

RhoA Function in Lamellae Formation and Migration Is Regulated by the $\alpha 6\beta 4$ Integrin and cAMP Metabolism

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Abstract. Clone A colon carcinoma cells develop fan-shaped lamellae and exhibit random migration when plated on laminin, processes that depend on the ligation of the $\alpha 6\beta 4$ integrin. Here, we report that expression of a dominant negative RhoA (N19RhoA) in clone A cells inhibited $\alpha 6\beta 4$ -dependent membrane ruffling, lamellae formation, and migration. In contrast, expression of a dominant negative Rac (N17Rac1) had no effect on these processes. Using the Rhotekin binding assay to assess RhoA activation, we observed that engagement of $\alpha 6\beta 4$ by either antibody-mediated clustering or laminin attachment resulted in a two- to threefold increase in RhoA activation, compared with cells maintained in suspension or plated on collagen. Antibody-mediated clustering of $\beta 1$ integrins, however, actually suppressed

RhoA activation. The $\alpha 6\beta 4$ -mediated interaction of clone A cells with laminin promoted the translocation of RhoA from the cytosol to membrane ruffles at the edges of lamellae and promoted its colocalization with $\beta 1$ integrins, as assessed by immunofluorescence microscopy. In addition, RhoA translocation was blocked by inhibiting phosphodiesterase activity and enhanced by inhibiting the activity of cAMP-dependent protein kinase. Together, these results establish a specific integrin-mediated pathway of RhoA activation that is regulated by cAMP and that functions in lamellae formation and migration.

Key words: carcinoma • protein kinase A • G-protein • phosphodiesterase • cytoskeleton

Introduction

The organization and remodeling of the actin cytoskeleton are controlled by the Rho family of small GTPases, which includes Rho, Rac, and cdc42. These proteins have been implicated in the formation of stress fibers, lamellipodia, and filopodia, respectively (reviewed in Hall, 1998). Although much of our knowledge on their function has been obtained from studies with fibroblasts, other activities for these Rho GTPases have been observed recently in cells of epithelial origin. For example, Rac and cdc42 are required to maintain apical-basal polarity in epithelia (Jou and Nelson, 1998). Rho, interestingly, has been implicated in membrane ruffling in epithelial cells (Nishiyama et al., 1994; Fukata et al., 1999), a process attributed to Rac in fibroblasts (Hall, 1998). These findings are of particular interest with respect to our understanding of epithelial cell migration. The migration and invasion of epithelial-derived carcinoma cells are important phenomena that require the

involvement of Rho GTPases (Keely et al., 1997; Shaw et al., 1997; Yoshioka et al., 1998; Itoh et al., 1999). For these reasons, it is essential to define the factors that regulate the function of Rho GTPases in carcinoma cells and to characterize the mechanisms by which they contribute to the dynamics of migration. For example, although cell adhesion has been reported to activate RhoA (Barry et al., 1997; Ren et al., 1999), little is known about the involvement of specific integrins in adhesion-dependent RhoA activation or in the regulation of RhoA-dependent functions.

Recent studies by our group have highlighted a pivotal role for the integrin $\alpha 6\beta 4$ in the migration and invasion of carcinoma cells, as well as in epithelial wound healing (Lotz et al., 1997; Rabinovitz and Mercurio, 1997; Shaw et al., 1997; O'Connor et al., 1998; Rabinovitz et al., 1999). Although it is well established that $\alpha 6\beta 4$ functions in the formation and stabilization of hemidesmosomes (Borradori and Sonnenberg, 1996; Green and Jones, 1996), our findings revealed a novel role for this integrin in the formation of actin-rich cell protrusions at the leading edges of carcinoma cells and in the migration of these cells

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(Rabinovitz and Mercurio, 1997; Rabinovitz et al., 1999). Moreover, we demonstrated the importance of cAMP metabolism in these events (O'Connor et al., 1998). Given the recent interest in the participation of RhoA in migration, we examined the hypothesis that RhoA is essential for the formation of actin-rich cell protrusions, the migration of carcinoma cells and, more importantly, that the activity of RhoA is regulated by the $\alpha 6 \beta 4$ integrin. In addition, we assessed the involvement of cAMP metabolism in these events.

Materials and Methods

Cells and Antibodies

Clone A cells, originally isolated from a poorly differentiated colon adenocarcinoma (Dexter et al., 1979), were used in all experiments. For each experiment, adherent cells were harvested by trypsinization, rinsed three times with RPMI medium containing 250 $\mu\text{g/ml}$ heat-inactivated BSA (RPMI/BSA), and resuspended in RPMI/BSA. Where indicated, cells were treated with 1 mM isobutylmethylxanthine (IBMX)¹ or 15 mM H-89 (Calbiochem-Novabiochem, Int.) for 15 min before use. The following antibodies were used in this study: MC13, mouse anti- $\beta 1$ integrin mAb (obtained from Steve Akiyama, National Institutes of Health, Research Triangle Park, NC); K20, mouse anti- $\beta 1$ integrin mAb (Immunotech); 439-9B, rat anti- $\beta 4$ integrin mAb (obtained from Rita Falcioni, Regina Elena Cancer Institute, Rome, Italy); mouse anti-HA mAb (Roche Biochemicals); rabbit anti-RhoA polyclonal antibody (Santa Cruz Biotechnology); and anti-Rac1 (Transduction Laboratories).

To obtain expression of N19RhoA and N17Rac1, adherent cells were harvested using trypsin, rinsed with PBS, and suspended in electroporation buffer (20 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 5 mM glucose). Cells were cotransfected with 1 mg of either pCS2-(n) β -gal or pGFP (green fluorescent protein) and 4 μg of either control vector or vector containing HA-tagged N19RhoA (provided from Alex Toker, Beth Israel Deaconess Medical Center, Boston, MA) or GST-tagged N17Rac1 (obtained from Margaret Chou, University of Pennsylvania) by electroporation at 250V and 500 μF . Subsequently, cells were plated in complete growth medium containing 0.05% sodium butyrate and used for experiments 48 h after the initial transfection. Expression of the recombinant proteins was confirmed by concentrating extracts of transfected cells with an HA-specific mAb or glutathione-coupled beads and subsequent immunoblotting for RhoA or Rac1, respectively.

Microscopic Analyses

Glass coverslips were coated overnight at 4°C with collagen I (50 $\mu\text{g/ml}$; Collagen Corp.) or laminin-1 purified from EHS tumor (20 $\mu\text{g/ml}$; provided by Hinda Kleinman, NIDR, Bethesda, MD) and then blocked with BSA (0.25% in RPMI). Cells were plated on these coverslips for 30–40 min, rinsed with PBS, fixed, and then permeabilized as described previously (O'Connor et al., 1998). For immunofluorescence, cells were incubated with 1 $\mu\text{g/ml}$ of K20 (anti- $\beta 1$) and anti-RhoA antibody diluted in block solution (3% BSA/1% normal donkey serum in PBS) for 30 min, rinsed four times with PBS, and then incubated for 30 min with a 1:400 dilution of anti-mouse IgG Cy2- and anti-rabbit IgG Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Images of cells were captured digitally, analyzed, and lamellar area quantified as described previously (Rabinovitz and Mercurio, 1997; O'Connor et al., 1998).

Migration Assays

The lower compartments of Transwell chambers (6.5-mm diam, 8- μm pore size; Costar) were coated for 30 min with 15 $\mu\text{g/ml}$ laminin-1 diluted

in RPMI medium. RPMI/BSA was added to the lower chamber and cells (1×10^5) suspended in RPMI/BSA were added to the upper chamber. After incubating for 5 h at 37°C, cells were removed from the upper chamber with a cotton swab and cells that had migrated to the lower surface of the membrane were fixed, stained with crystal violet or for β -galactosidase (β -gal), and quantified as described previously (Shaw et al., 1997).

RhoA Activity

RhoA activity was assessed using the Rho-binding domain of Rhotekin as described (Ren et al., 1999). In brief, cells (3×10^6) were plated onto 60-mm dishes coated with LN-1 (20 $\mu\text{g/ml}$) or collagen I (50 $\mu\text{g/ml}$) for 30 min and extracted with RIPA buffer (50 mM Tris, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS, 10 mM MgCl_2 , 0.5 $\mu\text{g/ml}$ leupeptin, 0.7 $\mu\text{g/ml}$ pepstatin, 4 $\mu\text{g/ml}$ aprotinin, and 2 mM PMSF). Alternatively, cells were incubated with 8 μg of anti- $\beta 1$ mAb mc13 or anti- $\beta 4$ rat mAb 439-9B for 30 min, rinsed, plated on 60-mm dishes coated with 50 μg of either anti-mouse or anti-rat IgG, respectively, for 30 min, and then extracted. After centrifugation at 14,000 g for 3 min, the extracts were incubated for 45 min at 4°C with glutathione beads (Pharmacia Biotech) coupled with bacterially expressed GST-RBD (Rho-binding domain of Rhotekin) fusion protein (provided by Martin Schwartz, Scripps Research Institute, La Jolla, CA), and then washed three times with Tris buffer, pH 7.2, containing 1% Triton X-100, 150 mM NaCl, and 10 mM MgCl_2 . The RhoA content in these samples was determined by immunoblotting samples using rabbit anti-RhoA antibody.

Results

Clone A colon carcinoma cells develop fan-shaped lamellae and exhibit random migration when plated on laminin-1, processes that are dependent on both the $\alpha 6 \beta 4$ and $\beta 1$ integrins. In contrast, the $\beta 1$ integrin-mediated adhesion and spreading of these cells on collagen I does not induce significant lamellae formation or migration (Rabinovitz and Mercurio, 1997; Shaw et al., 1997). To examine the hypothesis that RhoA functions in $\alpha 6 \beta 4$ -dependent lamellae formation, clone A cells were cotransfected with a GFP construct and either a dominant negative RhoA (N19RhoA) or a control vector. Subsequently, the cells were plated onto laminin-1 and examined by phase-contrast microscopy. Clone A cells that expressed the control vector developed large lamellae with ruffled edges (Fig. 1 A). In contrast, cells that expressed N19RhoA developed only a few small, fragmented lamellae that were devoid of membrane ruffles (Fig. 1 B). Quantitative analysis of these images revealed that expression of N19RhoA reduced lamellar area by 80% in comparison to cells that expressed the control vector (Fig. 1 D). Interestingly, expression of a GST-tagged, dominant negative Rac1 (N17Rac1) did not inhibit either lamellae formation or membrane ruffling in clone A cells (Fig. 1, C and D), although this construct has been shown to inhibit p70 S6 kinase (Chou and Blenis, 1996) and invasion (Shaw et al., 1997).

Expression of N19RhoA inhibited the migration of clone A cells on laminin-1 by 70% (Fig. 2 A). In contrast, expression of N17Rac did not inhibit the migration of clone A cells (Fig. 2 A), although it did inhibit the migration of 3T3 cells by 85% (data not shown). Importantly, expression of N19RhoA had only a modest effect on cell spreading because cells expressing N19RhoA plated on collagen-I spread to $\sim 80\%$ of the surface area occupied by control cells (Fig. 1 E). Expression of N19RhoA and N17Rac1 in clone A cells was confirmed by immunoblotting (Fig. 1, F and G).

Our observation that RhoA functions in lamellae for-

¹Abbreviations used in this paper: β -gal, β -galactosidase; GFP, green fluorescent protein; IBMX, isobutylmethylxanthine; LPA, lysophosphatidic acid; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; RBD, Rho-binding domain of Rhotekin.

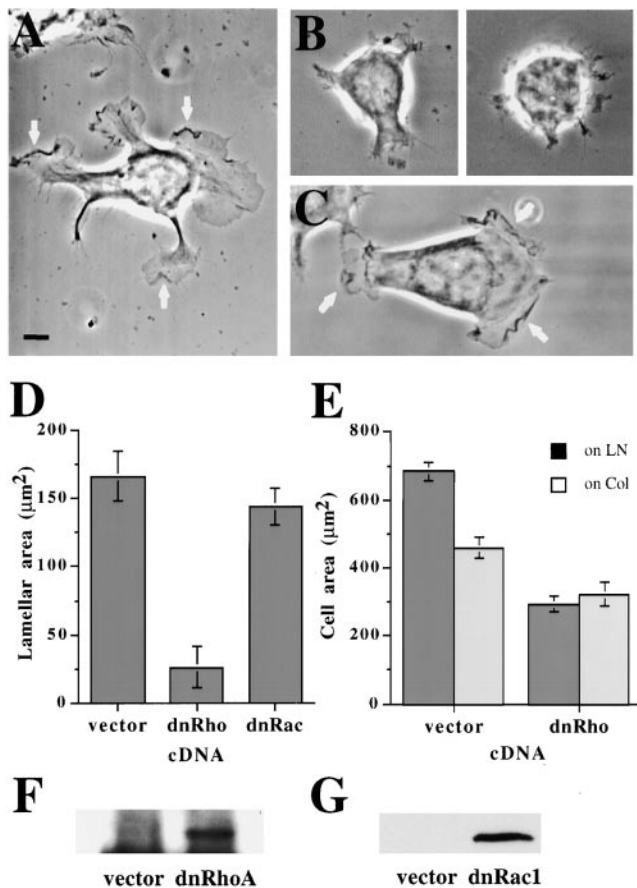


Figure 1. Dominant negative RhoA inhibits membrane ruffling and lamellae formation in clone A cells in response to laminin-1. Clone A cells were cotransfected with a GFP construct and either a control vector or a vector encoding N19RhoA or N17Rac as described in Materials and Methods. Cells were plated onto laminin-coated coverslips for 40 min, fixed, and assessed by phase-contrast microscopy. A–C, Phase-contrast microscopy of vector control (A), N19RhoA (B, two panels), or N17Rac (C) transfected cells. Note large lamellae and membrane ruffles in control and N17Rac transfected cells (open arrow in A and C), but not in cells that express N19RhoA (B). Representative GFP-positive cells are shown. D, Quantitative analysis of the lamellar area of transfected, GFP-positive cells was obtained by digital imaging. Lamellae are defined as broad, flat cellular protrusions rich in F-actin and devoid of membrane-bound vesicles. E, Quantitative analysis of total area covered by cells transfected with either vector control or N19RhoA when plated on laminin-1 (dark bars) or collagen I (light bars). Bars represent mean area \pm SEM in which $n > 20$ (D, E). F and G, Transfected cells were extracted with RIPA buffer and either immunoprecipitated with HA-specific mAb and immunoblotted for RhoA (F), or concentrated using glutathione-Sepharose and immunoblotted for Rac1 (G). Representative blots are shown.

mation and the migration of clone A cells, in conjunction with our previous finding that these events require the engagement of the $\alpha 6 \beta 4$ integrin (Rabinovitz and Mercurio, 1997), indicated that $\alpha 6 \beta 4$ may mediate the activation of RhoA. To assess RhoA activation, we used the RBD to capture GTP-bound RhoA from cell extracts (Ren et al., 1999). As shown in Fig. 3, the interaction of clone A cells

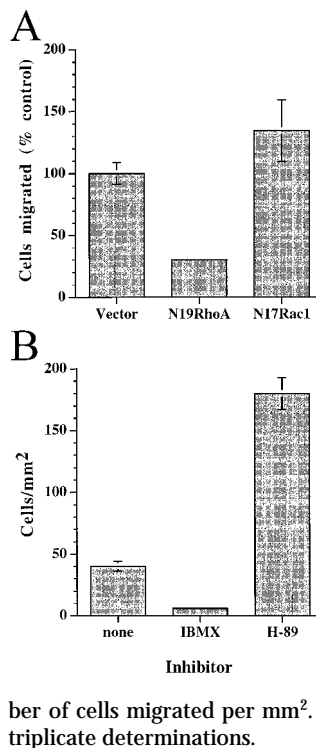


Figure 2. Effects of dominant negative RhoA (N19RhoA) and cAMP metabolism on laminin-1 stimulated migration. A, Clone A cells that had been cotransfected with a β -gal cDNA and either N19RhoA, N17Rac1, or control vector were assayed for migration on laminin-1 as described in Materials and Methods. Migration was scored as the relative number of β -gal staining cells migrated compared with the vector only control. Transfection rates were comparable. B, Clone A cells were left untreated or treated with 1 mM IBMX or 15 μ M H-89 for 15 minutes and then assayed for laminin-1 mediated migration as described in Materials and Methods. Migration rates were reported as the number of cells migrated per mm^2 . Bars represent mean \pm SD from triplicate determinations.

with laminin-1, which requires $\alpha 6 \beta 4$, resulted in a significant amount of RhoA retained by RBD in comparison to the interaction of these cells with collagen I, which does not involve $\alpha 6 \beta 4$ directly. These experiments were performed with cells that had been attached to laminin for 30 min because membrane ruffling was most apparent at this time. Quantitative analysis of the results obtained in four independent experiments revealed a threefold greater increase in RhoA activation in cells plated on laminin-1 than in cells plated on collagen (Fig. 3 B). To establish the ability of $\alpha 6 \beta 4$ to activate RhoA more definitively, we used integrin-specific mAbs to cluster both $\alpha 6 \beta 4$ and $\beta 1$ integrins. As shown in Fig. 3, C and D, clustering of $\alpha 6 \beta 4$ resulted in an approximate two- to threefold higher level of RhoA activity in comparison to cells maintained in suspension. Interestingly, clustering of $\beta 1$ integrins actually decreased RhoA activation in comparison to cells maintained in suspension (Fig. 3), even though clone A cells express similar surface levels of both integrins (Lee et al., 1992). Similar results were obtained between 5 and 30 min of antibody clustering (data not shown).

The involvement of cAMP metabolism in migration, lamellae formation, and $\alpha 6 \beta 4$ -mediated RhoA activation was investigated using both IBMX, a phosphodiesterase (PDE) inhibitor, and H-89, a cAMP-dependent protein kinase (PKA) inhibitor. IBMX treatment, which prevents cAMP breakdown, inhibited the migration of clone A cells on laminin-1 almost completely (Fig. 2 B). In contrast, inhibition of PKA with H-89 increased the rate of migration by fourfold (Fig. 2 B). Together, these data indicate that cAMP inhibits or “gates” carcinoma migration and lamellae formation, in agreement with our previous findings (O’Connor et al., 1998). To establish the involvement of cAMP metabolism in the $\alpha 6 \beta 4$ -mediated activation of

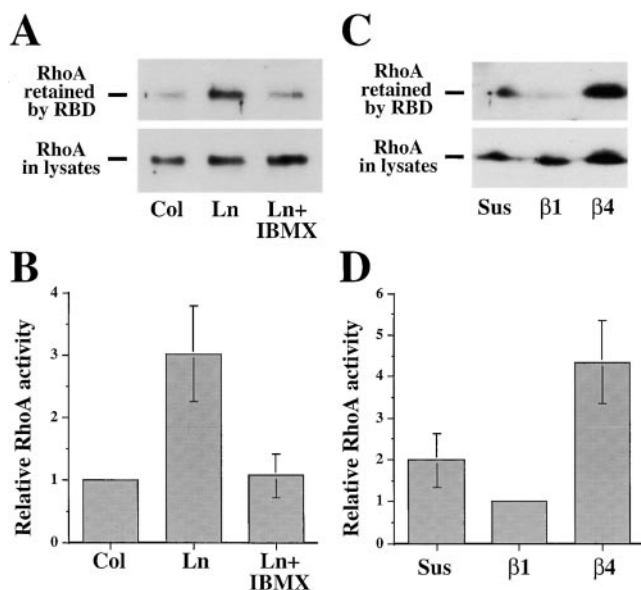


Figure 3. Engagement of the $\alpha 6 \beta 4$ integrin by either laminin-1 or antibody-mediated clustering activates RhoA. A and B, Clone A cells were plated on either collagen or laminin for 30 min or pretreated with 1 mM IBMX for 15 min and then plated on laminin for 30 min. Cell extracts were assayed for Rhotekin binding activity as described in Materials and Methods. C and D, Cells were either left in suspension (sus) or clustered with either $\beta 1$ - or $\beta 4$ -specific antibodies for 30 min as described in Materials and Methods. Cell extracts were assessed for RhoA activity by RBD binding. For these experiments, the total RhoA bound to the RBD (top panels in A and C) was normalized to the RhoA content of cell extracts (bottom panels in A and C). A and C, Representative immunoblots from these experiments are shown. B and D, Quantitative analysis of the results obtained by densitometry is provided. Bars represent mean of four separate experiments \pm SEM.

RhoA, we used IBMX in the RBD assay. As shown in Fig. 3 A, pretreatment of clone A cells with IBMX before plating on laminin-1 reduced the level of RhoA activation to that observed in cells plated on collagen. Importantly, IBMX did not inhibit either cell adhesion or spreading (Fig. 4, C and E). Similar results were obtained with integrin clustering (data not shown). These observations implicate cAMP metabolism in the $\alpha 6 \beta 4$ -mediated activation of RhoA.

The data reported here raise the possibility that $\alpha 6 \beta 4$ influences RhoA localization because activation of RhoA is thought to involve its translocation to membranes (Bokoch et al., 1994). To address this issue, clone A cells plated on either laminin-1 or collagen I were immunostained with a RhoA-specific antibody, as well as a $\beta 1$ -integrin-specific antibody to mark membranes. In cells plated on collagen I, RhoA immunostaining was confined largely to the cytosol and it was distinct from the $\beta 1$ -integrin staining of the plasma membrane (Fig. 4 A). In contrast, the $\alpha 6 \beta 4$ -dependent interaction of clone A cells with laminin-1 resulted in the translocation of RhoA to membrane ruffles at the edges of lamellae where it colocalized with $\beta 1$ integrin staining (Fig. 4 B). However, RhoA did not colocalize with $\beta 1$ integrins on the plasma membrane

along the cell body (Fig. 4 B). To assess the influence of cAMP metabolism on RhoA localization, clone A cells were pretreated with either IBMX or H-89 before plating on laminin-1. Inhibition of PDE activity with IBMX dramatically inhibited membrane ruffling and abolished RhoA localization in the few ruffles that persisted after IBMX treatment (Fig. 4, C and E). Conversely, inhibition of cAMP-dependent PKA with H-89 resulted in an apparent increase in membrane ruffling and RhoA localization in membrane ruffles (Fig. 4, D and F).

Discussion

Recently, we established that the $\alpha 6 \beta 4$ integrin stimulates the migration of carcinoma cells and enhances the formation of actin-rich protrusions, including lamellae and membrane ruffles (Shaw et al., 1997; Rabinovitz and Mercurio, 1997; O'Connor et al., 1998; Rabinovitz et al., 1999). In this study, we advance our understanding of the mechanism by which $\alpha 6 \beta 4$ functions in these dynamic processes by demonstrating that ligation of $\alpha 6 \beta 4$ with either antibody or laminin-1 results in the activation of RhoA and its translocation from the cytosol to membrane ruffles at the leading edges of migrating carcinoma cells. Importantly, we also provide evidence that the $\alpha 6 \beta 4$ -mediated activation of RhoA is necessary for lamellae formation, membrane ruffling, and migration. Furthermore, we establish that these events are regulated by cAMP metabolism and that they can occur independently of Rac1 involvement.

Our findings strengthen the evidence that integrins can participate in the activation of RhoA. Much of the evidence supporting integrin activation of RhoA had been based largely on the observation that integrin activation leads to the Rho-dependent formation of stress fibers and focal adhesions (Ren et al., 1999; Schoenwaelder and Burridge, 1999). Recently, the development of a biochemical assay for RhoA activation using the ability of GTP-bound RhoA to associate with the Rho-binding domain of Rhotekin has enabled a more rigorous and sensitive assessment of the mechanism of RhoA activation (Ren et al., 1999). Using this assay, cell attachment to fibronectin was shown to activate RhoA and that the level of activation was augmented by serum or lysophosphatidic acid (LPA). In our study, we extend this observation by providing evidence that a specific integrin, $\alpha 6 \beta 4$, can activate RhoA, as assessed by both Rhotekin binding and translocation to membrane ruffles. An interesting and unexpected finding obtained in our study is that the $\alpha 6 \beta 4$ integrin is a more effective activator of RhoA than $\beta 1$ integrins in clone A cells. In fact, antibody-mediated ligation of $\beta 1$ integrins actually suppressed RhoA activation. Because we used carcinoma cells in our study, the potent activation of RhoA we observed in response to $\alpha 6 \beta 4$ ligation could have resulted from a cooperation of $\alpha 6 \beta 4$ with a secreted growth factor or activated oncogene. However, if cooperative signaling between integrins and such factors occurs in these cells, it is specific for $\alpha 6 \beta 4$ because clustering of $\beta 1$ integrins did not activate RhoA.

Our findings implicate an important role for RhoA in the formation of membrane ruffles and lamellae. Specifically, the expression of N19RhoA in clone A cells attached to laminin resulted in the appearance of frag-

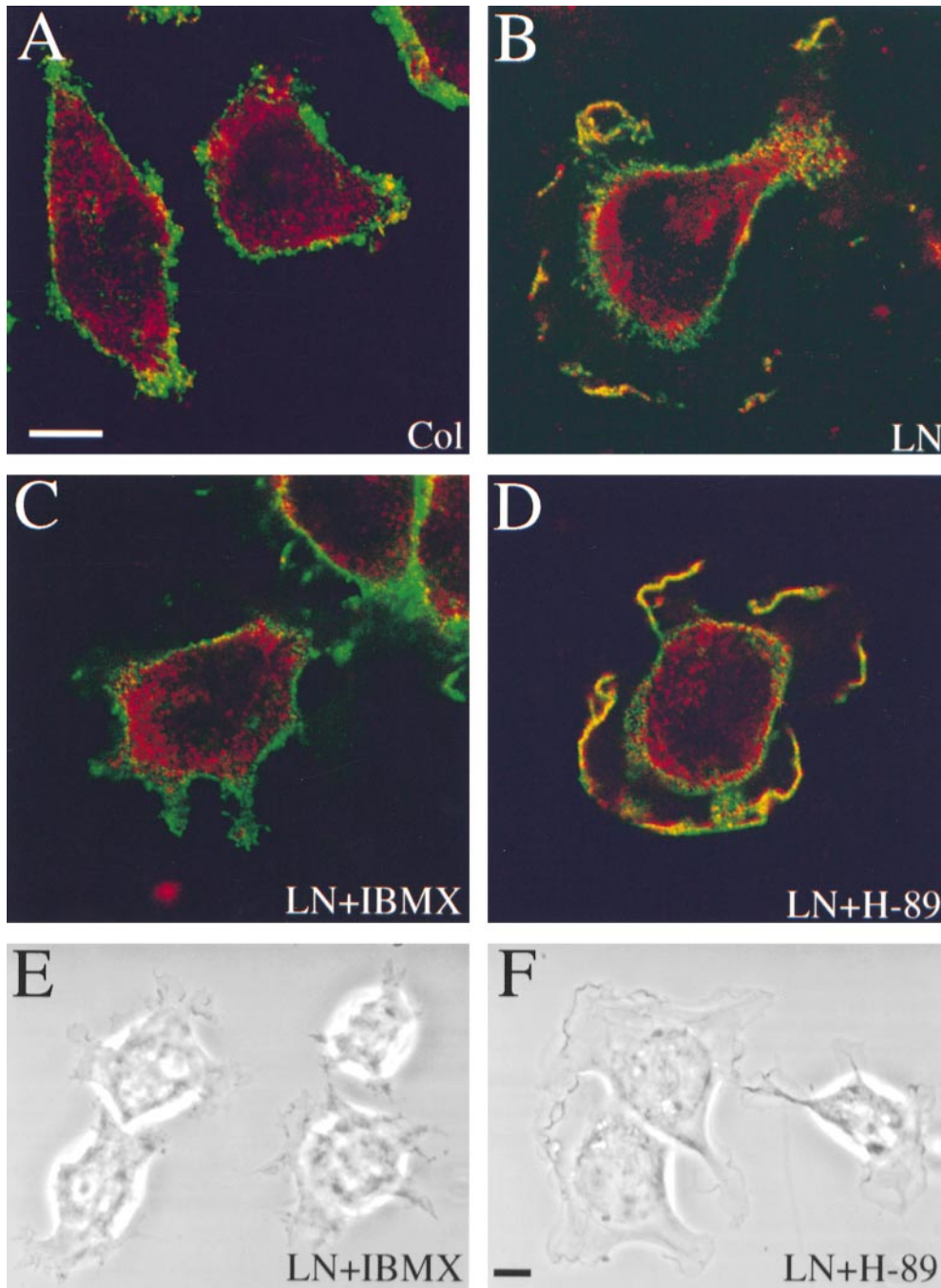


Figure 4. Laminin-1, but not collagen-I, promotes the colocalization of RhoA and $\beta 1$ integrin in membrane ruffles in a cAMP-sensitive manner. A–D, Clone A cells were plated on either collagen I (A) or laminin-1 (B–D) for 30 min, fixed, and stained for both $\beta 1$ integrin and RhoA using indirect immunofluorescence as described in Materials and Methods. To assess the impact of cAMP signaling on recruitment of RhoA to the plasma membrane, cells were pretreated with 1 mM IBMX (C) or 15 μ M H-89 (D) for 15 min before plating cells on laminin-1. Images were captured digitally $\sim 1 \mu$ m from the basal surface using a Bio-Rad confocal microscope. Red color represents RhoA; green, $\beta 1$ integrin; yellow, $\beta 1$ and RhoA colocalization. E and F, Phase-contrast micrographs of cells treated with IBMX (E) or H-89 (F) and plated on laminin depict the general impact of cAMP metabolism on membrane ruffling. Bars, 10 μ m.

mented, immature lamellae and a loss of membrane ruffles. These results are of interest in light of recent reports that Rho kinase, a downstream effector of Rho, promotes membrane ruffling in epithelial-derived cells (Nishiyama et al., 1994; Fukata et al., 1999) by a mechanism that involves Rho kinase-mediated phosphorylation of adducin (Fukata et al., 1999). Moreover, both Rho and Rho kinase have been implicated in tumor cell invasion (Yoshioka et al., 1998; Itoh et al., 1999). Together, these findings along with our previous work that established the ability of $\alpha 6\beta 4$ to promote carcinoma migration and invasion (Rabinovitz and Mercurio, 1997; Shaw et al., 1997; O'Connor et al., 1998), suggest that $\alpha 6\beta 4$ -mediated regulation of the Rho/

Rho kinase pathway is an important component of carcinoma progression. It is also possible that the $\alpha 6\beta 4$ -mediated activation of RhoA contributes to migration and invasion by activating the adhesive functions of other integrins. RhoA can activate integrin-mediated adhesion in leukocytes (Laudanna et al., 1997) and is believed to participate in adhesion in other cell types (Nobes and Hall, 1999). Our observation that RhoA and $\beta 1$ integrins colocalize in membrane ruffles in response to $\alpha 6\beta 4$ ligation raises the possibility that RhoA influences the function of $\beta 1$ integrins, which are essential for migration and invasion.

Interestingly, expression of the dominant negative

N17Rac in clone A cells had no inhibitory effect on either membrane ruffling, lamellae formation, or migration. Although it is well established that Rac functions in lamellipodia formation in fibroblasts (Hall, 1998) and in the migration of several cell types (e.g., see Keely et al., 1997; Shaw et al., 1997; Nobes and Hall, 1999), recent studies have highlighted the complexity of Rac involvement in these dynamic processes. For example, Rac activation can also inhibit migration by promoting cadherin-mediated cell-cell adhesion (Hordijk et al., 1997; Sander et al., 1999) and by downregulating Rho activity (Sander et al., 1999). Nonetheless, Rac activation stimulates membrane ruffling under conditions in which it also promotes cell-cell adhesion (Sander et al., 1999). Clone A cells, therefore, may represent the first example of a cell type in which both membrane ruffling and migration are Rac-independent.

Our results highlight the importance of cAMP metabolism in the activation and localization of RhoA. Our finding that cAMP inhibits RhoA activation and translocation to membrane ruffles is consistent with our previous report that linked the ability of $\alpha 6\beta 4$ to promote carcinoma migration with its ability to alter cAMP metabolism (O'Connor et al., 1998). In addition, these results substantiate other studies that indicated an inhibitory effect of cAMP on RhoA activity (Lang et al., 1996; Laudanna et al., 1997; Dong et al., 1998). The basis for this inhibition may be the direct phosphorylation of RhoA by PKA (Lang et al., 1996). In this context, $\alpha 6\beta 4$ may contribute to RhoA activation by increasing the activity of a cAMP-dependent PDE and subsequently reducing PKA activity, as we have suggested previously (O'Connor et al., 1998). However, the fact that we observed RhoA activation in response to antibody-mediated clustering of $\alpha 6\beta 4$ suggests this integrin can be linked directly to RhoA activation. This direct activation of RhoA would permit $\alpha 6\beta 4$ to augment pathways, such as LPA signaling, that involve RhoA activation. In conclusion, the results reported here establish a specific integrin-mediated pathway of RhoA activation that is regulated by cAMP and functions in lamellae formation and migration.

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References

Barry, S.T., H.M. Flinn, M.J. Humphries, D.R. Critchley, and A.J. Ridley. 1997. Requirement for Rho in integrin signaling. *Cell Adhes. Comm.* 4:387-398.

- Bokoch, G.M., B.P. Bohl, and T.-H. Chuang. 1994. Guanine nucleotide exchange regulates membrane translocation of Rac/Rho GTP-binding proteins. *J. Biol. Chem.* 269:31674-31679.
- Borradori, L., and A. Sonnenberg. 1996. Hemidesmosomes: roles in adhesion, signaling and human disease. *Curr. Opin. Cell Biol.* 8:647-658.
- Chou, M.M., and J. Blenis. 1996. The 70 kDa S6 kinase complexes with and is activated by the rho family G proteins cdc42 and Rac. *Cell.* 85:573-583.
- Dexter, D.L., J.A. Barbosa, and P. Calabresi. 1979. N', N-dimethylformamide-induced alteration of cell culture characteristics and loss of tumorigenicity in cultured human colon carcinoma cells. *Cancer Res.* 39:1020-1025.
- Dong, J.-M., T. Leung, E. Manser, and L. Lim. 1998. cAMP-induced morphological changes are counteracted by the activated RhoA small GTPases and the Rho kinase ROK-a. *J. Biol. Chem.* 273:22554-22562.
- Fukata, Y., N. Oshiro, N. Kinoshita, Y. Kawano, Y. Matsuoka, V. Bennett, Y. Matsuura, and K. Kaibuchi. 1999. Phosphorylation of adducin by Rho-kinase plays a crucial role in cell motility. *J. Cell Biol.* 145:347-361.
- Green, K.J., and J.C.R. Jones. 1996. Desmosomes and hemidesmosomes: structure and function of molecular components. *FASEB J.* 10:871-881.
- Hall, A. 1998. Rho GTPases and the actin cytoskeleton. *Science.* 279:509-514.
- Hordijk, P.L., J.P. ten Klooster, R.A. van der Kammen, F. Michiels, L.C. Oomen, and J.G. Collard. 1997. Inhibition of invasion of epithelial cells by Tiam1-Rac signaling. *Science.* 278:1464-1466.
- Itoh, K., K. Yoshioka, H. Akedo, M. Uehata, T. Ishizake, and S. Narumiya. 1999. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. *Nature Med.* 5:221-225.
- Jou, T.-S., and W.J. Nelson. 1998. Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. *J. Cell Biol.* 142:85-100.
- Keely, J.P., J.K. Westwick, I.P. Whitehead, C.J. Der, and L.V. Parise. 1997. Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature.* 390:632-636.
- Lang, P., F. Gesbert, M. Delespine-Carmagnat, R. Stancou, M. Pouchet, and J. Bertoglio. 1996. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:510-519.
- Laudanna, C., J.M. Campbell, and E.C. Butcher. 1997. Elevation of intracellular cAMP inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemoattractants. *J. Biol. Chem.* 272:24141-24144.
- Lee, E.C., M.M. Lotz, G.D. Steele, and A.M. Mercurio. 1992. The integrin $\alpha 6\beta 4$ is a laminin receptor. *J. Cell Biol.* 117:671-678.
- Lotz, M.M., C.A. Korzelius, and A.M. Mercurio. 1997. Intestinal epithelial restitution. Involvement of specific laminin isoforms and integrin receptors in wound closure of a transformed model epithelium. *Am. J. Pathol.* 150:747-760.
- Nishiyama, T., T. Sasaki, K. Takaishi, M. Kato, H. Yaku, K. Araki, Y. Matsuura, and Y. Takai. 1994. *rac p21* is involved in insulin-induced membrane ruffling and *rho p21* is involved in hepatocyte growth factor- and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced membrane ruffling in KB cells. *Mol. Cell. Biol.* 14:2247-2456.
- Nobes, C.D., and A. Hall. 1999. Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J. Cell Biol.* 144:1235-1244.
- O'Connor, K.L., L.M. Shaw, and A.M. Mercurio. 1998. Release of cAMP gating by the $\alpha 6\beta 4$ integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. *J. Cell Biol.* 143:1749-1760.
- Rabinovitz, I., and A.M. Mercurio. 1997. The integrin $\alpha 6\beta 4$ functions in carcinoma cell migration on laminin-1 by mediating the formation and stabilization of actin-containing motility structures. *J. Cell Biol.* 139:1873-1884.
- Rabinovitz, I., A. Toker, and A.M. Mercurio. 1999. Protein kinase C-dependent mobilization of the $\alpha 6\beta 4$ integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. *J. Cell Biol.* 146:1147-1159.
- Ren, X.-D., W.B. Kiosses, and M.A. Schwartz. 1999. Regulation of small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:578-585.
- Sander, E.E., J.P. ten Klooster, S. van Delft, R.A. van der Kammen, and J.G. Collard. 1999. Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior. *J. Cell Biol.* 147:1009-1021.
- Schoenwaelder, S.M., and K. Burridge. 1999. Bidirectional signaling between the cytoskeleton and integrins. *Curr. Opin. Cell Biol.* 11:275-286.
- Shaw, L.M., I. Rabinovitz, H.H.-F. Wang, A. Toker, and A.M. Mercurio. 1997. Activation of phosphoinositide 3-OH kinase by the $\alpha 6\beta 4$ integrin promotes carcinoma invasion. *Cell.* 91:949-960.
- Yoshioka, K., F. Matsumura, H. Akedo, and K. Itoh. 1998. Small GTP-binding protein Rho stimulates the actomyosin system, leading to invasion of tumor cells. *J. Biol. Chem.* 273:5146-5154.