

R-Ras Signals through Specific Integrin α Cytoplasmic Domains to Promote Migration and Invasion of Breast Epithelial Cells

Patricia J. Keely,*[§] Elena V. Rusyn,^{‡§} Adrienne D. Cox,*^{‡§} and Leslie V. Parise*[§]

*Department of Pharmacology, [‡]Department of Radiation Oncology, and [§]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599

Abstract. Specificity and modulation of integrin function have important consequences for cellular responses to the extracellular matrix, including differentiation and transformation. The Ras-related GTPase, R-Ras, modulates integrin affinity, but little is known of the signaling pathways and biological functions downstream of R-Ras. Here we show that stable expression of activated R-Ras or the closely related TC21 (R-Ras 2) induced integrin-mediated migration and invasion of breast epithelial cells through collagen and disrupted differentiation into tubule structures, whereas dominant negative R-Ras had opposite effects. These results imply novel roles for R-Ras and TC21 in promoting a transformed phenotype and in the basal migration and polarization of these cells. Importantly, R-Ras induced an increase in cellular adhesion and migration on collagen but not fibronectin, suggesting that R-Ras signals to specific integrins. This was further supported by experiments in which R-Ras enhanced the migration of cells expressing integrin chimeras containing the $\alpha 2$,

but not the $\alpha 5$, cytoplasmic domain. In addition, a transdominant inhibition previously noted only between integrin β cytoplasmic domains was observed for the $\alpha 2$ cytoplasmic domain; $\alpha 2\beta 1$ -mediated migration was inhibited by the expression of excess $\alpha 2$ but not $\alpha 5$ cytoplasmic domain-containing chimeras, suggesting the existence of limiting factors that bind the integrin α subunit. Using pharmacological inhibitors, we found that R-Ras induced migration on collagen through a combination of phosphatidylinositol 3-kinase and protein kinase C, but not MAPK, which is distinct from the other Ras family members, Rac, Cdc42, and N- and K-Ras. Thus, R-Ras communicates with specific integrin α cytoplasmic domains through a unique combination of signaling pathways to promote cell migration and invasion.

Key words: integrins • Ras • cell migration • transformation • signaling

INTEGRINS are a family of α/β heterodimers that mediate cellular interactions with components of the extracellular matrix, resulting in regulation of cell differentiation, growth control, and cellular migration and invasion. Integrins participate in a number of signaling events in cells. Signaling events induced by the extracellular matrix through integrins, termed outside-in signaling, include activation of the mitogen-activated protein kinase (MAPK)¹ pathway, cytoplasmic tyrosine kinases such as focal adhesion kinase, phosphoinositide turnover, Ca^{2+} influx, Na^+/H^+ exchange, and alterations in gene expression and cellular growth (for review see Clark and Brugge, 1995; Schwartz et al., 1995; Yamada and Miyamoto, 1995;

Burridge and Chrzanowska-Wodnicka, 1996; Howe et al., 1998). Signaling events also impinge upon integrin function by regulating the ability of integrins to bind their ligands, a process termed inside-out signaling or integrin activation. Regulation of integrin–ligand interactions by inside-out signaling helps determine the nature of cellular responses to the extracellular matrix, influencing decisions such as whether a cell will remain stationary or migrate (Palecek et al., 1997). Integrin cytoplasmic domains, though relatively short, are necessary for their participation in both outside-in and inside-out signaling events. Despite much recent progress, the current understanding of integrin-related signaling events is still in its infancy.

One of the most intriguing, but poorly understood, aspects of integrin function is how specific integrin subunits link to specific signaling pathways to create the appropriate cellular response. A handful of studies have begun to address this question (Sastry et al., 1996; Wary et al., 1996; Wei et al., 1998). For example, differences in integrin effects have been correlated to the ability of certain integrin subunits ($\alpha 1\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 3$) but not others ($\alpha 2\beta 1$,

Address correspondence to Patricia Keely, Department of Pharmacology, 1106 ME Jones Building, CB 7365, University of North Carolina, Chapel Hill, NC 27599. Tel.: (919) 962-1058. Fax: (919) 966-5640. E-mail: pkeely@med.unc.edu

1. *Abbreviations used in this paper:* MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PI3K, phosphatidylinositol 3-kinase; PI4K, phosphatidylinositol 4-kinase; PKC, protein kinase C.

$\alpha 3\beta 1$, and $\alpha 6\beta 1$) to recruit Shc and activate the MAPK pathway (Wary et al., 1996). Moreover, integrin subunit specificity has been shown for association of integrins with transmembrane-4 superfamily proteins and activation of phosphatidylinositol 4-(PI4) kinase (Yauch et al., 1998). Although these studies begin to address specificity of outside-in signaling pathways, less well-studied is the specificity of inside-out signaling pathways.

Recently, it has become evident that both outside-in and inside-out integrin signaling pathways can involve small GTPases of the Ras superfamily (for review see Howe et al., 1998; Keely et al., 1998). Integrins signaling collaborates with growth factor-induced signaling through the Ras-MAPK pathway (Miyamoto et al., 1996; Lin et al., 1997; Renshaw et al., 1997), and can directly activate the MAPK pathway by both Ras-dependent and -independent means (P. Chen et al., 1994; Schlaepfer et al., 1994; Morino et al., 1995; Zhu and Assoian, 1995; Chen et al., 1996; Clark and Hynes, 1996; Miyamoto et al., 1996). Signaling through integrins also activates signaling pathways involving members of the Rho family of GTPases (Hotchin and Hall, 1995; Laudanna et al., 1996; Renshaw et al., 1996; Schwartz et al., 1996; Udagawa and McIntyre, 1996). The bidirectional nature of these signaling events is demonstrated by the finding that integrin clustering into focal complexes can be regulated by Rho family members, Rho, Rac, and Cdc42 (Hotchin and Hall, 1995; Nobes and Hall, 1995; Clark et al., 1998) or by Ras (Kinch et al., 1995). Additionally, activation of Rac and Cdc42 stimulates integrin-mediated migration and invasion across collagen matrices (Keely et al., 1997). Ras family members have direct effects on integrin function, since H-Ras suppresses integrin activation (Hughes et al., 1997), whereas R-Ras activates integrins, resulting in increased cell adhesion and matrix assembly (Zhang et al., 1996).

R-Ras is a 23-kD GTP-binding molecule ~55% homologous to H-, N-, and K-Ras, with an extra 26 amino acids at its NH₂ terminus (Lowe et al., 1987). Despite this homology, the biological function of R-Ras is distinct from H-Ras (Cox et al., 1994; Rey et al., 1994; Marte et al., 1996; Huff et al., 1997). R-Ras binds to many of the same effectors as H-, N-, or K-Ras, including Raf and Ral-GDS (Spaargaren and Bischoff, 1994; Spaargaren et al., 1994), but is unable to activate these effectors efficiently (Herrmann et al., 1996; Marte et al., 1996). However, R-Ras is able to activate the phosphatidylinositol 3-kinase (PI3K)-Akt/PKB pathway (Marte et al., 1996). The transforming properties of R-Ras remain under investigation, and are partially dependent on the specific cell in question (Lowe and Goeddel, 1987; Cox et al., 1994; Saez et al., 1994). TC21 (R-Ras2), the closest relative of R-Ras, is also implicated in cellular transformation of fibroblasts and human breast carcinomas (Clark et al., 1996; Graham et al., 1996), although TC21 shares more functional similarity to H-Ras than R-Ras (Huff et al., 1997). Other than PI3K activation, the signaling pathways and full range of biological effects downstream of R-Ras function are not known.

We previously showed that stable expression of constitutively active Cdc42 or Rac alters the response of breast epithelial cells to a collagen matrix by disrupting polarization and promoting a migratory invasive phenotype (Keely et al., 1997). The effect of Cdc42 and Rac is medi-

ated by PI3K (Keely et al., 1997). Additionally, the ability of these cells to polarize or migrate in collagenous matrices is related to $\alpha 2\beta 1$ integrin expression levels (Keely et al., 1995; Zutter et al., 1995). Since R-Ras has been shown to have direct effects on both integrin function and PI3K activity, we determined in this study whether R-Ras can alter breast cell response to the extracellular matrix. We find that R-Ras activation disrupts the polarized differentiation of breast epithelial cells in collagen matrices and stimulates migration and invasion through collagen, suggesting new roles for R-Ras. Additionally, we find that R-Ras specifically stimulates migration and adhesion to collagen but not fibronectin. Using integrin chimeras, we demonstrate cross-talk between R-Ras and the $\alpha 2$, but not the $\alpha 5$, integrin cytoplasmic tail, resulting in enhanced migration of breast epithelial cells. Surprisingly, R-Ras-induced migration was only partially dependent on PI3K activity, suggesting that PI3K-independent signaling pathways also contribute to the effects of R-Ras. We find that one of these pathways involves protein kinase C (PKC). Migration induced by R-Ras did not involve the MAPK kinase (MEK)/MAPK pathway, which differed from K-Ras-induced migration. Thus, R-Ras affects specific integrin α cytoplasmic domains through multiple distinct combinations of signaling pathways to activate cell migration and transformation.

Materials and Methods

Cell Lines

T47D cells (American Type Culture Collection) were transfected with constitutively active constructs for R-Ras(87L) or (38V), TC21(72L), N-Ras(12D), or K-Ras(12V), or with dominant negative constructs for R-Ras(41A) or (43N), or TC21(26A) that were expressed in pZIP. Cells were selected in G418 as described (Keely et al., 1995) and expanded as pools of stably transfected cells. Control cells were transfected with pZIP alone and selected in parallel with the other cells. Expression of constructs was determined by immunoblotting. Because these different molecules could not be probed by the same antibody on the same blot, it was impossible to determine absolute expression levels of R-Ras compared with TC21, N-Ras, or K-Ras. However, a comparison of each molecule to endogenous levels was made: R-Ras(87L) was 8.8-fold above endogenous R-Ras (or 8.8 \times), R-Ras(43N) = 10.7 \times , R-Ras(41A) = 1.8 \times , TC21(72L) = 4.0 \times , TC21(26A) = 1.0 \times , N-Ras(12D) = 0.9 \times , and K-Ras(12V) = 2.1 \times . For double transfectants to create X4 chimeric cell lines, cells were cotransfected with X4 chimeras expressed in pSFneo and pCGN (20:1), and selected in hygromycin-containing growth medium as described (Keely et al., 1997). Expression of $\alpha 4$ integrin on the surface of the cell, indicating expression of the X4 chimeras, was determined by flow cytometry (see below). X4 chimeras were a gift of Dr. Martin Hemler (Dana-Farber Cancer Institute).

Cell Migration, Invasion, and Morphogenesis

Migration and invasion across transwells were performed as described previously (Santoro et al., 1994; Keely et al., 1995, 1997). Transwells were coated from the underside with 6 μ g/ml collagen I or 25 μ g/ml fibronectin (Collaborative Biomedical Products). For studies with X4 chimeras, transwells were coated with specific $\alpha 4$ ligands: 6 μ g/ml of the 40-kD chymotryptic heparin binding fragment of fibronectin (GIBCO BRL) (McCarthy et al., 1986) or 50 μ g/ml of the CS-1 peptide (EILDVPSST; Peninsula Labs Inc.) (Humphries et al., 1987). CS-1 peptide was coupled to ovalbumin as described (Haugen et al., 1990) and the best coating concentration for cell migration was determined. Cell migration was performed in the absence of serum or growth factors for 16 h. For inhibitor assays, cells were pretreated for 15 min with DMSO (control), Wortmannin (30 nM), LY294002 (25 μ M), bisindolylmaleimide (1 μ M) all from Calbiochem-Novabiochem Corp., or PD98059 (25 μ M; Alexis Biochemicals), and al-

lowed to migrate across collagen in the continued presence of the inhibitor for 5 h.

For antibody inhibition assays, cells were preincubated with control IgG or anti- $\alpha 2$ integrin antibody, P1E6 (GIBCO BRL) at a final concentration of 1:1,000. Migration was performed in the continued presence of the antibody for 16 h. Invasion was assessed on serum starved cells that were seeded onto the top of 2-mm collagen gels and allowed to migrate in response to 10% serum placed in the lower chamber. Invasion assays were allowed to proceed for 26–36 h. Unless otherwise noted, values presented are the mean \pm SEM of at least three experiments normalized to control values. Data were analyzed by performing ANOVA using Microsoft Excel software. Morphogenesis in three-dimensional collagen gels was assessed after 7 and 16 d as described (Santoro et al., 1994; Keely et al., 1995).

Adhesion Assays

Adhesion to Immobilon plates (Dynatech) coated with various concentrations of collagen I or fibronectin was performed and adherent cells quantitated by hexosaminidase activity as described (Haugen et al., 1990; Keely et al., 1995). Values given represent the average of two separate experiments, each of triplicate determinations, \pm SEM.

Flow Cytometry

Cells were detached in versene, washed in PBS containing 5 mg/ml BSA, and incubated with primary antibody on ice. To determine cell surface integrin expression, cells were incubated with the following mAbs: P4C2 against the $\alpha 4$, P1D5 against the $\alpha 5$, P1E6 against the $\alpha 2$, or P1B5 against the $\alpha 3$ subunits (all from GIBCO BRL), or TS2-7 against the $\alpha 1$, or 4F10 against the $\alpha 6$ subunits (Serotec). Primary antibody was diluted 1:1000. After a 20-min incubation, cells were washed once in PBS/BSA, and incubated with FITC-conjugated donkey anti-mouse secondary antibody at 1:100 (Jackson Labs) for 20 min on ice. Cells were washed again and analyzed on a Beckman FACScan[®]. For conformation studies, cells were either treated with EDTA or with manganese at 2 mM before incubation with 12G10 antibody (Serotec), which detects conformational changes in the $\beta 1$ integrin subunit. After this primary antibody incubation, analysis proceeded as above.

PKC Kinase Assay

Cells were pretreated with DMSO or bisindolylmaleimide (1 μ M) for 20 min, lysed in buffer containing 0.05% Triton-X 100, and the *in vitro* activity of PKC was determined on crude lysates using the PKC assay system (SignaTECT; Promega Corp.), according to manufacturer's directions. In brief, a single purification step over a DEAE cellulose column was used to generate a crude PKC-containing fraction. Background activity (nonspecific) was determined in the absence of phosphatidylserine and diacylglycerol, and compared with PKC activity determined in the presence of phosphatidylserine and diacylglycerol.

MAPK Activity

Cells expressing R-Ras(87L), R-Ras(38V), TC21(72L), K-Ras(12V), N-Ras(12D), or control vectors were cultured overnight either in the presence or absence of serum, treated for 20 min with DMSO (control) or PD98059 (25 μ M), and lysed. Lysates were electrophoresed, immunoblotted with anti-p42/44 ERK antibody (Santa Cruz Biotech), and the activation of MAPK was determined by gel-shift (Cox et al., 1994). Determination of the mean image density was made using NIH image software.

Results

R-Ras or TC21 (R-Ras2) Expression Affects the Differentiation of Mammary Epithelial Cells in Three-dimensional Collagen Gel Culture

Well differentiated human breast carcinoma T47D cells maintain the ability to polarize into tubule and ductlike structures when cultured in three-dimensional collagen gels (Keely et al., 1995). This differentiation is dependent on the $\alpha 2\beta 1$ integrin (Keely et al., 1995; Zutter et al.,

1995). One of the characteristics of T47D differentiation into tubules is the multicellular nature of the structure, such that individual cell-cell boundaries become more difficult to distinguish, as can be noted in Fig. 1 A. Stable expression of activated R-Ras(87L) in T47D cells disrupted the ability of these cells to differentiate and polarize in collagen gel culture, instead the cells grew as disorganized clumps and sheets of individual, nonpolarized cells (Fig. 1 B). Similar results were obtained with cells stably expressing another activated R-Ras isoform, R-Ras(38V) (not shown). Thus, activation of R-Ras results in a less differentiated, more transformed phenotype. In contrast, stable expression of dominant negative R-Ras(43N) enhanced differentiation; tubule structures developed earlier and were more extensive (Fig. 1 D). This suggests that endogenous R-Ras may play a negative role in regulating the rate or extent of normal epithelial differentiation. Like R-Ras, expression of constitutively active TC21(72L) disrupted differentiation in collagen gels (Fig. 1 C), whereas expression of dominant negative TC21(26A) enhanced differentiation in a qualitative manner (Fig. 1 E). This suggested that TC21, like R-Ras, is a negative regulator of breast cell differentiation.

R-Ras and TC21 Stimulate Migration and Invasion Specifically across Collagen

Breast carcinoma progression is accompanied by the loss of polarization and differentiation, and the acquisition of a migratory invasive phenotype. Migration was determined using a Boyden chamber haptotactic assay in which transwells were coated on the underside with collagen. Expression of activated R-Ras isoforms, R-Ras(87L) or R-Ras(38V), significantly enhanced cell migration relative to control cells (Fig. 2 a), indicating that R-Ras activation promotes a more migratory phenotype. Dominant negative R-Ras(41A) inhibited migration by \sim 45–50% (Fig. 2 a), suggesting a contribution of R-Ras to basal migratory mechanisms. Activated TC21(72L) also enhanced cell migration, albeit to a somewhat lesser extent than R-Ras (Fig. 2 a). Expression of activated isoforms of other Ras family members, N-Ras(12D) and K-Ras(12V), also enhanced cell migration across collagen, but to a lesser extent than R-Ras (Fig. 2 a).

The migration induced by R-Ras(87L) across collagen-coated filters was dependent on the $\alpha 2\beta 1$ integrin, since it could be completely inhibited by anti- $\alpha 2\beta 1$ blocking antibodies (Fig. 2 b). Similarly, TC21(72L)-induced cell migration was also $\alpha 2\beta 1$ integrin-dependent (Fig. 2 b). These results are consistent with the finding that the $\alpha 2\beta 1$ integrin is a major collagen receptor in breast epithelial cells that mediates many of their responses to collagenous matrices (Keely et al., 1995; Zutter et al., 1995).

To examine whether the effect of R-Ras was specific for collagen, cell migration across fibronectin was determined. Surprisingly, expression of R-Ras(87L) or R-Ras(38V) did not enhance and, in fact, partially inhibited migration across fibronectin by \sim 40% (Fig. 2 c). R-Ras(87L) did not enhance or inhibit migration of cells across laminin (not shown). Similar results were obtained for TC21(72L), which also inhibited migration across fibronectin by 25%, although this difference was not statistically significant

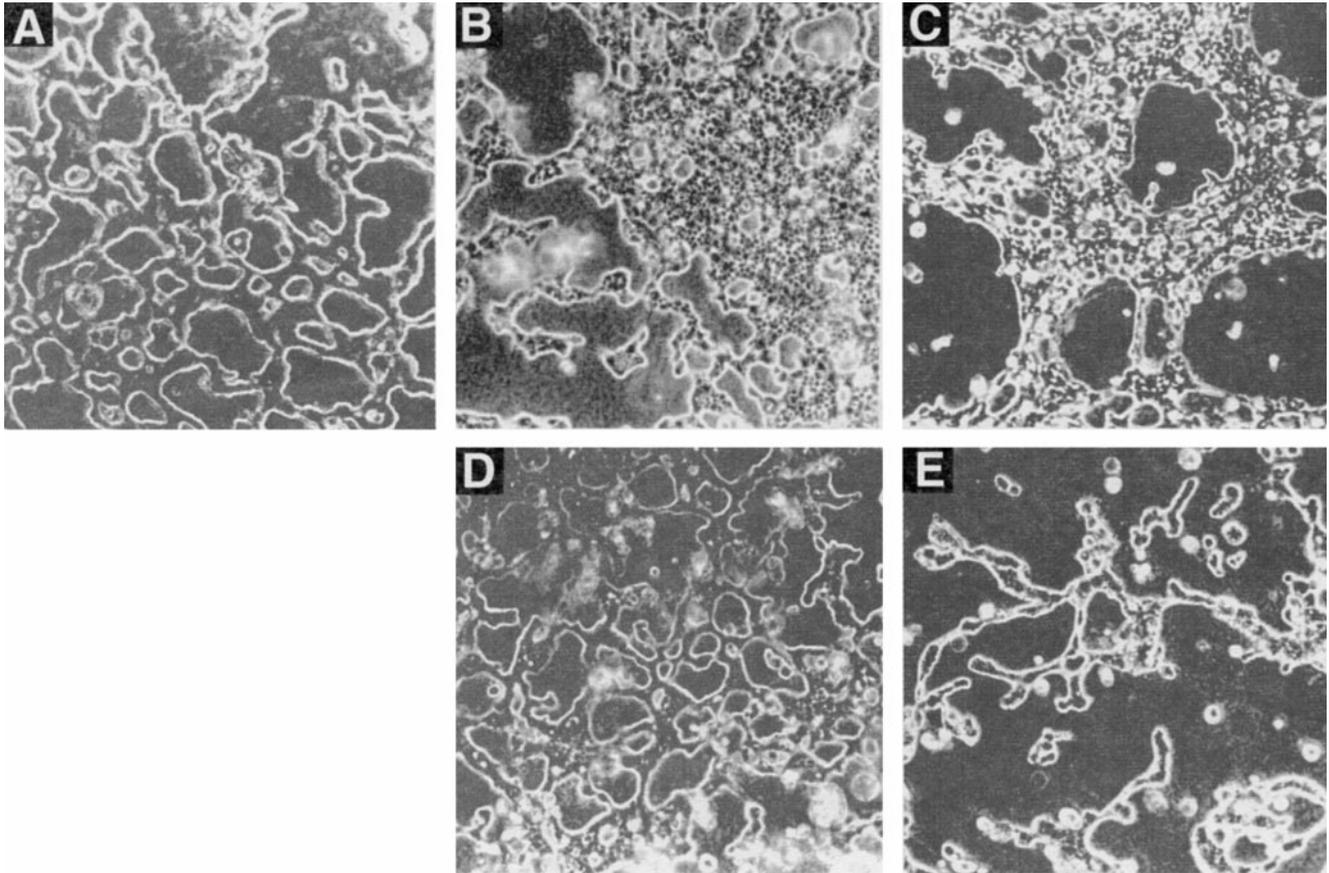


Figure 1. Expression of activated R-Ras or TC21 (R-Ras 2) disrupts the organized polarization of breast epithelia. Cells were cultured in three-dimensional collagen gels and the formation of polarized tubule structures was assessed by phase-contrast microscopy. Cells were transfected with: (A) vector only, (B) constitutively active R-Ras(87L), (C) constitutively active TC21(72L), (D) dominant negative R-Ras(43N), or (E) dominant negative TC21(26A). Constitutively active R-Ras and TC21 disrupt tubule formation, whereas dominant negative R-Ras and TC21 enhance formation in a qualitative manner.

(Fig. 2 c). The difference between migration across collagen and fibronectin is not due to dramatically different levels of migration, since similar numbers of control cells migrate across each substratum, as shown by a representative experiment in Table I. In contrast to the results with R-Ras and TC21, expression of either N-Ras(12D) or K-Ras(12V) enhanced migration across fibronectin (Fig. 2 c). Thus, R-Ras and TC21 differ from N- and K-Ras in the specificity of substrata on which they enhance cell migration.

To metastasize, cells must not only migrate but must also invade through extracellular matrices. Therefore, we examined the ability of transfected cells to invade through

a 2-mm-thick collagen gel in response to a serum gradient. Consistent with their more migratory phenotype, cells expressing activated R-Ras(87L) or R-Ras(38V) were significantly more invasive through collagen than control cells (Fig. 3). Similarly, activated TC21(72L), N-Ras(12D), and K-Ras(12V) induced cell invasion (Fig. 3). Because basal invasion levels of T47D cells are already so low, we did not further investigate the effect of dominant negative R-Ras or TC21 on invasion. Therefore, we find that expression of activated R-Ras or TC21 converts cells from a polarized, differentiated phenotype into a migratory and invasive phenotype.

R-Ras Specifically Affects the $\alpha 2$ Integrin Cytoplasmic Domain

Next, we examined the effect of R-Ras on adhesion of cells to collagen and fibronectin since R-Ras activation specifically enhanced cell migration across collagen, but decreased migration across fibronectin. This was of particular interest since it has been shown that expression of activated R-Ras(38V) increases the adhesion of myeloid cells to fibronectin and vitronectin through activation of integrin subunits (Zhang et al., 1996). Similar to these re-

Table I. Migration of Cells Expressing Activated R-Ras and TC21 on Collagen and Fibronectin

	Collagen*	Fibronectin*
Control	93 (\pm 3)	121 (\pm 8)
R-Ras(87L)	246 (\pm 21)	50 (\pm 4)
TC21(72L)	255 (\pm 16)	58 (\pm 5)

*Average number of cells per microscopic field for a representative experiment from Fig. 2. Values represent an average of 6 fields \pm SD.

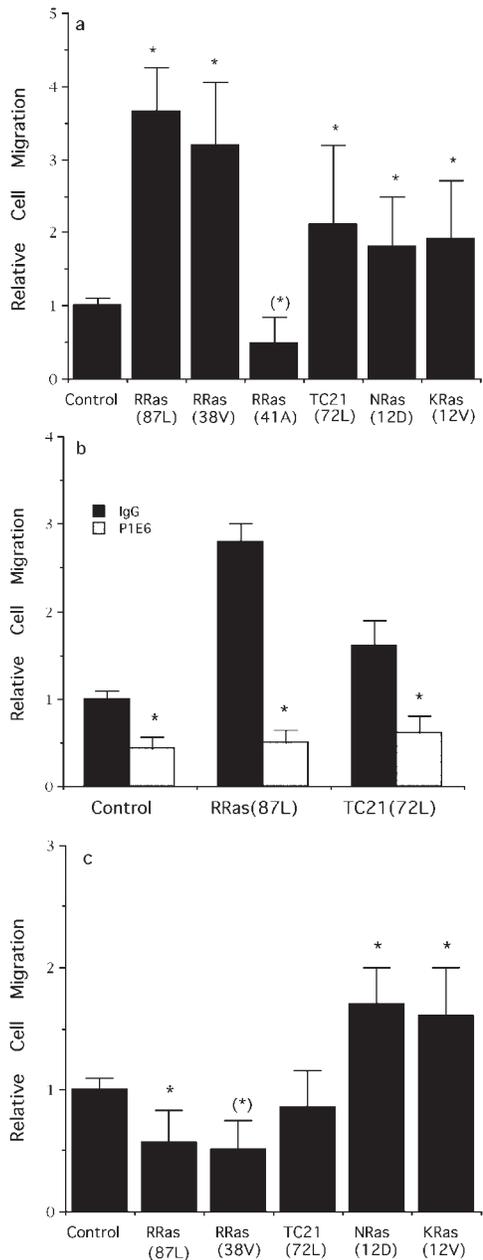


Figure 2. R-Ras and TC21 stimulate integrin-mediated cell migration across collagen but not fibronectin. (a) Migration of cells transfected with Ras family members across collagen I. Expression of constitutively active R-Ras(87L) and R-Ras(38V) stimulate cell migration, whereas dominant negative R-Ras(41A) inhibits migration. Expression of constitutively active isoforms of Ras family members, TC21(72L), N-Ras(12D), and K-Ras(12V) also stimulate cell migration. Haptotactic cell migration was assayed in the absence of serum across filters coated from the underside with collagen I. Values are relative to migration of cells expressing vector alone: * $P < 0.05$, compared with control cells; (*) $P < 0.1$. (b) Migration across collagen is mediated by the $\alpha 2\beta 1$ integrin. Cells were preincubated with control IgG or P1E6, an anti- $\alpha 2$ integrin antibody, and allowed to migrate across filters coated with collagen I. P1E6 significantly inhibits migration of control cells as well as cells expressing activated R-Ras(87L) or TC21(72L). * $P < 0.05$ for P1E6 compared with control IgG. (c) Cell migration across fibronectin. Expression of constitutively active R-Ras(87L) or (38V) decreases migration, whereas constitutively active TC21(72L) does not. In contrast, expression of

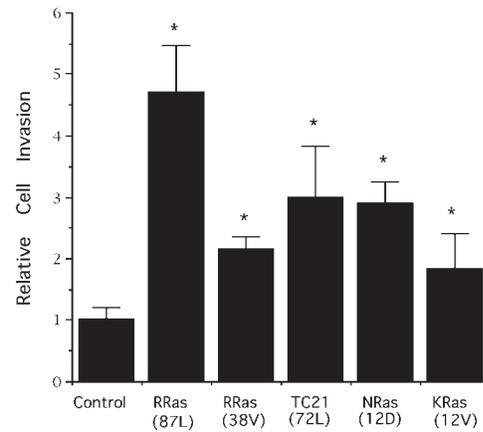


Figure 3. R-Ras and Ras family members stimulate invasion. Cells expressing constitutively active R-Ras(87L) or (38V), TC21(72L), N-Ras(12D), or K-Ras(12V) are significantly more invasive across collagen gels in response to a serum gradient. Values are relative to cells transfected with vector alone (control). * $P < 0.05$ compared with control invasion.

sults, we found that expression of activated R-Ras(87L) enhanced adhesion to collagen by a modest but consistent amount (Fig. 4 a), demonstrating an effect of R-Ras activation on the avidity of cells for collagen. Dominant negative R-Ras(41A) decreased adhesion to collagen (Fig. 4 a) by a similar amount, demonstrating a contribution of endogenous R-Ras to cell adhesion. The other dominant negative isoform of R-Ras, R-Ras(43N), did not affect cell adhesion (not shown). Expression of TC21 isoforms had no consistent or significant effect on adhesion of cells to collagen (not shown). In contrast to the effect on cell adhesion to collagen, expression of R-Ras(87L) or R-Ras(41A) did not affect cell adhesion to fibronectin (Fig. 4 b). This is in contrast to the results of Zhang et al. (1996) who found increased adhesion of myeloid cells to fibronectin substrata. This suggests that there are cell-type specific differences in the effects of R-Ras on different integrin subunits.

To determine whether changes in cell migration and adhesion could be explained by altered expression of endogenous integrin subunits, integrin α subunit expression was examined by flow cytometry. Cells expressing activated R-Ras(87L) or dominant negative R-Ras(41A) or R-Ras(43N) showed no change in the expression of integrin subunits $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, or $\alpha 6$ (not shown). Additionally, there were no changes noted in expression of $\alpha 4$ (not shown), an integrin subunit not normally expressed in these cells. Therefore, R-Ras-induced changes in cell migration and adhesion are not due to changes in integrin subunit expression.

To examine if an apparent change in integrin avidity was reflected in a detectable change in integrin conformation,

constitutively active N-Ras(12D) or K-Ras(12V) stimulates migration across fibronectin. * $P < 0.05$, (*) $P < 0.1$, compared with control cells transfected with vector alone.

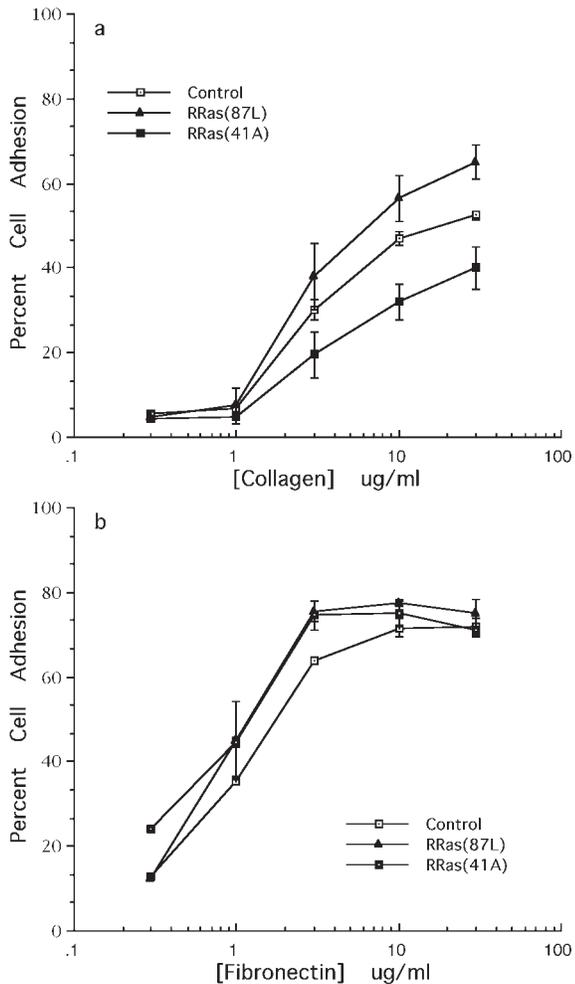


Figure 4. Activated R-Ras enhances adhesion to collagen but not fibronectin. (A) Cells expressing activated R-Ras(87L) (triangles) are more adherent to collagen I than control cells expressing vector alone (open squares). In contrast, cells expressing dominant negative R-Ras(41A) (filled squares) are less adherent to collagen I. (b) Cells expressing activated or dominant negative R-Ras are no different from control cells in their adhesion to fibronectin. Values in a and b are an average of two separate experiments, each of triplet determinations, \pm SD.

we used a conformationally sensitive anti- β 1 integrin antibody, 12G10. The 12G10 epitope is known to be expressed on integrins that are activated by manganese. We found no increase in 12G10 binding, as determined by flow cytometry, in cells expressing R-Ras(87L) compared with control cells (not shown). This result suggests that changes in apparent integrin avidity because of R-Ras activation either do not correlate to conformational changes, or that R-Ras changes integrin conformation in a manner not detected by this antibody.

The R-Ras-enhanced migration and adhesion of cells to collagen via α 2 β 1, but not fibronectin via α 5 β 1, suggests that, in these cells, R-Ras affects specific integrin subunits. Since the β subunits are identical, we speculated that R-Ras specifically signals to the α 2 cytoplasmic domain. To test this hypothesis, the effects of R-Ras on a chimeric

integrin containing the α 2 cytoplasmic domain were compared with its effects on a similar chimera containing the α 5 cytoplasmic domain. Chimeric integrins with the extracellular domain of the α 4 subunit and the cytoplasmic domain of either the α 2 subunit (X4C2) or the α 5 subunit (X4C5) were transfected into control and R-Ras(87L)-expressing cells. Some cells were instead transfected with the complete α 4 subunit (X4C4). We selected α 4 integrin chimeras since T47D cells normally do not express the α 4 subunit (Fig. 5 A, mock profile) which allowed us to monitor the expression of the chimeras in our control and R-Ras-transfected cells. Each of the α 4 chimeras was expressed at similar levels on the surface of control and R-Ras-expressing cells (Fig. 5, A–F). Since individual integrin α subunits are not expressed on the cell surface, this indicates that α 4 β 1 heterodimers were formed. Importantly, expression of α 4 chimeras did not affect the expression of endogenous α 2 (Fig. 5, G and H) or α 5 subunits (not shown).

The ligand for the α 4 β 1 integrin is the CS-1 sequence found in the COOH-terminal 40-kD heparin-binding fragment of fibronectin (McCarthy et al., 1986; Humphries et al., 1987). This 40-kD fragment does not contain the RGD sequence that is the ligand for the α 5 β 1 integrin in fibronectin. Therefore, to specifically assess the effects of R-Ras expression on the X4 chimeras, cell migration across membranes coated with either the CS-1 peptide or the 40-kD fragment was determined, since untransfected T47D cells do not migrate across these substrata (not shown). We found that expression of the X4 chimeras in T47D cells conferred the ability to migrate on the 40-kD fragment and the CS-1 peptide (Fig. 6 a). Interestingly, R-Ras(87L) enhanced migration of cells expressing X4C2 and decreased migration of cells expressing X4C5 (Fig. 6 a). This is consistent with our finding that R-Ras enhances migration across collagen and decreases migration across fibronectin (Fig. 2). R-Ras(87L) also enhanced migration of X4C4-expressing cells, suggesting that R-Ras not only signals to the α 2, but also the α 4 cytoplasmic domains in breast epithelial cells (Fig. 6 a). Cells expressing an α 4 chimera containing no α cytoplasmic domain, X4C0, were unable to migrate across the 40-kD and CS-1 substrata (not shown), indicating the importance of an intact α cytoplasmic domain for cell migration. These results suggest that R-Ras activation enhances α 2 β 1 integrin function and diminishes α 5 β 1 integrin function, at least with regard to cell migration.

We also determined whether cells expressing α 4 chimeras had altered migration across collagen. Since the α 4 chimeras do not bind collagen, any effect will be due to pleiotropic effects on endogenous integrin subunits. Expression of X4C2, but not X4C4 or X4C0, blocked the increase in migration of cells across collagen that is induced by activated R-Ras(87L) (Fig. 6 b), suggesting that there is some form of competition between the X4C2 subunit and the endogenous α 2 subunit. Thus, the effects of R-Ras on endogenous α 2 subunits can be antagonized specifically by de novo expression of α 2 cytoplasmic domains. Such dominant negative effects of integrin cytoplasmic domains have been noted previously with expression of exogenous β cytoplasmic domains (Y.P. Chen et al., 1994; LaFlamme et al., 1994; Lukashev et al., 1994). However, this is the first

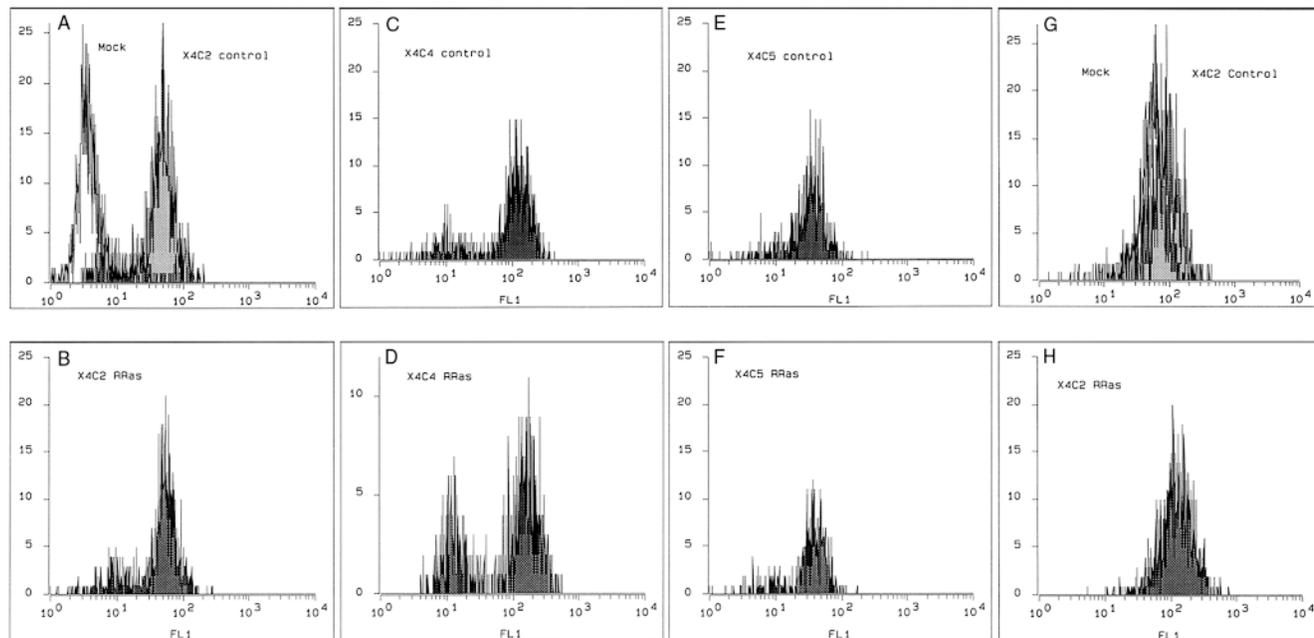


Figure 5. Flow cytometry of cells expressing $\alpha 4$ integrin chimeras. (A–F) De novo expression of $\alpha 4$ integrin on the surface of control or R-Ras(87L)-expressing cells double-transfected with $\alpha 4$ chimeras. Chimeras consist of the extracellular domain of the $\alpha 4$ subunit and the cytoplasmic domain of $\alpha 2$ (X4C2), $\alpha 4$ (X4C4), or $\alpha 5$ (X4C5). All cell lines expressed similar levels of the X4 chimeras as determined by surface $\alpha 4$ integrin expression. (A) Control cells transfected with X4C2 express $\alpha 4$ on their surface (shaded histogram). In contrast, mock-transfected cells, like untransfected cells, do not express $\alpha 4$ on their surface (open histogram). (B) R-Ras(87L) cells transfected with X4C2 express similar levels of $\alpha 4$ as control X4C2 cells in A. (C) Control cells transfected with X4C4. (D) R-Ras(87L) cells transfected with X4C4. (E) Control cells transfected with X4C5. (F) R-Ras(87L) cells transfected with X4C5. (G and H) Cell surface expression of $\alpha 2\beta 1$ integrin does not change in cells transfected with X4C2. (G) Control cells transfected with X4C2 (shaded histogram) have similar levels of $\alpha 2$ expression on their surface as mock-transfected cells (open histogram). (H) R-Ras(87L) cells transfected with X4C2 also have similar $\alpha 2$ expression levels.

demonstration of transdominant inhibition by a specific α integrin subunit. This suggests that there may be competition for a limited supply of α cytoplasmic domain binding factors. Conversely, X4C5 expression slightly enhanced the R-Ras(87L)-induced migration across collagen (Fig. 6 b), although this enhancement is not statistically significant when compared to the results obtained with cells expressing X4C4 or X4C0. These results provide additional evidence that R-Ras signaling involves specific integrin subunits.

PI3K and PKC Contribute to R-Ras- and TC21-induced Cell Migration

We previously found that migration induced by the small GTPases, Rac and Cdc42, was dependent on PI3K (Keely et al., 1997). Like Rac and Cdc42, R-Ras also has been shown to activate PI3K (Marte et al., 1996). Therefore, we determined whether pharmacological inhibitors of PI3K, Wortmannin, and LY294002, could inhibit the migration induced by R-Ras. Wortmannin (30 nM) and LY294002 (25 μ M) significantly inhibited the migration induced by activated R-Ras(87L) or R-Ras(38V) (Fig. 7 a). However, unlike the total inhibition previously noted with Rac and Cdc42-expressing cells (Keely et al., 1997), the effect of PI3K inhibition on R-Ras-induced migration was only partial; migration was inhibited only by $\sim 50\%$. Wortman-

nin and LY294002 also only partially inhibited the migration induced by activated TC21(72L) (Fig. 7 a). Thus, PI3K activity contributes to R-Ras-induced cell migration, but other signaling pathways are probably also involved.

PKC has been implicated in the migration of some cell types (Vuori and Ruoslahti, 1993; Derman et al., 1997; Battle et al., 1998). Pretreatment of cells with 1 μ M bisindolylmaleimide, an inhibitor of most isoforms of PKC, significantly inhibited the migration of R-Ras(87L) or R-Ras(38V)-expressing cells (Fig. 7 b). Once again, inhibiting PKC produced only partial inhibition of R-Ras-induced cell migration. Bisindolylmaleimide also partially inhibited the migration of cells expressing TC21(72L) and K-Ras(12V), albeit to an even lesser extent than the effect on R-Ras cells (not shown). The concentration of bisindolylmaleimide used was adequate, since pretreatment of cells with this concentration inhibited PKC activity to background levels in an in vitro PKC kinase assay (data not shown). Since certain isoforms of PKC (δ , ϵ , and η) can be activated downstream of PI3K (Toker et al., 1994; Derman et al., 1997), we wished to determine if PKC and PI3K might be part of the same signaling pathway leading to cell migration, or separate pathways that both contribute to migration. Treatment of cells with Wortmannin and bisindolylmaleimide had an additive effect and completely inhibited the increased migration induced by R-Ras ex-

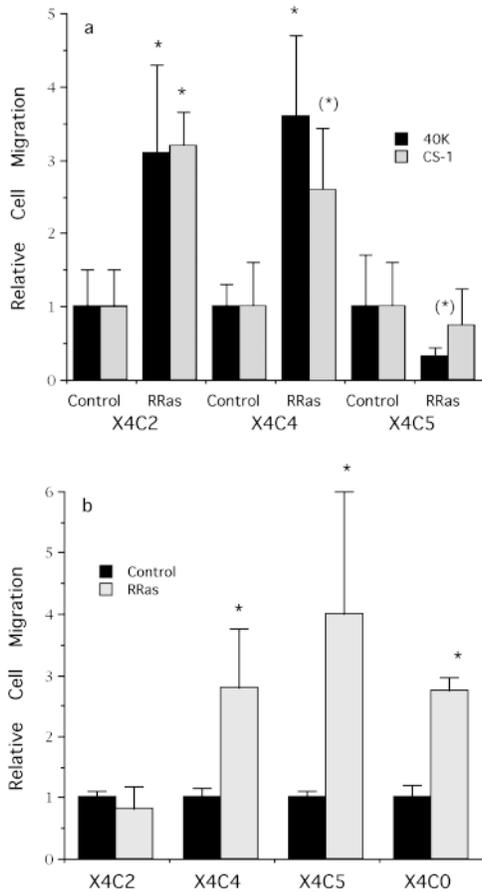


Figure 6. R-Ras specifically enhances migration through effects on the $\alpha 2$ integrin cytoplasmic domain. (a) Migration of cells across ligands specific for the $\alpha 4\beta 1$ integrin: the 40-kD fragment of fibronectin or the CS-1 peptide. Cell lines expressing control vector or activated R-Ras(87L) were double-transfected with $\alpha 4$ chimeras that consist of the extracellular domain of the $\alpha 4$ subunit and the cytoplasmic domain of $\alpha 2$ (X4C2), $\alpha 4$ (X4C4), or $\alpha 5$ (X4C5). R-Ras(87L) enhances migration only for cells expressing the X4C2 or X4C4 chimeras, but not cells expressing the X4C5 chimera. Values are normalized for each chimera set and substratum to control values. * $P < 0.05$ compared with control cells for that chimera. (b) Migration of cells expressing X4 chimeras across collagen I, which is not a ligand for the $\alpha 4$ extracellular domain with no cytoplasmic domain) did not inhibit R-Ras(87L)-induced cell migration across collagen (i.e., R-Ras still induces a statistically significant increase in the migration of these cells). Values are normalized for each chimera set. * $P < 0.05$ compared with control values (solid bars) for that chimera. Expression of X4C5 actually increases the effect of R-Ras, but this is not statistically significant, when compared with X4C4, or X4C0-expressing cells.

pression (Fig. 7 b), suggesting that PI3K and PKC each contribute to R-Ras-induced cell migration through separate pathways.

Other pharmacological inhibitors of signaling pathways potentially downstream of R-Ras were also tested. The MEK inhibitor, PD98059 (Dudley et al., 1995), had no effect on migration induced by activated R-Ras or TC21

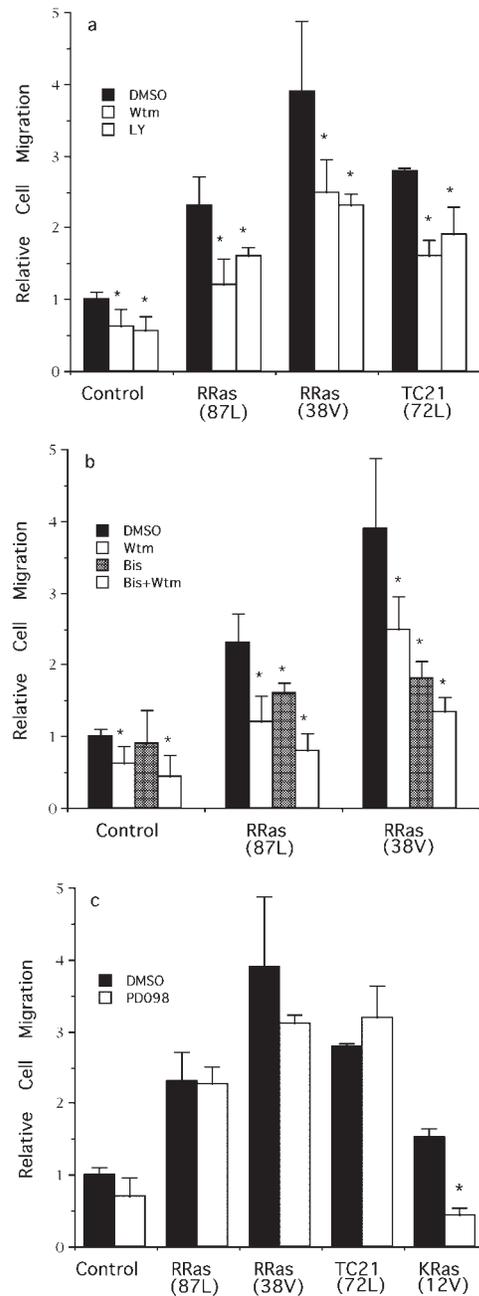


Figure 7. PI3K and PKC contribute to R-Ras induced cell migration. (a) Pretreatment with PI3K inhibitors Wortmannin (Wtm, 30 nM) or LY294002 (LY, 25 μ M) partially inhibits the migration of cells expressing activated R-Ras(87L) or (38V), or TC21(72L) across collagen. * $P < 0.05$ compared with DMSO-treated samples for each cell line shown. (b) Pretreatment of cells with the PI3K inhibitor, Wortmannin (Wtm, 30 nM), or the PKC inhibitor, bisindolylmaleimide (Bis, 1 μ M), or both. Wortmannin and bisindolylmaleimide alone each partially inhibits R-Ras-induced migration of cells across collagen. Together, Wortmannin and bisindolylmaleimide have an additive effect and completely inhibit R-Ras-induced migration. * $P < 0.05$ compared with DMSO-treated samples for each cell line. (c) Pretreatment of cells with the MEK inhibitor, PD98059 (PD98, 25 μ M) has no effect on the migration across collagen of cells expressing activated R-Ras(87L) or (38V) or TC21(72L). In contrast, PD98059 completely inhibits migration induced by activated K-Ras(12V). * $P < 0.05$ compared with DMSO-treated samples for each cell line.

(Fig. 7 c), despite inhibiting MAPK activation 50–60% in cells expressing R-Ras(38V), R-Ras(87L), or TC21(72L) (data not shown). In sharp contrast, PD98059 significantly inhibited K-Ras-induced cell migration (Fig. 7 c), demonstrating important differences in the mechanism by which R-Ras and K-Ras induce migration. Rapamycin, an inhibitor of p70 S6 kinase, also did not inhibit cell migration (not shown), which is consistent with previous results with Rac and Cdc42 (Keely et al., 1997).

Discussion

In this study, we show that activated R-Ras disrupts mammary epithelial polarization in three-dimensional collagen gels and induces migration and invasion across collagenous matrices. These results implicate a role for R-Ras in the transformation of mammary epithelial cells, a role that has not been described previously. We find similar results for a molecule closely related to R-Ras, TC21, which extends previous results by Clark et al. (1996) demonstrating that TC21 can alter breast epithelial morphology. We also find that R-Ras enhances cell adhesion to collagen, which is consistent with its previously described role in promoting increased integrin avidity (Zhang et al., 1996). Importantly, R-Ras enhances cell migration and adhesion specifically on collagen, but not fibronectin. This substratum specificity reflects a difference in the effect of R-Ras on the $\alpha 2$ versus the $\alpha 5$ integrin cytoplasmic domains. Such specificity has not been described previously for the effects of R-Ras on integrin subunits, and could have important implications for inside-out integrin signaling events that lead to migration and invasion on collagenous matrices. Finally, migration induced by R-Ras is partially blocked by inhibitors of PI3K and PKC, but not by a MEK inhibitor, suggesting that unique combinations of signaling pathways are activated by R-Ras compared with other members of the Ras superfamily.

Currently, it is not understood how different integrin subunits specifically link to different inside-out or outside-in signaling pathways. Here, we demonstrate that R-Ras can stimulate cellular migration that is dependent on the $\alpha 2\beta 1$ or $\alpha 4\beta 1$ integrin, but not the $\alpha 5\beta 1$ integrin. In fact, R-Ras activation seems to have a slight inhibitory effect on $\alpha 5\beta 1$ -mediated cell migration. The effect of R-Ras is specifically on the α cytoplasmic domain since only this domain differs between the X4C2 and X4C5 chimeras. Our results are consistent with other observations that α subunits appear to regulate the specificity and appropriateness of integrin response. For example, swapping integrin α cytoplasmic domains on the $\alpha 2$ integrin changes the response of cells to collagen (Chan et al., 1992), whereas deletion of the $\alpha 2$ cytoplasmic domain causes unregulated recruitment of integrins into focal complexes, even when the cells are attached to fibronectin (Kawaguchi et al., 1994). Here we are able to link a specific signaling pathway involving R-Ras to the $\alpha 2\beta 1$ integrin, which may help explain these previous observations regarding specific $\alpha 2$ subunit effects. Activation of MAPK by integrins also depends on specific integrin subunits (Wary et al., 1996; Wei et al., 1998). Specifically, $\alpha 5\beta 1$ integrin, but not $\alpha 2\beta 1$ integrin, associates with Shc and activates the MAPK pathway in osteosarcoma cells (Wary et al., 1996). The failure of

$\alpha 2\beta 1$ to couple to the MAPK pathway may partially explain why R-Ras-induced migration through $\alpha 2\beta 1$ was unaffected by MEK inhibition. Our results imply that cell migration will differ on different matrices, depending in part on the signaling pathway that has been activated.

Our finding that R-Ras enhances cell adhesion to collagen suggests that R-Ras enhances $\alpha 2\beta 1$ integrin avidity for its ligand. An increase in integrin avidity is consistent with the findings of Zhang et al. (1996). However, we find that R-Ras enhances $\alpha 2\beta 1$ - but not $\alpha 5\beta 1$ -mediated adhesion, whereas Zhang et al. noted that R-Ras enhances adhesion to fibronectin through $\alpha 4\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 3$ integrins (Zhang et al., 1996). This difference could relate to the difference between the myeloid cells used in those studies and the epithelial cells used here. The R-Ras-induced increase in avidity determined by cell adhesion could not be correlated to conformational changes of the integrin as detected by the 12G10 anti- $\beta 1$ antibody. Other investigators have also noted a lack of correlation between ligand binding capability and $\beta 1$ conformation as detected by various conformationally sensitive antibodies (Bazzoni and Hemler, 1998; Crommie and Hemler, 1998). Our result could mean that R-Ras alters integrin conformation in a manner not detected by this antibody. Alternatively, changes in integrin avidity after R-Ras activation may not be due to obvious conformational changes but to alterations in integrin clustering, adhesion strengthening, or cytoskeletal attachments.

It is not clear at this point how R-Ras stimulation differentiates between the $\alpha 2$ and $\alpha 5$ integrin subunits. An attractive model invokes proteins or factors that bind specifically to either the $\alpha 2$ or $\alpha 5$ integrin subunits. Stimulation of R-Ras may alter the binding properties of such molecules, perhaps through phosphorylation/dephosphorylation events or membrane targeting. These molecules could bind to specific integrin subunits and activate their ligand binding properties, or could alter integrin association with the cytoskeleton. Alternatively, these factors could be inhibitory, such that R-Ras activation releases the integrin subunit from inhibition by these factors. Our finding that the X4C2 chimera blocks the R-Ras-induced migration of cells on collagen, which is not a ligand for X4C2, suggests that X4C2 acts as a dominant negative molecule in this case, presumably through competition for these putative integrin binding molecules. Such transdominant inhibition has been suggested to explain how one integrin β subunit can inhibit the function of another (Balzac et al., 1994; Diaz-Gonzalez et al., 1996; Fenczik et al., 1997) or how chimeras expressing the cytoplasmic tail of the β subunit suppress integrin function (Y.P. Chen et al., 1994; LaFlamme et al., 1994; Lukashev et al., 1994). Interestingly, transdominant $\beta 1$ integrin suppression can be overcome by expression of CD98 (Fenczik et al., 1997), lending support to the notion that accessory binding molecules regulate integrin function. To our knowledge, ours is the first demonstration of transdominant inhibition on α integrin subunits and implies that factors bind not only to the β , but also the α cytoplasmic domains. Future studies should identify factors specific for the $\alpha 2$ cytoplasmic domain that may play a role in R-Ras-enhanced integrin adhesion and migration.

The fact that R-Ras specifically enhances $\alpha 2\beta 1$ integrin-mediated migration and disrupts tubulogenesis is interest-

ing in light of previous work specifically implicating the $\alpha 2\beta 1$ integrin in these events in mammary epithelial cells (Keely et al., 1995; Zutter et al., 1995). Thus affecting $\alpha 2\beta 1$ function, either through changes in expression levels or by inside-out signaling events, has important consequences for the phenotype of these cells. It is intriguing that apparent increased function of the $\alpha 2\beta 1$ integrin by R-Ras results in the same outcome of increased migration and decreased cell polarization that is noted when $\alpha 2\beta 1$ levels are decreased (Keely et al., 1995). This could indicate that R-Ras activation fundamentally changes the signaling pathways used by the $\alpha 2\beta 1$ integrin, disrupting the normal signaling events that cause the cell to differentiate and polarize in response to the extracellular matrix. These same normal signaling events could be disrupted or diminished when $\alpha 2\beta 1$ levels are decreased, as is noted in breast carcinomas (Zutter et al., 1990). It will be interesting in future studies to determine exactly how cross-talk between the $\alpha 2\beta 1$ integrin and R-Ras affects cellular differentiation, migration, and invasion.

We find a new role for endogenous R-Ras and TC21 in the differentiation and migration of breast epithelial cells. Dominant negative R-Ras inhibits basal cell migration, suggesting that endogenous R-Ras plays a role in the migration of breast epithelial cells. Additionally, we find that inhibitors of PI3K and PKC partially inhibit basal migration, consistent with our model that these molecules are part of the mechanism by which R-Ras contributes to cell migration, whether basal or induced. This further suggests that the signaling pathways under investigation here are likely relevant to understanding the migration of spontaneously occurring breast carcinomas. Dominant negative isoforms of R-Ras or TC21 also enhance tubulogenesis in three-dimensional collagen culture, indicating that antagonizing activated R-Ras or TC21 promotes differentiation. This suggests that activated R-Ras and TC21 are negative regulators of breast cell differentiation, consistent with the finding that expression of activated R-Ras or TC21 isoforms disrupt tubulogenesis. These results could suggest that activation of R-Ras and TC21 turns on signaling pathways that are incompatible with the decision of a cell to slow proliferation and differentiate. Thus, inappropriate activation of R-Ras or TC21 could result in a cell with a more migratory and less differentiated phenotype, which is consistent with a role in transformation.

Our results add to the growing body of evidence pointing to an important role for PI3K in cellular migration events (Kundra et al., 1994; Keely et al., 1997; Shaw et al., 1997; Khwaja et al., 1998). The effect of R-Ras on PI3K possibly is direct since it has been shown that R-Ras binds to PI3K *in vitro* and activates PI3K in Cos-7 cells (Marte et al., 1996). Since R-Ras has direct effects on integrin avidity, it is interesting that a role for PI3K in affecting integrin activation has been shown in lymphocytes (Shimizu et al., 1995) and platelets (Kovacsics et al., 1995). Other Ras superfamily members, including H-Ras, Cdc42, and Rac, also bind and activate PI3K (Rodriguez-Viciano et al., 1994; Zheng et al., 1994; Tolias et al., 1995), which in the case of H-Ras plays an important role in cellular transformation (Hu et al., 1995; Rodriguez-Viciano et al., 1997). We previously found a role for PI3K in migration induced by Rac and Cdc42 (Keely et al., 1997) as well as by N- and

K-Ras (Keely, P., unpublished observation). Consistent with these observations, expression of activated PI3K promotes migration and invasion (Keely et al., 1997; Shaw et al., 1997).

Although R-Ras is similar to Ras, Rac, and Cdc42 in its use of a PI3K signaling pathway, it differs in its use of other signaling pathways. Migration induced by R-Ras or TC21 was only partially blocked by PI3K inhibitors; this effect was much less dramatic than for migration induced by Rac and Cdc42, which could be completely blocked by the same concentrations of these PI3K inhibitors (Keely et al., 1997). This suggests that PI3K-independent signaling pathways also contribute to R-Ras-stimulated migration in these cells. Although MEK and MAPK activation have been implicated in cell migration in various cells (Klemke et al., 1997), we found that MEK inhibition did not affect migration induced by R-Ras or TC21. In contrast, MEK inhibition completely abolished migration induced by K- and N-Ras. These results are consistent with observations that R-Ras activates PI3K but not Raf (Herrmann et al., 1996; Marte et al., 1996; Huff et al., 1997), unlike H-, N-, and K-Ras, which activate both PI3K and the Raf-MAPK pathway (Vojtek et al., 1993; Rodriguez-Viciano et al., 1997). Since a recent report suggests that the Raf-MAPK pathway might be involved in transformation by TC21 (Rosario et al., 1999), our results suggest that the mechanisms by which TC21 induces migration and transformation differ. Moreover, it appears that different small GTPases stimulate cell migration by activating different combinations of downstream signaling pathways. The use of different signaling pathways may explain why R-Ras differs from N- and K-Ras in stimulating migration across fibronectin.

We find a role for PKC, in addition to PI3K, in migration induced by R-Ras. Although certain isoforms of PKC (δ , ϵ , and η) can be activated downstream of PI3K and contribute to cell migration (Toker et al., 1994; Derman et al., 1997), the additive effect of PI3K and PKC inhibitors in our assays suggests that PKC is on a separate pathway from PI3K in these cells. Whether PKC is downstream of R-Ras, or part of an independent obligate pathway remains to be determined. To this end, we did not observe an increase in *in vitro* PKC activity in cells expressing R-Ras in preliminary experiments (our unpublished observation), although translocation of PKC to the membrane and subsequent activation in R-Ras-expressing cells cannot be ruled out. Dominant negative R-Ras was unable to block cell migration induced by PMA (our unpublished results), suggesting PKC is not upstream of R-Ras in our system. In addition to R-Ras, we find a role for PKC in migration induced by K-Ras. Others also have noted synergy between PKC and small GTPases. The Rac exchange factor, Tiam-1, can be activated by PKC (Fleming et al., 1997), which would place PKC upstream of Rac activation. Similarly, PKC is upstream of Ras activation in platelets (Shock et al., 1997). Additionally, PKC activation synergizes with Rho to induce focal adhesion kinase phosphorylation, cell spreading, and actin assembly (Lewis et al., 1996; Defilippi et al., 1997). Interestingly, activation of the $\alpha 2\beta 1$ integrin in monocytes by ligand binding to $\alpha 5\beta 1$ requires PKC (Pacifci et al., 1994), which would be consistent with a model in which PKC is part of the pathway by

which R-Ras enhances avidity of the $\alpha 2\beta 1$ integrin. Moreover, PKC activation is involved in activation of the $\alpha \text{IIb}\beta 3$ integrin by a number of agonists in platelets (Karniguan et al., 1990). Our results are also consistent with other observations that PKC activity contributes to cell migration (Vuori and Ruoslahti, 1993; Derman et al., 1997; Batlle et al., 1998).

In summary, we find that activation of R-Ras stimulates unique combinations of downstream signaling pathways compared with other GTPases of the Ras superfamily. These unique signaling combinations lead to specific effects on certain integrin α subunits to alter cellular responses to collagen such as polarization, migration, and invasion. Such specificity will decide how a cell might respond to different extracellular environments, depending on which Ras family member is activated, and could ultimately determine if a given carcinoma is metastatic or not. These differences have important implications for targeting signaling pathways in antimetastatic therapies since affecting certain signaling pathways may not have the desired effects on all neoplastic cells.

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