Oncogene (2003) 22, 98–108

www.nature.com/onc

Melanoma cell migration to type IV collagen requires activation of NF-κB

Louis Hodgson[1], Andrew J Henderson[2] and Cheng Dong*[,1]

[1]Department of Bioengineering, 229 Hallowell, The Pennsylvania State University, University Park, PA 16802, USA; [2]Department of Veterinary Science, 115 Henning, The Pennsylvania State University, University Park, PA 16802, USA

Chemotaxis is the consequence of environmental factors engaging their receptors to initiate signaling cascades. However, the biochemical mechanisms controlling this phenomenon are not clear. We employed an *in vitro* modified Boyden 48-well chemotaxis migration system to characterize the role of signal transducers in type IV collagen (CIV) induced A2058 human melanoma cell migration. Using specific pharmacological inhibitors and a series of dominant-negative and constitutively active signaling proteins, we show that Ras and Rac GTPases, PI-3K, and PKC participate in cell migration mediated by β1 integrins. Collagen also induces a time-dependent degradation of IκB-α and an increase in nuclear translocation of NF-κB which is dependent on PKC pathway. More importantly, collagen-stimulated melanoma cell migration directly correlated with an increase in NF-κB transactivation. Furthermore, CIV induced an increase in β1 integrin mRNA levels. Specific NF-κB inhibitors Helenalin and SN-50 inhibited melanoma cell migration to collagen, indicating a novel requirement for NF-κB transactivation in cell chemotaxis mediated by β1 integrin signals. These results describe signal transduction events that are initiated by type IV collagen through β1 integrins and demonstrate an important role for NF-κB in regulating melanoma chemotaxis.
Oncogene (2003) **22,** 98–108. doi:10.1038/sj.onc.1206059

Keywords: Chemotaxis; Integrin; GTPase; Transactivation; A2058

Introduction

Chemotactic cell movement is critical for a number of processes including embryogenesis, hematopoesis, immune function, and cancer metastasis (Condeelis, 1993; Stossel, 1993). A wide array of intercellular signaling molecules including cytokines, chemokines and extracellular matrix (ECM) proteins can serve as chemotactic agents (Aznavoorian *et al*., 1990; Lester and McCarthy, 1992; Schnaper and Kleinman, 1993). For example, ECM proteins including collagens, laminin, fibronectin, and vitronectin, have been shown to influence transvascular homing of tumor cells (Aznavoorian *et al*., 1990; Stracke *et al*., 1994). Recently, it was demonstrated that a soluble ECM protein, type IV collagen (CIV), which is highly prevalent within the basement lamina and intima underlining the endothelium, induced chemotaxis of a number of human melanoma cell lines in vitro by engaging α2β1 integrin (Aznavoorian *et al*., 1990; Boukerche *et al*., 2000; Pasco *et al*., 2000; Hodgson and Dong, 2001).

CIV-mediated chemotaxis has been shown to involve α2β1 integrins (Hodgson and Dong, 2001), however, the downstream signal transduction mechanism regulating cell migration remains undefined. Integrin ligation and clustering alter the cytoskeleton and initiates the assembly of a molecular platform upon which signaling molecules including src-kinases, GTPases, and various adapters are recruited to form focal adhesion complexes (Schwartz and Ingber, 1994; Schaller and Parsons, 1994; Schwartz *et al*., 1995; Meredith *et al*., 1996; Renshaw *et al*., 1996; Keely *et al*., 1998; Price *et al*., 1998; Giancotti and Ruoslahti, 1999; Parsons *et al*., 2000; Salazar and Rozengurt, 2001). Furthermore, the engagement of α2β1 integrin by CIV has been shown to increase intracellular Ca^{2+} (Savarese *et al*., 1992; Leavesley *et al*., 1993; Hodgson and Dong, 2001). In addition, A2058 cell migration in response to CIV was pertussis toxin (PT) sensitive, implicating the involvement of G-proteins (Aznavoorian *et al*., 1990). Some signaling events immediately downstream of integrin receptors are Ras-ERK, which influences cell proliferation, and Rac/cdc42/Rho pathways, which regulate cell spreading, polarization, and stress fiber formation (Ridley and Hall, 1992; Chant and Stowers, 1995; Nobes and Hall, 1995; Denhardt, 1996; Zigmond, 1996; Machesky and Hall, 1997; Keely *et al*., 1997; Aspenström, 1999; Tapon and Hall, 1997). Therefore, by influencing cytoskeleton organization and cell shape, signaling through integrin receptors is thought to be critical for cell motility (Price *et al*., 1998; Schaller and Parsons, 1994; Giancotti and Ruoslahti, 1999; Sarner *et al*., 2000).

Many of these signal transduction events target transcriptional regulators such as NF-κB, AP-1 and C/EBPs, which in turn regulate genes that alter cellular activities (Perona *et al*., 1997; Van Aelst and D'Souza-Schorey, 1997; Karin, 1994;

*Correspondence: C Dong, Department of Bioengineering, 229 Hallowell, The Pennsylvania State University, University Park, PA 16802, USA; E-mail: cxd23@psu.edu
Received 17 April 2002; revised 18 September 2002; accepted 24 September 2002

Minden *et al*., 1995). Although the induction of various transcription factors in response to integrin ligation is well documented, the significance of these factors, especially with regard to chemotaxis, has not been demonstrated. Recently, NF-κB has been shown to be required for cell migration mediated by some ECM proteins. For example, NF-κB was necessary for the migration of a pancreatic cell line in response to vitronectin, as well as the chemotaxis of keratinocytes in response to insulin (Yebra *et al*., 1995; Benoliel *et al*., 1997). Whether β1 integrin-dependent tumor cell migration requires the induction of transcription factors, including NF-κB, needs further elucidation.

In this report, we use an *in vitro* migration system based on a 48-well modified Boyden chemotaxis chamber to show a functional correlation between integrin signaling and melanoma cell migration. We demonstrate that GTPases Ras and Rac, as well as various downstream intermediates participate in regulating cell migration. More importantly, we show that NF-κB activation is required for cell migration associated with β1 integrin-initiated signaling in human melanoma cells.

Results

Ras and Rac GTPase participate in collagen-dependent cell migration

Ligation of integrin receptors leads to recruitment of GTPase, such as Ras, Rho, Rac, and cdc42, forming a signaling complex, which potentially influences cell migration to chemoattractants. To test whether specific GTPases are critical during α2β1-mediated migration, A2058 cells were transiently transfected with expression constructs encoding dominant negative and constitutively active mutants of Ras and Rac, and assayed for their ability to migrate in response to CIV using a modified Boyden chamber. Addition of CIV to the lower chamber results in 4000–6000 cells/well migrating to the backside of the filter compared to only 100–200 cells/well migrating in the absence of CIV (Harvath *et al*., 1980; Aznavoorian *et al*., 1990; Stracke *et al*., 1994; Hodgson and Dong, 2001). Expression of mutant GTPases was confirmed by immunoblot (Figure 1a) and transfection efficiency, which was greater than 55%, was monitored by GFP (see the Materials and methods section). As shown in Figure 1b, cell migration was reduced by approximately 45% when dominant negative Ras and Rac were expressed in A2058 cells. Since inhibiting these pathways did not completely attenuate cell migration, other pathways appeared to be operative. Furthermore, signaling through Ras and Rac is not sufficient for enhancing chemotaxis as shown by cells transfected with constitutively active mutants, RasV12 and RacV14. Dominant negative Rho and cdc42 did not show any significant effect on cell migration to CIV (data not shown). These findings suggest a critical role for Ras and Rac GTPases in CIV-stimulated cell migration.

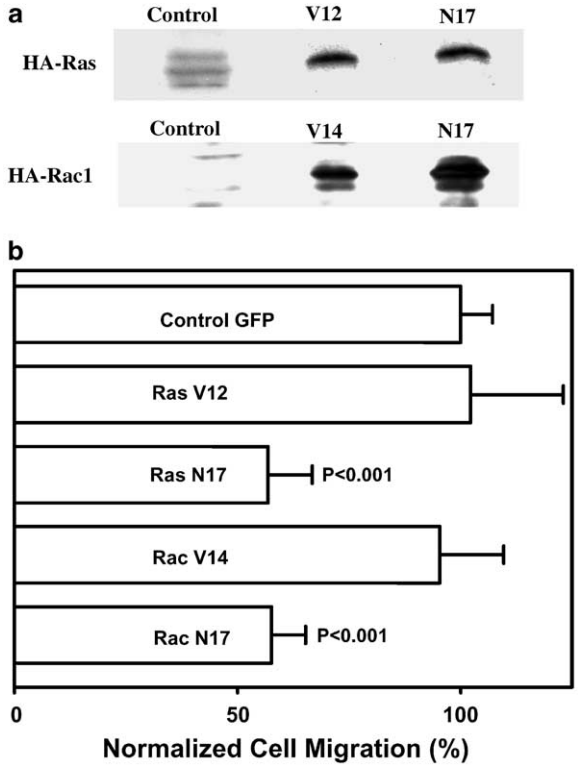


Figure 1 Ras and Rac GTPases are involved in A2058 cell migration to CIV. (**a**) Immunoblots confirming expression of mutant GTPases in A2058 cells. Cells were transfected with 25 μg of indicated expression constructs for mutant GTPases and recombinant protein was detected by staining with anti-HA antibody. We routinely observed that >55% of the cells were transfected. (**b**) A total of 25 μg of plasmids encoding constitutively active mutant GTPases (RasV12 and RacV14) and dominant negative expression constructs (RasN17 and RacN17) were transiently transfected into A2058 cells. Following transfection, cell migration in response to CIV was assessed with a 48-well-modified Boyden chemotaxis chamber. Cells migrated to the lower side of the microporous filter at the completion of a 4 h migration assay were stained and counted under ×10 magnification. Cell counts were normalized to the controls in which A2058 cells were transiently transfected with GFP and assayed for cell migration to CIV. The number of cells that migrated in the absence of CIV was considered the background and subtracted from all values. *P*-values are with respect to control GFP experiments. Each data point represents an average of three independent experiments ± s.e.m, in which 24 wells were sampled per experiment

PKC and PI-3K mediate cell migration in response to collagen

Since a potential role for PI-3K in mediating signaling between Ras and Rac has been previously suggested (Kjøller and Hall, 1999), we examined whether this pathway was participating in CIV-stimulated cell migration by using inhibitors LY294002 and Wortmannin. A2058 cells were treated with various concentrations of inhibitors and assayed for cell migration. LY294002 attenuated cell migration compared to the untreated control by 65% at the highest dose tested (50 μM; Figure 2a). Similarly, Wortmannin reduced cell migration to 20% of the untreated control (500 nM; Figure 2a). These results indicate PI-3K signaling is

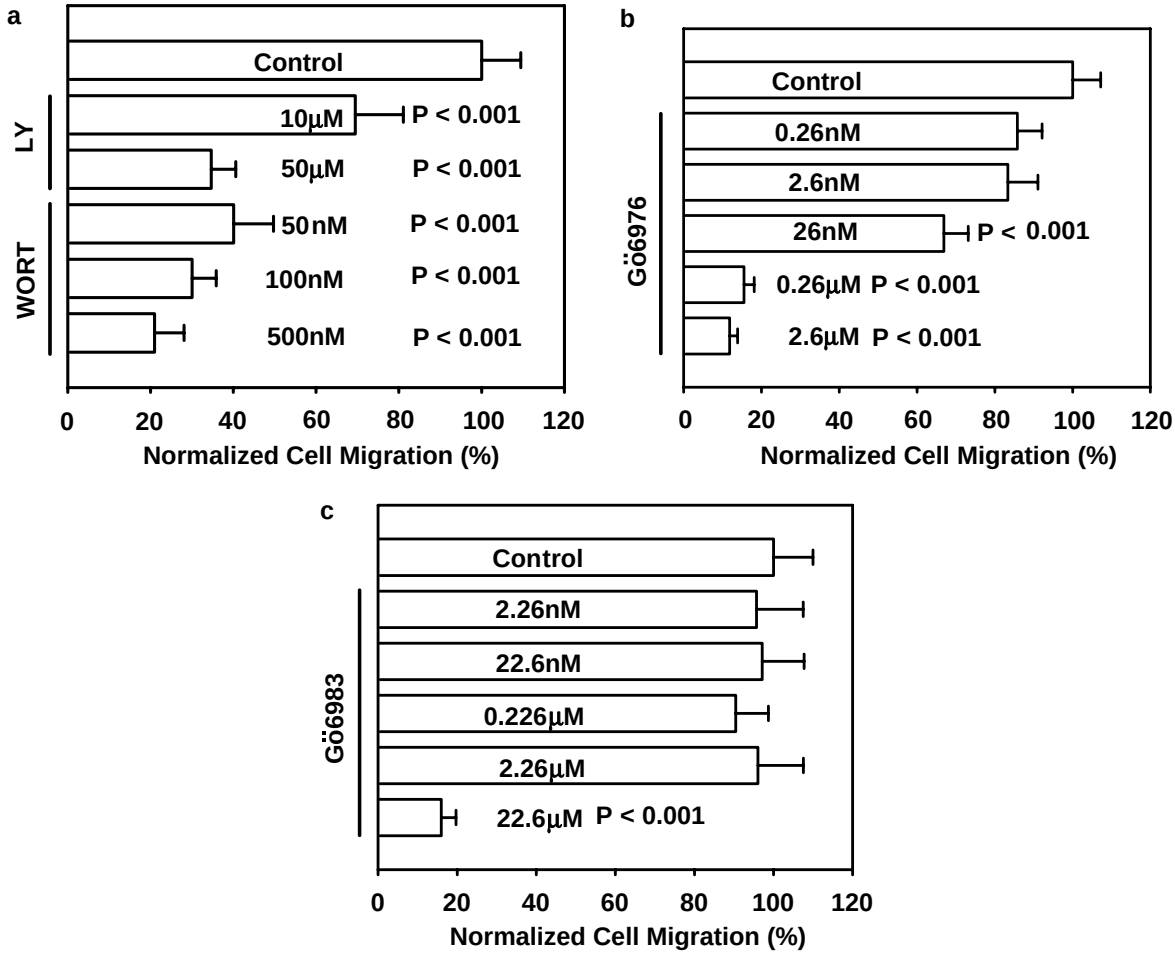


Figure 2 PI-3K and PKC participate in A2058 cell migration in response to CIV. (**a**) P1-3K is involved in mediating cell migration to CIV. LY294002 ('LY') was applied to cells at 10 or 50 μM for 2 h prior to and during cell migration assays. Wortmannin ('WORT') pretreatment involved a 30 min treatment prior to cell migration assays at the concentration range of 50–500 nM. Wortmannin was not included during the cell migration assays since the inhibition is irreversible within the time frame of migration assays. *P*-values are with respect to untreated control migrating in response to CIV. (**b**) Specific inhibitor of PKC, Gö6976, abrogates cell migration to CIV in a concentration-dependent manner. The inhibitor was applied to cells at the indicated concentrations 1 h prior to assays and was present during the 4 h migration assays. *P*-values are with respect to untreated controls migrating in response to CIV. (**c**) Specific inhibitor of PKC, Gö6983, significantly abrogates cell migration to CIV. The inhibitor was applied to cells at the specified concentrations 1 h prior to and during cell migration assays. *P*-values are with respect to untreated control. Each data point represents an average of three independent experiments $\pm$ s.e.m., in which 24 wells were sampled per experiment

regulating integrin-mediated melanoma chemotaxis in response to CIV.

We also examined whether PKC activity was necessary for A2058 cell migration stimulated by CIV. Specific PKC inhibitors Gö6976 and Gö6983 were used to target PKC. Gö6976 inhibited cell migration in a dose-dependent manner as shown in Figure 2b, with up to 90% inhibition at the highest dose (2.6 μM) tested. Furthermore, cell migration was inhibited by 85% when treated with 22.6 μM of Gö6983, but was not adversely affected by lower concentrations of this inhibitor (Figure 2c). Together, these results suggest a potential role for PKC in cell migration to CIV stimulation.

Collagen stimulation induces NF-κB activity

Multiple signaling pathways including those involving PKC, JNK, and Rac1 culminate at NF-κB (Karin, 1994; Minden *et al*., 1995; Perona *et al*., 1997; Van Aelst and D'Souza-Schorey, 1997). Therefore, we were interested in determining the role of NF-κB in CIV-dependent migration. Initial experiments addressed whether CIV treatment induced NF-κB activity. NF-κB activation requires the release of p65 from IκB, which is targeted for proteosome-dependent degradation by IKK phosphorylation. As an early indicator of NF-κB activation, we monitored the levels of IκB by immunoblots following CIV treatment. Whole-cell lysates were prepared from cells harvested at various times following the CIV treatment. As seen in Figure 3, the presence of IκB-α (37 kDa) decreased within 15 min, whereas no changes were observed in β-actin controls (44 kDa), indicating that degradation of cytoplasmic IκB-α and hence the release of p65 subunit of NF-κB is induced by CIV treatment.

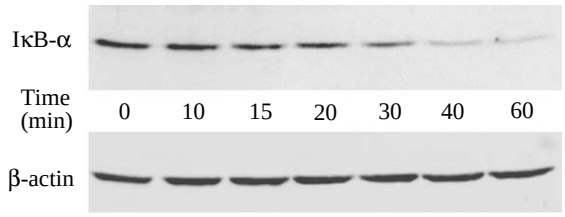


Figure 3 IκB-α is degraded in a time-dependent manner following CIV stimulation. Whole-cell lysates were prepared at indicated times following treatment with 100 μg/ml CIV treatment and IκB-α was detected by imunoblot as described in the Materials and methods section. β-actin(44 kDa) is shown as the loading control

In order to determine if CIV treatment of A2058 cells triggered nuclear translocation of NF-κB, electrophoretic mobility shift assays (EMSA) were performed. Using an oligonucleotide probe representing a canonical NF-κB binding site, a weak specific complex was observed in untreated melanoma cells (Figure 4a; lane 1). The intensity of this complex significantly increased in extracts prepared from CIV-treated A2058 cells (Figure 4a; lane 2). The specificity of these complexes was demonstrated by diminished binding in the presence of a cold competitor (Figure 4a; lanes 5 and 6), while the irrelevant competitor did not affect the binding (Figure 4a; lanes 9 and 10). Furthermore, the complex was supershifted by polyclonal sera against p50 and p65 (Figure 4b; lanes 1–4) but not control serum, which recognized C/EBPβ (Figure 4b; lanes 5 and 6). Treating cells with NF-κB inhibitor Helenalin (10 μM), which specifically and irreversibly alkylates the p65 subunit of NF-κB thus disrupting its binding activity (Lyss *et al*., 1997, 1998), attenuated NF-κB induction following CIV treatment (Figure 4a; lanes 3, 7, and 11). Decreased binding was also observed with a second inhibitor of NF-κB activation, SN-50 (50 μg/ml; Figure 4a; lanes 4, 8, and 12).

Ras N17- or Rac N17-transfected cells (FACS sorted based on EGFP coexpression; >98% positive population) were compromised in their ability to induce NF-κB binding activity in response to CIV (Figure 4c; lanes 4, 9, and 14: Ras N17 transfected; lanes 5, 10, and 15: Rac N17 transfected). Since these intermediates are early events in signaling cascades initiated by integrin activation, it is likely that other mechanisms may feed into this particular pathway. More importantly, the PKC inhibitor Gö6976 (0.26 μM; effective dose to inhibit cell migration in Figure 2b) prevented activation of NF-κB in response to CIV (Figure 4c; lanes 3, 8, and 13), thereby placing this signaling intermediate directly upstream of NF-κB activation in response to β1 integrin signals.

To ascertain that the signals emanating from β1 integrin are responsible for induction of NF-κB binding activity, we used blocking antibody specific for β1 integrin. Treatment with this antibody (25 μg/ml) significantly decreased CIV-dependent induction of NF-κB (Figure 4d; lanes 3, 6, and 9). These results indicate that one of the consequences of CIV stimulation of melanoma cells is the induction of NF-κB binding activity.

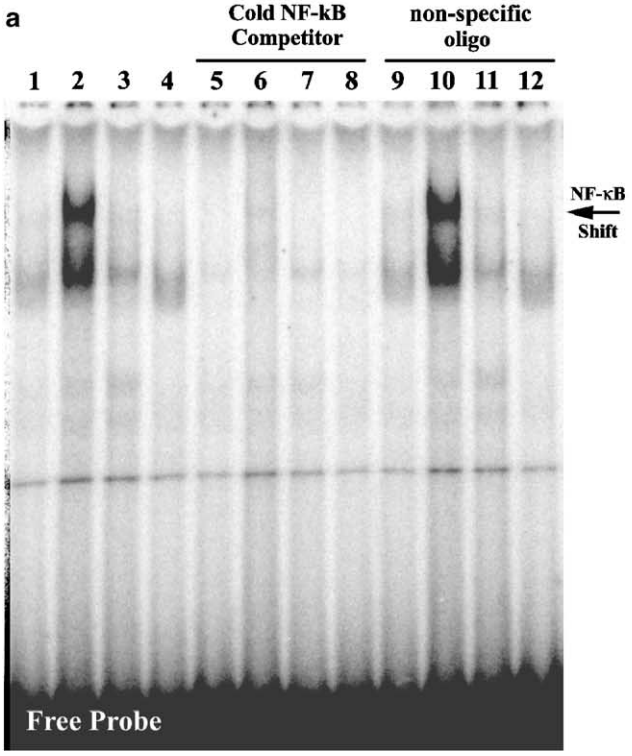
Cell migration to collagen induces transcriptional activation by NF-κB

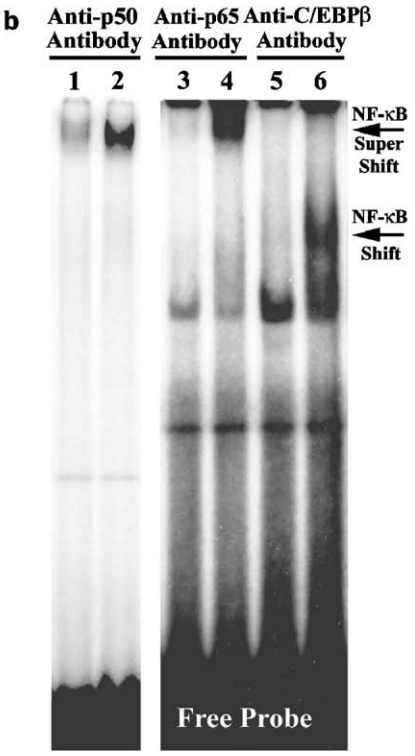
The cell migration assay was employed to evaluate whether NF-κB activity was induced during CIV-dependent A2058 cell migration. A luciferase reporter construct containing tandem NF-κB binding sites adjacent to a minimal promoter was transiently transfected into A2058 cells prior to their use in the migration assay system. At the end of the 4 h migration period, cells tightly associated with the microporous filter were collected and assayed for luciferase activity. Melanoma cells exposed to CIV (Figure 5a; +CIV) consistently had 190% greater luciferase activity compared to cells assayed in the absence of CIV (Figure 5a; −CIV) or untreated cells harvested prior to the migration assay (Figure 5a; Pre-mig). As a control for the specificity of transactivation mediated by NF-κB during cell migration, an AP-1-luciferase reporter construct was transiently transfected into A2058 cells and assayed for luciferase activity following the 4 h cell migration assay. A negligible difference in luciferase activity with this promoter was observed regardless of exposure to CIV (Figure 5a). These results further indicate that cell migration to CIV activates NF-κB.

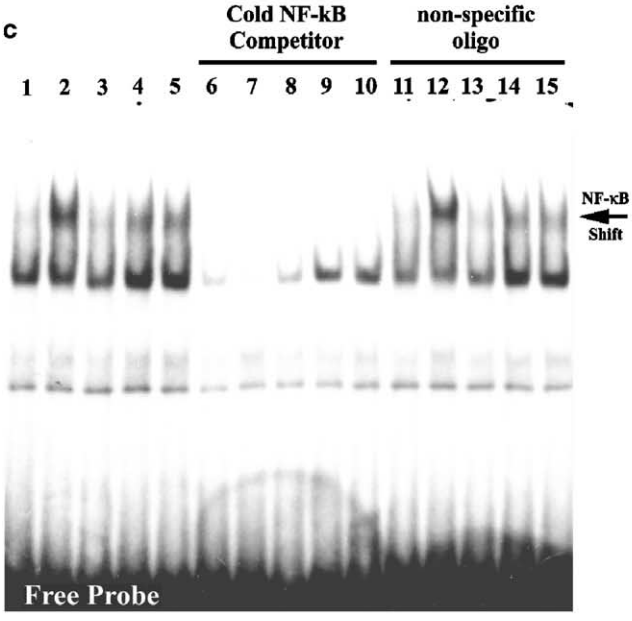
Melanoma cells assayed for migration in a chemotaxis chamber can be separated into two distinct groups: (1) those that migrated to the lower side of the filter in response to a chemotactic agent and (2) those that remain on the upper side of the filter even in the presence of a chemoattractant. We took advantage of this system to assess NF-κB activity in cells that successfully transversed the filter, thus obtaining a more accurate measurement of NF-κB activity in correlation with cell chemotaxis and migration to CIV. A2058 cells transiently transfected with NF-κB luciferase reporter were used in migration assay, and the migrated fraction, represented by cells scraped from the lower side of the microporous filter, were separated from cells that remained in the upper chamber after the 4 h migration assay. Since very few cells were recovered from the filter ($\sim 10^4$ cells/filter), it was difficult to detect significant luciferase activity; therefore, we used RT-PCR to detect luciferase mRNA. Cells that were not exposed to CIV or those that remained on the upper side of the filter following CIV exposure expressed modest levels of luciferase mRNA (Figure 5b; lanes 3 and 4), whereas cells that migrated to the lower side of the filter expressed significantly greater levels of luciferase mRNA (Figure 5b; lane 5). These data corroborate results from our luciferase assays and demonstrate that CIV-stimulated cell migration induces NF-κB activity.

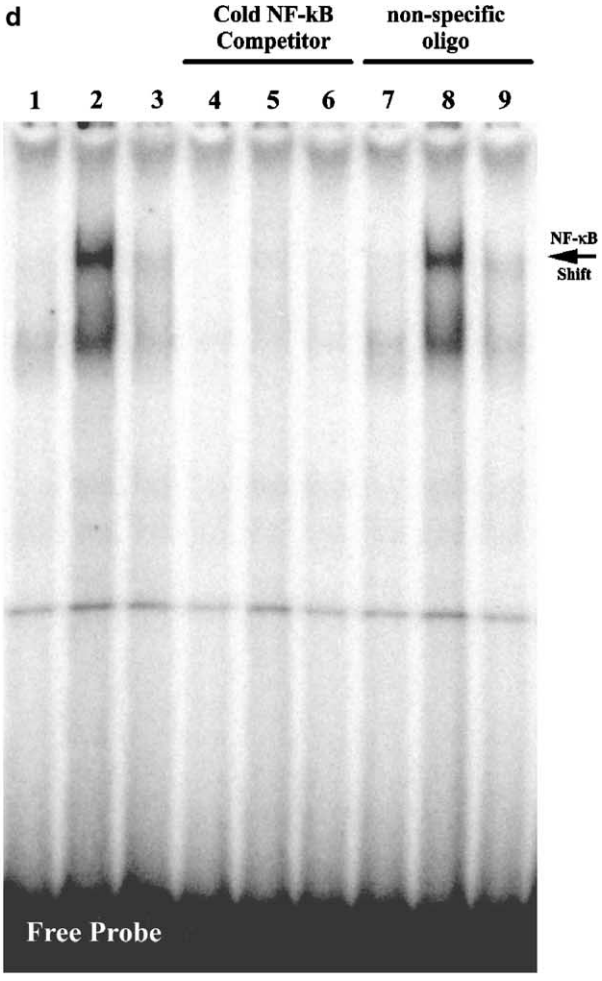
NF-κB is required for cell migration to type IV collagen

The above experiments demonstrate that NF-κB is activated in response to CIV-dependent A2058 cell migration, but do not address whether NF-κB activity is


a
Cold NF-kB Competitor
non-specific oligo
1 2 3 4 5 6 7 8 9 10 11 12
NF-κB Shift
Free Probe


b
Anti-p50 Antibody
Anti-p65 Antibody
Anti-C/EBPβ Antibody
1 2 3 4 5 6
NF-κB Super Shift
NF-κB Shift
Free Probe


c
Cold NF-kB Competitor
non-specific oligo
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
NF-κB Shift
Free Probe


d
Cold NF-kB Competitor
non-specific oligo
1 2 3 4 5 6 7 8 9
NF-κB Shift
Free Probe

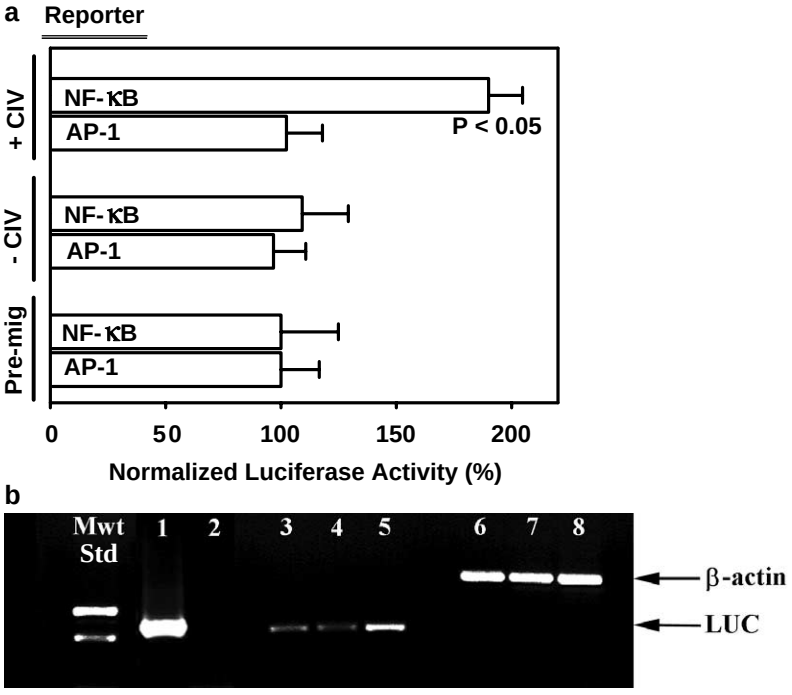


Figure 5 NF-κB-dependent transcription is induced in A2058 cells by CIV-mediated cell migration. (**a**) Luciferase-reporter activity following A2058 cell migration to CIV. A2058 cells were transiently transfected with 25 μg NF-κB-luciferase or AP-1-luciferase reporters, and cells associated with the filter were assayed for luciferase activity following the cell migration assays. '+CIV' were cells from migration chambers that contained CIV (100 μg/ml) for the duration of the 4 h migration assay, '−CIV' were cells from chambers that did not contain CIV during the 4 h assay, and 'Premig' were from cells collected and lysed prior to loading into the cell migration chambers. Experiments were conducted in triplicates and presented with ± s.e.m. *P*-values are with respect to '−CIV' case. (**b**) RT-PCR measuring luciferase mRNA at various stages of migration. Cells were isolated and collected from either the lower side (those cells that migrated) or the upper side (those did not migrate) of the filter membrane following the 4 h migration assays; total RNA was prepared for reverse transcription. Lane 3: cells collected from migration chamber that did not contain CIV during the 4 h assay; lane 4: cell fraction that remained in the upper chamber in presence of CIV during the migration assay; lane 5: those cells that sucessfully migrated to the underside of the microporous filter in the presence of CIV during the 4 h assay. Controls include β-actin mRNA loading control (lanes 6–8), luciferase vector (lane 1), and nonspecific target DNA (lane 2). Samples without reverse transcriptase served as controls to verify the absence of genomic DNA contamination (data not shown). These data represent three independent experiments

required. In order to determine whether the activation of NF-κB was critical to cell migration in response to CIV, A2058 cells were treated with NF-κB inhibitors Helenalin and SN-50 and assessed for migration. Helenalin, a plant-derived sesquiterpene lactone, specifically and irreversibly alkylates p65 subunit of NF-κB to block DNA binding (Lyss *et al*., 1997, 1998). SN-50 is a synthetic peptide that prevents the translocation of activated NF-κB complex to the nucleus (Lin *et al*., 1995). Helenalin application resulted in a dose-dependent attenuation of cell migration to CIV, with 80% inhibition of cell migration at the highest concentration tested (10 μM; Figure 6). Cell migration in response to CIV was attenuated by 50% when SN-50 was applied at 50 μg/ml, whereas no effect was observed with the negative control compound SN-50 M (Figure 6).

Figure 4 NF-κB binding is induced in CIV-treated A2058 melanoma cells. (**a**) Nuclear extracts were prepared from untreated cells (lanes 1, 5, and 9), or cells treated with 100 μg/ml CIV (2, 6 and 10), CIV with 10 μM NF-κB inhibitor Helenalin (lanes 3, 7 and 11), and CIV with 50 μg/ml NF-κB inhibitor SN-50 (lanes 4, 8, and 12). Controls included EMSA reactions with 50-fold excess cold NF-κB competitor (lanes 5–8) or nonspecific competitor (lanes 9–12). (**b**) Nuclear extracts were prepared from untreated cells (lanes 1, 3 and 5), or cells treated with 100 μg/ml CIV (lanes 2, 4, and 6). Polyclonal antibodies (0.5 μg/ml) against the p50 (lanes 1 and 2) or p65 subunits (lanes 3 and 4) of NF-κB were included in the EMSA reactions. Supershifted complexes are indicated by the arrow. Negative controls were reactions that included 0.5 μg/ml polyclonal antibodies against C/EBPβ (lanes 5 and 6). Arrows indicate specific NF-κB complex shifts. (**c**) EMSA demonstrating GTPase mutants inhibit NF-κB. Nuclear extracts were prepared from untreated cells (lanes 1, 6, and 11), or cells treated with 100 μg/ml CIV (lanes 2, 7, 12), CIV with 0.26 μM PKC inhibitor Gö6976 (lanes 3, 8, and 13), RasN17-transfected cells (positively selected for GFP expression; lanes 4, 9, and 14), and positively selected Rac N17-transfected cells (lanes 5, 10 and 15). To confirm specificity of the binding activity, EMSAs were performed in the presence of 50-fold excess cold-NF-κB (lanes 6–10) or nonspecific competitors (lanes 11–15). Arrow indicates the position of specific NF-κB complex shifts. (**d**) Nuclear extracts were prepared from untreated cells (lanes 1, 4, and 7), or cells treated with 100 μg/ml CIV (lanes 2, 5 and 8), and CIV with 25 μg/ml monoclonal blocking antibody against β1 integrin (lanes 3, 6 and 9). Controls included EMSA reactions with 50-fold excess cold NF-κB competitor (lanes 4–6) or nonspecific competitor (lanes 7–9). These data represent at least three independent experiments

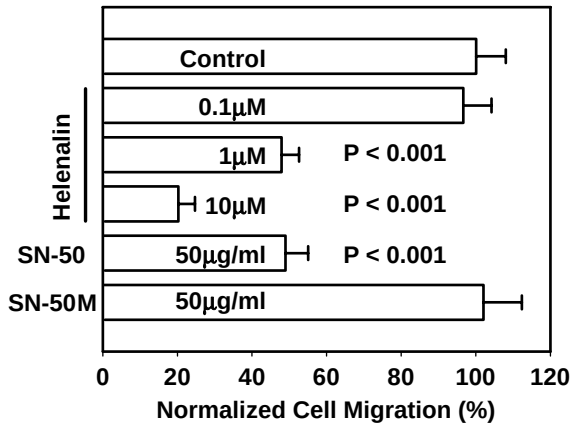


Figure 6 NF-κB activity is necessary for A2058 cell migration to CIV. Inhibitors Helenalin and SN-50 were applied to cells 1 h prior to and during migration assays at the indicated concentrations. Migration was assayed with 48-well modified Boyden chambers as described in the Materials and methods section. *P*-values are with respect to migration of untreated controls in response to CIV. Each data point represents an average of three independent experiments ± s.e.m., in which 24 wells were sampled per experiment

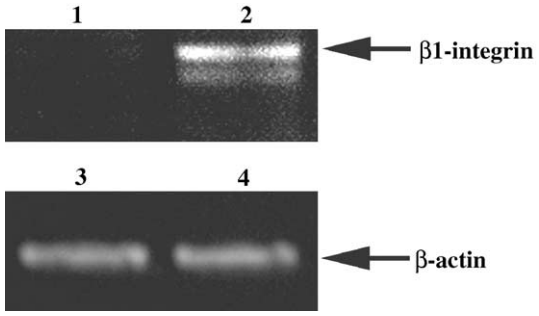


Figure 7 CIV induces transcription of β1 integrin in A2058 cells. Total RNA was prepared from untreated (lanes 1 and 3) or CIV-treated cells (lanes 2 and 4) and used in RT-PCR as described in the Materials and methods section. Lanes 1 and 2 are RT-PCR results using β1-integrin primers and lanes 3 and 4 show the β-actin control. Samples without the reverse transcriptase served as controls to verify the absence of genomic DNA contamination (data not shown). These data are representative of three independent experiments

Treating other human melanoma cell lines including C8161, WM9, and WM115, with the NF-κB inhibitors resulted in a similar reduction in cell migration in response to CIV (data not shown). These results demonstrate that induction of NF-κB is required for melanoma cells to migrate in response to a chemotactic stimulation by CIV.

Collagen induces β1 integrin expression

Since transcriptional regulation by NF-κB is required for melanoma migration in response to α2β1 integrin signals, changes in β1 integrin expression levels may play a critical role in regulating CIV-mediated chemotaxis. We monitored β1 integrin transcription by using RT-PCR. Control A2058 cells (in absence of CIV) did not show appreciable β1 integrin mRNA (Figure 7; lane 1), whereas CIV treatment (100 μg/ml; 4 h) induced a significant increase in β1 integrin mRNA (Figure 7; lane 2). These results were also corroborated using FACS analysis, which showed approximately 10% increase in β1 integrin receptor expression after 4 h of CIV treatment (data not shown). These findings suggest that β1 integrin is one gene that is targeted by signaling cascades initiated by CIV, and implicates this molecule in being directly involved in regulating CIV-induced cell migration.

Discussion

We report a novel requirement for NF-κB activity in human melanoma cell migration to CIV mediated by α2β1 integrin. We showed that signaling initiated by CIV requires Ras and Rac GTPases and utilizes PI-3K and PKC as intermediate messengers for cell chemotaxis. CIV-stimulated cell migration also causes time-dependent degradation of IκB-α to result in the induction of NF-κB transcriptional activation. We identified PKC pathway as a potential mechanism for this activation. More importantly, the induction of activated NF-κB is required for cell chemotaxis to CIV in this experimental system. Since we have shown previously, by using blocking antibodies, that CIV-mediated cell migration acts through α2β1 integrin (Hodgson and Dong, 2001), we conclude that these signaling events emanate from β1 integrin-ligation. Finally, we demonstrate that one consequence of CIV stimulation is increased β1 integrin expression, which potentially will make cells more responsive to CIV-mediated chemotaxis.

Expression of dominant negative mutant GTPases Ras and Rac caused a partial attenuation of cell migration in response to CIV. However, constitutively active variants of Ras and Rac did not affect cell migration when overexpressed. Taken together, these results indicate that Ras and Rac are necessary for efficient melanoma cell migration, but not sufficient for this activity. It has been shown that Ras activates Rac through PI-3 K (Kjøller and Hall, 1999). Although we present evidence suggesting the involvement of GTPases Ras and Rac in CIV-mediated cell migration, we did not directly address the cooperative effects of these GTPases in mediating the cell response. Cells transiently cotransfected with combinations of dominant negative and constitutively active Ras and Rac mutants do not rescue the effects of dominant negative GTPase (data not shown). Furthermore, cell migration to CIV is not further attenuated when dominant negatives of both Ras and Rac are cotransfected simultaneously (data not shown). These observations appear to rule out a linear pathway in which Ras and Rac operate, but suggest other potential signaling pathways being operative, such as PI-3K, which are also implicated by our data as being important in regulating melanoma migration. Certain limitations associated with using mutant GTPases as tools to address cellular functions such as in the case of cell motility should also be considered (Feig, 1999).

Using a combination of specific inhibitors, we were able to show that PKC is important in the cell migration

to CIV. Consistent with this finding, Palmantier *et al*. (2001) demonstrated a role for PKC in β1 integrin-mediated adhesion of human breast carcinoma cells to CIV. The results from our laboratory, as well as others, place PKC directly upstream of NF-κB activation (Anrather *et al*., 1999; Trushin *et al*., 1999; Khoshnan *et al*., 2000). Furthermore, some isoforms of PKC are activated by PT sensitive G_i protein in association with GTPases and, since A2058 cell migration and pseudopodial protrusions in response to CIV have been shown to depend on PT-sensitive G-proteins, it is possible that this pathway is utilized during β1 integrin-mediated cell migration (Aznavoorian *et al*., 1990; Paolucci *et al*., 2000). Taken together, these results suggest that PKC is an important signaling component for β1 integrin-dependent chemotaxis and cell migration in A2058 melanoma cells.

Signal transduction pathways initiated by integrin ligation ultimately target multiple transcription factors (Karin, 1994; Minden *et al*., 1995; Perona *et al*., 1997; Van Aelst and D'Souza-Schorey, 1997). Our data demonstrates that NF-κB activation is one consequence of β1 integrin binding and signaling. The signaling intermediates that we have identified as being critical for melanoma migration have been demonstrated to be upstream of NF-κB in multiple cellular responses. In addition, we have confirmed an altered NF-κB activity in response to changes in the activity of these intermediates; for example, our EMSA results indicate that PKC is directly upstream of NF-κB activation by β1 integrin ligation, and PKC has been shown to activate IKK which phosphorylates IκB, targeting this protein for degradation, and releasing NF-κB (Anrather *et al*., 1999; Trushin *et al*., 1999; Khoshnan *et al*., 2000). Furthermore, dominant negative Rac or Ras expression results in a modest reduction of CIV-induced NF-κB binding activity. PI-3K has also been shown to play a role in inducing NF-κB, by regulating Akt/PKB activity (Burow *et al*., 2000; Rong *et al*., 2002), and our preliminary data suggest that PI-3K influences NF-κB activity as well as cell migration (data not shown). Therefore, NF-κB is a likely target of integrin-mediated signaling pathways.

Experiments in which NF-κB was inhibited using Helenalin, a plant-derived sesquiterpene lactone, resulted in a dose-dependent attenuation of cell migration in response to CIV. However, in addition to inhibiting NF-κB, Helenalin has also been shown to increase intracellular calcium ($[Ca^{2+}]_i$) through an IP_3-mediated $[Ca^{2+}]_i$ release pathway (Powis *et al*., 1994). Furthermore, we have demonstrated previously that elevated $[Ca^{2+}]_i$ can significantly attenuate melanoma migration in response to CIV, whereas cell migration to CIV was unaffected following $[Ca^{2+}]_i$ depletion using EGTA in combination with ionophores (Hodgson and Dong, 2001). Therefore, it is possible that results obtained with Helenalin, in part, reflect changes in $[Ca^{2+}]_i$ rather than inhibition of NF-κB. We do not feel that this is the case, since depleting $[Ca^{2+}]_i$ at the time of Helenalin treatment resulted in a similar inhibition of migration (data not shown). Although these observations do not rule out Ca^{2+} as an important regulator of melanoma migration, they do support a model in which NF-κB is the primary target of integrin signaling. Our findings suggest a model for outside-in signaling through β1 integrin, which initially requires Ras and Rac GTPases, recruits activated PKC, and ultimately targets NF-κB-mediated transactivation of genes that participate in an efficient cell migration. Furthermore, PI-3K may directly and/or indirectly influence this pathway, although its exact role in regulating cell migration through an NF-κB-dependent mechanism requires further study.

Although other groups have suggested that NF-κB is involved in the migration of pancreatic carcinoma or keratinocytes (Yebra *et al*., 1995; Benoliel *et al*., 1997), these previous studies provide little insight into events responsible for NF-κB induction. More importantly, our data indicated a significant increase in transcription of β1 integrin mRNA following the CIV treatment. These results point toward regulation of β1 integrin expression as one possible mechanism that may affect cell motility in response to β1 integrin signals. Other potential genes regulated by NF-κB that could influence migration include actin-binding proteins, chemokines, and their receptors (Liotta *et al*., 1986; Lu and Kerbel, 1993). Moreover, induction of NF-κB upon successful extravasation to secondary sites could provide cell survival and proliferative signals, in part, through ectopic expression of mitogenic/chemotactic factors and adhesion receptors, thus imparting an advantage for these tumor cells.

Materials and methods

Cell culture and preparation

A2058 human melanoma cells (passages 14–18) were maintained in Dulbecco's modified Eagle medium (DMEM) (Biofluids Inc., Rockville, MD, USA) supplemented with 10% fetal bovine serum (Biofluids Inc.). For migration assays, cells were detached and suspended when subconfluent by treating with 0.05% trypsin/2 mM EDTA in phosphate-buffered saline (PBS) and gently rocked for 1 h in culture media at 37°C. Cells were resuspended in serum-free DMEM containing 0.1% w/v fraction V bovine serum albumin with 0.02 M HEPES (Sigma Chemical Co., St Louis, MO, USA), at 1.3×10^6 cells/ml and gently rocked for an additional 1 h prior to migration assays.

Migration assay

Chemotaxis assays using 48-well microchemotaxis chambers were described previously (Harvath *et al*., 1980). In brief, 10 μm pore size polycarbonate filters (Neuro Probe, Inc., Cabin John, MD, USA) were used. CIV (Becton Dickinson Labware, Bedford, MD, USA) was dispersed into media at a concentration of 100 μg/ml as the chemotactic solution (pH 7.4). The chemotactic solution was placed into the bottom wells of the chamber and the cell suspension was placed into the top wells, separated by a filter. The chamber assembly was placed into a 5% CO_2/37°C environment for 4 h. The filter was stained with the DiffQuik Staining Kit (Dade International Inc., Miami, FL, USA) and cells on the underside of the filter were visually counted using a bright field microscope. Negative

controls included samples that lacked CIV in the lower chamber. These background (negative control) values were subtracted from all of the migration results with CIV to reflect ligand-specific cell migration. Cell viability was monitored and confirmed at the end of migration assays by dye exclusion (data not shown).

Pharmacological inhibitors and antibodies

For inhibiting PKC activity, Gö6976 (Calbiochem, San Diego, CA, USA) was diluted to a concentration range of 26 nM–2.6 μM, and applied to cells 1 h prior to assays. Gö6983 was diluted to a concentration range of 0.226 nM–22.6 μM and applied to cells 1 h prior to experiments. PI-3K inhibitor LY294002 (Calbiochem) was applied to cells at 10–50 μM and 2 h prior to assays. Wortmannin (Calbiochem) was used in the concentration range of 50–500 nM, and applied to cells for a duration of 30 min, immediately prior to experiments. For inhibiting NF-κB, Helenalin (1–10 μM) and SN-50 (50 μg/ml) (Biomol Research Labs Inc., Plymouth Meeting, PA, USA) were applied to cells 2 h prior to experiments. Additionally, SN-50 M (inactive, negative control compound; Biomol Research Labs Inc.) was used at 50 μg/ml to serve as the negative control in experiments involving SN-50. Cell migration assays in which inhibitors were not included (vehicle control) served as the negative control for those agents where inactive forms of the inhibitors were not available. In all migration assays involving inhibitor treatments (except Wortmannin, irreversible inhibitor of PI-3K), both the cell suspension and the chemotactic solution contained the inhibitor at the specified concentration during the 4hr duration of migration assays. For all migration experiments involving inhibitors, cell viability was monitored and confirmed at the end of the migration assays by dye exclusion (data not shown).

The blocking antibody for β1 integrin (clone 4B4; Beckman Coulter Inc., Miami, FL, USA) was used at 25 μg/ml, and applied to monolayer of cells 40 min prior to addition of CIV. Antibody was present at the indicated concentration during the assay.

Plasmid DNA expression constructs

Mammalian expression constructs that encode the constitutively active and dominant negative mutants of GTPases RacV14/N17, RhoV12/N17, and cdc42V14/N19 (pcDNA backbone) were generously provided by Dr MA Schwartz (The Scripps Research Institute, La Jolla, CA, USA). RasN17 was provided by Dr K Calame (Columbia University, New York, NY, USA). NF-κB-luciferase reporter construct and RasV12 were obtained from Dr A August (Penn State University, University Park, PA, USA). Expression construct for enhanced green fluorescent protein (EGFP) was purchased (pEGFP-N3; Clontech Inc., Palo Alto, CA, USA).

Cell transfections

A2058 cells were transiently transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). A total of 25 μg of expression DNA construct encoding mutant GTPase or luciferase reporter was mixed with 5 μg of EGFP (Clontech Inc.) and transfected following the manufacturer's protocols. Transfection efficiency was monitored by EGFP expression 48 h post-transfection by fluorescence microscopy and flow cytometry. Viability was also confirmed by dye exclusion following 48 h post-transfection.

Western blots

Whole-cell extracts were prepared by resuspending 2×10^7 cells in 250 μl of lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA [pH 8.0], 2 mM Na_3VO_3, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1% NP-40, 1 mM PMSF, 2 ng/ml pepstatin A). Lysates were incubated on ice for 30 min followed by a centrifugation at 16 000*g* for 5 min at 4°C. The pellet was discarded and the supernatant was mixed with 2 × SDS running buffer (0.2% bromophenol blue, 4% SDS, 100 mM Tris [pH 6.8], 200 mM DTT, 20% glycerol) in 1 : 1 ratio. 30 μl was loaded onto a 10% SDS-PAGE gel and the protein was transferred to 0.2 μm nitrocellulose filter (Schleicher and Schuell, Keene, NH, USA) by electroblotting. Primary antibodies included anti-H-Ras (Rat monoclonal IgG; Zymed Labs Inc., South San Francisco, CA, USA), anti-Rac1 (Sigma Chemical Co.), anti-IκB-α (rabbit polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-β-actin IgG1 (Sigma Chemical Co.), and anti-HA (Rat polyclonal IgG; Roche Diagnostics Co., Indianapolis, IN, USA). Secondary antibodies were peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit IgG, or goat anti-rat IgG. Proteins were detected using the Enhanced Chemiluminescence Detection System (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). Filters were subsequently stripped and reprobed using the anti-β-actin or anti-HA antibody as described above.

Nuclear extract preparation and electromobility shift assays (EMSA)

Nuclear extracts from A2058 cells were prepared by lysing 2×10^6 cells with 10% nonidet NP-40 in Buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). Lysates were pelleted at 16 000 r.p.m. for 30 s and resuspended in Buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). Nuclear extracts were recovered by centrifugation at 16 000 rpm for 5 min at 4°C following a 15 min agitation of the suspension at 4°C (Schreiber *et al*., 1989). A total amount of 50 ng of annealed NF-κB binding site oligonucleotides (5′ AGCTAAGGGACTTTCCGCTGGGGACTTTCCAGG 3′ and 5′ AGCTCCTGGAAAGTCCCCAGCGGAAAGTCCCTT 3′) were end-filled with [α-^{32}P]dCTP using bacterial Klenow fragment (Promega, Madison, WI, USA). The DNA probe was used at a specific activity of 10^8–10^9 cpm/μg and incubated with 5 μg nuclear extract in a reaction mixture containing 3 μg dI-dC (Amersham Pharmacia Biotech), 0.25 M HEPES [pH 7.5], 0.6 M KCl, 50 mM $MgCl_2$, 1 mM EDTA, 7.5 mM DTT, and 9% glycerol for 20 min at 25°C. 50-fold excess of unlabeled NF-κB or irrelevant oligonucleotides (C/EBPβ; 5′ GATCGCCTAGCATTTCATCACACGT 3′ and 5′ GATCACGTGTGTGATGAAATGCTAGGC 3′) were used as specific and nonspecific competitors. A total of 0.5 μg of anti-p50 or anti-p65 (NF-κB), or anti-C/EBPβ antibody (Santa Cruz Biotechnology) was added to the reaction mixture during the 20 min incubation to supershift complexes. The samples were run on a 6% polyacrylamide gel prepared in 0.5 × TBE and visualized by autoradiography.

Luciferase assays

In all, 25 μg of NF-κB-LUC reporter construct was mixed with 5 μg of EGFP and transiently transfected into A2058 cells. Cells were harvested 48 h post transfection and prepared for cell migration assays as described. At the end of 4 h migration assays, cells associated with both upper and lower sides of the

microporous filter were collected by scraping, lysed and assayed for luciferase activity using the Promega Reporter Assay System (Promega). The lysate concentration was adjusted and normalized by spectrophotometric measurements using the Bradford protein quantification kit (Sigma Chemical Co.) prior to measuring the luciferase activity.

RNA extraction and RT-PCR

Total RNA was prepared by resuspending cells in PBS at 1.5×10^5 cells/ml and lysing with 4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarkosyl and 0.1 M β-mercaptoethanol followed by a phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The aqueous fractions containing RNA were treated with 10 U of DNase I for 20 min at 24°C to remove any contaminating genomic DNA, and further purified with phenol–chloroform extraction. cDNA was prepared from 2 μg of RNA using murine leukemia virus reverse transcriptase and random primers. Samples which did not include the reverse transcriptase served as controls for genomic DNA contamination. To amplify the cDNA, a 30-cycle polymerase chain reaction of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min was performed with 0.2 μg of cDNA using the target primers. Firefly luciferase cDNA was amplified using the primer pair 5′ GGAGAGCAACTG-CATAAGGC 3′ and 5' CATCGACTGAAATCCCTGGT3′. β1 integrin cDNA was amplified using the primer pair 5′ CCGAACGCGAGGGTC3′ and 5′CTCCCACCTGCGCTTG CCACCTGCGCTTG 3′. β-actin cDNA was amplified using the primer pair 5′ CCTAAGGCCAACCGTGAAAAG 3′ and 5′ TCTTCATGGTGCTAGGAGCCA 3′.

Acknowledgments
The authors thank Dr MA Schwartz (The Scripps Research Institute), Dr K Calame (Columbia University), Dr A August, Dr P Correll, and Dr JM Tarbell (Penn State University) for their generous gifts of cDNA constructs and pertinent reagents. The authors also thank Dr A August for his critical reading, helpful comments and discussions. Technical assistance of Eileen S Lee, Julie A Cook (Penn State University), and Elaine L Kunze (Flowcytometry facility, Penn State University) are also appreciated. This work was supported by NIH-CA76434 (CD), and NIH-AI46261 (AJH).

References

Anrather J, Csizmadia V, Soares MP and Winkler H. (1999). *J. Biol. Chem.*, **274,** 13 594–13 603.
Aspenström P. (1999). *Exp. Cell Res.*, **246,** 20–25.
Aznavoorian S, Stracke ML, Krutzsch H, Schiffmann E and Liotta LA. (1990). *J. Cell Biol.*, **110,** 1427–1438.
Benoliel AM, Kahn-Peries B, Imbert J and Verrando P. (1997). *J. Cell Sci.*, **110,** 2089–2097.
Boukerche H, Baril P, Tabone E, Berard F, Sanhadji K, Balme B, Wolf F, Perrot H and Thomas L. (2000). *Cancer Res.*, **60,** 5848–5856.
Burow ME, Weldon CB, Melnik LI, Duong BN, Collins-Burow BM, Beckman BS and McLachlan JA (2000). *Biochem. Biophys. Res. Commun.*, **271,** 342–345.
Chant J and Stowers L. (1995). *Cell*, **81,** 1–4.
Chomczynski P and Sacchi N. (1987). *Anal. Biochem.*, **162,** 156–159.
Condeelis J. (1993). *Annu. Rev. Cell Biol.*, **9,** 411–444.
Denhardt DT. (1996). *Biochem. J.*, **318,** 729–747.
Feig LA. (1999). *Nat. Cell Biol.*, **1,** E25–E27.
Giancotti FG and Ruoslahti E. (1999). *Science*, **285,** 1028–1032.
Harvath L, Falk W and Leonard EJ. (1980). *J. Immunol. Methods*, **37,** 39–45.
Hodgson L and Dong C. (2001). *Am. J. Physiol.*, **281,** C106–C113.
Karin M. (1994). *Curr. Opin. Cell Biol.*, **6,** 415–424.
Keely PJ, Parise LV and Juliano R. (1998). *Trends Cell Biol.*, **8,** 101–106.
Keely PJ, Westwick JK, Whitehead IP, Der CJ and Parise LV. (1997). *Nature*, **390,** 632–636.
Khoshnan A, Bae D, Tindell CA and Nel AE. (2000). *J. Immunol.*, **165,** 6933–6940.
Kjøller L and Hall A. (1999). *Exp. Cell Res.*, **253,** 166–179.
Leavesley DI, Schwartz MA, Rosenfeld M and Cheresh DA. (1993). *J. Cell Biol.*, **121,** 163–170.
Lester BR and McCarthy JB. (1992). *Cancer Metastasis Rev.*, **11,** 31–44.
Lin YZ, Yao SY, Veach RA, Torgerson TR and Hawiger J. (1995). *J. Biol. Chem.*, **270,** 14 255–14 258.
Liotta LA, Mandler R, Murano G, Katz DA, Gordon RK, Chiang PK and Schiffmann E. (1986). *Proc. Natl. Acad. Sci. USA*, **83,** 3302–3306.
Lu C and Kerbel RS. (1993). *J. Cell Biol.*, **120,** 1281–1288.
Lyss G, Knorre A, Schmidt TJ, Pahl HL and Merfort I. (1998). *J. Biol. Chem.*, **273,** 33 508–33 516.
Lyss G, Schmidt TJ, Merfort I and Pahl HL. (1997). *Biol. Chem.*, **378,** 951–961.
Machesky LM and Hall A. (1997). *J. Cell Biol.*, **138,** 913–926.
Meredith Jr JE, Winitz S, Lewis JM, Hess S, Ren XD, Renshaw MW and Schwartz MA. (1996). *Endocr. Rev.*, **17,** 207–220.
Minden A, Lin A, Claret FX, Abo A and Karin M. (1995). *Cell*, **81,** 1147–1157.
Nobes CD and Hall A. (1995). *Cell*, **81,** 53–62.
Palmantier R, George MD, Akiyama SK, Wolber FM, Olden K and Roberts JD. (2001). *Cancer Res.*, **61,** 2445–2452.
Paolucci L, Sinnett-Smith J and Rozengurt E. (2000). *Am. J. Physiol.*, **278,** C33–C39.
Parsons JT, Martin KH, Slack JK, Taylor JM and Weed SA. (2000). *Oncogene*, **19,** 5606–5613.
Pasco S, Han J, Gillery P, Bellon G, Maquart FX, Borel JP, Kefalides NA and Monboisse JC. (2000). *Cancer Res.*, **60,** 467–473.
Perona R, Montaner S, Saniger L, Sanchez-Perez I, Bravo R and Lacal JC. (1997). *Genes Dev.*, **11,** 463–475.
Powis G, Gallegos A, Abraham RT, Ashendel CL, Zalkow LH, Grindey GB and Bonjouklian R. (1994). *Cancer Chemother. Pharmacol.*, **34,** 344–350.
Price LS, Leng J, Schwartz MA and Bokoch GM. (1998). *Mol. Biol. Cell*, **9,** 1863–1871.
Renshaw MW, Toksoz D and Schwartz MA. (1996). *J. Biol. Chem.*, **271,** 21691–21694.
Ridley AJ and Hall A. (1992). *Cell*, **70,** 389–399.
Rong R, He Q, Liu Y, Sheikh MS and Huang Y. (2002). *Oncogene*, **21,** 1062–1070.

Salazar EP and Rozengurt E. (2001). *J. Biol. Chem.*, **276,** 17788–17795.
Sarner S, Kozma R, Ahmed S and Lim L. (2000). *Mol. Cell Biol.*, **20,** 158–172.
Savarese DMF, Russell JT, Fatatis A and Liotta LA. (1992). *J. Biol. Chem.*, **267,** 21928–21935.
Schaller MD and Parsons JT. (1994). *Curr. Opin. Cell Biol.*, **6,** 705–710.
Schnaper HW and Kleinman HK. (1993). *Pediatr. Nephrol.*, **7,** 96–104.
Schreiber E, Matthias P, Muller MM and Schaffner W. (1989). *Nucl. Acids Res.*, **17,** 6419–6420.
Schwartz MA and Ingber DE. (1994). *Mol. Biol. Cell*, **5,** 389–393.
Schwartz MA, Schaller MD and Ginsberg MH. (1995). *Annu. Rev. Cell Dev. Biol.*, **11,** 549–599.
Stossel TP. (1993). *Science*, **260,** 1086–1094.
Stracke ML, Murata J, Aznavoorian S and Liotta LA. (1994). *In vivo* **8,** 49–58.
Tapon N and Hall A. (1997). *Curr. Opin. Cell Biol.*, **9,** 86–92.
Trushin SA, Pennington KN, Algeciras-Schimnich A and Paya CV. (1999). *J. Biol. Chem.*, **274,** 22 923–22 931.
Van Aelst L and D'Souza-Schorey C. (1997). *Genes Dev.*, **11,** 2295–2322.
Yebra M, Filardo EJ, Bayna EM, Kawahara E, Becker JC and Cheresh DA. (1995). *Mol. Biol. Cell*, **6,** 841–850.
Zigmond SH. (1996). *Curr. Opin. Cell Biol.*, **8,** 66–73.