

The Small GTP-binding Protein R-Ras Can Influence Integrin Activation by Antagonizing a Ras/Raf-initiated Integrin Suppression Pathway

Tariq Sethi,* Mark H. Ginsberg,[†] Julian Downward,[‡] and Paul E. Hughes^{†§}

*Department of Respiratory Medicine, University of Edinburgh Medical School, Edinburgh EH8 9AG, United Kingdom; [†]Department of Vascular Biology, The Scripps Research Institute, La Jolla, California 92037; and [‡]Signal Transduction Laboratory, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom

Submitted November 18, 1998; Accepted March 29, 1999
Monitoring Editor: Joan Brugge

The rapid modulation of ligand-binding affinity (“activation”) is a central property of the integrin family of cell adhesion receptors. The small GTP-binding protein Ras and its downstream effector kinase Raf-1 suppress integrin activation. In this study we explored the relationship between Ras and the closely related small GTP-binding protein R-Ras in modulating the integrin affinity state. We found that R-Ras does not seem to be a direct activator of integrins in Chinese hamster ovary cells. However, we observed that GTP-bound R-Ras strongly antagonizes the Ras/Raf-initiated integrin suppression pathway. Furthermore, this reversal of the Ras/Raf suppressor pathway does not seem to be via a competition between Ras and R-Ras for common downstream effectors or via an inhibition of Ras/Raf-induced MAP kinase activation. Thus, R-Ras and Ras may act in concert to regulate integrin affinity via the activation of distinct downstream effectors.

INTRODUCTION

Integrins are heterodimeric cell–cell and cell–matrix adhesion receptors that play a key role in cell growth, survival, migration, and tumor metastasis (Hynes, 1992; Schwartz *et al.*, 1995). A characteristic feature of specific integrins is their ability to modulate dynamically their affinity for ligand in response to intracellular signals, a process referred to as “inside-out” signaling or “activation” (Hughes and Pfaff, 1998). Integrin activation is a cell type–specific and energy-dependent process, requiring both the α and β subunit cytoplasmic domains (O’Toole *et al.*, 1994; Hughes *et al.*, 1996; Hughes and Pfaff, 1998).

Currently the cytoplasmic-signaling pathways regulating integrin affinity are incompletely understood. However, a number of recent studies indicate that the Ras family of small GTP-binding proteins and their downstream effectors play a central role in regulating integrin affinity (Shimizu and Hunt, 1996; Z. Zhang *et al.*, 1996; Hughes *et al.*, 1997). The Ras family of pro-

teins functions as molecular switches that are controlled by a GDP/GTP-binding cycle (Bos, 1997). H-Ras and its downstream effector kinase Raf-1 can suppress integrin activation in Chinese hamster ovary (CHO) cells. This suppressive effect is independent of protein synthesis and mRNA transcription and correlates with the activation of the ERK MAP kinase pathway (Hughes *et al.*, 1997). Furthermore R-Ras, a small GTP-binding protein homologous to H-Ras, influences integrin activation. In contrast to H-Ras, activated R-Ras stimulates ligand binding to integrins (Z. Zhang *et al.*, 1996).

R-Ras was originally identified because of its similarity to the H-Ras, K-Ras, and N-Ras oncogenes, being ~55% identical to each (Lowe *et al.*, 1987). Currently, little is known about R-Ras function and how it compares with that of the Ras proteins. Despite the considerable sequence similarity to the other Ras proteins, several observations suggest that the functions of R-Ras are distinct. For example, activating mutations in H-Ras, K-Ras, or N-Ras will induce the morphological transformation of a variety of fibroblasts and epithelial cell lines (Bos, 1997). In contrast, acti-

[§] Corresponding author. E-mail address: phughes@scripps.edu.

vated R-Ras causes the transformation of a much more limited spectrum of cell types (Cox *et al.*, 1994; Huff *et al.*, 1997).

R-Ras and the other Ras proteins have highly homologous effector-binding domains; consequently both GTP-bound R-Ras and Ras bind to several common effectors. Like Ras, R-Ras interacts with the p110 catalytic subunit of phosphatidylinositol 3-kinase (PI 3-kinase) *in vitro* and induces the elevation of the levels of PI 3-kinase lipid products *in vivo* (Marte *et al.*, 1996). R-Ras also interacts with the Raf serine/threonine kinases and exchange factors for the Ras-related Ral small GTP-binding proteins (Vojtek *et al.*, 1993; Spaargaren and Bischoff, 1994; Spaargaren *et al.*, 1994). However, in contrast to Ras, R-Ras does not activate Raf or Ral guanine nucleotide exchange activity *in vivo* (Marte *et al.*, 1996; Urano *et al.*, 1996). The GTP-bound state of R-Ras seems to be regulated differently from that of Ras. Both Ras and R-Ras interact with the GTPase-activating proteins p120 Ras GAP, neurofibromin, and p98 R-Ras GAP (Garrett *et al.*, 1989; Rey *et al.*, 1994; Yamamoto *et al.*, 1995). However, *in vivo* it is thought that p120 GAP and neurofibromin primarily serve as GAPs for Ras and that p98 R-Ras GAP primarily serves as a R-Ras GAP. The physiological stimuli that activate R-Ras *in vivo* are not known. R-Ras is not activated by SOS or GRF1 and GRF2, guanine nucleotide exchange factors for Ras, and currently no R-Ras-specific guanine nucleotide factors have been identified (Shou *et al.*, 1995; Fam *et al.*, 1997; Huff *et al.*, 1997).

In this study we explored the relationship between Ras and R-Ras in integrin affinity modulation. We found that R-Ras does not seem to be a direct activator of integrins in CHO cells. However, we observed that GTP-bound R-Ras strongly antagonizes the Ras/Raf-initiated integrin suppression pathway. Furthermore, this reversal of the Ras/Raf suppressor pathway does not seem to be via a competition between Ras and R-Ras for common downstream effectors or via an inhibition of Ras/Raf-induced MAP kinase activation. Thus, these observations suggest that R-Ras and Ras could act in concert to regulate integrin affinity via the activation of distinct and novel effectors.

MATERIALS AND METHODS

Antibodies and Reagents

The isolation and characterization of the anti- $\alpha_{\text{IIb}}\beta_3$ monoclonal antibodies anti-LIBS6 and D57 have been described previously (O'Toole *et al.*, 1994). The activation-dependent anti- $\alpha_{\text{IIb}}\beta_3$ monoclonal antibody PAC1 was a generous gift of Dr. S. Shattil (Scripps Research Institute, La Jolla, CA) (Shattil *et al.*, 1985). The anti-Tac antibody 7G7B6 was obtained from the American Type Culture Collection (Rockville, MD). Antibodies 7G7B6 and D57 were biotinylated with biotin-*N*-hydroxysuccinimide (Sigma, St. Louis, MO) according to the manufacturer's directions. The $\alpha_{\text{IIb}}\beta_3$ -specific pep-

tidomimetic inhibitor Ro43-5054 was a generous gift of Dr. Beat Steiner (Hoffmann La Roche, Basel, Switzerland).

cDNA Constructs, Transfection, and Cell Lines

The CDM8 expression constructs encoding the α_{IIb} chimera $\alpha_{\text{IIb}}\alpha_{6A}$, composed of the α_{IIb} extracellular and transmembrane domain fused to the cytoplasmic domains of α_{6A} , and the chimera $\beta_3\beta_1$, composed of the β_3 extracellular and transmembrane domain fused to the cytoplasmic domain of β_1 , were constructed as described (O'Toole *et al.*, 1994). The plasmid pDCR-H-Ras(G12V) was a generous gift of Dr. M. H. Wigler (Cold Spring Harbor laboratory, Cold Spring Harbor, NY). pcDNA3-R-Ras(G38V) was a gift of Dr. K. Vuori and Dr. E. Ruoslahti (The Burnham Institute, La Jolla, CA). The plasmids pMT2-HA-Rlf-CAAX, pMT2-HA-RalA, pMT2-HA-RalA(T28N), and pMT2-HA-RalA(G23V) were generous gifts of Dr. Rob Wolthuis (Utrecht University, Utrecht, Netherlands) (Wolthuis *et al.*, 1997). The expression vectors encoding Tac- α_5 , Raf-BXB, hemagglutinin (HA)-tagged ERK2, HA-tagged Akt, and Myc-tagged R-Ras have been described previously (Marte *et al.*, 1996; Hughes *et al.*, 1997).

CHO-K1 cells were obtained from the American Type Culture Collection. The $\alpha\beta$ -py cells were generated as described (Baker *et al.*, 1997). All cell lines were grown in DMEM (BioWhittaker, Walkersville, MD) containing 10% fetal bovine serum, 1% nonessential amino acids, 2 mM glutamine (Sigma), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Transient transfections were undertaken using lipofection (lipofectamine; Life Technologies, Gaithersburg, MD) as described previously (Hughes *et al.*, 1996).

Flow Cytometry

For single-color FAC analysis, 5×10^5 cells were incubated on ice for 30 min with the primary antibody, washed, and then incubated on ice for a further 30 min with an FITC-conjugated goat anti-mouse IgG (Tago, Burlingame, CA) secondary antibody. Cells were pelleted, resuspended, and analyzed on a FACScan (Becton Dickinson, Mountain View, CA). PAC1 binding was analyzed by two-color flow cytometry. Cell staining was performed in DMEM and 1 mg/ml BSA (Sigma). Single-cell suspensions were obtained by incubating cells for 5 min in trypsin and EDTA (Worthington, Freehold, NJ) and diluting with an equal volume of DMEM containing 10% FCS. After washing, 5×10^5 cells were incubated in a final volume of 50 μl containing 0.1% PAC1 ascites in the presence or absence of the competitive inhibitor Ro43-5054 at 1 μM . After a 30-min incubation at room temperature, cells were washed with cold DMEM solution and then incubated on ice with DMEM containing either the biotinylated anti-Tac antibody 7G7B6 or biotinylated D57. After 30 min on ice, the cells were washed and incubated with 10% FITC-conjugated goat anti-mouse IgM (Tago) and 4% phycoerythrin-streptavidin (Molecular Probes, Eugene, OR). Thirty minutes later, cells were washed with 0.5 ml of cold PBS and resuspended in 0.5 ml of cold PBS. The cells were then analyzed on a FACScan (Becton Dickinson) flow cytometer as described (Hughes *et al.*, 1996).

In transiently transfected $\alpha\beta$ -py cells, PAC1 binding (FITC staining) was analyzed only on a gated subset of cells positive for Tac- α_5 expression (phycoerythrin staining). To define the affinity state, histograms depicting PAC1 staining in the absence or presence of the competitive inhibitor Ro43-5054 were superimposed. Because the peptide mimetic Ro43-5054 is an inhibitor of ligand binding to $\alpha_{\text{IIb}}\beta_3$, a leftward shift in the histogram in the presence of inhibitor is indicative of the presence of high-affinity $\alpha_{\text{IIb}}\beta_3$.

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 of PAC1 binding, F_r is the median fluorescence intensity of PAC1 binding in the presence of competitive inhibitor (Ro43-5054, 1 μM), and $F_o \text{LIBS6}$ is the median fluorescence intensity of PAC1 binding in the presence

of 2 μM anti-LIBS6. Percent inhibition was calculated by $100(\text{AI}_0 - \text{AI})/\text{AI}_0$, 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 Akt Activity

For ERK2 assays, 2×10^5 cells were transfected using lipofectamine (Life Technologies) with 2 μg of pCMV5 HA-ERK2. The cells were also transfected with 2 μg of the test plasmid [e.g., pDCR-H-Ras(G12V)]. In some experiments, 4–6 μg of a second plasmid [e.g., R-Ras(G38V)] were cotransfected, and the total amount of DNA was standardized at 10 μg , by the addition of pcDNA3, for each transfection. Transfections were done in duplicate to allow parallel analysis of both ERK2 kinase activity and PAC1 binding by flow cytometry, as described above. Forty-eight hours after transfection, cells were harvested and lysed in 0.5% Nonidet P-40 (NP-40) buffer containing phosphatase inhibitors (10 mM sodium pyrophosphate, 10 mM NaF, 3 mM β glycerophosphate, and 1 mM Na_3VO_4) in addition to protease inhibitors. The HA-ERK2 was immunoprecipitated by the anti-HA antibody 12CA5, and its activity was assessed by an immune-complex kinase assay using myelin basic protein as a substrate. ERK2 expression and recovery were monitored by fractionating 25 μg of whole-cell lysate or one-fifth of the 12CA5 immunoprecipitate on 12.5% SDS-polyacrylamide gels, transferring to Immobilon (Millipore, Bedford, MA) membranes, and immunoblotting with the anti-HA antibody 12CA5 or polyclonal anti-ERK2 (Santa Cruz Biotechnology, Tebu, France).

For Akt kinase assays, 2×10^5 cells were transfected using the lipofectamine method with 2 μg of pSG5-HA-AKT. The cells were also transfected with the appropriate test plasmids, and the total amount of DNA in each transfection was then adjusted to 8 μg by the addition of pcDNA3. Transfections were done in duplicate to allow parallel analysis of both Akt activity and PAC1 binding. Forty-eight hours after transfection, cells were lysed with 1.0% NP-40 buffer containing phosphatase inhibitors (10 mM sodium pyrophosphate, 10 mM NaF, 3 mM β glycerophosphate, and 1 mM Na_3VO_4) in addition to protease inhibitors. HA-Akt was immunoprecipitated with the anti-HA antibody 12CA5, as described for the ERK2 kinase assay. The immunoprecipitates were washed two times in cell lysis buffer, followed by two washes in high-salt buffer (0.5 M LiCl, 0.1 M Tris, pH 8.0, and 1 mM EDTA) and a final wash in nonreducing kinase buffer (50 mM Tris, pH 7.5, and 10 mM MgCl_2). The immunoprecipitates were resuspended in kinase buffer (50 mM Tris, pH 7.5, 10 mM MgCl_2 , and 1 mM DTT) and reacted with 2.5 μg of histone 2B, as described (Marte *et al.*, 1996). After incubation at room temperature for 20 min, the reaction was stopped with SDS sample buffer. The samples were then subjected to SDS-PAGE on 16% gels; the gels were dried down and visualized by autoradiography. HA-Akt expression was monitored by fractionating 25 μg of whole-cell lysate on 4–20% SDS-polyacrylamide gels, transferring to Immobilon (Millipore) membranes, and immunoblotting with the anti-HA antibody 12CA5.

Ral Activation Assay

The GTP-bound form of Ral was specifically pulled down from clarified cell lysates by incubation with the GST-tagged form of the Ral-binding domain (RalBD) of RLIP76, as described (Wolthuis *et al.*, 1998). Cells (2×10^5) were transfected using the lipofectamine method with the indicated HA-Ral and HA-Rlf-CAAX constructs and with the total amount of DNA in each transfection adjusted to 8 μg by the addition of pcDNA3. The cells were then washed after transfection, and after 24 h the cells were maintained in media containing 0.5% FCS. The cells were then washed twice with cold PBS and lysed on ice in Ral-binding buffer (15% glycerol, 1% NP-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM MgCl_2) containing protease inhibitors. Lysates were then clarified by centrifugation, and the supernatants of each sample were incubated with 15

μg of GST-RalBD precoupled to glutathione beads. Samples were then incubated for 1 h on a tumbler at 4°C followed by four washes in Ral-binding buffer. The beads were boiled in Laemmli sample buffer and subjected to SDS-PAGE and Western blotting with the anti-HA antibody 12CA5 to assay the recovery of HA-Ral. In parallel HA-Ral and HA-Rlf-CAAX expression was monitored by fractionating 20 μg of whole-cell lysate on a 4–20% SDS-polyacrylamide gel, followed by transfer to Immobilon (Millipore) membranes and immunoblotting with the anti-HA antibody 12CA5.

RESULTS

R-Ras Does Not Directly Activate the Integrin $\alpha_{\text{IIb}}\beta_3$ in CHO Cells

To gain further insight into the role of R-Ras in integrin affinity modulation, we tested the effect of transfecting activated R-Ras on the affinity state of resting and active integrins expressed in CHO cells. When stably expressed in CHO cells (A5 cells), the platelet-specific integrin $\alpha_{\text{IIb}}\beta_3$ fails to bind activation-specific ligands with high affinity (O'Toole *et al.*, 1990). To determine whether the expression of an activated variant of R-Ras [R-Ras(G38V)] could activate the $\alpha_{\text{IIb}}\beta_3$ in these cells, an expression vector encoding R-Ras(G38V) was transiently transfected into A5 cells. We assessed activation by the binding of PAC1, an antibody specific for the active conformation of $\alpha_{\text{IIb}}\beta_3$ (Shattil *et al.*, 1985). Expression of activated R-Ras in A5 cells did not induce the activation of $\alpha_{\text{IIb}}\beta_3$ as determined by PAC1 binding (Figure 1). However, PAC1 binding to these A5 cells could be induced by the addition of an activating monoclonal antibody, anti-LIBS6 (our unpublished observations). We also tested the effect of transfecting R-Ras(G38V) into CHO cells stably expressing the active chimeric integrin $\alpha_{\text{IIb}}\alpha_{6A}\beta_3\beta_1$ ($\alpha\beta$ -py cells). We found that transfection of activated R-Ras did not lead to a significant increase in PAC1 binding to $\alpha\beta$ -py cells (Figure 1). Western blot analysis of lysates from both the transfected A5 and $\alpha\beta$ -py cells revealed that R-Ras(G38V) was well expressed in both cell types (Figure 1).

The A5 and $\alpha\beta$ -py cells are clonal cell lines. To ensure these results were not caused by artifacts of clonal selection, we examined PAC1 binding to parental CHO cells transiently transfected with expression vectors encoding wild-type $\alpha_{\text{IIb}}\beta_3$ and $\alpha_{\text{IIb}}\alpha_{6A}\beta_3\beta_1$ in the presence or absence of activated R-Ras. In agreement with the observations made in stably transfected cells, activated R-Ras had no significant effect on PAC1 binding (our unpublished observations). Thus, from these data we conclude that activated R-Ras does not directly influence the ligand-binding affinity of $\alpha_{\text{IIb}}\beta_3$ in CHO cells.

R-Ras Can Reverse the Suppressive Effect of Activated H-Ras and Raf-1

GTP-bound R-Ras did not activate the integrins tested in CHO cells. However, its activating effects might be

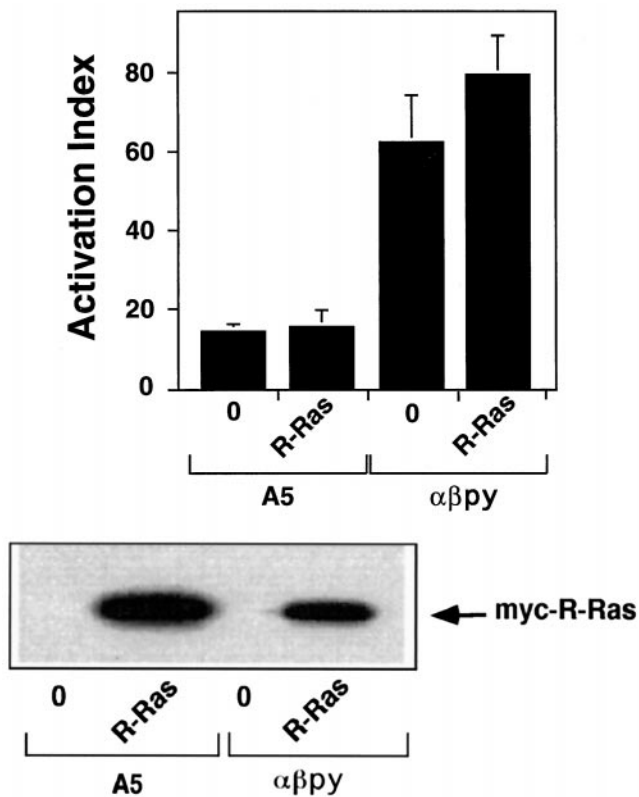


Figure 1. R-Ras(G38V) does not directly stimulate integrin $\alpha_{IIb}\beta_3$ activation in CHO cells. Both $\alpha\beta$ -py and A5 cells were transiently transfected with 3 μ g of an expression vector encoding R-Ras(G38V) and/or an equivalent amount of an empty control vector. After 48 h, PAC1 binding was analyzed by flow cytometry as described in MATERIALS AND METHODS. Top, The mean activation indices \pm SD of three independent experiments. Bottom, Immunoblot analysis of cell lysates illustrating the expression of myc-tagged R-Ras(G38V). Twenty micrograms of cell lysate from each transfection were separated on a 4–20% gradient gel and immunoblotted with the anti-Myc antibody 9E10.

attributable to it antagonizing the suppressive effect of Ras. To test this hypothesis, we examined the effect of R-Ras(G38V) on suppression caused by activated H-Ras. $\alpha\beta$ -py cells were transiently transfected with H-Ras(G12V) in the presence or absence of R-Ras(G38V). H-Ras(G12V) alone caused marked inhibition of PAC1 binding (Figure 2, A and B); however, cotransfection with an expression vector encoding activated R-Ras completely reversed this suppression (Figure 2A). The average median fluorescence intensity (MFI) of PAC1 binding from five independent experiments in the control transfected $\alpha\beta$ -PY cells was 37.8 ± 7.44 ; after transfection with H-Ras(G12V), this was reduced to 15.6 ± 3.92 . The cotransfection of activated R-Ras restored the mean MFI to 41.4 ± 8.72 . These MFIs are representative of those seen in subsequent experiments.

We also tested the ability of R-Ras to reverse suppression mediated by an activated membrane-targeted variant of Raf, Raf-CAAX, and results similar to those observed with H-Ras(G12V) were seen (Figure 2B). Thus R-Ras(G38V) reversed the suppressive effect of activated H-Ras or Raf-1 on integrin affinity, suggesting that R-Ras can regulate the integrin activation state by modulating the activity of the Ras/Raf-1-dependent suppression pathway.

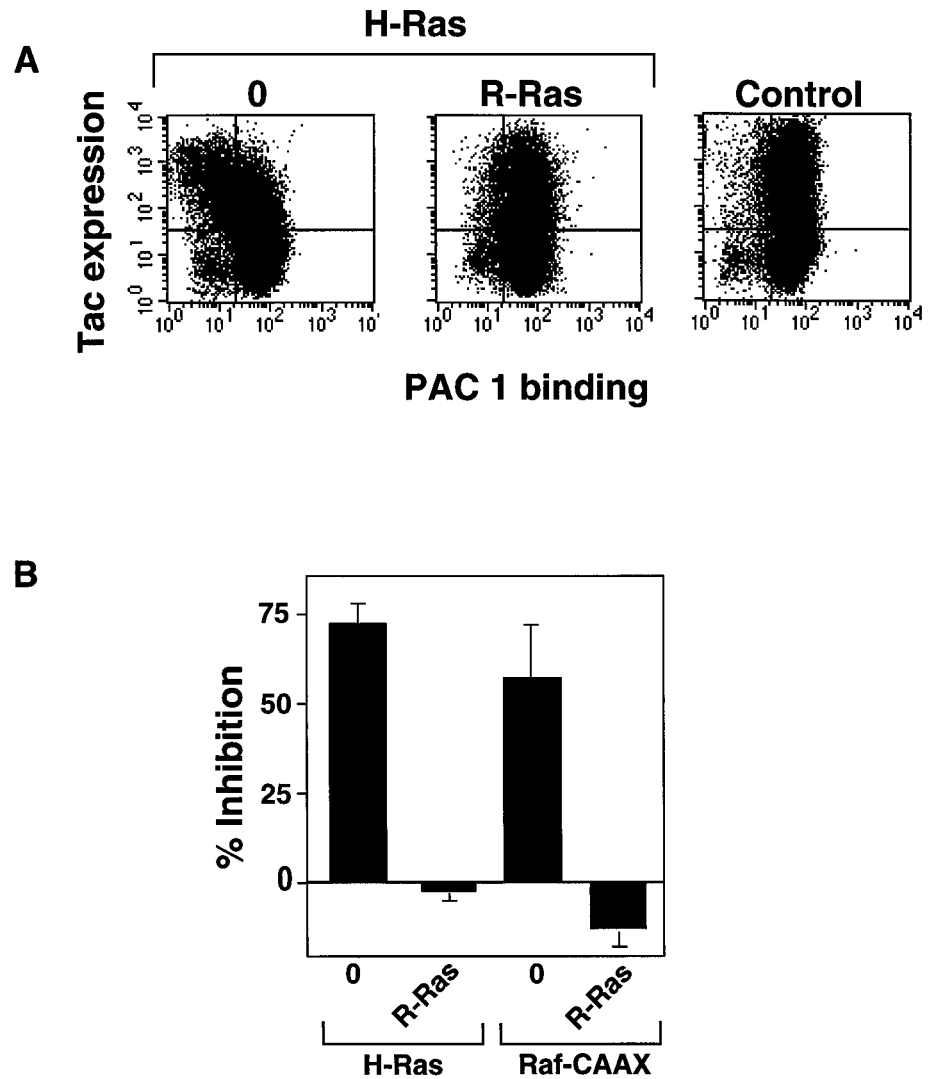
Small GTP-binding proteins must be in the GTP-bound conformation to bind and activate their downstream effectors (Bos, 1997). To determine whether R-Ras needs to be activated to reverse H-Ras suppression, we examined the effects of wild-type and putative dominant-negative R-Ras(S43N) on H-Ras suppression of integrin affinity in $\alpha\beta$ -py cells (Figure 3). In contrast, transfection of activated R-Ras(G38V) caused a concentration-dependent reversal of H-Ras(G12V) suppression. The cotransfection of R-Ras(G38V) (0.5–4 μ g), at ratios of plasmid DNA as low as 1 to 8 with respect to H-Ras(G12V), still antagonized H-Ras suppression (Figure 3). Furthermore, Western blot analysis showed that increasing amounts of transfected R-Ras(G38V) cDNA had no effect on H-Ras expression (Figure 3), eliminating the possibility that the R-Ras “rescue” is caused by a reduction in the expression of H-Ras(G12V). Thus, these data suggest that R-Ras may influence integrin activation via a competition for a common downstream effector.

R-Ras Reversal of H-Ras Suppression Is Not Caused by Simple Competition between H-Ras and R-Ras for the Common Effector Raf-1

Suppression of integrin activation by H-Ras and its downstream effector kinase Raf-1 correlates with the activation of the ERK MAP kinase pathway. Furthermore, R-Ras binds to Raf in a GTP-dependent manner but fails to stimulate Raf kinase activity. Consequently, reversal of H-Ras suppression by R-Ras could be the result of R-Ras competition with H-Ras for Raf-1. To test this idea, we assessed the capacity of R-Ras(G38V) to reverse the suppressive effect of Raf-BXB, an activated variant of Raf-1 that, in contrast to Raf-CAAX, lacks a Ras-binding domain. As expected, transient transfection of $\alpha\beta$ -py cells with Raf-BXB caused a marked suppression of integrin activation. However, the cotransfection of R-Ras(G38V) completely reversed suppression by Raf-BXB (Figure 4A). These data suggest that the R-Ras reversal of H-Ras-induced suppression is not caused by simple competition between the two G-proteins for the common effector Raf-1.

As described previously, H-Ras- and Raf-1-mediated suppression correlates with the activation of

Figure 2. Activated R-Ras rescues the suppression of integrin activation by H-Ras(G12V) and Raf-CAAX. (A) $\alpha\beta$ -py cells were transiently transfected with 2 μg of an expression vector encoding Tac- α_5 alone and Tac- α_5 plus H-Ras(G12V). In a separate transfection Tac- α_5 plus H-Ras(G12V) was cotransfected with 3 μg of a plasmid encoding R-Ras(G38V). After 48 h, cells were harvested and stained for Tac expression (y -axis) and PAC1 binding (x -axis). Left, In the H-Ras(G12V)-transfected cells, there is a leftward shift of the dot plot in the upper quadrants representing a reduction in PAC1 binding. Middle, This shift is reversed by the cotransfection with activated R-Ras(G38V). Right, In the empty vector control transfection, there was no suppression of PAC1 binding in the Tac- α_5 -expressing cells. (B) $\alpha\beta$ -py cells were cotransfected with 4 μg of an expression vector encoding Raf-CAAX and H-Ras(G12V). In separate transfections Raf-CAAX or H-Ras(G12V) expression vectors were simultaneously cotransfected with 3 μg of a plasmid encoding R-Ras(G38V). After 48 h, integrin activation was determined by PAC1 binding. Depicted is the mean percent inhibition of integrin activation relative to that of the empty vector \pm SE of three independent determinations.



Raf and the ERK MAP kinase pathway. Consequently, activated R-Ras could rescue suppression by affecting the ability of H-Ras and Raf-1 to activate ERK2. However, the coexpression of R-Ras(G38V) with H-Ras(G12V) or Raf-BXB did not influence the ability of either to activate ERK2 kinase (Figure 4B). In addition, the coexpression of R-Ras(G38V) did not effect ERK2 activation induced by Raf-CAAX (our unpublished observations). As reported previously, transfection of R-Ras(G38V) alone did not activate ERK2 (Marte *et al.*, 1996). These results indicate that the ability of R-Ras(G38V) to rescue the suppressive effects of H-Ras(G12V) and Raf-BXB is not caused by an inactivation of the ERK MAP kinase pathway and further argue against the notion that R-Ras competes with H-Ras for binding to Raf.

The Small GTP-binding Protein Ral and PI 3-Kinase Do Not Play a Role in H-Ras–dependent Suppression or R-Ras–mediated Rescue

The previously described experiments excluded competition for Raf binding as the mechanism for R-Ras reversal of Ras suppression. GTP-bound R-Ras and H-Ras can also bind to the p110 catalytic subunit of PI 3-kinase and Rlf, a guanine nucleotide exchange factor for the Ral family of small GTP-binding proteins. Consequently, we examined the role of these effectors in integrin affinity modulation.

We used a PI 3-kinase inhibitor, LY294002, to test the role of PI 3-kinase in R-Ras's capacity to oppose H-Ras as a suppressor of integrin activation. Pretreatment of $\alpha\beta$ -py cells with 20 μM LY294002 for 24 h had no effect on basal activation of the $\alpha_{\text{IIb}}\beta_3$ chimera (see Figure 6A). Furthermore 20 μM LY294002 had little

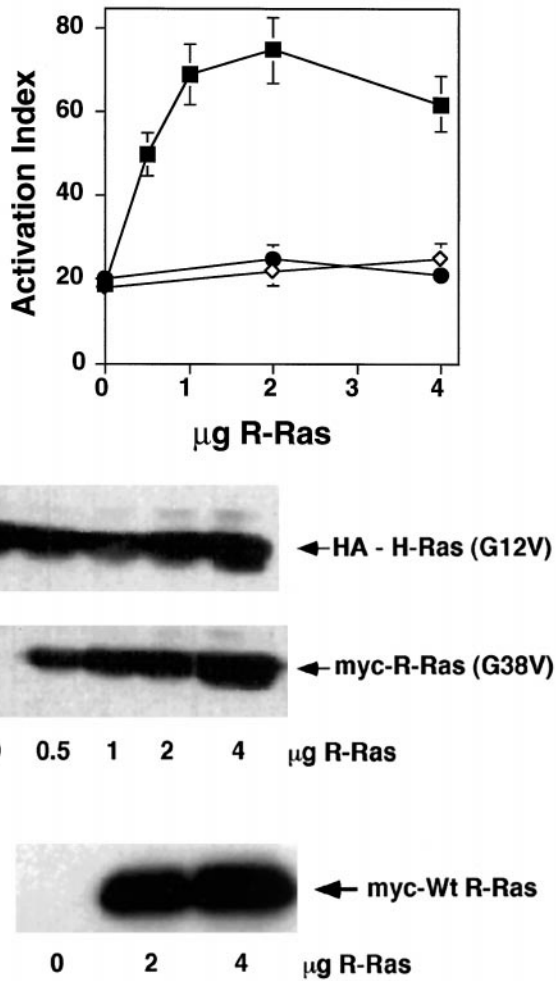


Figure 3. Activated but not wild-type or dominant-negative R-Ras can reverse the suppressive effect of activated H-Ras(G12V). Top, $\alpha\beta$ -py cells were cotransfected with an expression vector encoding H-Ras(G12V). They were simultaneously cotransfected with 2 and 4 μg of a plasmid encoding wild-type R-Ras (●) or R-Ras(S43N) (◇) and 0.5, 1, 2, and 4 μg of a plasmid encoding R-Ras(G38V) (■). After 48 h, integrin activation was determined by PAC1 binding. Depicted is the activation index in the presence of H-Ras(G12V) \pm SE of three independent determinations. Bottom, The immunoblot analysis of cell lysates illustrates the expression of wild-type (Wt) R-Ras and R-Ras(G38V) in the presence of H-Ras(G12V). Twenty micrograms of cell lysate from each transfection were separated on a 4–20% gradient gel and immunoblotted with either an anti-HA antibody, 12CA5, or the anti-Myc antibody 9E10. The R-Ras(S43N) was also well expressed, as determined by immunoblot analysis of lysates from R-Ras(S43N)-transfected cells (our unpublished observations).

effect on the H-Ras(G12V)-induced suppression or the R-Ras rescue of integrin activation in $\alpha\beta$ -py cells (Figure 5A). In parallel experiments, PI 3-kinase activity was assessed by measuring the *in vitro* kinase activity of the PI 3-kinase effector protein kinase B (PKB, Akt). LY294002 inhibited PI 3-kinase activity, and in the absence of detectable PI 3-kinase activation, R-Ras(G38V) was still able to reverse H-Ras(G12V)

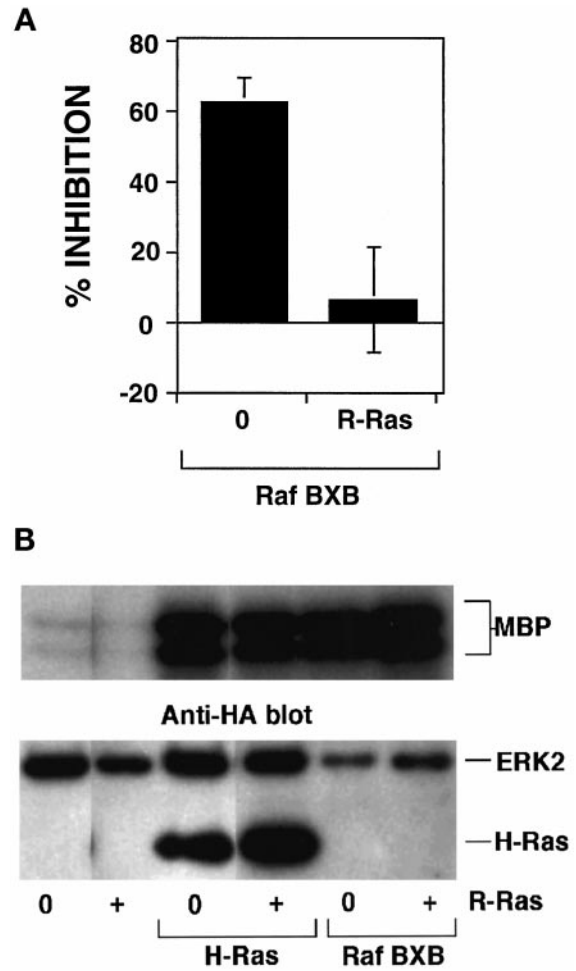


Figure 4. R-Ras reversal of H-Ras- and Raf-1-initiated suppression is not caused by inhibition of Ras/Raf activation of ERK MAP kinase. (A) $\alpha\beta$ -py cells were cotransfected with an expression vector encoding either Raf-BXB or a control cDNA. In a separate transfection Raf-BXB cDNA was cotransfected with a plasmid encoding activated R-Ras(G38V). After 48 h, integrin activation was determined by PAC1 binding. Depicted is the mean percent inhibition relative to that of the empty vector control \pm SE of three independent determinations. (B) $\alpha\beta$ -py cells were cotransfected with an expression vector encoding HA-tagged ERK2. The cells were also cotransfected with a control expression vector or vectors containing inserts encoding either R-Ras(G38V), Raf-BXB, or H-Ras(G12V). In separate transfections expression vectors encoding either Raf-BXB or H-Ras(G12V) were simultaneously cotransfected with a plasmid encoding R-Ras(G38V). The transfected ERK2 kinase was immunoprecipitated with the anti-HA antibody 12CA5. ERK-2 activity was measured by phosphorylation of myelin basic protein (MBP) using an immunocomplex kinase assay. Top, The relative ERK activation is shown. Bottom, Immunoblots with the anti-HA (12CA5) antibody illustrate the comparable expression of HA-tagged ERK2 in all transfections. The H-Ras(G12V) construct bore an HA-tag and was detected as the lower band in lanes transfected with that construct. Note the similar expression of recombinant activated H-Ras(G12V) in both the control and R-Ras(G38V)-cotransfected cells.

suppression (Figure 5, A and B). These results demonstrate that basal integrin activation in CHO cells is

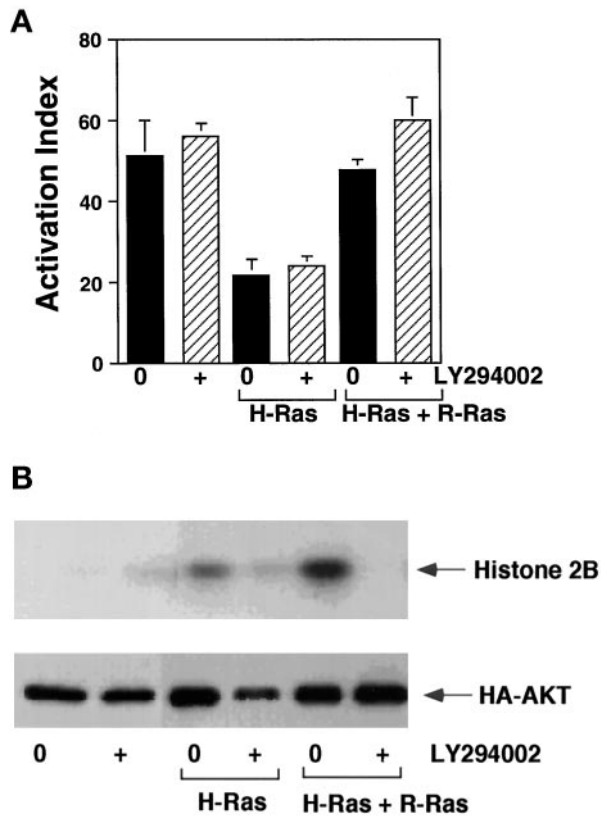


Figure 5. PI 3-kinase activation does not mediate H-Ras- or R-Ras-dependent regulation of integrin affinity. (A) $\alpha\beta$ -py cells were transiently transfected with either a control expression vector or vectors containing inserts encoding either R-Ras(G38V) and H-Ras(G12V) or H-Ras(G12V). Each transfection was performed in duplicate; 24 h after transfection, the PI 3-kinase inhibitor LY294002 was added at a final concentration of 20 μ M to one of the duplicates. After 48 h, integrin activation was determined by PAC1 binding. Depicted are the activation indices \pm SE of three independent determinations. (B) $\alpha\beta$ -py cells were transiently transfected with an expression vector encoding HA-tagged Akt and either a control expression vector or vectors containing inserts encoding either R-Ras(G38V) and H-Ras(G12V) or H-Ras(G12V). Each transfection was performed in duplicate, and 24 h after transfection, the PI 3-kinase inhibitor LY294002 was added at a final concentration of 20 μ M to one of the duplicates. Forty-eight hours after transfection, the cells were lysed, and the transfected Akt was immunoprecipitated with the anti-HA antibody 12CA5. Akt activity was then assayed using an immunocomplex kinase assay with histone 2B as a substrate. Top, The relative Akt activation is depicted; note the inhibition of Akt activity by LY294002. Bottom, Immunoblots with the anti-HA (12CA5) antibody illustrate comparable expression of HA-tagged Akt in all transfections.

not affected by inhibition of PI 3-kinase. Furthermore, the modulation of integrin affinity by H-Ras and R-Ras is not via the activation of PI 3-kinase in these cells.

The transient transfection of Rlf-CAAX [an activated membrane-targeted variant of the Ral guanine nucleotide exchange factor (Ral-GEF [Rlf])] or dominant-negative RalA(T28N) into $\alpha\beta$ -py cells had no effect on

basal integrin activation, as measured by PAC1 binding (Figure 6, A and B). Moreover, there was no effect of Rlf-CAAX or RalA(T28N) coexpression on the ability of H-Ras(G12V) to suppress activation of the chimeric integrin or of R-Ras(G38V) to rescue suppression (Figure 6, A and B). Furthermore, an H-Ras effector loop mutant, H-Ras(G12V, T35S), which interacts with Raf-1 but not with Ral-GEFs (Rodriguez-Viciana *et al.*, 1997), was a potent suppressor of integrin activation in CHO cells (our unpublished observations), providing further evidence that the suppression of integrin activation by Ras is independent of Ral activation. The overexpression of an activated variant of RalA(G23V) was also tested as an alternative to an activated Ral exchange factor in these experiments, and this construct produced results similar to those observed with Rlf-CAAX (our unpublished observations).

In parallel experiments the activity of Rlf-CAAX and dominant-negative RalA(T28N) was measured by an affinity precipitation assay for GTP-bound Ral using the Ral-binding domain of the RLIP76 (Wolthuis *et al.*, 1998). The cotransfection of Rlf-CAAX with RalA led to a substantial precipitation of GTP-bound RalA, compared with that observed after the transfection of RalA alone, demonstrating that Rlf-CAAX is a potent activator of Ral in CHO cells (Figure 6C). In contrast, in the presence of Rlf-CAAX, the dominant-negative RalA(T28N) was not precipitated by the GST-RalBD (Figure 6C), demonstrating that this variant exists in the GDP-bound state in CHO cells. Thus, activation or inhibition of the Ral arm of the Ras effector pathway does not contribute to modulation of integrin affinity by H-Ras and R-Ras in CHO cells.

DISCUSSION

H-Ras and its downstream effector kinase Raf-1 suppress integrin activation. Here we report that the small GTP-binding protein R-Ras regulates integrin affinity by modulating the activity of the Ras/Raf-initiated suppression pathway. The major findings of this article are as follows. First, activated R-Ras does not seem to be a direct activator of integrins in CHO cells. Second, GTP- but not GDP-bound R-Ras can reverse the suppressive effect of both activated H-Ras and its effector kinase Raf-1. Third, this property of activated R-Ras is not caused by simple competition between H-Ras and R-Ras for Raf-1 or guanine nucleotide exchange factors for the small GTP-binding protein Ral. Fourth, the ability of activated R-Ras to rescue H-Ras-initiated suppression did not correlate with the activation of PI 3-kinase. Furthermore, the inhibition of PI 3-kinase and Ral activity had no effect on basal integrin activation or the ability of activated H-Ras to suppress integrin activation in these cells. Taken together, these data suggest that R-Ras and H-Ras could

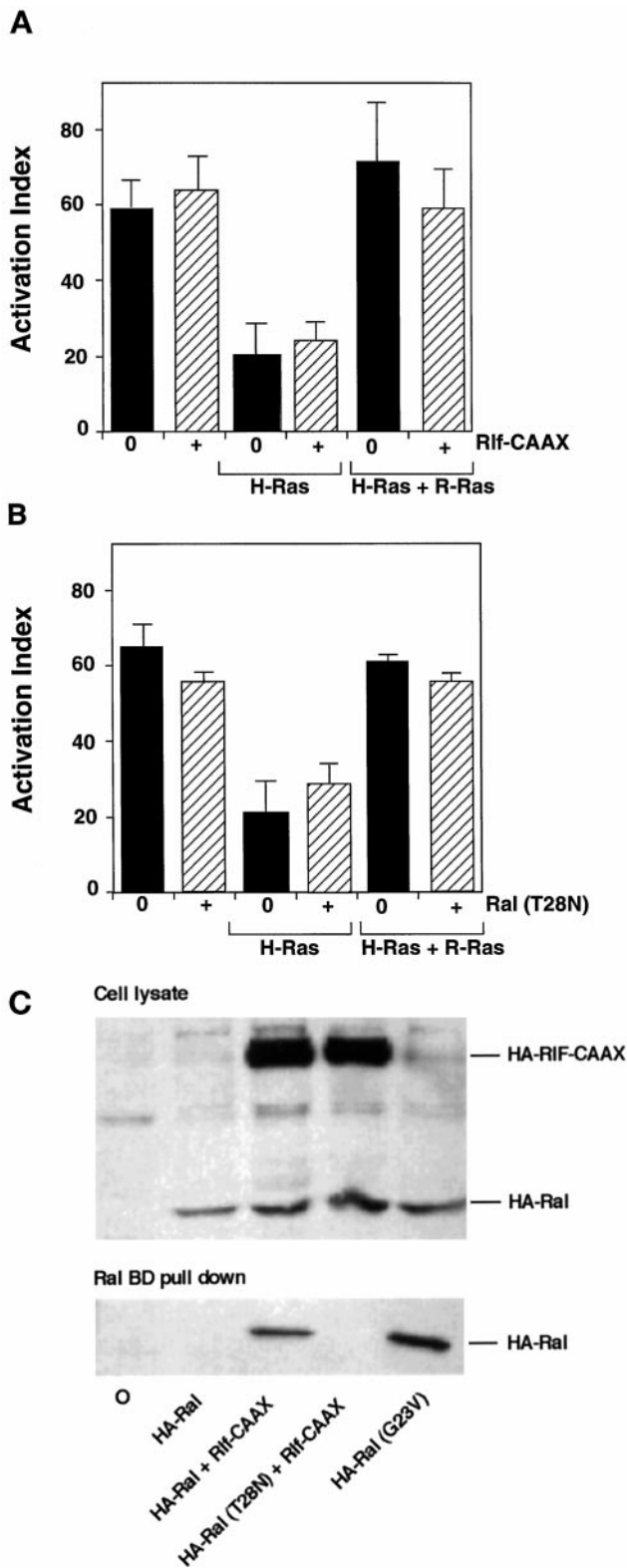


Figure 6. Ral activation does not mediate H-Ras- or R-Ras-dependent regulation of integrin affinity. (A) $\alpha\beta$ -py cells were cotransfected with expression vectors encoding H-Ras(G12V),

act in concert to regulate the ligand-binding affinities of integrins via the activation of specific H-Ras and R-Ras effectors.

The expression of an activated variant of the small GTP-binding protein R-Ras [R-Ras(G38V)] did not stimulate high-affinity ligand binding to either wild-type $\alpha_{IIb}\beta_3$ or an active $\alpha_{IIb}\beta_3$ chimera. These data were obtained by transfecting R-Ras(G38V) into CHO cells stably expressing $\alpha_{IIb}\beta_3$ and the chimeric integrin $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$. When expressed in CHO cells (A5 cells), the platelet-specific integrin $\alpha_{IIb}\beta_3$ is the low-affinity conformation, as measured by the binding of activation-specific ligands such as PAC1 and fibrinogen (O'Toole *et al.*, 1991). We found that the transfection of R-Ras(G38V) did not stimulate significant PAC1 binding to A5 cells, even though R-Ras(G38V) was well expressed. In agreement with our observation that R-Ras is not a direct activator of integrins in CHO cells, we also found that activated R-Ras failed to increase further PAC1 binding to CHO cells expressing the active integrin chimera $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$. We also found that R-Ras failed to stimulate PAC1 binding to parental CHO cells transiently transfected with $\alpha_{IIb}\beta_3$. This result is in contrast to that observed by Z. Zhang *et al.* (1996), who reported that activated R-Ras could stimulate PAC1 binding to CHO cells stably expressing $\alpha_{IIb}\beta_3$. It is possible that this apparent difference is caused by clonal variations in the CHO cell lines.

R-Ras(G38V) could reverse the suppressive effects of activated variants of both H-Ras and Raf-1. These results suggest that R-Ras could modulate integrin affinity by antagonizing the H-Ras/Raf-1-dependent suppressor pathway. The Ras GTPases function as molecular switches controlled by a GDP/GTP-binding cycle, binding downstream effectors only in the activated, GTP-bound conformation (Bos, 1997). R-Ras and the other Ras proteins have highly homologous effector-binding domains; consequently both GTP-bound R-Ras and H-Ras bind to several common effectors. Like H-Ras, R-Ras binds the p110 catalytic

Figure 6 (cont). R-Ras(G38V), or Rlf-CAAX in the combinations depicted on the *y*-axis. After 48 h, integrin activation was determined by PAC1 binding. Depicted are the activation indices \pm SE of three independent determinations. Immunoblot analysis of cell lysates demonstrated that HA-tagged Rlf-CAAX was well expressed in all conditions (our unpublished observations). (B) $\alpha\beta$ -py cells were cotransfected with expression vectors encoding H-Ras(G12V), R-Ras(G38V), or dominant-negative RalA(T28N) in the combinations depicted on the *y*-axis. After 48 h, integrin activation was determined by PAC1 binding. Depicted are the activation indices \pm SE of three independent determinations. (C) CHO cells were transfected with HA-RalA, HA-RalA(G23V), HA-RalA(T28N), and HA-Rlf-CAAX as indicated and grown in media containing 0.5% FCS before cell lysis. Ral-GTP was precipitated from the clarified cell lysates with glutathione-sepharose-bound GST-RalBD. Precipitated HA-Ral (bottom) and HA-Ral present in the cell lysate (top) were then identified by Western analysis using the anti-HA monoclonal 12CA5.

subunit of PI 3-kinase in vitro and induces an elevation in the levels of PI 3-kinase lipid products in vivo (Marte *et al.*, 1996). R-Ras also binds the Raf serine/threonine kinases and Ral-GDS, an exchange factor for the Ras-related Ral GTP-binding proteins (Spaargaren and Bischoff, 1994; Marte *et al.*, 1996). However, in contrast to Ras, R-Ras does not activate Raf or Ral-GDS in vivo (Marte *et al.*, 1996; Urano *et al.*, 1996). The observation that R-Ras can reverse the suppressive effect of activated H-Ras and its downstream effector kinase Raf-1 suggested that a possible mechanism for this effect of activated R-Ras is via a competition with H-Ras for common effectors. A precedent for such a model is illustrated by the Rap family of small GTP-binding proteins that have been reported to function as suppressors of Ras-mediated downstream signaling (Kitayama *et al.*, 1989; Zhang *et al.*, 1990; Cook *et al.*, 1993). The antagonism between Ras and Rap function seems to be attributable to the ability of Rap and Ras to interact with the same downstream effectors, so that the GTP-bound Rap sequesters Ras effectors in inactive complexes (Bos, 1997). For example, Rap1 can suppress the activation of the ERK MAP kinase via the inactivation of Raf-1, which occurs upon its association with Rap1 (Boussiotis *et al.*, 1997).

To explore this hypothesis further, we examined whether R-Ras needs to be in the activated, GTP-bound conformation to antagonize suppression mediated by activated H-Ras. We found that activated R-Ras(G38V) but not wild-type or the putative dominant-negative R-Ras(T43N) was able to reverse suppression. R-Ras(T43N) has a higher affinity for GDP than GTP, which indicates that this variant is in the inactive GDP-bound conformation and as such is unable to bind to downstream effectors (Huff *et al.*, 1997). This result is consistent with the hypothesis that activated R-Ras is mediating reversal either via a competition with H-Ras for a common effector or via the activation of a specific downstream effector that stimulates an integrin activation pathway.

The R-Ras rescue of H-Ras-mediated suppression was not caused by competition between H-Ras and R-Ras for the common downstream effector Raf-1. The inhibition of integrin activation by activated H-Ras and Raf-1 correlates with activation of the ERK MAP kinase pathway. We found that whereas R-Ras potentially reversed the suppressive effect of H-Ras and Raf, ERK2 activation induced by activated Raf and H-Ras was unaffected by cotransfection of activated R-Ras. This result indicates that R-Ras reversal is not caused by an inhibition of Ras- and Raf-induced MAP kinase activation. This result, combined with the observation that R-Ras can reverse suppression induced by an active Raf variant that lacks a Ras-binding domain, clearly demonstrates that R-Ras reversal is not a result of competition between H-Ras and R-Ras for Raf-1. We have shown previously that the MAP kinase

phosphatase-1 can reverse Ras- and Raf-mediated suppression (Hughes *et al.*, 1997). The observation that R-Ras reverses suppression without affecting MAP kinase activation demonstrates that reversal is not the result of R-Ras activating a MAP kinase phosphatase.

R-Ras reversal is not dependent on the activation of PI 3-kinase. GTP-bound R-Ras can bind to the p110 subunit of PI 3-kinase and stimulate the production of PI 3-lipids, demonstrating that PI 3-kinase is a downstream effector of R-Ras (Marte *et al.*, 1996). Studies on integrin function in platelets and leukocytes have identified a role for PI 3-kinase in regulating the activation of β_1 , β_2 , and β_3 integrins (Shimizu and Hunt, 1996; J. Zhang *et al.*, 1996). These observations combined with the fact that R-Ras can stimulate PI 3-kinase activity provided a possible explanation for the involvement of R-Ras in integrin activation. We used the PI 3-kinase inhibitor LY294002 to examine the role of PI 3-kinase in integrin affinity modulation. The inhibition of PI 3-kinase, as measured by the activation of the downstream effector Akt, had little effect on basal integrin affinity or on H-Ras-induced suppression and R-Ras rescue. Also, the overexpression of an activated variant of PI 3-kinase, p110-CAAX, did not reverse suppression by Raf-CAAX even though it was a potent activator of Akt (our unpublished observations). These data would appear to indicate that PI 3-kinase does not play a role in integrin affinity modulation, at least in CHO cells.

The small GTP-binding protein Ral is not involved in integrin affinity modulation in CHO cells. Both GTP-bound H-Ras and R-Ras can bind to GEFs for the small GTP-binding protein Ral; however, only H-Ras is capable of stimulating Ral-GEF activity in vivo (Urano *et al.*, 1996). This suggested that R-Ras may reverse suppression by competing with H-Ras for Ral-GEFs. To investigate this possibility, we examined the effect on integrin affinity modulation of both blocking and stimulating Ral activity by the coexpression of an activated Ral-GEF and a Ral dominant negative. Our results indicated that Ral does not contribute to the modulation of integrin affinity by H-Ras and R-Ras.

These data suggest that R-Ras and H-Ras mediate their opposing effects on integrin affinity via the activation of a distinct effector. Figure 7 illustrates a model that fits current data, demonstrating how R-Ras and H-Ras could act in concert to regulate integrin affinity. GTP-bound R-Ras could activate an effector that stimulates an undefined signaling pathway that impacts on the integrin suppressor pathway at a point downstream of MAP kinase, inactivating the integrin suppressor pathway. H-Ras can be activated via the dimerization of growth factor receptors and by the ligation and clustering of integrins. In both cases, Ras activation is mediated by the translocation of a complex between the adapter protein GRB2 and the Ras-GEF SOS to the plasma membrane.

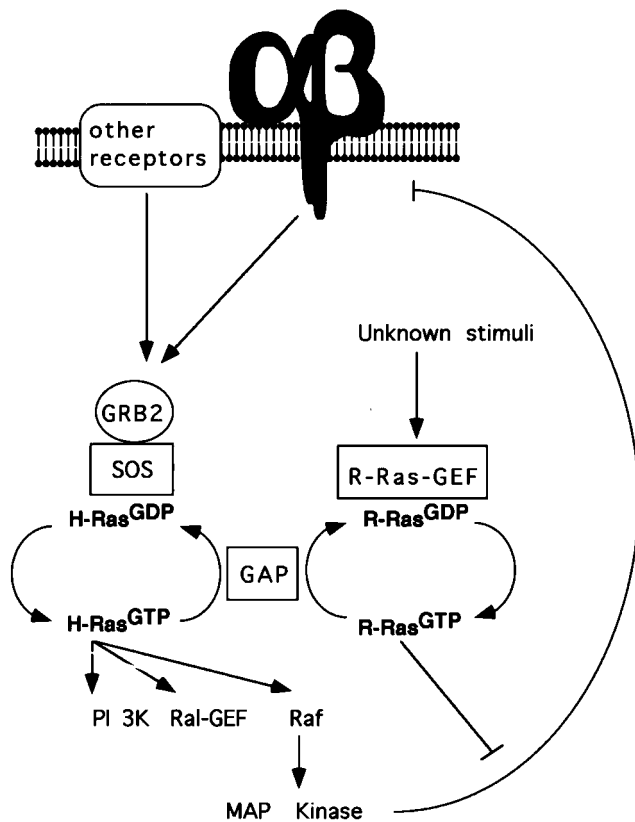


Figure 7. A hypothetical scheme of how the GTP-binding cycles of Ras and R-Ras could influence integrin affinity modulation. In this model, ligation of integrins and other cell surface receptors leads to the formation of the GRB2–SOS complex necessary for Ras activation. Ras can then activate its downstream effectors, including Raf-1, stimulating the MAP kinase–dependent negative feedback loop that can suppress activation of integrin. The activation of R-Ras, by as yet undefined stimuli, could then antagonize the Ras/Raf suppressor pathway downstream of MAP kinase.

In contrast to that of Ras, the stimuli that lead to the activation of R-Ras *in vivo* have yet to be fully defined. Recently, Ramos *et al.* (1998) have demonstrated that the overexpression of PEA-15, a small death effector domain–containing protein enriched in astrocytes, is able to reverse the suppressive effect of activated H-Ras. Significantly, the activity of PEA-15 is blocked by dominant-negative R-Ras (Ramos *et al.*, 1998), suggesting that the activation of endogenous R-Ras is capable of reversing H-Ras suppression. This observation suggests that PEA-15 may be a component of a signal transduction pathway that regulates the activity of R-Ras, and in the future it will be of interest to characterize the relationship between PEA-15 and R-Ras. In addition, there is a preliminary report suggesting that thrombin can induce a clear activation of R-Ras in megakaryoblasts (Bos, 1997). Until the stimuli and guanine-nucleotide exchange factors that activate R-Ras *in vivo* are identified, it will not be possible to

test the model outlined in Figure 7 and to define further the physiological role for R-Ras in integrin affinity modulation. It is possible that H-Ras and R-Ras are activated by distinct stimuli that induce either positive or negative effects on integrin affinity. Alternatively, the same stimuli may activate both H-Ras and R-Ras, with integrin affinity reflecting the ratio of the GTP-bound state of these two small G-proteins.

The deregulation of the MAP kinase pathway is often associated with oncogenic transformation. Unregulated activity of the MAP kinase–dependent integrin suppressor pathway can lead to the loss of the fibronectin matrix assembly and changes in integrin-dependent cell morphology, which may explain some of the integrin-dependent defects associated with the transformed phenotype. Indeed, such defects may account for the high metastatic potential of certain tumors. However, it is unclear whether these defects are primarily caused by the suppression of integrin activation or whether additional factors contribute to these phenotypes. Because R-Ras reverses H-Ras– and Raf-1–mediated suppression of integrin affinity, it will be of interest to determine whether the activation of R-Ras can also reverse these phenotypic defects.

ACKNOWLEDGMENTS

We thank Sandy Shattil and Martin Schwartz for their critical review of the manuscript and Rob Wolthuis for his help with the Ral activation assays. T.S. was supported by a Medical Research Council (United Kingdom) traveling fellowship. P.E.H. is the recipient of a senior fellowship from the Leukemia Society of America. M.H.G. is supported by grants from the National Institutes of Health. J.D. is supported by the Imperial Cancer Research Fund.

REFERENCES

- Baker, E.K., Tozer, E.C., Pfaff, M., Shattil, S.J., Loftus, J.C., and Ginsberg, M.H. (1997). A genetic analysis of integrin function: Glanzmann thrombasthenia *in vitro*. *Proc. Natl. Acad. Sci. USA* 94, 1973–1978.
- Bos, J.L. (1997). Ras-like GTPases. *Biochim. Biophys. Acta* 1333, 19–31.
- Boussiotis, V.A., Freeman, G.J., Berezovskaya, A., Barber, D.L., and Nadler, L.M. (1997). Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. *Science* 278, 124–148.
- Cook, S.J., Rubinfeld, B., Albert, I., and McCormick, F. (1993). RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. *EMBO J.* 12, 3475–3485.
- 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. *Oncogene* 9, 3281–3288.
- Fam, N.P., Fan, W.T., Wang, Z., Zhang, L., Chen, H., and Moran, M.F. (1997). Cloning and characterization of Ras-GRF2, a novel guanine nucleotide exchange factor for Ras. *Mol. Cell. Biol.* 17, 1396–1406.
- Garrett, M.D., Self, A.J., van Oers, C., and Hall, A. (1989). Identification of distinct cytoplasmic targets for ras/R-ras and rho regulatory proteins. *J. Biol. Chem.* 264, 10–13.

- Huff, S.Y., Quilliam, L.A., Cox, A.D., and Der, C.J. (1997). R-Ras is regulated by activators and effectors distinct from those that control Ras function. *Oncogene* 14, 133–143.
- Hughes, P.E., Diaz-Gonzalez, F., Leong, L., Wu, C., McDonald, J., 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.
- Hughes, P.E., and Pfaff, M. (1998). Integrin affinity modulation. *Trends Cell Biol.* 8, 359–364.
- Hughes, P.E., Renshaw, M.W., Pfaff, M., Forsyth, J., Keivens, V.M., Schwartz, M.A., and Ginsberg, M.H. (1997). Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. *Cell* 88, 521–530.
- Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69, 11–25.
- Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y., and Noda, M. (1989). A ras-related gene with transformation suppressor activity. *Cell* 56, 77–84.
- Lowe, D.G., Capon, D.J., Delwart, E., Sakaguchi, A.Y., Naylor, S.L., and Goeddel, D.V. (1987). Structure of the human and murine R-ras genes, novel genes closely related to ras proto-oncogenes. *Cell* 48, 137–146.
- Marte, B.M., Rodriguez-Viciana, P., Wennstrom, S., Warne, P.H., and Downward, J. (1996). R-Ras can activate the phosphoinositide 3-kinase but not the MAP kinase arm of the Ras effector pathways. *Curr. Biol.* 7, 63–70.
- 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., Loftus, J.C., Du, X.P., Glass, A.A., Ruggeri, Z.M., Shattil, S.J., Plow, E.F., and Ginsberg, M.H. (1990). Affinity modulation of the $\alpha_{IIb}\beta_3$ integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor. *Cell Regul.* 1, 883–893.
- O'Toole, T.E., Mandelman, D., Forsyth, J., Shattil, S.J., Plow, E.F., and Ginsberg, M.H. (1991). Modulation of the affinity of integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) by the cytoplasmic domain of α_{IIb} . *Science* 254, 845–847.
- Ramos, J.W., Kojima, T.K., Hughes, P.E., Fenczik, C.A., and Ginsberg, M.H. (1998). The death effector domain of PEA-15 is involved in its regulation of integrin activation. *J. Biol. Chem.* 273, 33897–33900.
- Rey, I., Harris-Taylor, P., van Earp, H., and Hall, A. (1994). R-ras interacts with rasGAP, neurofibromin and c-raf but does not regulate cell growth or differentiation. *Oncogene* 9, 685–692.
- Rodriguez-Viciana, P., Warne, P.H., Khwaja, A., Marte, B.M., Pappin, D.J., Das, P., Waterfield, M.D., Ridley, A., and Downward, J. (1997). Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* 89, 457–467.
- 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., and Hunt, S. (1996). Regulating integrin-mediated adhesion: one more function for PI 3-kinase? *Immunol. Today* 17, 565–573.
- Shou, C., Wurmser, A., Suen, K.L., Barbacid, M., Feig, L.A., and Ling, K. (1995). Differential response of the Ras exchange factor Ras-GRF to tyrosine kinase and G protein mediated signals. *Oncogene* 10, 1887–1893.
- Spaargaren, M., and Bischoff, J.R. (1994). Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-ras, H-ras, K-ras, and Rap. *Proc. Natl. Acad. Sci. USA* 91, 12609–12613.
- Spaargaren, M., Martin, G.A., McCormick, F.M., Fernandez, S.M., and Bischoff, J.R. (1994). The Ras-related protein R-ras interacts directly with Raf-1 in a GTP-dependent manner. *Biochem. J.* 300, 303–307.
- 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.
- Vojtek, A.B., Hollenberg, S.M., and Cooper, J.A. (1993). Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* 74, 205–214.
- Wolthuis, R.M., de Ruiter, N.D., Cool, R.H., and Bos, J.L. (1997). Stimulation of gene induction and cell growth by the Ras effector Rlf. *EMBO J.* 16, 6748–6761.
- Wolthuis, R.M., Franke, B., van Triest, M., Bauer, B., and Cool, R.H. (1998). Activation of the small GTPase Ral in platelets. *Mol. Cell Biol.* 18, 2486–2491.
- Yamamoto, T., Matsui, T., Nakafuku, M., Iwamatsu, A., and Kaibuchi, K. (1995). A novel GTPase-activating protein for R-Ras. *J. Biol. Chem.* 270, 30557–30561.
- Zhang, J., Zhang, J., Shattil, S.J., Cunningham, M.C., and Rittenhouse, S.E. (1996). Phosphoinositide 3-kinase γ and p85/phosphoinositide 3-kinase in platelets. Relative activation by thrombin receptor or β -phorbol myristate acetate and roles in promoting the ligand-binding function of $\alpha_{IIb}\beta_3$ integrin. *J. Biol. Chem.* 271, 6265–6272.
- Zhang, K., Noda, M., Vass, W.C., Papageorge, A.G., and Lowy, D.R. (1990). Identification of small clusters of divergent amino acids that mediate the opposing effects of ras and Krev-1. *Science* 249, 162–165.
- Zhang, Z., Vouri, K., Wang, H., Reed, J.C., and Ruoslahti, E. (1996). Integrin activation by R-ras. *Cell* 85, 61–69.