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Brief Report

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; Y oshio 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.
(Rabinovitz and Mecurio, 1997; Rabinovitz et al., 1999). Moreover, we demonstrated the importance of cAMP metabolism in these events (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 αβ4 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, resuspended three times with RPMI medium containing 250 μg/ml heat-inactivated BSA (RPMI/BSA), and resuspended in RPMI/BSA. Where indicated, cells were treated with 1 mM isobutylmethylxanthine (IBMX) or 15 mM H-89 (Calbiochem-Novabiochem, Int.) for 15 min before use. The following antibodies were used in this study: M13, mouse anti-β1 integrin mAb b (obtained from Steve Akiyama, National Institutes of Health, Research Triangle Park, NC); K20, mouse anti-β1 integrin mAb (ImmunoTech); 439-9B, rat anti-β4 integrin mAb b (obtained from R. Fortini, Regina Elena Cancer Institute, Rome, Italy); mouse anti-α-HA mAb (Roche Biochemicals); rabbit anti-Rho-polyclonal antibody (Santa Cruz Biotechnology); and anti-Rac1 (Transduction Laboratories).

To obtain expression of N19 RhoA and N17 Rac1, 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 Na2HPO4, 7 mM KH2PO4, 5 mM glucose). Cells were cotransfected with 1 μg of either pCS2-(n)β1-gal or pGFP (green fluorescent protein) and 4 μg of either control vector or vector containing HA-tagged N19 RhoA (provided by A. Toker, Beth Israel Deaconess Medical Center, Boston, MA) or GST-tagged N17 Rac1 (obtained from M. Chou, University of Pennsylvania) by electroporation at 250V and 500 μFd. 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 μg/ml; Collagen Corp.) or laminin-1 purified from EHS tumor (20 μg/ml; provided by Hinda K Leiman, N.I.D., Bethesda, MD) and then blocked with BSA (0.2% 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 μg/ml of K20 (anti-β1) 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 Mecurio, 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 μg/ml laminin-1 diluted in RPMI medium. RPMI/BSA was added to the lower chamber and cells (1×10⁶) 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 Rho kinase as described (Ren et al., 1999). In brief, cells (3×10⁶) were plated onto 60-mm dishes coated with LN-1 (20 μg/ml) or collagen I (50 μg/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 MgCl2, 0.5 μM leupeptin, 0.7 μM pepstatin, 4 μM aprotinin, and 2 mM PMSE). Alternatively, cells were incubated with 8 μg of anti-p1β mAb mc13 or anti-pβ4 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. A fluorcentification 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 Rho kinase) 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 MgCl2. 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 αβ4 and β1 integrins. In contrast, the β1 integrin-mediated adhesion and spreading of these cells on collagen I does not induce significant lamellae formation or migration (Rabinovitz and Mecurio, 1997; Shaw et al., 1997). To examine the hypothesis that RhoA functions in αβ4-dependent lamellae formation, clone A cells were cotransfected with a GFP construct and either a dominant negative RhoA (N19 RhoA) 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 N19 RhoA 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 N19 RhoA 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 (N17 Rac1) 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 56 kinase (Chou and Blenis, 1996) and invasion (Shaw et al., 1997).

Expression of N19 RhoA inhibited the migration of clone A cells on laminin-1 by 70% (Fig. 2 A). In contrast, expression of N17 Rac 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 N19 RhoA had only a modest effect on cell spreading because cells expressing N19 RhoA plated on collagen-1 spread to ~80% of the surface area occupied by control cells (Fig. 1 E). Expression of N19 RhoA and N17 Rac1 in clone A cells was confirmed by immunoblotting (Fig. 1, F and G).

Our observation that RhoA functions in lamellae for-
mation and the migration of clone A cells, in conjunction with our previous finding that these events require the engagement of the α6β4 integrin (Rabinovitz and Mercurio, 1997), indicated that α6β4 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). A, As shown in Fig. 3, the interaction of clone A cells with laminin-1, which requires α6β4, 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 α6β4 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 α6β4 to activate RhoA more definitively, we used integrin-specific mAbs to cluster both α6β4 and β1 integrins. A, As shown in Fig. 3, C and D, clustering of α6β4 resulted in an approximate two- to threefold higher level of RhoA activity in comparison to cells maintained in suspension. Interestingly, clustering of β1 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 α6β4-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 α6β4-mediated activation of...
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 α6β4-mediated activation of RhoA.

The data reported here raise the possibility that α6β4 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 β1-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 β1-integrin staining of the plasma membrane (Fig. 4 A). In contrast, the α6β4-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 β1 integrin staining (Fig. 4 B). However, RhoA did not colocalize with β1 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 α6β4 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 α6β4 functions in these dynamic processes by demonstrating that ligation of α6β4 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 α6β4-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, α6β4, 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 α6β4 integrin is a more effective activator of RhoA than β1 integrins in clone A cells. In fact, antibody-mediated ligation of β1 integrins actually suppressed RhoA activation. Because we used carcinoma cells in our study, the potent activation of RhoA we observed in response to α6β4 ligation could have resulted from a cooperation of α6β4 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 α6β4 because clustering of β1 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-
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 α6β4 to promote carcinoma migration and invasion (Rabinovitz and Mercurio, 1997; Shaw et al., 1997; O’Connor et al., 1998), suggest that α6β4-mediated regulation of the Rho/Rho kinase pathway is an important component of carcinoma progression. It is also possible that the α6β4-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 β1 integrins colocalize in membrane ruffles in response to α6β4 ligation raises the possibility that RhoA influences the function of β1 integrins, which are essential for migration and invasion.

Interestingly, expression of the dominant negative Rho kinase pathway is an important component of carcinoma progression. It is also possible that the α6β4-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 β1 integrins colocalize in membrane ruffles in response to α6β4 ligation raises the possibility that RhoA influences the function of β1 integrins, which are essential for migration and invasion.

Interestingly, expression of the dominant negative
N17R ac in clone A cells had no inhibitory effect on either membrane ruffling, lamellae formation, or migration. Although it was well established that R ac 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 R ac involvement in these dynamic processes. For example, R ac 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, R ac 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 R ac-independent.

Our results highlight the importance of C a M P metabolism in the activation and localization of RhoA. Our finding that C a M P inhibits RhoA activation and translocation to membrane ruffles is consistent with our previous report that linked the activity of α6β4 to promote carcinoma migration with its ability to alter C a M P metabolism (O’Connor et al., 1998). In addition, these results substantiate other studies that indicated an inhibitory effect of C a M P on RhoA activity (Lang et al., 1996; Laudanna et al., 1997; D’ong et al., 1998). The basis for this inhibition may be the direct phosphorylation of RhoA by P K A (Lang et al., 1996). In this context, α6β4 may contribute to RhoA activation by increasing the activity of a C a M P-dependent PDE and subsequently reducing P K A 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 α6β4 suggests this integrin can be linked directly to RhoA activation. This direct activation of RhoA would permit α6β4 to augment path-ways, 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 C a M P and functions in lamellae formation and migration.

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