Recent studies indicate that angiogenesis depends, in part, on ligation of integrin $\alpha_\beta_1$ by fibronectin. Evidence is now provided that integrin $\alpha_\beta_1$ regulates the function of integrin $\alpha_\beta_3$ on endothelial cells during their migration in vitro or angiogenesis in vivo. Secretion of fibronectin by endothelial cells leads to the ligation of integrin $\alpha_\beta_3$, which potentiates $\alpha_\beta_3$-mediated migration on vitronectin without influencing $\alpha_\beta_3$-mediated cell adhesion. Endothelial cell attachment to vitronectin suppresses protein kinase A (PKA) activity, while addition of soluble anti-$\alpha_\beta_3$ restores this activity.

Moreover, agents that activate intracellular PKA, such as forskolin, dibutyryl cAMP or $\alpha_\beta_3$ antagonists, suppress endothelial cell migration on vitronectin in vitro or angiogenesis in vivo. In contrast, inhibitors of PKA reverse the anti-migratory or anti-angiogenic effects mediated by $\alpha_\beta_3$ antagonists. Therefore, $\alpha_\beta_3$-mediated endothelial cell migration and angiogenesis can be regulated by PKA activity, which depends on the ligation state of integrin $\alpha_\beta_1$. 

The growth of new blood vessels, angiogenesis, contributes to the adverse effects of solid tumor cancer, arthritis, psoriasis, and blindness. Angiogenesis is a complex biological process that depends on growth factors and the extracellular matrix (1–6). A potential role for the extracellular matrix protein fibronectin in vascular development was initially suggested by studies demonstrating the presence of oncogenes fibronectin around blood vessels during development and wound healing and in tumor tissue (7–10). Interestingly, mice lacking fibronectin die early in development from a collection of defects, which includes an improperly formed vasculature (11). Additionally, mice lacking the fibronectin receptor $\alpha_\beta_1$, one of several fibronectin receptors (12), also die early in development and exhibit some vascular and cardiac defects (13, 14). This circumstantial evidence suggests potential roles for fibronectin and its receptor, integrin $\alpha_\beta_1$ in angiogenesis.

We have recently shown that integrin $\alpha_\beta_1$ and its ligand fibronectin are expressed at significantly increased levels in neovessels induced by growth factors or solid tumors (15). Notably, antibody, peptide, and small molecule antagonists of integrin $\alpha_\beta_1$ and fibronectin block growth factor- and tumor-induced angiogenesis (15). Interestingly, antagonists of another fibronectin-binding integrin, $\alpha_\beta_3$, also inhibit angiogenesis (16–22). Antagonists of both of these integrins substantially block angiogenesis induced by basic fibroblast growth factor (bFGF)$^1$ but not by vascular endothelial growth factor, suggesting that these integrins regulate similar pathways of angiogenesis (15, 22). Since antagonists of both integrins independently and substantially block the same pathway of angiogenesis, it is possible that these two integrins interact during angiogenesis.

Previous studies have indicated that the occupancy of one integrin by its ligand can inhibit the functions of other integrins (23–28). These studies have indicated that cross-talk between integrins regulates such properties as integrin-mediated cell migration (23, 24, 26–28) and ligand binding (25).

For example, ligation of integrin $\alpha_\beta_3$ has been shown to inhibit cell migration and phagocytosis mediated by integrin $\alpha_\beta_3$ (23). Interactions between individual integrins are known as cross-talk. Integrin cross-talk appears to depend on the actions of signal transduction molecules (23, 24, 28). For example, integrin $\alpha_\beta_3$ suppression of $\alpha_\beta_3$-mediated migration is dependent on calcium/calmodulin-dependent protein kinase II (28).

In this report, we provide evidence that integrin $\alpha_\beta_3$ regulates the functions of integrin $\alpha_\beta_3$ in vitro and in vivo. Antagonists of integrin $\alpha_\beta_3$ suppress $\alpha_\beta_3$-mediated focal contact formation and cell migration without inhibiting cellular attachment. This inhibition of $\alpha_\beta_3$ function is dependent on the activation of protein kinase A (PKA) and can be blocked by inhibitors of PKA.
minopropionic acid methyl ester. Ten-day-old chicken eggs were purchased from McIntyre Poultry (Ramona, CA). Basic fibroblast growth factor was purchased from Genzyme, Inc. (Cambridge, MA). PKA assay kits were purchased from Life Technologies, Inc.

**Immunohistochemical Analysis of Focal Contacts**—Round 1-mm-thick eggs were incubated with 1 μg/ml fibronectin, vitronectin, collagen, or poly-L-lysine for 1 h at room temperature, then blocked with 3% bovine serum albumin for 2 h at 37 °C. HUVECs were removed from culture dishes by trypsinization, washed in serum-free culture medium, and resuspended in Hanks’ balanced salt solution supplemented with 10 mM HEPES, pH 7.4, 2 mM MgCl₂, 2 mM CaCl₂, 0.2 mM MnCl₂, 1% BSA. Cells were then incubated for 1 h at 37 °C on coverslips. Cells were incubated with the presence of specific antibodies, or function-blocking antibodies, or no antibodies for 60 min. Coverslips were then washed to remove unbound cells, fixed for 5 min in 3.7% paraformaldehyde and permeabilized in 0.3% Triton X-100 for 3 min. Coverslips were incubated with 1.25 μg/ml anti-paxillin antibodies, 0.2 μg/ml anti-vinculin antibodies, 1:500 dilution of anti-integrin β₁ monoclonal antibodies, or 2.7 μg/ml anti-phosphotyrosine antibodies in 20 mM HEPES, pH 7.4, 2 mM MgCl₂, 2 mM CaCl₂, 0.2 mM MnCl₂, 1% BSA. Cells were then incubated for 1 h at 37 °C. Additional plates were coated with 25 μg/ml goat anti-mouse fluorescein isothiocyanate for 1 h at room temperature. Coverslips were well washed in phosphate-buffered saline and incubated in 10 μg/ml anti-mouse fluorescein isothiocyanate for 1 h at room temperature. Coverslips were well washed in phosphate-buffered saline and mounted in Gelvatol, prior to confocal fluorescence microscopy.

**Cell Adhesion Assays**—Cell adhesion assays were performed as described (15). In brief, the wells of 48-well non-tissue culture-treated culture dishes (Costar, Inc.) were coated with 5 μg/ml vitronectin, fibronectin, Del-1, or collagen for 1 h at 37 °C and blocked with 2% heat-denatured bovine serum albumin in phosphate-buffered saline for 1 h. Fifty thousand cells in 25 μg/ml anti-α₁β₁ function blocking antibody (JSB5), 25 μg/ml anti-α₁β₁ function blocking antibody (LM609), 25 μg/ml anti-α₁β₁ function blocking antibody, 25 μg/ml anti-β₁ function blocking antibodies (PAC1/10), or 25 μg/ml control antibody (W6/32) in adhesion buffer (Hepe-buffer-Hanks balanced salt solution containing 1% bovine serum albumin, 2 mM MgCl₂, 2 mM CaCl₂, and either 0.2 mM or 0.2 mM MnCl₂) were allowed to adhere to dishes for 20 min at 37 °C. In some experiments, dibutyryl cAMP was used at 500 μM and forskolin was used at 20 μM/ml. Each experiment was performed in triplicate, with triplicate samples per condition. The data are presented as percentage of adhesion exhibited by the positive control (adhesion triplicate, with triplicate samples per condition. The data are presented

**Migration Assays**—Migration assays were performed essentially as described (15). The lower side of 8-mm pore Transwell inserts (Costar, Inc.) were coated with 5 μg/ml fibronectin, vitronectin, Del-1, or collagen, or no protein for 1 h and were blocked with 2% bovine serum albumin in phosphate-buffered saline for 1 h. HUVECs (25,000) were added to Transwell inserts in adhesion buffer (Hepe-buffer-Hanks balanced salt solution containing 1% bovine serum albumin, 2 mM MgCl₂, 2 mM CaCl₂, and either 0.2 mM or 0.2 mM MnCl₂) and either 10 μM SJ749 or 0.2 μM anti-α₁β₁ function blocking antibodies against integrin α₁β₁ or α₁β₃, or 2.7 μg/ml antibodies, or 0.2 mM MnCl₂, 1% BSA). Cells were then incubated for 1 h at 37 °C, and incubated in 10 μM/l anti-α₁β₁ or control antibodies or control antibodies overnight at 4 °C. HUVECs were trypsinized and resuspended in adhesion buffer (Hanks’ balanced salt solution supplemented with 10 mM HEPES, pH 7.4, 2 mM MgCl₂, 2 mM CaCl₂, 0.2 mM MnCl₂, 1% BSA). Cells were plated on matrix protein or antibody-coated plates in medium or the presence of 25 μM anti-α₁β₁, or control antibodies (W6/32, anti-MHC) for 60 min. Plates were washed with phosphate-buffered saline and extracted with cold extraction buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 25 μg/ml aprotinin, and 25 μg/ml leupeptin). Protein kinase A activity was assessed using a kit from Life Technologies, Inc. Briefly, equal volumes of lysate and reaction mixture containing final concentrations of 50 mM Tris, pH 7.5, 10 mM MgCl₂, 0.25 mM MgCl₂, 10 mM ATP, 50 μM PKI-(6–22) amide, 0.2 μM H8, 500 μM H89, 25 μM SJ749 and 10 mM MnCl₂ were combined for 5 min at 30 °C. Total PKA in each sample was measured by performing the assay in the presence of 10 μM BAP. Background PKA activity in test and total PKA samples were determined by performing the assay in the presence of a PKA inhibitor peptide, PKI-(6–22) amide. Twenty μl samples of reaction mixtures were spotted onto phosphocellulose discs, air-dried, and washed repeatedly with 1% (v/v) phosphoric acid in water. Incorporation radioactivity was determined by scintillation counting. Activated PKA was calculated from the ratio of pmol/min test sample PKA to pmol/min total PKA. PKA activity in triplicate samples was measured and experiments were repeated at least three times. Statistical analyses were performed using Student’s t test.

**Western Blotting**—HUVECs were detached from culture dishes by trypsinization, washed, and resuspended in serum-free culture medium in the presence or absence of 200 μg/ml cycloheximide. Cells were plated on cell culture plates that had been coated with vitronectin, collagen, or fibronectin and blocked with bovine serum albumin. Cells were incubated for 4 h at 37 °C prior to lysis with boiling SDS sample buffer. Fifty micrograms of protein from each lysate was electrophoresed on 10% SDS-polyacrylamide gels. The separated proteins were electrophoretically transferred to nitrocellulose and then incubated in anti-fibronectin, anti-integrin α₁, or β₁, cytoplasmic tail, or anti-protein kinase A catalytic subunit antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. Blots were incubated in a chemiluminescent substrate for horseradish peroxidase and then exposed to film.

**RESULTS**

**Antibody Antagonists of Integrin α₁β₁, Block α₁β₃-dependent Cellular Migration**—Recent studies show that antagonists of integrin α₁β₁, or α₁β₃, can substantially block growth factor and tumor-induced angiogenesis in chicken embryos and in human skin transplanted onto SCID mice (15–18, 32). Furthermore, antagonists of these adhesion receptors block angiogenesis induced by bFGF but not by vascular endothelial growth factor, suggesting that these integrins may cooperate by regulating the same angiogenic pathways (15, 22). To evaluate the interactions of α₁β₁ and α₁β₃ in angiogenesis, experiments were designed to examine the contribution of each to collagen cell migration in vitro, as endothelial cell migration is an essential feature of angiogenesis. As shown in Fig. 1 (A and B), function blocking antibodies selective for integrin α₁β₁ blocked both the adhesion and migration of HUVECs on fibronectin, while antibodies against integrin α₁β₃ had little effect. Surprisingly, both anti-α₁β₁ and anti-α₁β₃ inhibited endothelial cell migration on vitronectin (Fig. 1C), yet only anti-α₁β₃ blocked adhesion to vitronectin (Fig. 1D). In contrast, anti-α₁β₃ had no impact on collagen-mediated cell migration, which depends on...
Cross-talk between Integrin α₅β₁ and Integrin α₅β₃

integron α₅β₁ (Fig. 1E). Similar results were observed for adult dermal microvascular endothelial cells (data not shown). Anti-α₅β₁ blocked α₅β₃-mediated cell migration to similar extents when measured after 2, 4, or 18 h of incubation time (data not shown). Anti-α₅β₁ blocked α₅β₃-mediated migration but not adhesion to the same extent in the absence or in the presence of high levels (200 μM) of Mn²⁺, a divalent cation that enhances the affinity of α₅β₃ for its ligand (data not shown). Therefore, α₅β₃ function is required for endothelial cell migration on vitronectin and other substrates of integrin α₅β₃, as well as on fibronectin.

Interestingly, both antibody and small molecule antagonists of integrin α₅β₁ block cell migration on vitronectin (Fig. 1F) without affecting cell attachment (Fig. 1C; Ref. 15). Thus, antibodies directed against α₅β₁ block α₅β₃-mediated cell migration by antagonizing α₅β₁ ligation, rather than by cross-linking cell surface α₅β₁ molecules. These results indicate that integrin α₅β₁ selectively impacts α₅β₃-mediated cellular migration without influencing α₅β₃-dependent adhesion, suggesting that these integrins may function in a cooperative manner on the surface of endothelial cells.

Ligation of Integrin α₅β₁ by Fibronectin Potentiates Integrin α₅β₃ Function—Endothelial cells readily secrete fibronectin during angiogenesis (8, 15). In fact, endothelial cell-derived oncostatin fibronectin specifically accumulates around tumor-associated blood vessels (7–10). Recent studies suggest that antibodies directed to the cell-binding domain of fibronectin block angiogenesis (15). The indirect inhibition of endothelial cell migration on vitronectin by α₅β₁ antagonists (Fig. 1) suggests the possibility that endothelial cell-derived fibronectin may influence cell migration on vitronectin. Indeed, cell migration on vitronectin is partially inhibited by function-blocking antibodies directed against the cell-binding domain of fibronectin, but not by antibodies directed against the N terminus of fibronectin (Fig. 2A). In contrast, anti-fibronectin antibodies (Fig. 2B) do not block cellular migration on collagen, even though endothelial cells cultured on either vitronectin (Fig. 2C) or collagen (data not shown) synthesize fibronectin. These results suggest that ligation of α₅β₁ by endothelial cell-secreted fibronectin specifically regulates α₅β₃ function but not that of other integrins.

To determine further whether ligation of α₅β₁ by endothelial cell secreted fibronectin potentiates integrin α₅β₃ function, endothelial cells were treated with cycloheximide to inhibit protein synthesis and then evaluated for their abilities to migrate on fibronectin, vitronectin, or collagen substrates. Endothelial cell synthesis of fibronectin is inhibited by cycloheximide pretreatment of endothelial cells (Fig. 2C). Inhibition of protein synthesis results in an overall reduction in cellular migration rates compared with untreated cells (Fig. 2, D–F). In contrast to untreated cells, the migration of cycloheximide-treated cells on vitronectin is not inhibited by anti-α₅β₁ antibodies (Fig. 2E). However, migration on vitronectin is inhibited to the same extent by anti-α₅β₁ antibodies in treated and untreated cells. In addition, in contrast to untreated cells, migration of cycloheximide-treated cells on vitronectin is refractory to anti-fibronectin antibodies (Fig. 2F). Migration on collagen is equally sensitive to anti-α₅β₁ and insensitive to anti-α₅β₃ antibodies in cycloheximide-treated and untreated cells (data not shown). In fact, expression levels of α₅ and β₃ integrins measured by immunoblotting with anti-cytosplasmic tail antibodies in treated and untreated cells remains unchanged as does expression of protein kinase A (data not shown). Taken together, these results suggest that fibronectin synthesized by HUVECs contributes to vitronectin migration based on its ability to ligate integrin α₅β₁.

Indirect Inhibition of Focal Adhesion Formation—Temporal prerequisites to cellular migration are the attachment and spreading of cells on the extracellular matrix, with concomitant formation of stress fibers and focal adhesions. Since antagonists of α₅β₁ do not impact α₅β₃-mediated cell attachment but do inhibit migration, we examined the effect of α₅β₁ antagonists on cell spreading and focal contact formation to establish the earliest integrin α₅β₃-mediated events that are affected by inhibition of α₅β₁. During spreading on vitronectin (within 30 min after initial cell attachment), endothelial cells form stress fibers (Fig. 3A) as well as focal adhesions incorporating paxillin, vinculin, integrin β₃ (Fig. 3B), and phosphotyrosine (data not shown). Integrin-mediated signal transduction during this time leads to the tyrosine phosphorylation of focal adhesion proteins such as paxillin and focal adhesion kinase.

In the presence of integrin α₅β₁ antagonists, endothelial cells generally appear less polarized than in control cells (Fig. 3A). Instead, these cells acquire a rounded, pancake-like morphology. Notably, stress fiber formation in cells attached to vitronectin but not to collagen is attenuated in the presence of antagonists of integrin α₅β₁ (Fig. 3A). Stress fiber formation is not attenuated in the presence of control antagonists (data not shown). Although some anti-α₅β₁-treated cells display thin,
Wispy stress fibers, 75% of the cells display no apparent stress fibers. Most appear to have accumulations of actin in membrane ruffles. Furthermore, formation of focal adhesions incorporating paxillin, vinculin, integrin \( \alpha_5 \beta_1 \), and phosphotyrosine on vitronectin is also inhibited in the presence of antagonists of integrin \( \alpha_5 \beta_1 \) (Fig. 3B). Antagonists of integrin \( \alpha_5 \beta_3 \) also block the tyrosine phosphorylation of paxillin (data not shown). In anti-\( \alpha_5 \beta_1 \)-treated cells, accumulations of paxillin and vinculin are found in small patches at the periphery of the cells but not in focal adhesions. As expected from cell migration studies, anti-\( \alpha_5 \beta_1 \) treatment does not block focal adhesion formation in cells attached to collagen (Fig. 3). Control antibody-treated cells also display normal focal adhesions (data not shown).

These results indicate that antagonists of integrin \( \alpha_5 \beta_1 \) block the ability of integrin \( \alpha_5 \beta_1 \) to form focal adhesions and engage the actin cytoskeleton, thus accounting for the inhibition of migration.

**Regulation of Integrin Cross-talk by Protein Kinase A**—Previous studies have demonstrated that cross-talk may take place between integrins (23–28), allowing one integrin to regulate
the functions of another. Furthermore, this cross-talk may depend on serine/threonine protein kinases (23, 28). Therefore, migration assays were performed in the presence of several different protein kinase inhibitors to determine if the functional cooperativity between integrin α5β1 and αβ3 during cellular migration was dependent on signal transduction. HUVEC migration on vitronectin was no longer inhibited by anti-αβ3 when cells were incubated in the presence of H8, a general inhibitor of protein kinases A, G, and C (Fig. 4A). In contrast, autokamitide II (AC2), an inhibitor of calcium/calmodulin-dependent protein kinase II, had no effect on αβ3 inhibition of vitronectin migration (Fig. 4A). These results indicate that protein kinase A, G, or C function may be required for the inhibition of integrin αβ3 by antagonists of integrin αβ1.

To determine which of these kinases might be responsible for the inhibition of integrin αβ3 function, endothelial cells were treated with selective inhibitors of these kinases. In the presence of calphostin C, an inhibitor of protein kinase C, anti-αβ1 antibodies maintained their abilities to inhibit cell migration (Fig. 4B). These results indicate that ligation of endothelial cell integrin αβ1 by fibronectin suppresses the activity of protein kinase A. To determine whether antagonists of integrin αβ3 influence αβ1 in a PKA-dependent manner, endothelial cells were plated onto vitronectin in the presence of anti-αβ3 or control antibodies (Fig. 5D). Even though anti-αβ3 does not block adhesion to vitronectin, it led to a significant elevation of PKA activity over that of cells incubated in control antibodies (p < 0.009). Taken together, these results suggest that protein kinase A activation by inhibition of integrin αβ3 ligation suppresses αβ3-mediated migration in vitro.

Protein kinase A typically becomes activated upon binding cAMP (33). Thus, agents that elevate cAMP in cells, such as forskolin and dibutyryl cAMP, activate protein kinase A. When endothelial cells were incubated in dibutyryl cAMP or forskolin, cell migration on vitronectin was blocked (Fig. 6A). Like anti-αβ3, neither of the cAMP-elevating reagents inhibited cell attachment to vitronectin (Fig. 6B), indicating that they specifically impact αβ3-mediated migration. Like anti-αβ3, activation of PKA by dibutyryl cAMP or forskolin also inhibits cell spreading and focal contact formation (data not shown). Taken together, these results confirm that the elevation of PKA activity results in inhibition of integrin αβ3-mediated focal contact formation and migration.

Integrin αβ3 and Protein Kinase A Regulation of αβ3-mediated Angiogenesis in Vivo—The in vitro studies described above implicate PKA in αβ3-dependent regulation of αβ3 function (Figs. 4 and 5). Recent evidence demonstrates a role for αβ3 (16–23) as well as αβ1 (15) in the process of angiogenesis in vivo. To evaluate the relationships between αβ3, αβ1, and PKA in angiogenesis, chick CAMs were stimulated with bFGF. These CAMs were then treated with anti-integrin αβ1 or control antibodies in the presence or absence of H8, an inhibitor of
the protein kinases A, C, and G. Anti-\(\alpha_5\beta_1\) treatment significantly blocked angiogenesis (Fig. 7A), and H8 reversed this anti-\(\alpha_5\beta_1\)-mediated inhibition (Fig. 7A). This reversal could be attributed to the inhibition of PKA activity since H89, a selective inhibitor of PKA, also reversed anti-\(\alpha_5\beta_1\)-mediated inhibition of angiogenesis (Fig. 7B). In contrast, PKC-selective inhibitors had no such effect (Fig. 7C). Inhibitors of PKA could not reverse the anti-angiogenic effects of anti-\(\alpha_5\beta_3\), indicating that PKA is downstream of \(\alpha_5\beta_1\) but not of \(\alpha_5\beta_3\) (data not shown). A role for PKA in the inhibition of angiogenesis was further supported by the observations that, like anti-\(\alpha_5\beta_1\), activators of PKA such as forskolin and cAMP also blocked angiogenesis (Fig. 7D). Taken together, these results demonstrate that ligation of integrin \(\alpha_5\beta_1\) by fibronectin suppresses protein kinase A activity, thereby influencing the function of integrin \(\alpha_5\beta_3\) during endothelial cell migration \textit{in vitro} and angiogenesis \textit{in vivo} (Fig. 8).

**DISCUSSION**

Antagonists of integrins \(\alpha_5\beta_1\) and \(\alpha_5\beta_3\) both substantially block angiogenesis induced by bFGF, but not by vascular endothelial growth factor, suggesting that these integrins regulate similar pathways of angiogenesis (15, 23). These two integrins may interact during angiogenesis, since antagonists of both integrins independently and substantially block the same pathway of angiogenesis.

Our studies indicate that integrin \(\alpha_5\beta_1\) regulates the function of integrin \(\alpha_5\beta_3\) on endothelial cells during their migration and spreading \textit{in vitro} and angiogenesis \textit{in vivo}. Ligation of integrin \(\alpha_5\beta_1\) potentiates \(\alpha_5\beta_3\)-mediated migration on vitronectin without influencing \(\alpha_5\beta_3\)-mediated cell adhesion. Endothelial cell attachment to vitronectin suppresses PKA activity, while addition of soluble anti-\(\alpha_5\beta_1\) restores this activity. Agents that activate intracellular PKA, such as forskolin, dibutyryl cAMP, or \(\alpha_5\beta_1\) antagonists, suppress endothelial cell migration on vitronectin \textit{in vitro} or angiogenesis \textit{in vivo}. In contrast, inhibitors of PKA reverse the anti-migratory or anti-angiogenic effects mediated by \(\alpha_5\beta_1\) antagonists. Antagonists of \(\alpha_5\beta_1\) and agonists of protein kinase A also inhibit \(\alpha_5\beta_3\)-mediated focal adhesion formation and cell spreading. These results suggest that \textit{unoccupied} \(\alpha_5\beta_1\) activates PKA, which inhibits focal adhesion formation and cell migration mediated by \(\alpha_5\beta_3\).

Our studies suggest that endothelial cell-secreted fibronectin ligates integrin \(\alpha_5\beta_1\), thereby suppressing protein kinase A function and permitting \(\alpha_5\beta_3\)-dependent migration, since antagonists of both fibronectin and integrin \(\alpha_5\beta_1\) indirectly inhibit \(\alpha_5\beta_3\)-mediated migration. Short term inhibition of protein synthesis suppresses fibronectin expression and secretion without impacting \(\alpha_5\beta_1\), \(\alpha_5\beta_3\), or protein kinase A expression. Protein synthesis inhibition prevents the inhibition of \(\alpha_5\beta_3\)-mediated migration by either anti-fibronectin or anti-\(\alpha_5\beta_1\) antibodies but has no impact on inhibition of migration by anti-\(\alpha_5\beta_3\) antibodies. These studies suggest that \(\alpha_5\beta_1\) interaction with cellular fibronectin promotes \(\alpha_5\beta_3\)-mediated migration. In contrast, if endothelial cell fibronectin were able serve as a ligand for integrin \(\alpha_5\beta_3\), inhibition of protein synthesis would enhance \(\alpha_5\beta_3\)-dependent migration on vitronectin, as more \(\alpha_5\beta_3\) would be able to interact with the vitronectin substrate. As the component of cellular migration on vitronectin that can be inhibited by anti-\(\alpha_5\beta_3\) remains unchanged in the presence or absence of cycloheximide, it is unlikely that cellular fibronectin interacts with \(\alpha_5\beta_3\). Thus, these studies suggest that endothelial cell fibronectin interacts with \(\alpha_5\beta_1\) and promotes \(\alpha_5\beta_3\)-dependent migration.

In many cells, activation of PKA induces dramatic morphological and biochemical alterations leading to inhibition of cell proliferation or cell survival. For example recent studies show that activation of PKA with cAMP or other PKA agonists leads to the inhibition of focal contact and stress fiber formation, with concomitant inhibition of mitogen-activated protein kinase activation as well as pp125 focal adhesion kinase tyrosine phosphorylation.
phosphorylation and paxillin phosphorylation (34). The induction of changes in \( \alpha_\beta_1 \)-mediated cell spreading and migration by antagonists of \( \alpha_\beta_1 \) may result from the inactivation of Rho A by PKA. Elevation of cAMP inhibits RhoA activity as well as integrin-mediated cell migration (35–37). For example, ligation of integrin \( \alpha_\beta_1 \) suppresses intracellular cAMP levels by activating cAMP-specific phosphodiesterases (36) and subsequently activating RhoA (37). cAMP-induced morphological changes can be inhibited by activation of the small GTPase RhoA (38). RhoA regulates integrin- and growth factor-mediated stress fiber and focal contact formation (39, 40). Rho is also thought to regulate cellular migration by activating myosin light chain kinase (41). Although the related GTPases Rac and cdc42 promote formation of lamellipodia and filopodia, respectively (42), cAMP inhibition of cell spreading cannot be rescued by these proteins. Thus, inactivation of Rho A by protein kinase A is a possible event in the regulation of \( \alpha_\beta_1 \)-mediated cell spreading and migration by integrin \( \alpha_\beta_1 \).

Previous studies have indicated that integrins can inhibit the functions of other integrins by a process called cross-talk (23–28). These studies indicate that cross-talk between integrins regulates integrin-mediated cell migration (23, 26–28) and ligand binding (25). For example, ligation of integrin \( \alpha_\beta_1 \) inhibits cell migration and phagocytosis mediated by integrin \( \alpha_\beta_1 \) (23). This integrin cross-talk depends on the actions of signal transduction molecules (23–24, 28). For example, suppression of \( \alpha_\beta_1 \)-mediated migration by integrin \( \alpha_\beta_1 \) depends on calcium calmodulin-dependent protein kinase II (28).

Recent studies linking cAMP levels with regulation of integrin-mediated chemotaxis or haptotaxis (32–34, 43) suggest that phosphodiesterases (33, 34) or heterotrimeric G proteins (43) may be regulated by integrins. In vascular smooth muscle cells, \( \alpha_\beta_1 \) chemotaxis is regulated by integrin-associated protein. Integrin-associated protein activates the inhibitory heterotrimeric G protein \( \gamma_0 \), which suppresses adenylyl cyclase activity (43). Thus, inactivation of \( \alpha_\beta_1 \) by antagonists of \( \alpha_\beta_1 \) likely involves regulation of either adenylyl cyclase and/or G protein-coupled receptors.

In conclusion, our results suggest that ligation of \( \alpha_\beta_1 \) by fibronectin suppresses protein kinase A activation and permits the association of \( \alpha_\beta_3 \) with the actin cytoskeleton as well as cellular migration. In contrast, inhibiting \( \alpha_\beta_1 \) ligation with antagonists of \( \alpha_\beta_1 \) or the fibronectin cell-binding domain promotes the dissociation of the catalytic subunit from the regulatory subunit of protein kinase A, thereby activating the kinase and inhibiting \( \alpha_\beta_1 \)-mediated focal contact and stress fiber formation, as well as migration (see Fig. 8). Our results suggest that \( \alpha_\beta_1 \) exerts this type of control over \( \alpha_\beta_3 \) in vivo. In vivo, fibronectin and integrins \( \alpha_\beta_1 \) and \( \alpha_\beta_3 \) are simultaneously expressed on the endothelial cells of tumor blood vessels and of tissues exposed to angiogenic growth factors (15). Integrin \( \alpha_\beta_1 \) is largely selective for fibronectin, which is a component of the provisional matrix expressed by endothelial cells during wound healing or tumor angiogenesis (7–10, 15). Integrin \( \alpha_\beta_3 \), in contrast, is a promiscuous integrin with the

**FIG. 5.** Protein kinase A activity is elevated by \( \alpha_\beta_1 \)-antagonism. A, protein kinase A activity was measured in endothelial cells plated for 1 h on poly-L-lysine, vitronectin collagen, or fibronectin. B, protein kinase A was measured in endothelial cells plated for 1 h on tissue culture plastic coated with anti-\( \alpha_\beta_1 \) antibodies or control antibodies. C, PKA activity was measured in cells plated on poly-L-lysine, fibronectin, or fibronectin in the presence of antibody antagonists to integrin \( \alpha_\beta_1 \). D, PKA was measured in cells plated on vitronectin in the presence of anti-\( \alpha_\beta_1 \) or control antibodies. Activity was calculated as a percentage of total cellular PKA activity.

**FIG. 6.** Activation of PKA inhibits \( \alpha_\beta_1 \)-dependent migration but not adhesion. The migration (A) and adhesion (B) of endothelial cells on vitronectin or collagen in the presence of medium, 25 \( \mu \)g/ml anti-\( \alpha_\beta_1 \), 25 \( \mu \)g/ml anti-\( \alpha_\beta_3 \), 25 \( \mu \)g/ml control antibodies, 20 \( \mu \)g/ml forskolin, and 500 \( \mu \)M cAMP was evaluated. The number of cells that migrated in a 4-h period was evaluated by counting triplicate 200× microscope fields after staining of cells with crystal violet. Adhesion was determined by measuring the absorbance at 650 nm of crystal violet-stained cell layers attached to matrix proteins.
potential to mediate migration on a host of extracellular matrix proteins with arginine-glycine-aspartic acid moieties, such as vitronectin, fibrinogen, collagen, von Willebrand’s factor, and others. To maintain orderly outgrowth of blood vessels, this ability of αvβ3 to promote migration in most extracellular matrix contexts must be regulated at the cellular level through precise mechanisms. Thus, it is likely that ligation of integrin αvβ3 by endothelial cell fibronectin and suppression of the inhibitory capabilities of protein kinase A is one means by which αvβ3 functions are regulated during angiogenesis in vivo.

The studies presented here are the first observations demonstrating a role for integrin cross-talk in vivo as well as the first indicating a role for integrin cross-talk during angiogenesis. Furthermore, they are the first studies demonstrating a significant role for protein kinase A in the suppression of angiogenesis. These studies also suggest the potential use of PKA...
agonists in the treatment of angiogenic diseases, including cancer and rheumatoid arthritis.

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