Cell, Vol. 88, 521–530, February 21, 1997, Copyright ©1997 by Cell Press

Suppression of Integrin Activation: A Novel Function of a Ras/Raf-Initiated MAP Kinase Pathway

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Summary

Rapid modulation of ligand binding affinity ("activation") is a central property of the integrin cell adhesion receptors. Using a screen for suppressors of integrin activation, we identified the small GTP-binding protein, H-Ras, and its effector kinase, Raf-1, as negative regulators of integrin activation. H-Ras inhibited the activation of integrins with three distinct α and β subunit cytoplasmic domains. Suppression was not associated with integrin phosphorylation and was independent of both mRNA transcription and protein synthesis. Furthermore, suppression correlated with activation of the ERK MAP kinase pathway. Thus, regulation of integrin affinity state is a novel, transcription-independent function of a Ras-linked MAP kinase pathway that may mediate a negative feedback loop in integrin function.

Introduction

Interactions between integrin cell adhesion receptors and their extracellular ligands are central to cell migration, growth, and survival (Hynes, 1992; Schwartz et al., 1995). A characteristic feature of certain integrins is their ability to modulate their affinity for extracellular ligands in response to intracellular signals, a process termed "activation" or "inside-out signaling" (reviewed by Williams et al., 1994). Integrin activation is energy-dependent and cell type-specific, requiring both the α and β subunit cytoplasmic domains (O'Toole et al., 1994; Hughes et al., 1996).

Several observations suggest that there are cytoplasmic signaling pathways that suppress integrin activation. For example, integrin activation in T cells is often transient, even with continued stimulation (Dustin and Springer, 1989; Shimizu et al., 1990). Cell migration requires coordinated adhesion and de-adhesion events, and the "freezing" of integrins in an activated state alters cell migration (Kuijpers et al., 1993). Platelet-derived growth factor inhibits platelet aggregation, suggesting that activation of a tyrosine kinase receptor impairs the function of integrin $\alpha_{IIb}\beta_3$ (Vassbotn et al., 1994). During mitosis, cells become rounded and appear to lose adhesion. Phosphorylation of a conserved serine residue in the β_1 cytoplasmic domain may be responsible for this loss of integrin function in mitotic cells (Hynes, 1992). Signaling events initiated by the occupancy of one integrin can suppress functions associated with other integrins (Blystone et al., 1995; Diaz-Gonzalez et al., 1996). Thus, there may be active cellular mechanisms to inhibit integrin activation.

Transformed fibroblasts often have defects in integrin function and localization. They are generally less well spread and have poorly developed focal adhesions and stress fibers (Burridge et al., 1988). In addition, fibronectin matrix assembly, an integrin activation-dependent process (Wu et al., 1995), is often lost in transformed cells (Hynes et al., 1978). Many oncogenes are members of or interact with cytoplasmic signaling cascades, and transformation is often a direct result of the deregulation of a cytoplasmic signal transduction pathway (Cantley et al., 1991). This raises the possibility that sustained activation of cytoplasmic signaling pathways in transformed cells could be responsible for the suppression of integrin activation.

Chimeric integrins in which the extracellular and transmembrane domains of the platelet-specific integrin, $\alpha_{IIb}\beta_3$, are joined to the cytoplasmic domains of α_5, α_{6A}, α_{6B}, and β_1 are constitutively active in CHO cells (O'Toole et al., 1994). The activation properties of these chimeric integrins reflect those of the cytoplasmic domain of the parent integrin. For example, $\alpha_{IIb}\beta_3$ is in a low affinity state when expressed in CHO cells. However, the replacement of the cytoplasmic domains of $\alpha_{IIb}\beta_3$ with those of $\alpha_5\beta_1$, which is in the high affinity state in CHO cells, produces a chimeric integrin, $\alpha_{IIb}\alpha_5\beta_3\beta_1$, that binds activation-dependent $\alpha_{IIb}\beta_3$ ligands such as the monoclonal antibody PAC1 and fibrinogen with high affinity (O'Toole et al., 1994). Moreover, the activation of this chimera is energy-dependent, suggesting a requirement for cellular signaling pathways in activating endogenous $\alpha_5\beta_1$. The monoclonal antibody PAC1 is specific for the high affinity conformation of $\alpha_{IIb}\beta_3$, mimicking the binding characteristics of the natural ligand, fibrinogen (Shattil et al., 1985). Thus, the combined properties of the active $\alpha_{IIb}\beta_3$ chimeras and the activation-dependent ligand, PAC1, permit the use of the extracellular domain of $\alpha_{IIb}\beta_3$ as a reporter of cytoplasmic signaling events involved in integrin activation.

To identify suppressors of integrin activation, we screened a collection of cDNAs for their ability to suppress PAC1 binding to a CHO cell line expressing an active $\alpha_{IIb}\beta_3$ chimera. We found that the expression of activated variants of the small GTP-binding protein, H-Ras, and its kinase effector, Raf-1, blocks integrin activation. Furthermore, this suppressive activity correlates with activation of the ERK MAP kinase pathway (extracellular signal–regulated protein kinase [ERK] mitogen-activated protein [MAP] kinase pathway) and does not require protein synthesis or mRNA transcription.

Results

H-Ras and Activated Raf-1 Kinase Suppress Integrin Activation

Using two-color flow cytometry, we screened a collection of 102 cDNAs for suppressors of integrin activation.

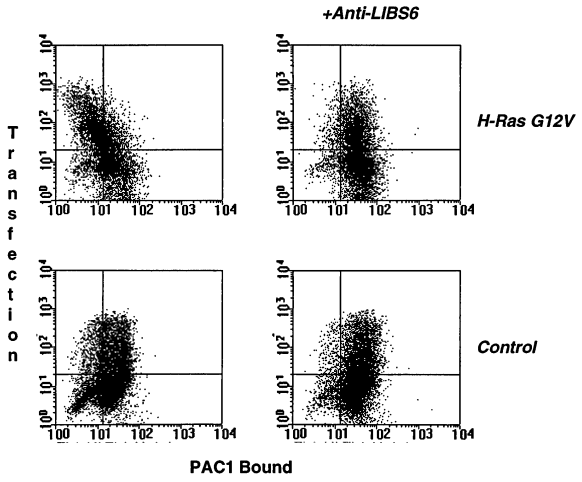

Figure 1. Suppression of Integrin Activation by Activated H-Ras

αβ-py cells were transiently transfected with cDNA encoding Tac-α_5 alone or Tac-α_5 plus H-Ras(G12V). After 48 hr, cells were harvested and stained for Tac expression (ordinate) and PAC1 binding (abscissa). In the Ras(G12V)-transfected cells there is a leftward shift of the dot plot in the upper but not lower quadrants, representing a reduction in PAC1 binding (top left). This shift is reversed by the addition of an activating monoclonal antibody, anti-LIBS6, establishing that it is due to "deactivation" of the integrin by the transfected Ras(G12V) cDNA (top right). In the empty vector transfection, there was no suppression of PAC1 binding in the Tac-α_5–expressing cells (bottom left and bottom right).

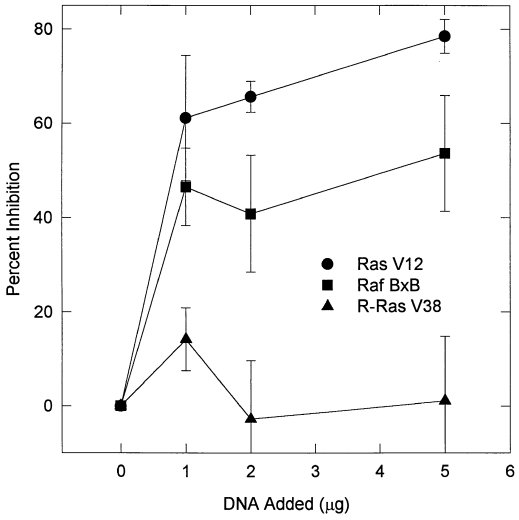

Figure 2. Suppression of Integrin Activation in Transient Transfections

CHO cells were cotransfected with 2 μg of cDNA encoding $\alpha_{IIb}\alpha_{6A}$ and 2 μg of cDNA encoding $\beta_3\beta_1$. They were also simultaneously cotransfected with 1, 2, or 4 μg of a cDNA encoding R-Ras(G38V), Raf-BXB, or H-Ras(G12V). After 48 hr, cells were harvested and analyzed for PAC1 binding. The reduction in specific PAC1 binding was used to calculate the percentage inhibition relative to the pcDNA3 control. Depicted is the mean percentage inhibition relative to empty vector ± SE of three independent determinations.

These cDNAs encoded cytoskeletal proteins relevant to integrin function (e.g., α-actinin and vinculin), protein kinases (e.g., focal adhesion kinase [FAK] and protein kinase C isoforms), and small G proteins (e.g., Rac-1, H-Ras, and R-Ras). The cDNAs were grouped into pools of six to eight clones and cotransfected into CHO cells stably expressing the active chimeric integrin $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ (αβ-py cells) along with a vector encoding the extracellular domain of the Tac subunit of the interleukin-2 receptor (Tac-α_5) (LaFlamme et al., 1992). After 48 hr, the cells were harvested and analyzed by flow cytometry for anti-Tac (to identify transfected cells) and PAC1 (to assess integrin affinity state) binding. From this screen we isolated three different cDNAs: those encoding both activated (G12V) and wild-type forms of the small GTP-binding protein H-Ras (Figure 1) and an activated variant of the serine/threonine kinase Raf-1 (Raf-BXB) (data not shown) that suppressed PAC1 binding (Figure 1). PAC1 binding to the H-Ras- or Raf-BXB-transfected cells could be restored by addition of an "activating" monoclonal antibody, anti-LIBS6 (Figure 1), showing that there was no reduction in the expression of the recombinant integrin following transfection with H-Ras(G12V) or Raf-BXB. Also, suppression was cell-autonomous, since only transfected cells showed a reduction in PAC1 binding (Figure 1).

To exclude the possibility that the observed effect depended on the use of a clonal cell line and the Tac marker, we transiently transfected CHO cells with expression vectors for both the chimeric integrin and the suppressor molecules. Both Raf-BXB and H-Ras suppressed integrin activation (Figure 2). The effect of activated H-Ras was not a general property of small G proteins, because the transfection of R-Ras(G38V) (Figure 2), Cdc42(Q61L), RhoA(Q63L), and Rac-1(Q61L) (data not shown) did not suppress integrin activation.

H-Ras Suppresses the Activation of Integrins with Distinct Cytoplasmic Domains

To test whether the suppressive effect of activated H-Ras was restricted to the β_1 and α_{6A} cytoplasmic domains, we transiently transfected CHO cells with different active $\alpha_{IIb}\beta_3$ chimeras in the presence or absence of 2 μg of H-Ras(G12V) cDNA. We tested chimeras composed of the cytoplasmic domains of α_{6A}, α_{6B}, and α_5 fused to the extracellular and transmembrane domains of α_{IIb}. These α_{IIb} subunit chimeras were then coexpressed with both native β_3 and a β_3 chimera containing the cytoplasmic domain of β_1. When coexpressed with each chimera, activated H-Ras suppressed PAC1 binding (Figure 3) by an average of 73% ± 4.2%. Therefore, the effects of activated H-Ras are not specific for α_{6A} and β_1 but also extend to other integrin cytoplasmic domains.

Activated H-Ras or Raf-1 could suppress integrin activation by activating downstream kinases that phosphorylate the α_{6A} or β_1 cytoplasmic tails. However, we found no detectable phosphorylation of $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ following Raf-1 activation even though integrin activation was suppressed (data not shown). This observation suggests that phosphorylation of the integrin cytoplasmic domains is not required for suppression of integrin activation. However, it is possible that rapid and transient phosphorylation occurs below the limits of detection or before the earliest time point examined (1 hr). Therefore, we wished to determine whether the mutation of certain known phosphorylation sites could block H-Ras–

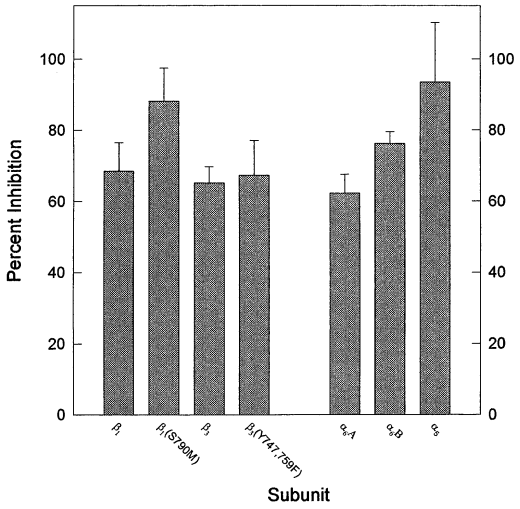

Figure 3. H-Ras(G12V) Suppresses the Activation of Integrins with Multiple Cytoplasmic Domains

CHO cells were cotransfected with 2 μg of cDNA encoding $\alpha_{IIb}\alpha_{6A}$ and 2 μg of cDNA encoding either wild-type β_3, β_3(Y747F, Y759F), or the chimeras $\beta_3\beta_1$ or $\beta_3\beta_1$(S790M). They were simultaneously cotransfected either with 2 μg of cDNA encoding H-Ras G12V or 2 μg of the plasmid pcDNA3 as a control. To determine the effect of different α subunit cytoplasmic domains, CHO cells were cotransfected with 2 μg of cDNA encoding the chimera $\beta_3\beta_1$ and 2 μg of cDNA encoding $\alpha_{IIb}\alpha_{6A}$, $\alpha_{IIb}\alpha_{6B}$, or $\alpha_{IIb}\alpha_5$. They were also simultaneously cotransfected with either 2 μg of cDNA encoding H-Ras(G12V) or 2 μg of the plasmid pcDNA3. After 48 hr, cells were harvested and analyzed for PAC1 binding. The reduction in specific PAC1 binding was used to calculate the percentage inhibition relative to the pcDNA3 control. Depicted is the mean percentage inhibition relative to pcDNA3 ± SE of three to ten independent determinations.

mediated suppression. We transiently transfected CHO cells with active variants containing mutations at known phosphorylation sites. The chimera $\beta_3\beta_1$(S790M) contains a methionine substituted for a serine residue phosphorylated in mitotic cells (Hynes, 1992). H-Ras(G12V) suppressed PAC1 binding to $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$(S790M) (Figure 3). Therefore, the ability of H-Ras(G12V) to suppress activation of this mutant is not the result of phosphorylation of S790. The β_3 cytoplasmic domain contains two tyrosine residues as part of a conserved NPXY and a more distal NXXY motif. One or both of these tyrosine residues are phosphorylated during thrombin-induced platelet aggregation (Law et al., 1996). We substituted both of these tyrosines with phenylalanine in β_3(Y747F, Y759F), eliminating the possible phosphorylation of these residues. H-Ras(G12V) suppressed PAC1 binding to $\alpha_{IIb}\alpha_{6A}\beta_3$(Y747F, Y759F) (Figure 3). Therefore, the ability of activated H-Ras to suppress the activation of this mutant was not mediated by the phosphorylation of tyrosine residues on the β_3 cytoplasmic domain. These data indicate that Ras- and Raf-mediated suppression of integrin activation is not due to the phosphorylation of the integrin cytoplasmic domains.

Integrin Suppression Correlates with Activation of a MAP Kinase

Ras plays a central role in the transduction of signals from growth factor and G protein–coupled receptors, in part via multiple distinct MAP kinase cascades. Raf-1 is a downstream effector of Ras, leading to the activation of the ERK1/ERK2 MAP kinases (Howe et al., 1992). Therefore, the suppressive effect of H-Ras and Raf-BXB on integrin activation may be mediated by the activation of one or more of the MAP kinase pathways. To test this hypothesis we examined the effect of MAP kinase phosphatase 1 (MKP-1) (Sun et al., 1993) on suppression mediated by H-Ras(G12V) and Raf-BXB. αβ-py cells were transiently transfected with H-Ras(G12V) or Raf-BXB in the presence or absence of MKP-1. Integrin activation (Figure 4A) and the activation of ERK2 kinase were then analyzed (Figure 4B). MKP-1 reversed the activation of ERK2 by H-Ras(G12V) and Raf-BXB and reduced the inhibition of integrin activation. These results indicate that the suppressive effects of H-Ras (G12V) and Raf-BXB are due to the activation of a MAP kinase pathway.

Since MKP-1 can inactivate both ERK and JNK, we sought to determine whether the activation of ERK or JNK correlated with the inhibition of integrins. αβ-py cells were transfected with H-Ras(G12V), Raf-BXB, or an activated variant of the small GTP-binding protein Cdc42(Q61L). Activated Cdc42 stimulates the c-Jun N-terminal kinase (JNK) but not the ERK MAP kinase pathway (Minden et al., 1995). The suppression of integrin activation (Figure 4A) and the activation of MAP kinase pathways were analyzed (Figure 4B). Both H-Ras(G12V) and Raf-BXB, but not Cdc42(Q61L), suppressed integrin activation and activated ERK2. In contrast, Cdc42(Q61L) activated JNK1 but not ERK2. These data suggest that the classic MAP kinase cascade leading to ERK1/ERK2 activation, rather than that leading to JNK activation, is involved in the suppression of integrin activation by Raf-BXB and H-Ras(G12V).

We wished to determine whether the activation of endogenous Ras could suppress integrin activation. The tyrosine kinase Src is known to activate the ERK MAP kinase pathway in a Ras-dependent manner (Brown and Cooper, 1996). Therefore, we examined the ability of two activated forms of Src, Src(E378G) and v-Src, to suppress integrin activation in αβ-py cells. We found that both Src(E378G) and v-Src suppressed PAC1 binding (Figure 4C). This suppression was dependent on the activation of the endogenous Ras-dependent MAP kinase pathway, as cotransfection of dominant negative H-Ras(S17N) (Figure 4C) or MKP-1 (data not shown) markedly reduced the suppressive effect of both forms of activated Src. Thus, the activation of an endogenous Ras can induce suppression of integrin activation.

Suppression of Integrin Activation Occurs without Transcription or Protein Synthesis

Activation of the ERK MAP kinase pathway induces immediate-early gene expression (Marshall, 1995). Thus, we wished to determine whether the suppression of integrin activation required the transcription and de novo synthesis of a suppressive factor. To test this possibility, we used Raf-ER cells that express the estrogen-dependent Raf-1, Raf-1.ER. Upon addition of estradiol, Raf-1.ER is rapidly activated in the absence of protein synthesis (Samuels et al., 1993).

In the absence of estradiol, the Raf-ER cells bound PAC1 with high affinity (Figure 5A). However, following the addition of estradiol, PAC1 binding was reduced (Figure 5A) but was restored by addition of the activating antibody anti-LIBS6. There was no reduction in the abundance of $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ following the addition of estradiol, as determined by simultaneous anti-$\alpha_{IIb}\beta_3$ staining (Figure 5A). In addition, PAC1 binding in the presence of anti-LIBS6 was the same whether estradiol was added (fluorescence intensity = 20 ± 5.1 units) or not (fluorescence intensity = 18 ± 5 units), further confirming that estradiol did not affect recombinant $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ expression. The addition of estradiol in the absence of Raf-1.ER had no effect on PAC1 binding to $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ (Figure 5A). These data indicate that the suppression of PAC1 binding to Raf-ER cells by estradiol is due to Raf-1 kinase activation.

We next examined the effect of Raf-1 activation in the presence of cycloheximide or actinomycin D, inhibitors of protein synthesis and mRNA transcription, respectively. In the absence of estradiol, both cycloheximide (20 μg/ml) and actinomycin D (2 μg/ml) had little effect on PAC1 binding to Raf-ER cells (Figure 5B). In the presence of both estradiol and either cycloheximide (20 μg/ml) or actinomycin D (2 μg/ml), PAC1 binding was nearly completely inhibited. Total protein synthesis was inhibited by 98% in the cells treated with 20 μg/ml cycloheximide either in the presence or absence of estradiol, as assessed by incorporation of [^{35}S]methionine into trichloroacetic acid–insoluble protein. Thus, the suppressive effect of activated Raf-1 does not require substantial mRNA transcription or protein synthesis.

We used the Raf-ER cells to determine the time course of suppression following the activation of Raf-1. Raf-ER cells were treated with cycloheximide to block upregulation of MKP-1 (Sun et al., 1993). PAC1 binding and ERK2 kinase activity were then assayed in parallel at hourly intervals from 0 to 4 hr after the addition of estradiol. After 1 hr, ERK2 activity was near maximal, as expected (Samuels et al., 1993). However, suppression of PAC1 binding was delayed (Figure 5C). After 2 hr, integrin activation was suppressed by 60%, with maximal suppression reached after 3 hr (Figure 5C). The addition of cycloheximide alone to Raf-ER cells had no effect on PAC1 binding to $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ (data not shown).

Activation of Raf-1 Suppresses Fibronectin Matrix Assembly and Changes Integrin-Dependent Cell Morphology

To determine whether this suppression pathway could inhibit other integrin-dependent functions, we examined the effect of activated Raf-1 on fibronectin matrix assembly and cell morphology. In these experiments, we used the Raf-ER cells in which Raf-1 kinase activity is controlled by the presence of exogenous estradiol.

We examined the effect of activated Raf-1 on $\alpha_5\beta_1$-mediated fibronectin matrix assembly, a process requiring an activated integrin (Wu et. al., 1995). Because the Raf-ER cells could use the recombinant $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ to assemble a matrix, we added saturating quantities of an $\alpha_{IIb}\beta_3$ specific inhibitor, lamifiban, to all samples to ensure that matrix assembly was mediated only by endogenous $\alpha_5\beta_1$. In the absence of estradiol, Raf-ER cells assembled an extensive fibronectin matrix that was inhibited by the function-blocking anti-α_5 antibody PB1 (Figure 6). Therefore, in the presence of lamifiban, endogenous $\alpha_5\beta_1$ mediated fibronectin matrix assembly. Addition of estradiol almost totally blocked fibronectin matrix assembly by these cells, whereas estradiol had no effect on CHO cells lacking Raf-1.ER. To test whether the inhibition of fibronectin matrix assembly was due to an estradiol-induced reduction in $\alpha_5\beta_1$ expression in Raf-ER cells, we measured the number of $\alpha_5\beta_1$ receptors in the presence and absence of estrogen with radiolabeled PB1 antibody. We found that there were $1.8 \times 10^5 \pm 2.2 \times 10^4$ molecules/cell in the presence of estradiol compared to $1.8 \times 10^5 \pm 1.3 \times 10^4$ molecules/cell in the absence of estradiol. Therefore, Raf-1 activation suppresses the capacity of integrin $\alpha_5\beta_1$ to mediate fibronectin matrix assembly without affecting the integrin's abundance.

We next examined the effect of activated Raf-1 on the morphology of Raf-ER cells plated on fibronectin and fibrinogen. In the absence of estradiol, Raf-ER cells adhered and spread normally on both fibrinogen and fibronectin. Activation of Raf-1 following the addition of estradiol to the culture medium resulted in a profound effect on cell shape (Figures 6E and 6F). The cells lost their spread phenotype and adopted a more rounded shape (Figure 6F). There were no changes in the morphology of the cells lacking activated Raf-ER.

Discussion

We report the identification and characterization of a signal transduction pathway that leads to the suppression of integrin activation. The major findings are as follows. First, this suppression pathway involves the small GTP-binding protein H-Ras and its downstream effector kinase Raf-1. Second, activated H-Ras suppresses the activation of integrins with multiple α and β subunit cytoplasmic domains. The suppressive effect is not associated with integrin phosphorylation, nor is it reversed by mutations that eliminate certain known phosphorylation sites in the β_3 or β_1 cytoplasmic domains. Third, the suppression of integrin activation by H-Ras(G12V) and Raf-BXB correlates with activation of the ERK but not the JNK MAP kinase pathway. Fourth, the suppressive effect of Raf-1 does not require mRNA transcription or protein synthesis. These data identify integrin affinity state as a novel, transcription-independent target of Ras-initiated MAP kinase pathways. They also provide a cogent explanation for the alteration of certain integrin-dependent functions in many transformed cells.

Expression of activated variants of the small GTP-binding protein H-Ras and the serine/threonine kinase Raf-1 blocked integrin activation. This result was observed with a series of chimeric integrins in which the extracellular and transmembrane domains of $\alpha_{IIb}\beta_3$ were joined to the cytoplasmic domains of α_{6A}, α_{6B}, and α_5 and β_1. Each of these α subunit cytoplasmic domains has a highly distinct sequence. In contrast, both the β_1 and β_3 tails share homologous sequence motifs in common with other β subunit cytoplasmic domains. This

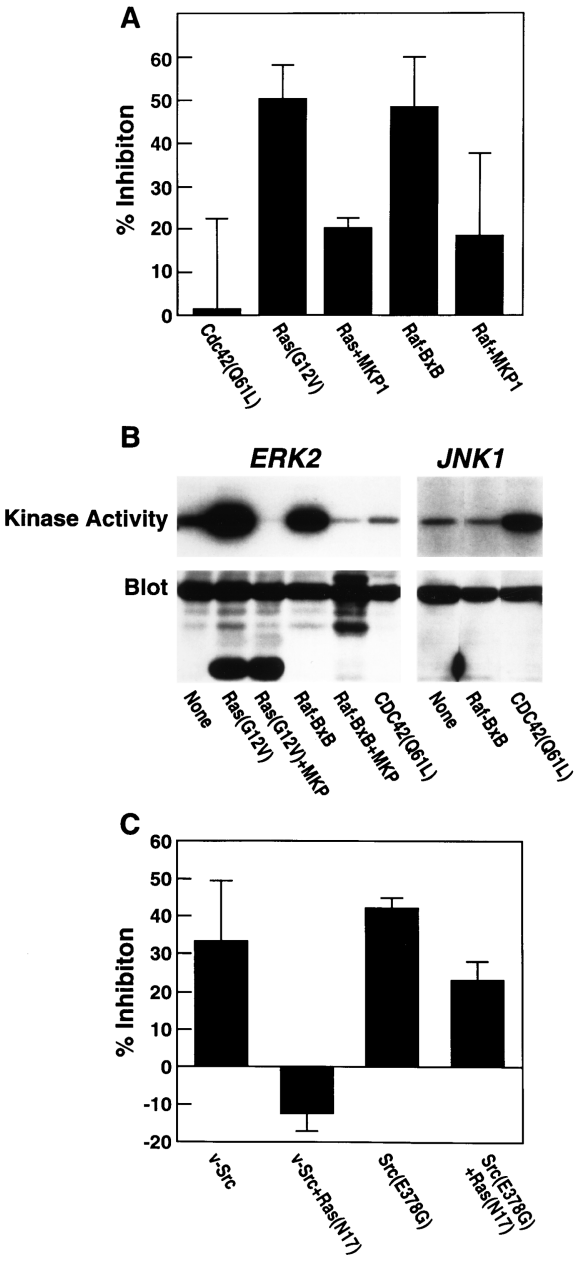

Figure 4. Suppression of Integrin Activation Correlates with Activation of the ERK1/ERK2 MAP Kinase Cascade

(A) $\alpha\beta$-py cells were cotransfected with 2 μg of expression vector or vectors bearing inserts encoding Cdc42(Q61L), Raf-BXB, or Ras(G12V). In separate transfections, 2 μg of Raf-BXB or Ras(G12V) cDNA were cotransfected with 6 μg of a plasmid encoding MKP-1. After 48 hr, PAC1 binding was determined by flow cytometry. Depicted is the mean percentage inhibition relative to empty vector $\pm$ SE of three independent determinations.

(B) $\alpha\beta$-py cells were cotransfected with 2 μg of an expression vector encoding HA-tagged ERK2 or HA-tagged JNK1. The cells were also cotransfected with 2 μg of expression vector (None) or vectors containing inserts encoding Cdc42(Q61L), Raf-BXB, or Ras(G12V). In separate transfections, 2 μg of expression vectors encoding either Raf-BXB or Ras(G12V) were simultaneously cotransfected with 6 μg of a plasmid encoding MKP-1. The transfected kinases were immunoprecipitated with anti-HA antibody 12CA5. ERK2 activity was measured by phosphorylation of myelin basic protein by an in-gel kinase assay, and JNK-1 activity was measured by the phosphorylation of GST-Jun (1-79). (Top) Relative ERK and JNK activity. Note the activation of ERK2 by Ras(G12V) and Raf-BXB. In addition, note the inhibition of ERK2 activity by MKP-1. Activated Cdc42 failed to activate ERK2 but activated JNK1. Immunoblotting revealed an equivalent amount of ERK2 and JNK1 in each of the kinase reactions (data not shown). (Bottom) Immunoblots with the anti-HA antibody 12CA5. There was a comparable expression of either HA-tagged ERK2 or JNK1 in all transfections. The Ras(G12V) construct bore an HA-tag and was detected as the the lower band in lanes transfected with that construct. Note the similar expression of recombinant activated Ras in both the control and MKP-1-cotransfected cells.

(C) $\alpha\beta$-py cells were transfected with 4 μg of an expression vector encoding either v-Src or Src(E378G). In separate transfections, 4 μg of expression vectors encoding either Src(E378G) or v-Src were simultaneously cotransfected with 1 μg of a plasmid encoding dominant negative Ras(S17N). After 48 hr, integrin activation was determined by PAC1 binding. Depicted is the mean percentage inhibition relative to the empty vector control $\pm$ SE of three independent determinations.

raises the possibility that the suppression pathway is acting in a β subunit–dependent manner or, alternatively, that it is capable of acting via multiple α subunit cytoplasmic domains. Earlier reports suggested that the tyrosine phosphorylation of either β_1 or β_3 subunit cytoplasmic domains can influence integrin function and localization (Johansson et al., 1994; Law et al., 1996). However, activated H-Ras was able to suppress the activation of the double mutant $\alpha_{IIb}\alpha_{6A}\beta_3$(Y747F, Y759F), demonstrating that suppression is not the result of the phosphorylation of tyrosine residues in the β_3 cytoplasmic domain. In addition, activated H-Ras was able to suppress the activation of $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$(S790M). The serine residue substituted with methionine in $\beta_3\beta_1$(S790M) corresponds to the serine phosphorylated in mitotic cells (Hynes, 1992). This result shows that this effect is distinct from the reported effects of S790 phosphorylation and represents a second potential mechanism for regulation of integrin function in concert with the cell cycle.

The inhibition of integrin activation by activated H-Ras and Raf-1 correlated with activation of the ERK but not the JNK MAP kinase pathway. Ras plays a pivotal role in the transduction of signaling events initiated by both tyrosine kinase growth factor receptors and G protein–coupled receptors. Activated Ras can interact with multiple downstream effectors, controlling both gene expression and the organization of the actin cytoskeleton (reviewed by Hall, 1992). Given that the other Ras family members, N-Ras and K-Ras, have downstream effectors similar to those of H-Ras, these Ras family members would also be expected to suppress integrin activation. Indeed, an activated variant of K-Ras(C12V) suppresses PAC1 binding to $\alpha\beta$-py cells (Ginsberg and Forsyth, unpublished data). Raf-1 is a downstream effector of H-Ras that induces the activation of the ERK1/ERK2 MAP kinases (Howe et al., 1992). Because Raf-1 had a suppressive effect similar to that of activated H-Ras, integrin suppression may be mediated by the activation of one or more of the MAP kinase pathways. Moreover, MKP-1, a MAP kinase phosphatase, partially reversed the suppressive effect of both activated H-Ras and Raf-1. In the same experiment, MKP-1 completely reversed activation of recombinant ERK2 induced by activated Raf

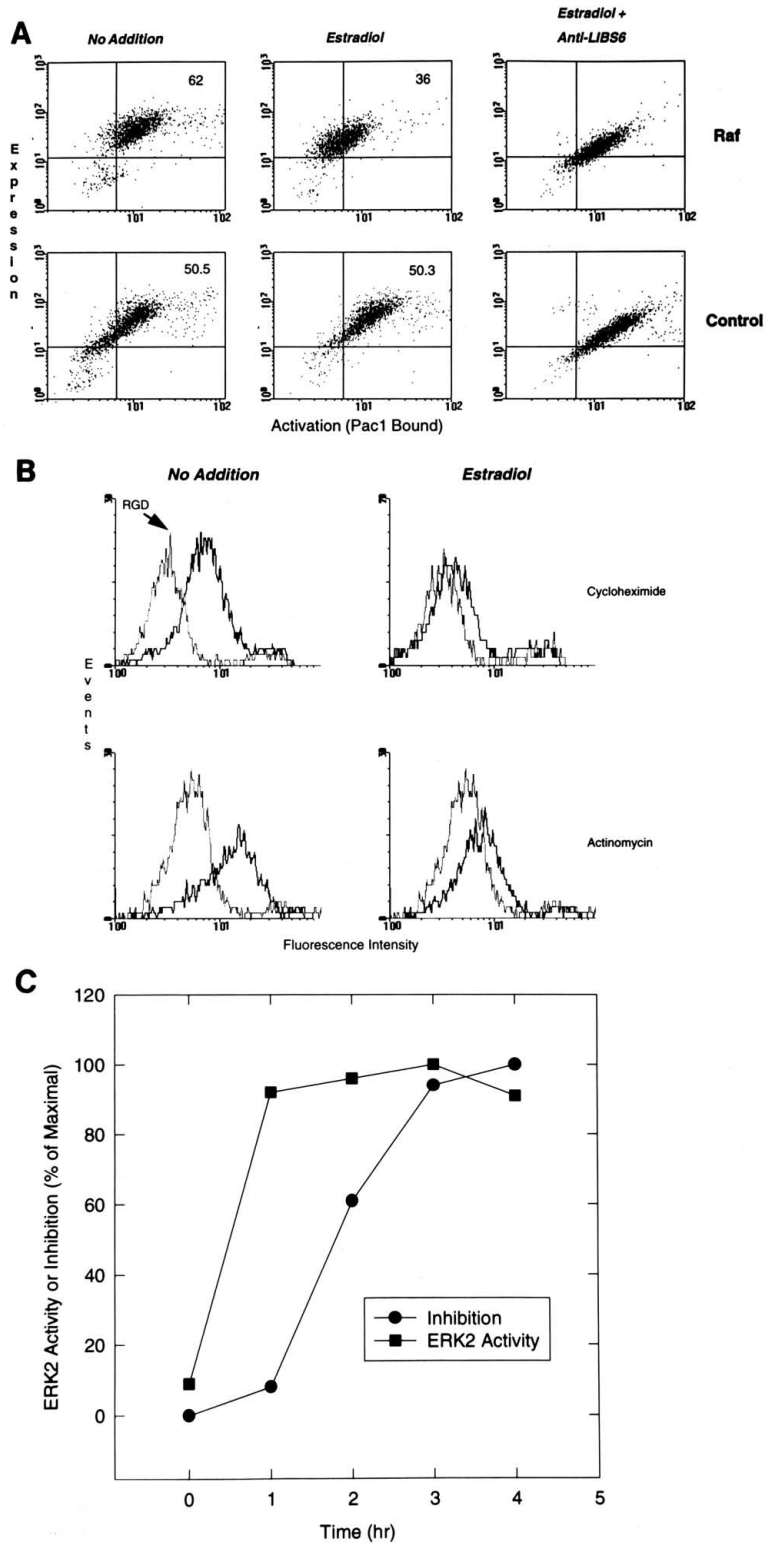

Figure 5. The Suppression of Integrin Activation by Raf-1 Is Independent of Transcription and Protein Synthesis

(A) Raf-ER cells or CHO$\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ cells were incubated in the presence or absence of estradiol for 24 hr. The cells were then harvested and double-stained for integrin expression (ordinate) and PAC1 binding (abscissa). In the Raf-ER cells (top graphs) there is a leftward shift of the dot plot following the addition of estradiol, representing a reduction in PAC1 binding. The reduction in PAC1 binding can be restored by the addition of the activating antibody anti-LIBS6. The number in the top right corner of the graphs represents the activation index in the absence or presence of estrogen. The addition of estrogen had no effect on PAC1 binding to CHO cells expressing $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ in the absence of Raf-1ER (bottom graphs).

(B) Flow cytometry histograms illustrating PAC1 binding to Raf-ER cells in the presence (shaded line) or absence (solid line) of 1 μM competitive inhibitor Ro43–5054 and in the presence of either cycloheximide or actinomycin D. In the absence of estradiol, both the cycloheximide and actinomycin D-treated cells bound PAC1, showing that the $\alpha_{IIb}\beta_3$ chimera was in the high affinity state. The presence of estradiol blocked PAC1 binding to both the cycloheximide and actinomycin D-treated cells.

(C) Raf-ER cells were cultured in the presence of estrogen and cycloheximide over a 4 hr period. The cells were then harvested at the time points indicated and analyzed for PAC1 binding and ERK2 kinase activity. The reduction in specific PAC1 binding was used to calculate the percentage inhibition of integrin activation relative to the cycloheximide-alone control. Integrin activation was suppressed within 2 hr after the addition of estradiol, with maximal suppression reached after 3 hr. There was at least a 2 hr lag between ERK2 activation and maximal suppression of integrin activation.

and Ras. This difference suggests the possibility of additional MAP kinase–independent pathway(s) that suppress integrin activation. Alternatively, endogenous ERK's or other MAP kinases may have been only partially inactivated by the transfected MKP-1. Nevertheless, it is clear that the effect of activated Ras and Raf was mediated by a MAP kinase–dependent integrin suppression pathway. MKP-1 can dephosphorylate and therefore inactivate ERK, JNK, and p38 kinases (Chu et al., 1996). However, we found that the suppression of integrin activation correlated with activation of the ERK but not the JNK MAP kinase pathway.

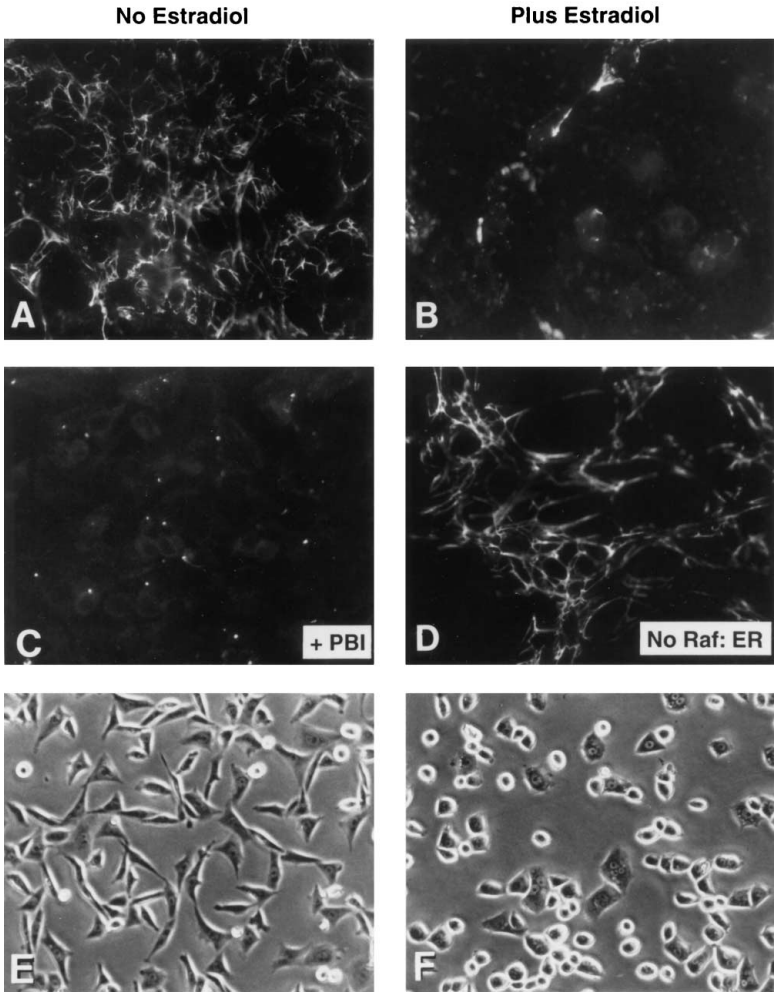

Figure 6. Changes in Fibronectin Matrix Assembly and Integrin-Dependent Cell Morphology Induced by Activated Raf-1

Raf-ER cells were cultured in medium supplemented with human fibronectin (150 μg/ml) and the $\alpha_{IIb}\beta_3$- specific inhibitor lamifiban (1 μM). Cells cultured in the absence of estradiol (A) assembled an extensive fibronectin matrix. In contrast, fibronectin matrix assembly was reduced in cells grown in the presence of estradiol (B). In the absence of estradiol, fibronectin matrix assembly was completely inhibited in Raf-ER cells following the addition of the anti-α_5 antibody PB1 (C). Estradiol had no effect in cells lacking Raf-1.ER (D). To determine the effect of activated Raf-1 on the adhesive phenotype, Raf-ER cells were plated on fibrinogen-coated coverslips (5 μg/ml) and cultured in the absence (E) or presence (F) of estradiol for 24 hr. Cells cultured in the absence of estradiol (E) exhibited a normal spread morphology. In contrast, cells plated on fibrinogen in the presence of estradiol were less well spread and displayed a more rounded phenotype (F). Identical results were obtained for cells plated on fibronectin (not shown).

Activated variants of the tyrosine kinase Src could also suppress integrin activation via a Ras-dependent MAP kinase pathway. These results establish that the activation of endogenous Ras can also inhibit integrin activation. In addition, the fact that overexpressed wild-type H-Ras also inhibited PAC1 binding establishes that the suppressive effect of H-Ras(G12V) is not due to its ability to interact with a downstream effector unique to the activated variant. Thus, transformation initiated by oncogenes upstream of Ras can also engage this integrin suppression pathway.

Zhang et al. (1996) reported that R-Ras, a Ras-related small G protein, could "activate" integrins. R-Ras and H-Ras have similar effector binding domains and bind many of the same effectors, including Raf and Ral-GDS (Urano et al., 1996). However, R-Ras and H-Ras have different morphological effects on cells (Cox et al., 1994), possibly because R-Ras may not activate some of the Ras effectors to which it binds. Thus, R-Ras could block the suppressive effect of H-Ras by competition for common effectors. Alternatively, activated H-Ras could act as a dominant negative inhibitor with respect to the specific effectors of R-Ras involved in integrin activation. However, Raf-1, a downstream effector of H-Ras, induced suppression of integrin activation. Furthermore, a MAP kinase phosphatase, MKP-1, reversed H-Ras–induced inhibition. These two findings suggest that suppression is the result of downstream signaling events initiated by H-Ras. Thus, H-Ras-mediated suppression does not appear to be the consequence of simple competition between H-Ras and R-Ras for common effectors. It would be of interest to determine whether R-Ras "activation" of integrins is the result of competition with Ras for its effectors.

Using a conditionally activated Raf-1, we found that the suppressive effect of activated Raf-1 was largely independent of de novo transcription and protein synthesis. The conditional Raf-1 was directly activated by the addition of exogenous estradiol, and this led to the suppression of integrin activation. In the presence of inhibitors of mRNA transcription and protein synthesis, the suppressive effect of Raf-1 was maintained. Thus, Raf-1-mediated integrin suppression was independent of protein synthesis. The estrogen-dependent Raf-1 allowed us to determine the time interval between the activation of Raf-1 and the loss of PAC1 binding. By monitoring both ERK2 kinase activity and integrin activation we found that maximal suppression was not reached until after 3 hr after Raf-1 activation. In contrast, ERK2 kinase activity was maximal 1 hr after addition of estradiol. Therefore, there appears to be a lag period of at least 2 hr between the activation of the ERK MAP

kinase pathway and the suppression of PAC1 binding. This assay measures the time interval between Raf-1 activation and the loss of PAC1 binding to a previously activated integrin. One explanation for this lag period is that the activation of the MAP kinase pathway may initiate the disassembly of an integrin "activation" complex. Alternatively, it is possible that integrins on the cell surface cycle between active and inactive states and that the integrin suppression pathway may act by suppressing only the activation step. Thus, the lag period could be due to the time required for the previously active receptors to lose the capacity to bind PAC1.

Our results indicate that integrin suppression is mediated by transcription-independent signaling through a MAP kinase pathway. There are examples of cytosolic signaling events downstream of MAP kinases. For example, MAP kinases can phosphorylate and thus activate cytoplasmic phospholipase A2 in vitro (Lin et al., 1993) and MAP kinase can phosphorylate the gap junction protein connexin 43 (Kanemitsu and Lau, 1993). However, the biological significance of these events has yet to be determined. We found that a cellular function, integrin activation, is regulated by transcription-independent signaling through a MAP kinase pathway. Thus, we provide evidence for novel cytoplasmic signaling targets of the MAP kinase pathway. It will be of great interest to identify the elements of this pathway.

The classic MAP kinase pathway can be activated by the dimerization of growth factor receptor tyrosine kinases and by the ligation and clustering of integrins (Schlaepfer et al., 1994; Marshall, 1995). In both events, Ras activation is thought to be mediated by the translocation of a complex between the adapter protein GRB-2 and the guanine nucleotide exchange factor SOS to the plasma membrane (Clark and Hynes, 1996). This suggests that integrin- and growth factor-derived signals could engage the MAP kinase-dependent integrin suppression pathway described in this work. Ras and Raf activation occurs at the plasma membrane (Leevers et al., 1994), and integrin suppression does not require protein synthesis. This raises the possibility that the integrin suppression pathway forms a local negative feedback loop for the regulation of integrin function. There are several observations that support this hypothesis. The integrin-dependent activation of ERK's is transient and may require Ras activation (Clark and Hynes, 1996). In this context Ras activation could occur via the formation of a complex of FAK, GRB-2, and SOS (Schlaepfer et al., 1994). Cells derived from FAK-deficient mice show enhanced focal adhesion formation, suggesting that these cells may have lost a negative regulator of integrin function (Illc et al., 1995). In addition, dominant negative Ras can enhance focal adhesion formation (Clark and Hynes, 1996). Further evidence for the existence of a negative feedback loop comes from observations that integrin occupancy or the expression of isolated β subunit cytoplasmic domains can suppress the function of other integrins (*trans*-dominant inhibition) (Chen et al., 1994; Lukashev et al., 1994; Blystone et al., 1995). Both occupied integrins and isolated integrin β subunit cytoplasmic domains can initiate cytoplasmic signaling cascades (Lukashev et al., 1994). This raises the possibility that some instances of *trans*-dominant inhibition could be the direct result of the activation of a MAP kinase–dependent integrin suppression pathway.

Deregulation of the MAP kinase pathway is often associated with oncogenic transformation, suggesting that malignant transformation could produce a sustained activation of this integrin suppression pathway. Unregulated action of an integrin suppression pathway may help to explain some of the characteristics of the transformed phenotype, such as the loss of fibronectin matrix assembly and altered cell migration and morphology. Indeed, we show that a conditionally active Raf-1 is able to inhibit integrin $\alpha_5\beta_1$–mediated fibronectin matrix assembly and induce changes in integrin-dependent cell morphology. Moreover, it is likely that a cytoplasmic substrate(s) of a MAP kinase are involved in suppression, since de novo protein synthesis is not required. Thus, these data point to a novel Ras-initiated effector pathway that is responsible for some of the phenotypic alterations frequently found in transformed cells.

Experimental Procedures

Antibodies and Reagents

The anti-$\alpha_{IIb}\beta_3$ monoclonal antibodies anti-LIBS6 and D57 have been described previously (O'Toole et al., 1994). The anti-$\alpha_{IIb}\beta_3$ monoclonal antibody PAC1 (Shattil et al., 1985) and the anti-hamster α_5 antibody PB1 were generous gifts from Dr. S. Shattil (Scripps Research Institute) and Dr. R. L. Juliano (University of North Carolina, Chapel Hill). The anti-Tac antibody, 7G7B6, was obtained from the American Type Culture Collection (Rockville, Maryland). Antibodies 7G7B6 and D57 were biotinylated with biotin-N-hydroxy-succinimide (Sigma) according to the manufacturer's directions. The $\alpha_{IIb}\beta_3$-specific peptidomimetic inhibitors Ro44–9883 (lamifiban) and Ro43–5054 (Alig et al., 1992) were generous gifts from Dr. B. Steiner (F. Hoffmann, La Roche, Basel).

cDNA Constructs, Transfection, and Cell Lines

The expression vectors encoding the α_{IIb} and β_3 chimeras, $\alpha_{IIb}\alpha_{6B}$, $\alpha_{IIb}\alpha_{6A}$, $\alpha_{IIb}\alpha_5$, $\beta_3\beta_1$, and $\beta_3\beta_1$(S790M), have been described previously (O'Toole et al., 1994, 1995). β_3(Y747F, Y759F) was constructed by splice-overlap polymerase chain reaction mutagenesis. pDCR-H-Ras(G12V) was a gift from Dr. M. H. Wigler (Cold Spring Harbor laboratory) (White et al., 1995). pcDNA3-R-Ras(G38V) (Zhang et al., 1996) was a gift from Dr. E. Ruoslahti (The Burnham Institute, La Jolla) with permission from Dr. Alan Hall (University of London). pCEP4 Raf-1:ER was a gift from Dr. A. Thorburn (University of Utah) with permission of Dr. M. McMahon (DNAX Research Institute). pGEX GST–Jun (1–79) and pSRα3 HA-JNK1 (Minden et al., 1994) were gifts from Dr. M. Karin (University of California, San Diego). The wild-type H-Ras cDNA was a gift from Dr. J. Jackson (University of California, San Diego). pCMV5 Cdc42(Q61L) was a gift from Dr. G. Bokoch (Scripps Research Institute). The vectors encoding Raf-BXB, HA-ERK2 and v-Src have been described previously (Bruder et al., 1992; Devary et al., 1992; Renshaw et al., 1996). pSG5 MKP-1 was a gift from Dr. N. Tonks (Cold Spring Harbor laboratory) (Sun et al., 1993). pBK-CMV Src(E378G) (Levy et al., 1986) was a gift from Dr. J. Brugge (Ariad Pharmaceuticals).

The $\alpha\beta$-py cells were generated by stably transfecting CHO-K1 cells with pPSVE-PyE (a generous gift from M. Fukuda, The Burnham Institute) encoding the polyoma large T antigen and replication-deficient CDM8 expression constructs encoding $\alpha_{IIb}\alpha_{6A}$ and $\beta_3\beta_1$. Raf-1.ER cells were generated by stably transfecting CHO-K1 cells with the hygromycin-resistant vector pCEP Raf-1:ER and expression constructs encoding $\alpha_{IIb}\alpha_{6A}$ and $\beta_3\beta_1$.

Flow Cytometry

PAC1 binding was analyzed by two-color flow cytometry as described (Hughes et al., 1996). In transiently transfected CHO-K1 or $\alpha\beta$-py cells, PAC1 binding was analyzed only on a gated subset of cells positive for $\alpha_{IIb}\beta_3$ or Tac-α_5 expression. To examine the effects

of inhibitors of protein synthesis and transcription on the suppressive effect of activated Raf-1, cycloheximide (20 μg/ml) or actinomycin D (2 μg/ml) was added to Raf-ER cells for 24 hr in the presence and absence of estradiol (13 μM). The cells were then harvested and assayed for PAC1 binding. In experiments measuring the time course of integrin suppression, cycloheximide (20 μg/ml) was added to the Raf-ER cells in the presence of estradiol (13 μM) for 4 hr. Aliquots of cells were then harvested at hourly intervals and assayed for both PAC1 binding and ERK2 kinase activity. As a control, PAC1 binding and ERK2 activity were assayed in Raf-ER cells incubated in the presence of 20 μg/ml cycloheximide for 4 hr.

To obtain numerical estimates of integrin activation we calculated an activation index (AI), defined as $100 \times (F_o - F_r)/(F_o\text{LIBS6} - F_r)$, where F_o is the median fluorescence intensity (MFI) of PAC1 binding; F_r is the MFI of PAC1 binding in the presence of competitive inhibitor (Ro43–5054, 1 μM); and F_oLIBS6 is the MFI of PAC1 binding in the presence of 2 μM anti-LIBS6. The percentage inhibition was calculated as 100 $(AI_0 - AI)/AI_0$, where AI_0 is the activation index in the absence of the cotransfected suppressor, and AI is the activation index in its presence.

Measurement of ERK2 and JNK1 Activity

For ERK2 and JNK1 kinase assays, 2×10^5 cells were transfected using Lipofectamine (Gibco–BRL) with 2 μg of pCMV5 HA-ERK2 or 2 μg of pSRα3HA-JNK1. The cells were also transfected with 2 μg of the test plasmid, such as pDCR H-Ras(G12V). In some experiments 4–6 μg of a second plasmid, such as MKP-1, were cotransfected and the total amount of DNA was standardized at 10 μg. Transfections were done in duplicate to allow parallel analysis of both kinase activity and PAC1 binding. Forty-eight hours after transfection the cells were harvested and lysed as described (Renshaw et al., 1996). HA-ERK2 activity was assayed either by an in-gel kinase assay (Renshaw et al., 1996) or by an immune-complex kinase assay with myelin basic protein as a substrate. In experiments monitoring the activity of endogenous ERK2, the ERK2 was immunoprecipated with polyclonal anti-ERK2 (Santa Cruz). ERK2 kinase activity was then assayed by an immune-complex kinase assay with myelin basic protein as a substrate (Minden et al., 1995). Relative ERK2 activity was determined by autoradiography followed by scanning densitometry.

For JNK1 kinase assays, HA-JNK1 was immunoprecipitated with antibody 12CA5 as described for the ERK2 kinase assay, resuspended in kinase buffer containing phosphatase inhibitors, and reacted with 2 μg of a GST fusion protein containing the first 79 residues of c-Jun as a substrate. After incubation at 30°C for 20 min, the reaction was stopped with SDS sample buffer. The samples were then subjected to SDS–polyacrylamide gel electrophoresis on 10% gels, and the gels were dried and visualized by autoradiography.

Fibronectin Matrix Assembly and Cell Spreading

Cells were plated on coverslips and cultured in complete medium. After 15 hr, estradiol (Sigma) was added to a final concentration of 13 μM. After a further 24 hr the medium was supplemented with 150 μg/ml human fibronectin and 2 μM of the $\alpha_{IIb}\beta_3$ antagonist lamifiban. The function-blocking anti-hamster α_5 monoclonal antibody PB1 and 13 μM estradiol were added to the samples as indicated. Twenty-four hours later, the cells were washed with cold PBS and incubated on ice for 30 min with a rabbit anti-human fibronectin, diluted 1:750 in PBS, 2% BSA, and 0.05% sodium azide (buffer A). The cells were then washed with cold PBS and stained with a fluorescein isothiocyanate–conjugated goat anti-rabbit antibody (TAGO) for 30 min on ice in buffer A. After washing with PBS, cells were fixed with 3.7% methanol-free formaldehyde (Polysciences) in PBS and permeabilized with 0.1% Triton X-100 in PBS. After counterstaining with rhodamine-phalloidin, coverslips were mounted in Fluorostab (Cappel) and analyzed on a Leitz Orthoplan immunofluorescence microscope at 400× magnification. Photographs were then taken on Tmax 400 film (Eastman Kodak).

For the cell-spreading experiments, Raf-ER cells were plated on coverslips coated with fibrinogen (5 μg/ml) or fibronectin (15 μg/ml) and cultured in DMEM supplemented with 13 μM estradiol or an ethanol vehicle control. Twenty-four hours later, the cells were examined by phase-contrast microscopy. Photographs were then taken on Tmax 400 film.

Acknowledgments

Correspondence should be addressed to M. H. G. (e-mail: ginsberg@scripps.edu). We thank our colleagues for generously providing the reagents acknowledged in Experimental Procedures. We thank Dr. Joe Ramos for critical review of the manuscript. P. E. H. is a postdoctoral fellow of the American Heart Association. M. W. R was supported by a postdoctoral training grant from the National Institutes of Health. M. P. was supported by a fellowship from the Deutsche Forschungsgemeinschaft. This work was supported by grants from the National Institutes of Health and Cor Therapeutics.

Received July 3, 1996; revised December 18, 1996.

References

Alig, L., Edenhofer, A., Hadvary, P., Hurzeler, M., Knopp, D., Muller, M., Steiner, B., Trzeciak, A., and Weller, T. (1992). Low molecular weight, non-peptide fibrinogen receptor antagonists. J. Med. Chem. *35*, 4393–4407.

Blystone, S.D., Lindberg, F.P., LaFlamme, S.E., and Brown, E.J. (1995). Integrin β_3 cytoplasmic tail is necessary and sufficient for regulation of $\alpha_5\beta_1$ phagocytosis by $\alpha_v\beta_3$ and integrin-associated protein. J. Cell Biol. *130*, 745–754.

Brown, M.T., and Cooper, J.A. (1996). Regulation, substrates and functions of src. Biochem. Biophys. Acta *1287*, 121–149.

Bruder, J.T., Heidecker, G., and Rapp, U.R. (1992). Serum, TPA, and ras induced expression from AP-1/Ets-driven promoters requires Raf-1 kinase. Genes Dev. *6*, 545–555.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu. Rev. Cell Biol. *4*, 487–525.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991). Oncogenes and signal transduction. Cell *64*, 281–302.

Chen, Y., O'Toole, T., Shipley, T., Forsyth, J., LaFlamme, S., Yamada, K., Shattil, S., and Ginsberg, M. (1994). "Inside-out" signal transduction inhibited by isolated integrin cytoplasmic domains. J. Biol. Chem. *269*, 18307–18310.

Chu, Y., Solski, P.A., Khosravi-Far, R., Der, C.J., and Kelly, K. (1996). The mitogen-activated protein kinase phosphatases PAC1, MKP-1 and MKP-2 have unique substrate specificities and reduced activity *in vivo* toward the ERK2 *sevenmaker* mutation. J. Biol. Chem. *271*, 6497–6501.

Clark, E.A., and Hynes, R.O. (1996). Ras activation is necessary for integrin-mediated activation of extracellular signal-regulated kinase 2 and cytosolic phospholipase A2, but not cytoskeletal organisation. J. Biol. Chem. *271*, 14814–14818.

Cox, A.D., Brtva, T.R., Lowe, D.G., and Der, C.J. (1994). R-Ras induces malignant, but not morphologic, transformation of NIH3T3 cells. Mol. Cell Biol. *9*, 3281–3288.

Devary, Y., Gottlieb, R.A., Smeal, T., and Karin, M. (1992). The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell *71*, 1081–1091.

Diaz-Gonzalez, F., Forsyth, J., Steiner, B., and Ginsberg, M.H. (1996). Trans-dominant inhibition of integrin function. Mol. Biol. Cell *7*, 1939–1951.

Dustin, M.L., and Springer, T.A. (1989). T cell receptor crosslinking transiently stimulates adhesiveness through LFA-1. Nature *341*, 619–624.

Hall, A. (1992). Ras-related GTPases and the cytoskeleton. Mol. Biol. Cell *3*, 475–479.

Howe, L.R., Leevers, S.J., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C.J. (1992). Activation of the MAP kinase pathway by the protein kinase raf. Cell *71*, 335–343.

Hughes, P.E., Diaz-Gonzalez, F., Leong, L., Wu, C., McDonald, J.A., Shattil, S.J., and Ginsberg, M.H. (1996). Breaking the integrin hinge:

a defined structural constraint regulates integrin signaling. J. Biol. Chem. *271*, 6571–6574.

Hynes, R.O. (1992). Integrins: versatility, modulation and signaling in cell adhesion. Cell *69*, 11–25.

Hynes, R., Ali, I., Destree, A., Mautner, V., Perkins, M., Senger, D., Wagner, D., and Smith, K. (1978). A large glycoprotein lost from the surfaces of transformed cells. Ann. NY Acad. Sci. *312*, 317–343.

Illc, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., et al. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature *377*, 539–544.

Johansson, M.W., Larsson, E., Luning, B., Pasquale, E.B., and Ruoslahti, E. (1994). Altered localization and cytoplasmic domain-binding properties of tyrosine-phosphorylated β1 integrin. J. Cell Biol. *126*, 1299–1309.

Kanemitsu, M.Y., and Lau, A.F. (1993). Epidermal growth factor stimulates the disruption of gap junctional communication and connexin43 phosphorylation independent of 12-0-tetradecanoylphorbol 13-acetate-sensitive protein kinase C: the possible involvement of mitogenactivated protein kinase. Mol. Biol. Cell *4*, 837–848.

Kuijpers, T.W., Mul, E.P., Blom, M., Kovach, N.L., Gaeta, F.C., Tollefson, V., Elices, M.J., and Harlan, J.M. (1993). Freezing adhesion molecules in a state of high-avidity binding blocks eosinophil migration. J. Exp. Med. *178*, 279–284.

LaFlamme, S.E., Akiyama, S.K., and Yamada, K.M. (1992). Regulation of fibronectin receptor distribution. J. Cell Biol. *117*, 437–447.

Law, D.A., Nannizzi-Alaimo, L., and Phillips, D.R. (1996). Outside-in integrin signal transduction. J. Biol. Chem. *271*, 10811–10815.

Leevers, S.J., Paterson, H.F., and Marshall, C.J. (1994). Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature *369*, 411–414.

Levy, J.B., Iba, H., and Hanafusa, H. (1986). Activation of the transforming potential of $p60^{c\text{-}src}$ by a single amino acid change. Proc. Natl. Acad. Sci. USA *83*, 4228–4232.

Lin, L.-L., Wartmann, M., Lin, A.Y., Knopf, J.L., Seth, A., and Davis, R.J. (1993). cPLA2 is phosphorylated and activated by MAP kinase. Cell *72*, 269–278.

Lukashev, M.E., Sheppard, D., and Pytela, R. (1994). Disruption of integrin function and induction of tyrosine phosphorylation by the autonomously expressed β_1 integrin cytoplasmic domain. J. Biol. Chem. *269*, 18311–18314.

Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell *80*, 179–185.

Minden, A., Lin, A., Claret, F.X., Abo, A., and Karin, M. (1995). Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell *81*, 1147–1157.

Minden, A., Lin, A., McMahon M., Lange-Carter, C., Derijard B., Davis R.J., Johnson G.L., and Karin, M. (1994). Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science *266*, 1719–1723.

O'Toole, T.E., Katagiri, Y., Faull, R.J., Peter, K., Tamura, R., Quaranta, V., Loftus, J.C., Shattil, S.J., and Ginsberg, M.H. (1994). Integrin cytoplasmic domains mediate inside-out signal transduction. J. Cell Biol. *124*, 1047–1059.

O'Toole, T.E., Ylanne, J., and Culley, B.M. (1995). Regulation of integrin affinity states through an NPXY motif in the β subunit cytoplasmic domain. J. Biol. Chem. *270*, 8553–8558.

Renshaw, M.W., Lea-Chou, E., and Wang, J.Y.J. (1996). Rac is required for v-Abl tyrosine kinase to activate mitogenesis. Curr. Biol. *6*, 76–83.

Samuels, M.L., Weber, M.J., Bishop, J.M., and McMahon, M. (1993). Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. Mol. Cell. Biol. *13*, 6241–6252.

Schlaepfer, D.D., Hanks, S.K., Hunter, T., and van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature *372*, 786–791.

Schwartz, M.A., Schaller, M.D., and Ginsberg, M.H. (1995). Integrins: emerging paradigms of signal transduction. Annu. Rev. Cell Biol. *11*, 549–599.

Shattil, S.J., Hoxie, J.A., Cunningham, M., and Brass, L.F. (1985). Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. J. Biol. Chem. *260*, 11107–11114.

Shimizu, Y., VanSeventer, G., Horgan, K., and Shaw, S. (1990). Regulated expression and binding of three VLA (β1) integrin receptors on T cells. Nature *345*, 250–253.

Sun, H., Charles, C.H., Lau, L.F., and Tonks, N.K. (1993). MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell *75*, 487–493.

Urano, T., Emkey, R., and Feig, L.A. (1996). Ral-GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. EMBO J. *15*, 810–816.

Vassbotn, F.S., Havnen, O.K., Heldin, C.H., and Holmsen, H. (1994). Negative feedback regulation of human platelets via autocrine activation of the plateletet derived growth factor alpha receptor. J. Biol. Chem. *269*, 13874–13879.

White, M.A., Nicolette, C., Minden, A., Polverini, A., Aelst, L.V., Karin, M., and Wigler, M.H. (1995). Multiple Ras functions can contribute to mammalian cell transformation. Cell *80*, 533–541.

Williams, M.J., Hughes, P.E., O'Toole, T.E., and Ginsberg, M.H. (1994). The inner world of cell adhesion: integrin cytoplasmic domains. Trends Cell Biol. *4*, 109–112.

Wu, C., Kievens, V., O'Toole, T.E., McDonald, J.A., and Ginsberg, M.H. (1995). Integrin activation and cytoskeletal interaction are critical steps in the assembly of a fibronectin matrix. Cell *83*, 715–724.

Zhang, Z., Vuori, K., Wang, H.G., Reed, J.C., and Ruoslahti, E. (1996). Integrin activation by R-ras. Cell *85*, 61–69.