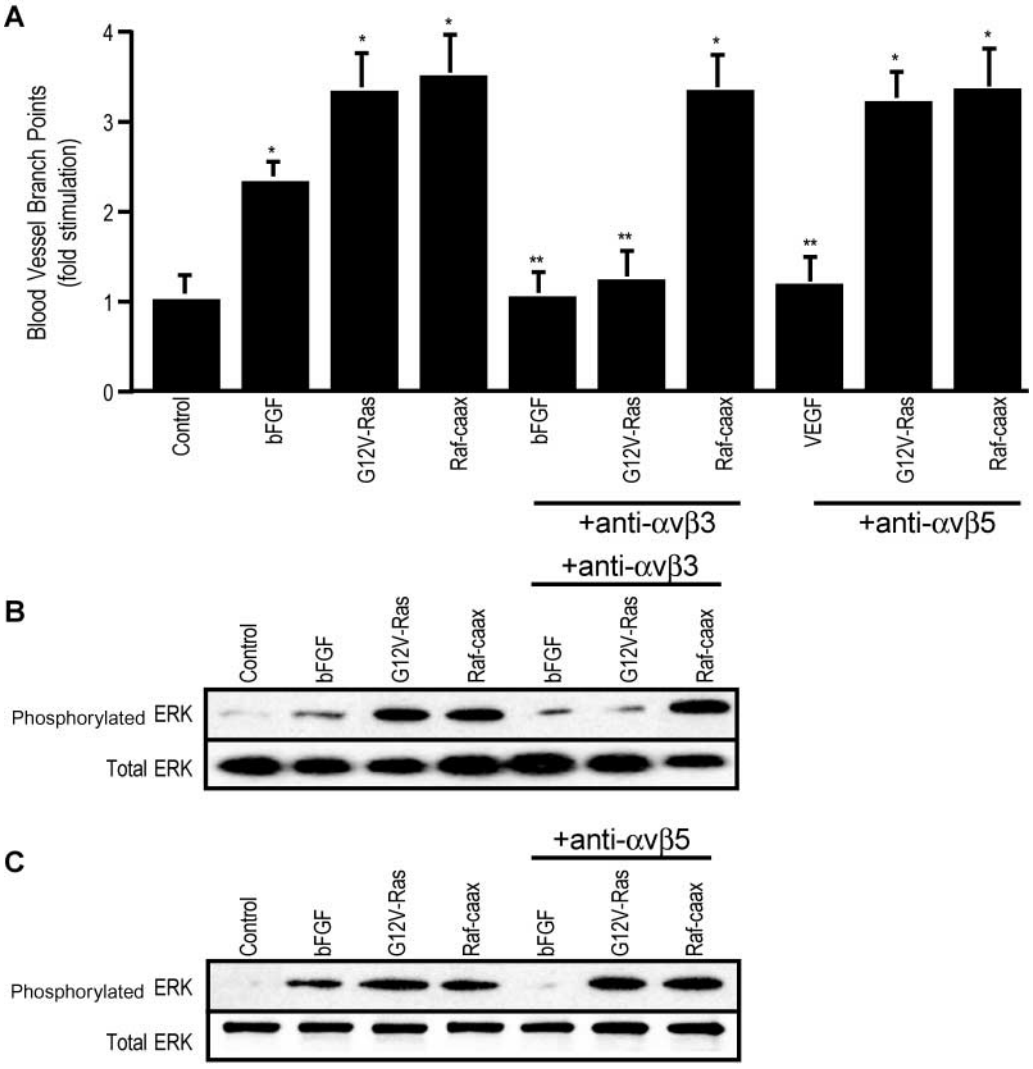

Figure 3. **Ras and Raf are differentially regulated by integrins αvβ3 and αvβ5 during bFGF- and VEGF-induced angiogenesis.** (A) 10-d-old chick CAMs were exposed to filter paper disks saturated with either bFGF, VEGF, RCAS-Ras G12V (active Ras), or RCAS-Raf-caax (active c-Raf). After 20 h, embryos were i.v. injected with function-blocking antibodies directed against integrins αvβ3 or αvβ5. After 72 h, blood vessels were enumerated by counting vessel branch points in a double-blinded manner. Each bar represents the mean ± SEM of 24 replicates. *, $P < 0.05$ relative to control; **, $P < 0.05$ relative to treatment. (B) Chick CAMs were treated as above with the exception that antibodies directed against integrin αvβ3 were injected after 19 h of growth-factor stimulation followed by excision and detergent extraction 1 h later. Lysates were then electrophoresed and probed with antibodies directed against the active, phosphorylated form of ERK or an anti-ERK antibody as a loading control. (C) Chick CAMs were treated as above with the exception that antibodies directed against integrin αvβ5 were injected after 19 h of growth-factor stimulation followed by excision and detergent extraction 1 h later. Lysates were then electrophoresed and probed with antibodies directed against the active, phosphorylated form of ERK or an anti-ERK antibody as a loading control.

caax), which promoted a strong angiogenic response in these tissues (Fig. 3 A). CAMs stimulated with active Ras or c-Raf were then treated with either anti-αvβ3 or -αvβ5 and analyzed for Ras or c-Raf activity after 20 h, or measured for angiogenesis after 72 h. Expression of active Ras or c-Raf induced an equivalent or greater angiogenic response compared with that seen with bFGF or VEGF (Fig. 3 A). Although anti-αvβ3 blocked bFGF as well as Ras-mediated angiogenesis, it had little effect on that induced with c-Raf, suggesting that αvβ3 potentiates signaling to ERK downstream of Ras, but at or upstream of c-Raf. In contrast, anti-αvβ5 blocked VEGF-induced angiogenesis, yet had no effect on angiogenesis induced with activated Ras or c-Raf, suggesting that the anti-angiogenic effects of anti-αvβ5 was upstream of both Ras and c-Raf. These results are consistent with the finding that anti-αvβ5 blocks VEGF-mediated Ras activity, whereas anti-αvβ3 disrupts bFGF-mediated c-Raf activation, but fails to block Ras activation.

To extend these experiments, ERK activity was assessed in extracts of CAMs stimulated as described in the previous paragraph. Consistent with the angiogenesis results (Fig. 3 A), anti-αvβ3 blocked bFGF as well as Ras-mediated ERK activation in these tissues, but failed to influence that induced by Raf-caax. However, anti-αvβ5 failed to block Ras- or c-Raf–induced ERK activity, but it did disrupt VEGF-mediated ERK activation (Fig. 3, B and C). Together, these results support the finding that integrins αvβ3 and αvβ5 influence the Ras-ERK pathway at distinct points (Fig. 1 and Fig. 2).

Src requirement for Raf-ERK activation during VEGF-induced (but not bFGF-induced) angiogenesis

Previous reports have documented that VEGF-mediated (but not bFGF-mediated) angiogenesis depends on the activity of Src family kinases (Eliceiri et al., 1999) and αvβ5 (Friedlander et al., 1995). To evaluate whether αvβ5 signaling is linked to Src's role in angiogenesis, CAMs stimulated with bFGF or VEGF and treated with anti-αvβ3 or -αvβ5, respectively, were lysed and analyzed for Src activity. Although both growth factors stimulated Src activity, neither antibody was able to suppress this response (Fig. 4 A), which is consistent with previous findings that Src activity is upstream of integrin signaling on the VEGF pathway (Eliceiri et al., 2002). Notably, Src can also regulate c-Raf activation by phosphorylating c-Raf on tyrosines 340/341 (Fabian et al., 1993), a site phosphorylated in response to stimulation by VEGF, but not bFGF (Alavi et al., 2003). To evaluate the role of αvβ5 in VEGF-induced c-Raf phosphorylation, CAMs stimulated with bFGF or VEGF were treated with either anti-αvβ3 or -αvβ5, respectively, and c-Raf was analyzed for phosphorylation of tyrosines 340/341 (Fig. 4 B). In agreement with earlier reports (Alavi et al., 2003), VEGF (but not bFGF) induced phosphorylation of Raf on tyrosines 340/341. However, this phosphorylation event was completely blocked by inhibition of integrin αvβ5. To confirm the role of Src in VEGF-induced c-Raf activation, CAMs stimulated with bFGF or VEGF were transduced with RCAS-Src251 or treated with the Src inhibitor PP1 and subsequently ana-

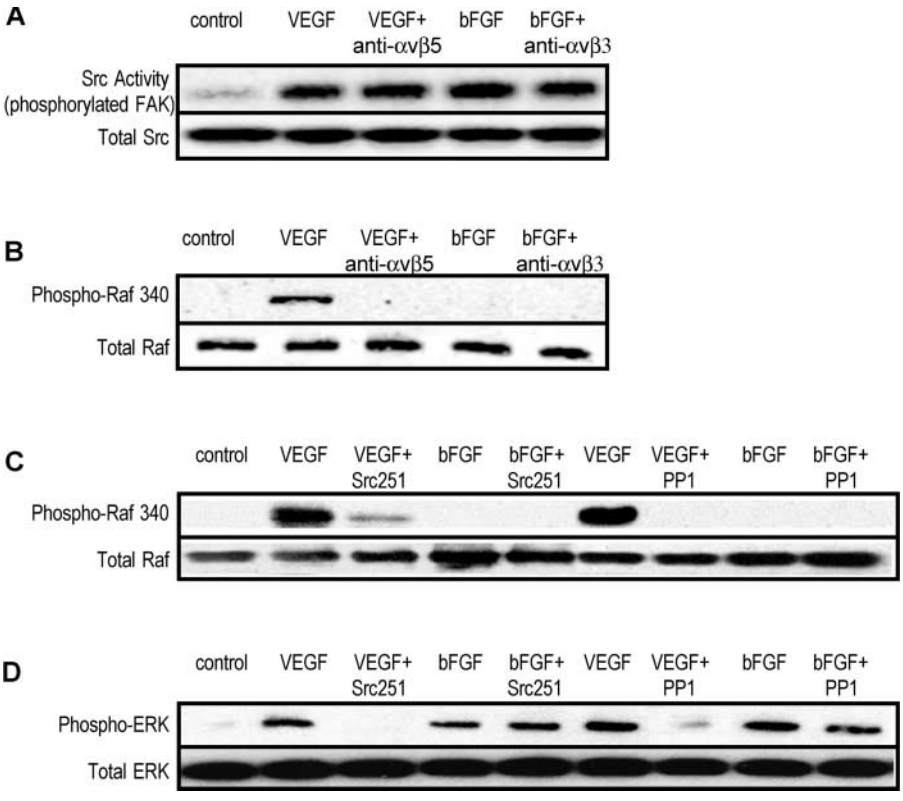

Figure 4. **Src requirement for Raf-ERK activation during VEGF-induced (but not bFGF-induced) angiogenesis.** (A) 10-d-old chick CAMs were exposed to filter paper disks saturated with either bFGF or VEGF for 20 h, followed by excision and detergent extraction of the tissues. 1 h before excision, the embryos were 1.v. injected with 30 μg function-blocking antibodies selective for either integrin αvβ3 or αvβ5 as indicated. Endogenous Src was immunoprecipitated and subjected to an in vitro kinase assay using a FAK-GST fusion protein as a substrate, electrophoresed, and anti-Src antibody was used as a loading control as described in Materials and methods. (B) 10-d-old chick CAMs were treated as described above with the exception that after excision, c-Raf was immunoprecipitated from the tissue extracts and probed with an antibody directed against phosphorylated tyrosine 340 on c-Raf. The above blot was then stripped and probed with an anti-c-Raf antibody as a loading control. (C) Chick CAMs were stimulated as described above with the exception that filter paper disks on the CAM were saturated with either the Src inhibitor PP1 or RCAS-Src251 (inactive Src), followed by blotting for phospho-Raf 340 or anti-c-Raf. (D) Chick CAMs were stimulated as described above with the exception that lysates were probed with antibodies directed against the active, phosphorylated form of ERK or an anti-ERK antibody as a loading control.

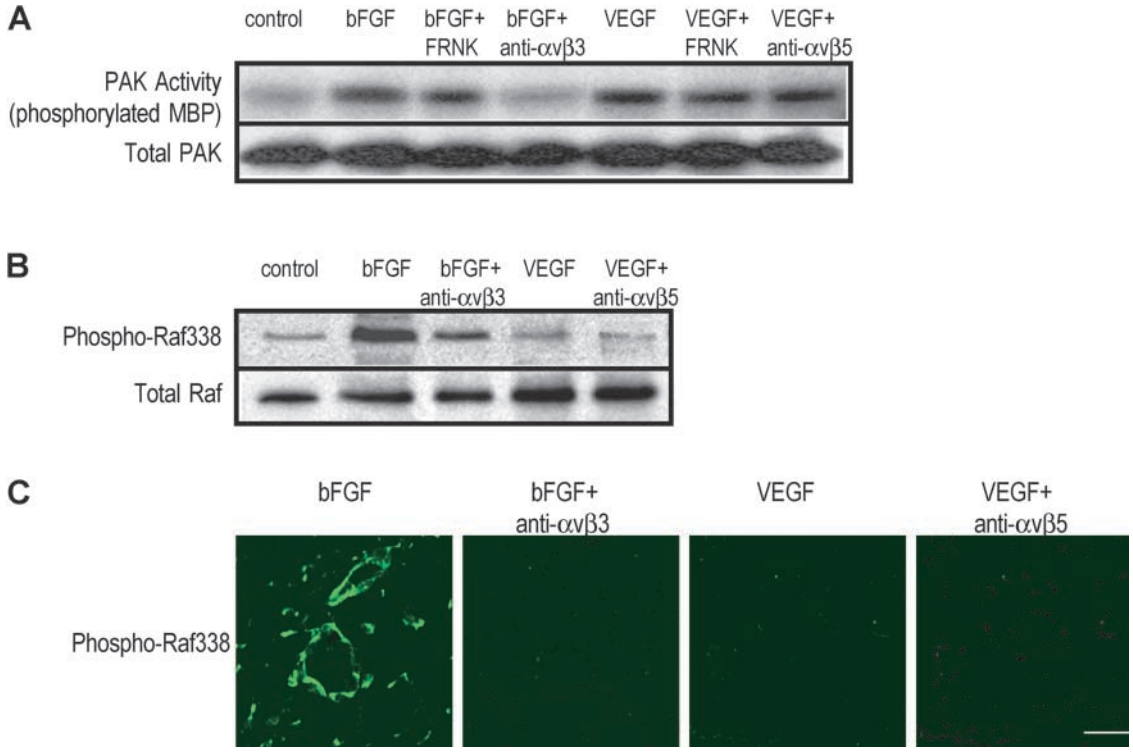

Figure 5. **Integrins αvβ3 and αvβ5 differentially influence PAK activity during angiogenesis.** (A) 10-d-old chick CAMs were exposed to filter paper disks saturated with RCAS-FRNK (inactive FAK), followed by stimulation with either 2 μg/ml bFGF or VEGF for 20 h. 1 h before tissue excision, function-blocking antibodies directed against integrin αvβ3 or αvβ5 were i.v. injected. Endogenous PAK was immunoprecipitated from equivalent amounts of total protein and subjected to a kinase assay using myelin basic protein as a substrate, electrophoresed, and transferred to nitrocellulose as described in Materials and methods. The above blot was probed with an anti-PAK antibody as a loading control. (B) Chick CAMs were treated as above with the exception that total lysates were probed with an antibody directed specific to c-Raf phosphorylated at serine 338. The above blot was probed with an anti-c-Raf antibody as a loading control. (C) Chick CAMs were treated as above with the exception that after 20 h, the angiogenic tissue was resected and snap frozen. Tissue sections were probed with an antibody directed against c-Raf phosphorylated at serine 338. Bar, 50 μm.

lyzed for phosphorylation of c-Raf at tyrosines 340/341 (Fig. 4 C). Consistent with a direct role for Src in VEGF-mediated c-Raf activation, VEGF-induced (but not bFGF-induced) phosphorylation of c-Raf on tyrosines 340/341 was blocked by treatment of tissues with Src251 or PP1. Furthermore, VEGF (but not bFGF) induced ERK activity that was completely abolished by Src251 or PP1 (Fig. 4 D). These findings reveal that VEGF/αvβ5 selectively use Src to phosphorylate c-Raf in vivo, leading to ERK activation and angiogenesis.

αvβ3 contributes to bFGF-induced PAK-1 activation, which is required for bFGF-mediated (but not VEGF-mediated) Raf-ERK activation and angiogenesis

The finding that αvβ3 appears to regulate c-Raf, but not Ras activity in response to bFGF prompted us to evaluate how αvβ3 contributes to c-Raf activation. Recently, we found that bFGF-mediated angiogenesis is dependent on PAK-1 signaling (Kiosses et al., 2002). Importantly, PAK-1 phosphorylates c-Raf on serine 338, leading to its activation (Zang et al., 2002). To evaluate whether αvβ3 signaling is linked to PAK-1's role in angiogenesis, CAMs stimulated with bFGF or VEGF and treated with anti-αvβ3 or -αvβ5 were lysed and analyzed for PAK-1 activity. Although both growth factors stimulated PAK activity, only anti-αvβ3 was able to suppress this response (Fig. 5 A). Accordingly, direct evaluation of the tissue lysates revealed that bFGF (but not VEGF) induced an αvβ3-dependent phosphorylation of c-Raf at its PAK phosphorylation site, S338 (Fig. 5 B). These findings were confirmed by in situ evaluation of CAM tissue sections, revealing that the phospho-Raf 338 staining was primarily associated with bFGF-stimulated blood vessels (Fig. 5 C).

Consistent with these results, transduction of CAMs with an RCAS retrovirus encoding the PAK-1 auto-inhibitory domain ($PAK_{83–149}$; Zhao et al., 1998) selectively disrupted bFGF-induced ERK activity (Fig. 6 A) in these tissues. This finding was confirmed in situ, using an antibody specific for phosphorylated (activated) ERK, which revealed that the suppression of PAK-1 selectively blocked bFGF-mediated ERK activation within angiogenic blood vessels (Fig. 6 B). These findings were extended by evaluating angiogenesis on CAMs stimulated with VEGF- and bFGF-mediated or bFGF and transduced with RCAS-$PAK_{83–149}$. Although both growth factors stimulated angiogenesis, inhibition of PAK-1 selectively suppressed bFGF-induced angiogenesis (Fig. 6 C). These findings reveal that integrin αvβ3 regulates PAK-1 activity during bFGF-induced angiogenesis in vivo, and that PAK-1 selectively mediates bFGF-induced ERK activation and angiogenesis.

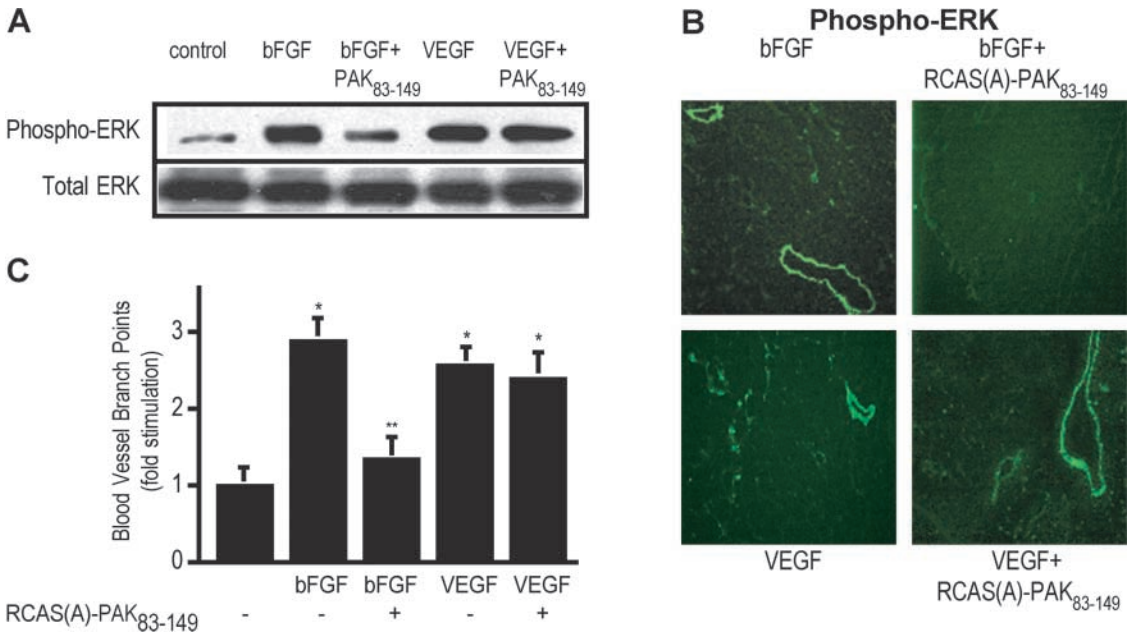

Figure 6. **PAK activity is required for bFGF-mediated ERK activation and angiogenesis.** (A) 10-d-old chick CAMs were exposed to filter paper disks saturated with RCAS-PAK$_{83–149}$ (inactive PAK), followed by stimulation with either 2 μg/ml bFGF or VEGF for 20 h. CAM tissue was excised, subjected to detergent extraction, electrophoresed, and probed with antibodies directed against the active phosphorylated form of ERK or an anti-ERK antibody as a loading control as described in Materials and methods. (B) Chick CAMs were treated as above with the exception that after 20 h, the angiogenic tissue was resected and snap frozen. Tissue sections were probed with an antibody directed against the active phosphorylated form of ERK. (C) 10-d-old chick CAMs were exposed to filter paper disks saturated with RCAS-PAK$_{83–149}$ (inactive PAK), followed by stimulation with either bFGF or VEGF for 72 h. Blood vessels were enumerated by counting vessel branch points in a double-blinded manner. Each bar represents the mean ± SEM of 36 replicates. *, $P < 0.05$ relative to control; **, $P < 0.05$ relative to treatment.

Discussion

Recent evidence suggests that activation of the Ras-ERK signaling pathway is critical for vasculogenesis and angiogenesis (Eliceiri et al., 1998; Giroux et al., 1999; Huser et al., 2001; Hood et al., 2002). Although it is established that cell interaction with growth factors and ECM components ultimately lead to Ras-ERK activation, little is known of how integrins and growth factor receptors coordinate their intracellular signaling pathways. The current analyses address how bFGF and VEGF and αv integrin-derived signals are coordinated within ECs undergoing angiogenesis in vivo.

In this report, evidence is provided that distinct integrin/growth factor receptor pairs differentially activate the Ras-ERK cascade during angiogenesis (Fig. 7). Consistent with earlier reports, blocking αv integrin ligation selectively inhibited the late-acting sustained ERK activity (Fig. 1) that is critical for angiogenesis (Eliceiri et al., 1998), and inhibition of integrin αvβ3 or αvβ5 selectively inhibited bFGF- or VEGF-mediated angiogenesis, respectively (Friedlander et al., 1995). Although αv integrins appear to control sustained/late Ras-ERK signaling, the initial signaling response to these angiogenic growth factors may be supported by preexisting EC integrins such as α2β1 and α1β1, which also contribute to angiogenesis (Senger et al., 2002). In the current work, we found that coordinated signals from integrins αvβ3 or αvβ5 together with bFGF or VEGF, respectively, induce angiogenesis in a manner that is dependent on activation of FAK, Ras, c-Raf, and ERK. However, there was surprising disparity in how these integrin-growth factor pairs activated the Ras-ERK pathway, and thereby likely influence EC survival (Alavi et al., 2003) and angiogenesis (Fig. 7).

Integrin ligation leads to activation of FAK, which is known to mediate downstream signaling (Schlaepfer et al., 1999), facilitating serum-induced activation of ERK in NIH 3T3s (Renshaw et al., 1999) and growth factor–dependent cell migration (Sieg et al., 2000). Notably, our results indicate that although disrupting FAK mirrors some of the effects of integrin antagonists, it had no impact on αvβ3-mediated PAK activation (Fig. 5), a requirement for bFGF-mediated c-Raf activity. These findings indicate that during angiogenesis, some critical integrin signaling is independent of FAK activity. In this case, it is possible that the small GTPase Rac, which has been reported to be both activated by integrins and essential for PAK activation (del Pozo et al., 2000), is responsible for the FAK-independent, αvβ3-induced activation of PAK after bFGF stimulation.

Although the importance of FAK in integrin signaling is well established, its role in angiogenesis is not entirely clear. Experiments presented here indicate that although FAK is required for c-Raf and ERK activation during bFGF-mediated angiogenesis, it is not required for bFGF-mediated Ras activation (Fig. 2). These results are consistent with reports indicating that integrins influence growth factor–induced activation of the ERK cascade in a manner downstream of Ras (Chen et al., 1996; Renshaw et al., 1997), and that FAK can signal directly to c-Raf (Barberis et al., 2000). However, this was not the case for VEGF activation of Ras because αvβ5 ligation (Fig. 1) and FAK activity (Fig. 2) were found to play a critical role in Ras activation. Therefore, the role of

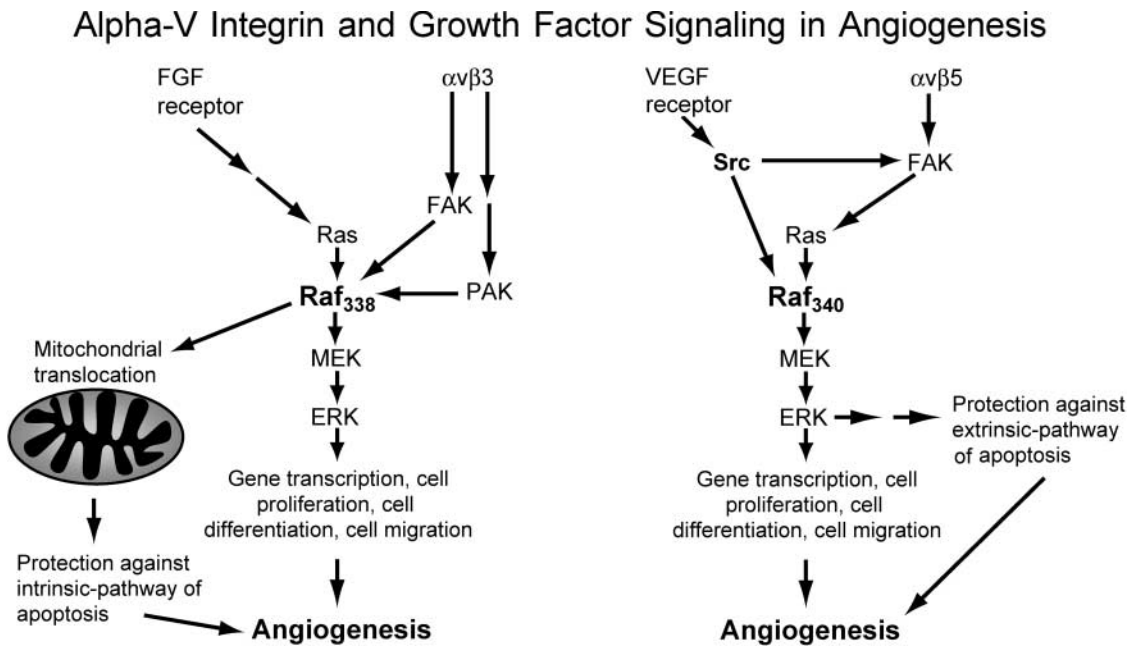

Figure 7. **bFGF/αvβ3 and VEGF/αvβ5 signaling pathways.** A summary of the signaling pathways outlined in this report as it relates to EC cell survival as recently described in Alavi et al. (2003). Evidence presented here reveals that bFGF/αvβ3 and VEGF/αvβ5 differentially activate Ras-Raf-ERK signaling. This, together with our recent work (Alavi et al., 2003), allows us to propose a model whereby each of these signaling pathways accounts for protection of EC from distinct mediators of apoptosis. The αvβ3 pathway promotes an ERK-independent survival mechanism preventing stress-mediated death based on Raf coupling to the mitochondria, whereas the αvβ5 pathway prevents receptor-mediated death in an ERK-dependent manner. In addition, ERK is likely playing a general role in both pathways of angiogenesis because it regulates gene transcription, cell cycle progression, and cell migration, which are critical to the growth and differentiation of new blood vessels.

αvβ5 in VEGF-induced Ras-ERK activation appears to be limited to signaling through FAK to Ras, which is in stark contrast to the role FAK and αvβ3 play in bFGF-induced Raf-ERK activation (Fig. 7).

Previous reports have revealed that although Ras activation is necessary for c-Raf activity, it is not sufficient (Yip-Schneider et al., 2000). For example, activation of c-Raf requires uncoupling of the 14–3–3 adaptor protein, association with activated Ras at the plasma membrane, and phosphorylation by various protein kinases. At the membrane, c-Raf activity can be modulated by phosphorylation of at least 11 tyrosine and serine/threonine residues. Depending on which sites are phosphorylated, c-Raf activity can be either elevated or diminished (Chong et al., 2003). Several kinases are capable of modulating c-Raf, yet the molecular mechanisms responsible for c-Raf activation within intact tissues remain unclear. Our findings reveal that the pathway leading to c-Raf activation varies within blood vessels undergoing angiogenesis, depending on the growth factor used to stimulate the process. For example, bFGF elicited an αvβ3-dependent, PAK-mediated phosphorylation of c-Raf at serine 338, a site at which VEGF-stimulated tissue showed minimal phosphorylation (Fig. 5). Integrin-mediated adhesion activates PAK through its effector Rac (del Pozo et al., 2000), which enables PAK to activate c-Raf by phosphorylation of serine 338 (King et al., 1998; del Pozo et al., 2000; Drogen et al., 2000). Notably, phosphorylation of these sites may be sufficient for c-Raf activity induced by integrin ligation (Chaudhary et al., 2000).

Although bFGF selectively used PAK to activate c-Raf, VEGF makes use of the tyrosine kinase Src for this purpose. Previous reports have shown that VEGF selectively uses Src during VEGF-induced permeability increases and angiogenesis (Eliceiri et al., 1999), but the role of Src activity on downstream signals such as Raf was unclear. Our work reveals that although αvβ5 is not required for VEGF activation of Src, it is required for the subsequent Src-mediated phosphorylation of Raf. Considering our current work and previous findings (Eliceiri et al., 1999), Src appears to function both upstream and downstream of αvβ5/FAK. Once the VEGF receptor (Flk) becomes ligated, it recruits and activates Src (He et al., 1999). This leads to tyrosine phosphorylation on several FAK sites, including tyrosine 861, allowing FAK to couple to αvβ5 (Eliceiri et al., 2002). This potentiates αvβ5/FAK signaling and activation of downstream signaling molecules including VEGF-induced FAK-dependent Ras activation (Fig. 2). Subsequent to Ras activation, Raf is recruited to the membrane where it is phosphorylated by Src on tyrosines 340/341 (Fig. 4 and Fig. 7). This phosphorylation appears critical to the role that Src plays in VEGF-mediated survival (Alavi et al., 2003). Together, these findings support a multi-layered role for Src in the VEGF/αvβ5 signaling cascade. Consistent with this conclusion, delivery of active Src to angiogenic tissues promotes angiogenesis that was blocked by anti-αvβ5 (unpublished data), and mice deficient in Src or αvβ5 show no VEGF-mediated vascular permeability increases, whereas mice lacking αvβ3 maintain their permeability response to VEGF (Eliceiri et al., 2002).

Although coordinated signals from either VEGF/αvβ5 or bFGF/αvβ3 result in angiogenesis, the differential activation of these signaling pathways may impact the distinct bio-

logical responses of VEGF and bFGF. For example, even though bFGF and VEGF both induce angiogenesis, only VEGF is a potent vascular permeability factor (Gautschi et al., 1986; Senger et al., 1986; Leung et al., 1989). However, mice lacking either αvβ3 or αvβ5 show no angiogenic defect (Reynolds et al., 2002), yet animals deficient in αvβ5 fail to undergo VEGF-mediated permeability (Eliceiri et al., 2002). Although it has been suggested that αv integrins negatively regulate angiogenesis (Hynes, 2002), evidence is presented here that anti-αv integrin function–blocking antibodies, which antagonize these integrins, block activation of Ras and/or Raf in response to angiogenic growth factors such as bFGF and VEGF. Moreover, this differential activation of Raf has been directly linked to two pathways of EC survival during angiogenesis (Alavi et al., 2003), as summarized in Fig. 7. Therefore, it is very possible that mice deficient in αvβ3 and/or αvβ5 may have up-regulated compensatory signaling mechanisms leading to EC cell survival. In fact, αvβ3-deficient mice up-regulate both VEGF and VEGF receptor (Reynolds et al., 2002), demonstrating that at least one survival factor and its receptor may be playing a compensatory role in αvβ3 KO mice. In addition, we recently determined that mice deficient in p53 showed no anti-angiogenic response when challenged with a cyclic peptide antagonist of αvβ3/αvβ5, indicating that p53-deficient mice may be genetically prone to using a survival pathway that does not depend on αv integrin ligation (Stromblad et al., 2002). In any event, removal of an integrin before development is quite distinct from blocking one present on fully formed blood vessels actively engaged in neovascularization.

We were interested to ask why there should be distinct signaling pathways leading to the formation of new blood vessels. To this end, we recently reported that bFGF and VEGF selectively protect ECs from stress- and receptor-mediated apoptosis, respectively, in a manner that is dependent on the differential phosphorylation of c-Raf by PAK and Src (Alavi et al., 2003). As depicted in Fig. 7, we have integrated our current findings with our recent work (Alavi et al., 2003), showing that FGF-mediated EC protection from stress-mediated death is independent of ERK, yet also dependent on phosphorylation of c-Raf at its PAK phosphorylation sites, S338, leading Raf translocation to the mitochondria. In contrast, VEGF-mediated protection (Fig. 7) is mediated in part by its phosphorylation of c-Raf at its Src phosphorylation sites, YY340/341 leading to an ERK-dependent protection against receptor-mediated apoptosis (Alavi et al., 2003). Intriguingly, although ERK likely plays a role in bFGF-mediated cell proliferation, migration, and differentiation (Eliceiri et al., 1998), it does not appear to be essential for survival (Alavi et al., 2003). What remains unclear is whether ERK itself is activated in the same manner by both growth factor/integrin signaling pathways or whether other ERK isoforms may be activated in response to either pathway.

In the current report, we show that bFGF-induced PAK activation and subsequent c-Raf activation are blocked by inhibitors of αvβ3 (Fig. 5). Intriguingly, stimulation by either bFGF or VEGF activates PAK, but only bFGF/αvβ3 signaling led to phosphorylation of Raf S338 (Fig. 5). Similarly, both bFGF and VEGF stimulation activates Src, but only VEGF signaling leads to an Src-dependent phosphorylation of c-Raf on Y340/341. This suggests that regulation of signaling enzymes and subsequent biological events are dependent on more than the sequential activation of enzymes in an on/off binary fashion, but is instead dependent on the entire context of signals within the cell, some of which are derived from specific ECM–integrin ligation events and propagated by distinct αv integrins.

What, then, is the need for two growth factor/integrin pairs to differentially influence EC survival during angiogenesis? This may be important for vascular pruning (Benjamin et al., 1999) or regulation of blood vessel morphology and/or function in specific microenvironments such as wounds or inflammatory sites (Alon et al., 1995). In addition to growth factor–mediated signals, ECM composition likely influences the fate and integrity of newly sprouting blood vessels based on the expression of specific integrins on the EC surface (Alon et al., 1995; Stupack et al., 2001). In fact, we recently demonstrated that certain unligated integrins could induce "integrin-mediated death" (Stupack et al., 2001), which may regulate angiogenesis by promoting EC apoptosis, thereby only permitting the survival of appropriately positioned ECs during vascular remodeling.

In summary, our results indicate that during angiogenesis, VEGF and αvβ5 or bFGF and αvβ3 cooperate to differentially regulate the activation of the Ras-ERK pathway. VEGF uses αvβ5 and FAK to activate Ras, along with Src to activate c-Raf, whereas bFGF uses αvβ3, FAK, and PAK downstream of Ras to activate c-Raf. Either of these pathways leads to sustained Ras-ERK activity and subsequent angiogenesis, and the distinct signaling molecules activated may play a role in the divergent vascular and survival responses elicited by bFGF and VEGF.

Materials and methods

Antibodies and reagents

Mouse mAbs raised against integrins αvβ3 and αvβ5 have been described previously (Friedlander et al., 1995). A mouse mAb raised against p21-Ras (Transduction Laboratories) was used for immunoblotting of loading controls. A mouse mAb raised against c-Raf (Transduction Laboratories) was used for immunoprecipitations for in vitro kinase assays and immunoblotting. A phosphospecific antibody raised against ERK phosphorylated at threonine 202/tyrosine204 (Cell Signaling Technologies) was used for immunoblots and immunofluorescence of active ERK. A rabbit pAb, C-14, raised against rat ERK2 (Santa Cruz Biotechnology, Inc.) was used for immunoblotting of loading controls. A phosphospecific rat mAb raised against c-Raf phosphorylated at serine 338 (Upstate Biotechnology) was used for immunoblots and immunofluorescence. A phosphospecific rabbit pAb raised against c-Raf phosphorylated at Serine 340 (Biosource International, Camarillo, CA) was used for immunoblots. A rabbit polyclonal antibody, N-20, raised against an NH_2-terminal sequence of rat PAK (Santa Cruz Biotechnology, Inc.) was used for immunoblotting and immunoprecipitations for in vitro kinase assays. The bFGF was a gift from Dr. J. Abraham (Scios, Mountain View, CA), and PD98059 was the gift of Dr. A. Saltiel (Parke-Davis, Ann Arbor, MI). All other reagents were from Sigma-Aldrich unless otherwise stated.

Constructs and retroviruses

The replication-competent RCASBP(A) (Hughes et al., 1987) retrovirus was used to express the described mutant cDNAs subcloned as NotI–ClaI. These constructs were transfected into the chicken immortalized fibroblast line DF-1. Viral supernatants were collected from DF-1 producer cell lines in serum-free CLM media. Viral supernatants were concentrated by ultracentrifugation at 4°C for 2 h at 22,000 rpm, and the pellets were resuspended in 1/100 the original volume in serum-free media with a titer of at

least 108 infectious units (i.u.)/ml and stored at −80°C. RCAS-Raf-caax, a dominant-active form of c-Raf generated by fusing the membrane targeting region of Ras to c-Raf (Leevers et al., 1994; Stokoe et al., 1994), was the gift of Sally Johnson (Pennsylvania State University, University Park, PA); Ras N17, a dominant-negative form of Ras in which amino acid 17 is changed to Asn (Feig and Cooper, 1988), was the gift of Janet Jackson (The Scripps Research Institute, La Jolla, CA); Raf-ATP$^{\mu}$, a dominant-negative form of c-Raf made by generating a point mutation in the ATP binding region (Kolch et al., 1991), was the gift of Deborah Morrison (National Cancer Institute, Bethesda, MD); Ras G12V, a dominant-active form of Ras made by generating a point mutation at amino acid 12 of Ras (Bos, 1989), was the gift of Janet Jackson; and FRNK, a dominant-negative form of FAK found endogenously lacking the kinase domain (Cobb et al., 1994), was the gift of TJ Parsons (University of Virginia, Charlottesville, VA). $PAK_{83–149}$ was generated as a PCR product from full-length PAK as described previously (Zhao et al., 1998).

Chicken CAM angiogenesis assay and treatments

Angiogenesis assays were performed essentially as described previously (Brooks et al., 1994b). Filter discs saturated with high titer RCAS retrovirus or anti-integrin antibodies (50 μg) along with 2.0 μg/ml bFGF or VEGF were placed on the CAMs of 10-d-old chick embryos. The growth factor doses were chosen based on dose-response studies in which saturable and maximal angiogenic responses were seen at 100 ng/ml of each growth factor. We chose a 20-fold higher dose to eliminate any issues with integrin specificity. After 72 h, filter discs and associated CAM tissues were harvested and quantified. Angiogenesis was assessed as the number of visible blood vessel branchpoints within the defined area of the filter discs. At least 20 CAMs were used for each treatment.

Immunoprecipitation and immunoblotting

Biochemical assays of chick angiogenic tissues were done essentially as described previously (Eliceiri et al., 1998). In brief, tissues were harvested and snap frozen in liquid nitrogen at the described time points, homogenized in radioimmunoprecipitation (RIPA) buffer, centrifuged at 15,000 *g*, and assayed for total protein using the BCA reagent (Pierce Chemical Co.). Equivalent amounts of protein (400–700 μg) were immunoprecipitated with either anti-c-Raf or anti-PAK antibodies bound to protein A–Sepharose beads. Whole-cell lysates or immunoprecipitates were washed with RIPA, separated using SDS-PAGE, transferred to nitrocellulose, blocked with 3% BSA in TBS with 0.1% Tween 20, and exposed to the described primary antibodies. Antibody binding was detected using HRP-conjugated goat anti–rabbit or anti–mouse antibodies and ECL (Amersham Biosciences).

Immunofluorescence and microscopy

Immunofluorescent assays were performed essentially as described previously, with minor modifications (Eliceiri et al., 1998). Cryosections of CAMs treated with either bFGF or VEGF were examined for the tissue distribution of phosphorylated ERK or c-Raf using primary antibodies directed against phosphorylated ERK (Cell Signaling Technologies) or c-Raf phosphorylated at S338 (Upstate Biotechnology). CAM sections were treated as described previously (Eliceiri et al., 1998), removed from embryos, washed in PBS, embedded in O.C.T. compound (Sakura Finetek), and snap frozen in liquid nitrogen. Sections of 4-μm thick CAM tissue were cut, fixed in acetone for 30 s, and stored at −70°C until use. Tissue sections were prepared for immunostaining by a brief rinse in PBS, a block in 1:5 dilution of block Hen (Aves Labs), and incubation in a 1:50 dilution of primary antibody for 1 h. After 20 min of PBS washes, slides were incubated in Alexa® 488–labeled secondary antibodies (Molecular Probes, Inc.) for 2 h at 1:500. Slides were mounted, and images were collected on a microscope (Axiovert 100; Carl Zeiss, MicroImaging, Inc.) with a 20× 0.7 NA lens with a cooled CCD camera (model CE200A; Photometrics) as 12-bit, 512 × 384 pixel arrays using fluorescein filter sets from Chroma Technology Corp.

Kinase assays for Ras, Raf, Src, and PAK

Ras activity was quantitated essentially as described previously (Taylor and Shalloway, 1996). In brief, amino acids 1–149 of c-Raf were expressed in pGEX-RBD in order to obtain a GST fusion protein expressing the active Ras-binding domain of c-Raf. GST–RBD expression in transformed *Escherichia coli* was induced with 1 mM IPTG for 1–2 h, and the fusion protein was purified on glutathione-Sepharose beads. The beads were washed in a solution containing 20 mM Hepes, pH 7.5, 120 mM NaCl, 10% glycerol, 0.5% NP-40, 2 mM EDTA, 10 μg ml^{-1} leupeptin, and 10 μg ml^{-1} aprotinin, stored in the same buffer at 4°C, and used within 2–3 d of preparation. For affinity precipitation, lysates were incubated with GST–RBD prebound to glutathione-Sepharose (∼15 μl packed beads; ∼15–30 μg protein) for 30 min at 4°C with rocking. Bound proteins were eluted with SDS–PAGE sample buffer, resolved on 11% acrylamide gels, and subjected to Western blotting with anti-pan Ras (Transduction Laboratories). c-Raf activity was quantitated essentially as described previously (Hood and Granger, 1998). In brief, c-Raf immunoprecipitates were incubated with kinase-inactive MEK-1-GST (Upstate Biotechnology) as a substrate for 20 min at 30°C in 40 μl reaction buffer (25 mM Hepes, pH 7.4, 25 mM glycerophosphate, 1 mM dithiothreitol, 10 mM $MnCl_2$, 100 μM ATP, and 10 μCi of [^{32}P]ATP (ICN Biomedicals). The assay was terminated by addition of Laemmli buffer and boiling, followed by size fractionation on 12% SDS-PAGE, gel drying, and autoradiography. Src activity was quantitated as described previously (Eliceiri et al., 1999). PAK activity was quantitated essentially as described previously (Zenke et al., 1999). In brief, immunoprecipitated Pak was incubated in kinase buffer (50 mM Hepes/NaOH, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM DTT, and 5 μg myelin basic protein) containing 20 μM ATP and 5 μCi [^{32}P]ATP. The reactions were incubated for 30 min at 30°C and stopped by addition of sample buffer, followed by size fractionation on 12% SDS-PAGE, gel drying, and autoradiography.

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