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13. ABSTRACT (Maximum 200 Words)
Breast carcinoma invasion is a complex process that involves directed migration and localized proteolysis, as well as the survival of cells in foreign tissue. Recent work by our group defined an integrin-mediated mechanism of breast carcinoma invasion that involves the stimulation of carcinoma migration by the dynamic association of $\alpha6\beta4$ with F-actin and the activation of a specific signaling pathway by this integrin. Studies carried out during Year 3 of this grant have extended our analysis of the contribution of the $\alpha6\beta4$ integrin to breast carcinoma progression significantly. A major finding is that $\alpha6\beta4$ is a potent activator of the AKT kinase that maintains the survival of metastatic breast carcinoma cells. Interestingly, this pathway is inhibited by p53, an observation which indicates that the ability of a6b4 to maintain survival will be enhanced in p53-deficient breast carcinomas. We also established that $\alpha6\beta4$ regulates the activity of RhoA GTPase that is necessary for the migration of invasive carcinoma cells. The significance of this finding is that it increases our understanding of the signaling pathways that are involved in breast cancer invasion. Indeed, it is widely assumed that such signaling pathways will be the target of the next generation of chemotherapeutic agents.

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FOREWORD

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5) INTRODUCTION:

Breast carcinoma invasion is a complex process that involves directed migration and localized proteolysis (1). Although the mechanistic basis of invasion had been elusive, recent advances in molecular cell biology have facilitated a much more rigorous analysis of this important and critical component of cancer progression. In particular, insight into the function and regulation of cell adhesion receptors, as well as proteases, has fueled significant progress in our understanding of the invasive process. Studies aimed at defining specific signal transduction pathways that determine the behavior of invasive carcinoma cells are also contributing to an uncovering of the molecular basis of invasion. Recent work by our group and others has implicated a key role for the $\alpha6\beta4$ integrin in breast carcinoma invasion (2-7). This dynamic function of $\alpha6\beta4$ in enhancing the migration of invasive carcinoma cells is quite distinct from its role in maintaining stable adhesive contacts in normal breast epithelia by associating with intermediate filaments. In fact, we have established that the ability of $\alpha6\beta4$ to stimulate breast carcinoma migration and invasion depends upon its preferential activation of a PI3-K/Rac signaling pathway that is necessary for invasion (6). In essence, our studies have defined an integrin-mediated mechanism of breast carcinoma invasion that involves the stimulation of carcinoma migration by the dynamic association of $\alpha6\beta4$ with F-actin and the activation of a specific signaling pathway by this integrin.

Studies carried out during Year 3 of this grant have extended our analysis of the contribution of the $\alpha6\beta4$ integrin to breast carcinoma progression significantly. The major findings are that $\alpha6\beta4$ is a potent activator of a signaling pathway that maintains the survival of metastatic breast carcinoma cells and that $\alpha6\beta4$ also regulates the activity of RhoA GTPase that is necessary for the migration of invasive carcinoma cells.

6) BODY

The $\alpha6\beta4$ integrin promotes the survival of p53-deficient breast carcinoma cells: Although the interaction of matrix proteins with integrins is known to initiate signaling pathways that are essential for cell survival, a role for tumor suppressors in the regulation of these pathways has not been established. We demonstrate here that p53 can inhibit the survival function of integrins by inducing the caspase-dependent cleavage and inactivation of the serine/threonine kinase AKT/PKB. Specifically, we show that the $\alpha6\beta4$ integrin promotes the survival of p53-deficient breast carcinoma cells by activating AKT/PKB. In contrast, this integrin does not activate AKT/PKB in carcinoma cells that express wild-type p53 and it actually stimulates their apoptosis, in agreement with our previous findings (Bachelder et al. 1999. JBC 274:20733-20737). Interestingly, we observed reduced levels of AKT/PKB protein following antibody clustering of $\alpha6\beta4$ in carcinoma cells that express wild-type p53. In contrast, $\alpha6\beta4$ clustering did not reduce the level of AKT/PKB in carcinoma cells that lack functional p53. The involvement of caspase 3 in AKT/PKB regulation was indicated by the ability of Z-DEVD-FMK, a caspase 3 inhibitor, to block the $\alpha6\beta4$-associated reduction in AKT/PKB levels *in vivo*, and by the ability of recombinant caspase 3 to promote the cleavage of AKT/PKB *in vitro*. In addition, the ability of $\alpha6\beta4$ to activate AKT/PKB can be restored in p53-wild-type carcinoma cells by inhibiting caspase 3 activity. These studies demonstrate that the p53 status of an $\alpha6\beta4$-expressing breast carcinoma cell influences its growth and survival potential. **NOTE: The data to support these conclusions can be found in the Bachelder et al. preprint provided in the Appendix. These research accomplishments are associated with Technical Objectives 2 and 4 of the original proposal.**

RhoA function in lamellae formation and migration of carcinoma cells is regulated by the $\alpha6\beta4$ integrin and cAMP metabolism: The integrin $\alpha6\beta4$ promotes the formation of actin-rich protrusions and stimulates the migration of breast carcinoma cells. In this study, we examined the involvement of RhoA in these events and the regulation of RhoA activation by integrin signaling. Clone A carcinoma cells develop fan-shaped lamellae and exhibit random migration when plated on laminin, processes that depend on $\alpha6\beta4$. Here, we report that expression of a dominant negative RhoA (N19RhoA) in clone A cells inhibited $\alpha6\beta4$-dependent membrane

ruffling and the formation of lamellae by 80%. Inhibition of RhoA also blocked the migration of clone A cells but had minimal effects on spreading. Using the Rhotekin binding assay to assess RhoA activation, we observed that engagement of $\alpha6\beta4$ by either laminin or antibody-mediated clustering resulted in a 3-4 fold greater increase in RhoA activation than engagement of $\beta1$ integrins. The $\alpha6\beta4$-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 $\beta1$ 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 A. 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. **NOTE: The data to support these conclusions can be found in the O'Connor et al. manuscript provided in the Appendix. These research accomplishments are associated with Technical Objectives 2 and 4 of the original proposal.**

7) KEY RESEARCH ACCOMPLISHMENTS:

- The $\alpha6\beta4$ integrin is associated with breast carcinoma progression
- The $\alpha6\beta4$ integrin activates the phopshoinositide 3-OH kinase/AKT survival pathway in p53-deficient breast carcinoma cells.
- In p53-wild-type carcinoma cells, a caspase-3 mediated cleavage of AKT kinase occurs that inhibits the $\alpha6\beta4$-mediated survival pathway.
- The RhoA GTPase is essential for the migration and invasion of carcinoma cells.
- The $\alpha6\beta4$ integrin regulates the activation of RhoA and its translocation from the cytosol to membrane ruffles at the leading edge of migrating carcinoma cells.
- cAMP inhibits the migration and invasion of breast carcinoma cells.
- The $\alpha6\beta4$ integrin activates a phosphodiesterase that degrades cAMP and facilitates migration and invasion.

8) REPORTABLE OUTCOMES:

Manuscripts:

O'Connor KL, Shaw LM and AM Mercurio. 1998. Release of cAMP gating by the $\alpha6\beta4$ integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. **J. Cell Biology**, 143:1749-1760.

Bachelder, R., Ribick, M., Marchetti, A., Falcioni, R., Soddu, S., Davis, K. and AM Mercurio. 1999. p53 inhibits $\alpha6\beta4$ integrin survival signaling by promoting the caspase-3 dependent cleavage of AKT/PKB. **J. Cell Biology**, In Press.

O'Connor, KL, Nguyen, B-K. and AM Mercurio. 1999. RhoA function in lamellae formation and migration is regulated by the $\alpha6\beta4$ integrin and cAMP metabolism. **J. Cell Biology**, Submitted.

Major Presentations: (All presentations were focused on the contribution of the $\alpha6\beta4$ integrin to breast cancer progression)

- Seventh International Congress of the Metastasis Research Society, San Diego, CA (10/98)
- 'Distinguished Visiting Scientist', Department of Physiology and Cell Biology, Albany Medical College (11/98)
- Istituto Regina Elena (Queen Elena Cancer Institute), Rome, Italy (2/99)
- Cancer Research Campaign Beatson International Cancer Conference: Invasion and Metastasis Glasgow, Scotland (6/99)
- AACR Special Conference "Molecular Aspects of Metastasis" Snowmass, CO (9/99)
- Ninth International Symposium on Basement Membranes Nice, France (11/99)

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9) CONCLUSIONS:

The research accomplished during Year 3 of this grant strengthen the evidence that the $\alpha6\beta4$ integrin contributes to the progression of human breast cancer. Our finding that this integrin promotes the survival of p53-deficient breast carcinoma cells is of particular importance because many advanced and aggressive breast tumors are p53-deficient and they express the $\alpha6\beta4$ integrin. The next phase of this analysis will be to extend these findings to mouse models of breast cancer progression as outlined in the original application.

In addition to our findings on the survival of breast carcinoma cells, we also implicated the Rho GTPase in the migration and invasion of these cells. The significance of this finding is that it increases our understanding of the signaling pathways that are involved in breast cancer invasion. Indeed, it is assumed by many cancer scientists and oncologists that such signaling pathways will be the target of the next generation of chemotherapeutic agents.

10) REFERENCES: (Note: Most references are cited in the three manuscripts that are included in the Appendix and they are not duplicated here)

1. Liotta, L. A., and W. G. Stetler-Stevenson. 1991. Tumor invasion and metastasis: An imbalance of positive and negative regulation. *Cancer Res.* 51:5054s-5059s.
2. Chao, C., M. M. Lotz, A. C. Clarke, and A. M. Mercurio. 1996. A function for the integrin $\alpha6\beta4$ in the invasive properties of colorectal carcinoma cells. *Cancer Res.* 56:4811-4819.
3. Falcioni, R., V. Turchi, P. Vittulo, G. Navarra, F. Ficari, F. Cavaliere, A. Sacchi, and R. Mariani- Costantini. 1994. Integrin $\beta4$ expression in colorectal cancer. *Int. J. Oncology.* 5:573-578.
4. Rabinovitz, I., and A. M. Mercurio. 1997. The integrin $\alpha6\beta4$ functions in carcinoma cell migration on laminin-1 by mediating the formation and stabilization of actin-containin motility structures. *J. Cell Biol.* 139:1873-1884.
5. Serini, G., L. Trusolino, E. Saggiorato, O. Cremona, M. Derossi, A. Angeli, F. Orlandi, and P. C. Marchisio. 1996. Changes in integrin and E-cadherin expression in neoplasatic verus normal thyroid tissue. *J. Natl. Cancer Inst.* 88:442-449.
6. Shaw, L. M., I. Rabinovitz, H. H.-F. Wang, A. Toker, and A. M. Mercurio. 1997. Activation of phosphoinositide 3-OH kinase by the $\alpha6\beta4$ integrin promotes carcinoma invasion. *Cell.* 91:949-960.
7. VanWaes, C., K. F. Kozarsky, A. B. Warren, L. Kidd, D. Paugh, M. Liebert, and T. E. Carey. 1991. The A9 antigen associated with aggressive human squamous carcinoma is structurally and functionally similar to the newt defined integrin $\alpha6\beta4$. *Cancer Res.* 51:2395-2402.

11) APPENDIX: The following manuscripts are included in the Appendix:

O'Connor KL, Shaw LM and <u>AM Mercurio</u>. 1998. Release of cAMP gating by the $\alpha6\beta4$ integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. **J. Cell Biology**, 143:1749-1760.

Bachelder, R., Ribick, M., Marchetti, A., Falcioni, R., Soddu, S., Davis, K. and <u>AM Mercurio</u>. 1999. p53 inhibits $\alpha6\beta4$ integrin survival signaling by promoting the caspase-3 dependent cleavage of AKT/PKB. **J. Cell Biology**, In revision.

O'Connor, KL, Nguyen, B-K. and <u>AM Mercurio</u>. 1999. RhoA Function in Lamellae Formation and Migration is Regulated by the $\alpha6\beta4$ Integrin and cAMP Metabolism. **J. Cell Biology**, Submitted.

RhoA Function in Lamellae Formation and Migration is Regulated by the α6β4 Integrin and cAMP Metabolism

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Abbreviations used in this paper are: β-gal, β-galactosidase; GFP, green fluorescent protein; IBMX, isobutylmethylxanthine; LPA, lysophosphatidic acid; PKA, cAMP-dependent protein kinase PI3-K, phosphoinositide 3-OH kinase; PDE; phosphodiesterase; RBD, Rho-binding domain of Rhotekin

Abstract:

The integrin α6β4 promotes the formation of actin-rich protrusions and stimulates the migration of carcinoma cells (Shaw et al., Cell 91:949-960; Rabinovitz and Mercurio, J. Cell. Biol. 139:1873-1884; O'Connor et al., J. Cell. Biol.143:1749-1760). In this study, we examined the involvement of RhoA in these events and the regulation of RhoA activation by integrin signaling. Clone A colon carcinoma cells develop fan-shaped lamellae and exhibit random migration when plated on laminin, processes that depend on α6β4. Here, we report that expression of a dominant negative RhoA (N19RhoA) in clone A cells inhibited α6β4-dependent membrane ruffling and the formation of lamellae by 80%. Inhibition of RhoA also blocked the migration of clone A cells but had minimal effects on spreading. Using the Rhotekin binding assay to assess RhoA activation, we observed that engagement of α6β4 by either laminin or antibody-mediated clustering resulted in a 3-4 fold greater increase in RhoA activation than engagement of β1 integrins. The α6β4-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 β1 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 A. 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.

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, lamellae and filopodia, respectively (reviewed in ref. 8). 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 (10). Rho, interestingly, has been implicated in membrane ruffling in epithelial cells (6, 15), a process attributed to Rac in fibroblasts (8). 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 (9, 11, 22, 23). 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 (1, 20), 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 $\alpha6\beta4$ in the migration and invasion of carcinoma cells, as well as in epithelial wound healing (14, 17-19, 22). Although it is well-established that $\alpha6\beta4$ functions in the formation and stabilization of hemidesmosomes (3, 7), 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 (18, 19). Moreover, we demonstrated the importance of cAMP metabolism in these events (17). 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 and the migration of carcinoma cells and that the activity of RhoA is regulated by the $\alpha6\beta4$ 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 (4), were used in all experiments. For each experiment, adherent cells were harvested by trypsinization, rinsed three times with RPMI medium containing 250μg/ml heat-inactivated BSA (RPMI/BSA), and resuspended in RPMI/BSA. Where indicated, cells were treated with 1mM isobutylmethylxanthine (IBMX) or 15μM H-89 (Calibiochem-Novabiochem, Int.) for 15 min prior to plating or use in migration assays. The following antibodies were used in this study: MC13 (mouse anti-β1 integrin mAb, 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, obtained from Rita Falcioni, Regina Elena Cancer Institute, Rome, Italy), mouse anti-HA mAb (Roche Biochemicals) and rabbit anti-RhoA pAb (Santa Cruz Biotechnology).

To obtain expression of N19RhoA, adherent cells were harvested using trypsin, rinsed with PBS and suspended in electroporation buffer (20mM HEPES, pH 7.05, 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4-$7H_2O$, 5mM glucose). Cells were co-transfected with 1μg of either pCS2-(n)β-gal or pGFP and 4μg of either control vector or vector containing HA-tagged N19RhoA (obtained from Alex Toker, Beth Israel Deaconess Medical Center, Boston, MA) by electroporation at 250V and 500μFd. Subsequently, cells were plated in complete growth medium containing 0.05% sodium butyrate and used for experiments 48hrs after the initial transfection. Expression of N19RhoA was confirmed by immunoprecipitating extracts of transfected cells with an HA-specific mAb and subsequent immunoblotting for RhoA.

Microscopic Analyses: Glass coverslips were coated overnight at 4°C with collagen I (50μg/ml; Collagen Corp.) or laminin-1 purified from EHS tumor (20μg/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 and fixed for 20 min with 4% paraformaldehyde containing 10mM PIPES, pH 6.8, 2mM EGTA, 2mM $MgCl_2$, 7% sucrose and 100mM KCl. Cells were then permeabilized by treating with 0.05% Triton X-100 for 15 sec, rinsed twice with PBS, and then incubated with blocking solution (3% BSA /1% normal donkey serum in PBS) for 30

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min. For immunofluorescence, cells were incubated with 1μg/ml of K20 (anti-β1) and anti-RhoA Ab for 30 min, rinsed 4 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 Abs (Jackson ImmunoResearch Laboratories, Inc.). Images of cells were captured digitally, analyzed and lamellar area quantified as described previously (17, 18).

Migration Assays: The lower compartments of Transwell chambers (6.5mm diameter, 8μm pore size; Costar) were coated for 30 min with 15μg/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 (22).

RhoA Activity: RhoA activity was assessed using the Rho binding domain of Rhotekin as described (20). Briefly, cells (3×10^6) were plated onto 60mm dishes coated with LN-1 (20μg/ml) or collagen I (50μg/ml) for 30 min. and extracted with RIPA buffer (50mM Tris, pH 7.2, 500mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS, 10mM $MgCl_2$, 0.5μg/ml leupeptin, 0.7μg/ml pepstatin, 4μg/ml aprotinin, and 2mM PMSF). Alternatively, cells were incubated with 8μg of anti-β1 mAb mc13 or anti-β4 rat mAb 439-9B for 30 min, rinsed, plated on 60mm dishes coated with 50μg of either anti-mouse or anti-rat IgG, respectively, for 30 min and then extracted. After centrifugation at 14,000 x 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 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, 150mM NaCl and 10mM $MgCl_2$. The RhoA content in these samples was determined by immunoblotting samples using rabbit anti-RhoA Ab.

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 α6β4 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 (18, 22). To examine the hypothesis that RhoA functions in α6β4-dependent lamellae formation, clone A cells were co-transfected 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. 1A). In contrast, cells that expressed N19RhoA did not form membrane ruffles nor did they extend large, fan-shaped lamellae. The lamellae that did form in these cells were small and fragmented (Fig. 1B). 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. 1C). In addition, expression of N19RhoA inhibited migration on laminin-1 by 70% (Fig. 2A). Importantly, expression of N19RhoA had only a modest effect on cell spreading because cells expressing N19RhoA plated on collagen–I spread to approximately 80% of the surface area occupied by control cells (Fig. 1D). Transient expression of N19RhoA in clone A cells was confirmed by immunoblotting (Fig. 1E).

Our observation that RhoA functions in lamellae formation and the migration of clone A cells, in conjunction with our previous finding that these events require the engagement of the α6β4 integrin (18), indicated that α6β4 may mediate the activation of RhoA. To assess RhoA activation, we used the Rho-binding domain of Rhotekin (RBD) to capture GTP-bound RhoA from cell extracts (20). 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 at this time point because optimal membrane ruffling was noted with cells that had interacted with laminin for 30 min. Quantitative analysis of the results obtained in four independent experiments revealed a three-fold greater increase in RhoA activation in cells plated on laminin-1

than cells plated on collagen (Fig. 3B). To establish the ability of α6β4 to activate RhoA more definitively, we used integrin-specific mAbs to cluster both α6β4 and β1 integrins. As shown in Figs. 3C and D, clustering of α6β4 resulted in an approximate four-fold higher level of RhoA activity in comparison to clustering of β1 integrins even though clone A cells express similar surface levels of both integrins (data not shown). In fact, clustering of β1 integrins actually decreased RhoA activation in comparison to cells maintained in suspension (Fig. 3).

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. 2B). In contrast, inhibition of PKA with H-89 increased the rate of migration by four-fold (Fig. 2B). Together, these data indicate that cAMP is a potent inhibitor of carcinoma migration, in agreement with our previous findings (17). 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. 3A, pretreatment of clone A cells with IBMX prior to plating on laminin-1 reduced the level of RhoA activation to that observed in cells plated on collagen. Importantly, IBMX did not inhibit adhesion or spreading (Fig. 4C, 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 above raise the possibility that α6β4 influences RhoA localization because activation of RhoA is thought to involve its translocation to the membrane (2). To address this issue, clone A cells plated on either laminin-1 or collagen I were immunostained with a RhoA-specific Ab, as well as a β1-integrin specific Ab 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. 4A). 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 co-localized with β1 integrin staining (Fig. 4B). However, RhoA did not co-localize with β1 integrins on the plasma membrane along the cell body (Fig. 4B). To assess the

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influence of cAMP metabolism on RhoA localization, clone A cells were pretreated with either IBMX or H-89 prior to 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. 4C, 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. 4D, F).

Discussion:

We established recently that the α6β4 integrin stimulates the migration of carcinoma cells and enhances the formation of actin rich protrusions, including lamellae and membrane ruffles (17-19, 22). 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, and that these events are regulated by cAMP metabolism.

Our findings strengthen the evidence that integrins can participate in the activation of RhoA. Much of the evidence obtained to date to support the integrin activation of RhoA had been indirect and based largely of the observation that integrin activation leads to the Rho-dependent formation of stress fibers and focal adhesions (20, 21). Recently, the development of a biochemical assay for RhoA activation that is based on the ability of GTP-bound RhoA to associate with the Rho-binding domain of the Rho effector Rhotekin has enabled a more rigorous and sensitive assessment of the mechanism of RhoA activation (20). Using this assay, cell attachment to fibronectin was shown to activate RhoA and the level of activation was augmented by serum or 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. 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 fragmented, 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 (6, 15) by a mechanism that involves Rho kinase-mediated phosphorylation of adducin (6). Moreover, Rho kinase has been implicated in tumor cell invasion (9, 23). Together, these findings, along with our previous work that established the ability of α6β4 to promote carcinoma migration and invasion (17, 18, 22), 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 (13) and is believed to participate in adhesion in other cell types (16). Our observation that RhoA and β1 integrins colocalize in membrane ruffles in response to α6β4 ligation raises this possibility that RhoA influences the function of β1 integrins, which are essential for migration and invasion. Moreover, the fragmented lamellae that we observed in response to expression of N19RhoA could result from the inability of lamellae to stabilize their attachment to matrix.

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 α6β4 to promote carcinoma migration with its ability to alter cAMP metabolism (17). In addition, these results substantiate other studies that indicated an inhibitory effect of cAMP on RhoA activity (5, 12, 13). The basis for this inhibition appears to be the direct phosphorylation of RhoA by PKA. In this context, α6β4 may contribute to RhoA activation by increasing the activity of a cAMP-dependent PDE, and subsequently reducing PKA activity, as we have suggested previously (17). 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 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 that functions in lamellae formation and migration.

Acknowledgements:

References:

1. 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.

2. 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.

3. Borradori, l., and A. Sonnenberg. 1996. Hemidesmosomes -- roles in adhesion, signaling and human disease. *Curr. Opin. Cell Biol.* 8:647-658.

4. Dexter, D. L., J. A. Barbosa, and P. Calabresi. 1979. N', N-dimethylformamide-induced alteration of cell culture characteristics and loss of tumorgenicity in cultured human colon carcinoma cells. *Cancer Res.* 39:1020-1025.

5. 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-α. *J. Biol. Chem.* 273:22554-22562.

6. 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.

7. Green, K. J., and J. C. R. Jones. 1996. Desmosomes and hemidesmosomes -- structure and function of molecular components. *FASEB J.* 10:871-881.

8. Hall, A. 1998. Rho GTPases and the actin cytoskeleton. *Science.* 279:509-514.

9. 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.

10. 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.

11. 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.

12. Lang, P., F. Gesbert, M. Delespine-Carmagnat, R. Stancou, M. Pouchelet, and J. Bertoglio. 1996. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. *EMBO J.* 15:510-519.

13. 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.

14. 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.

15. 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 12-0-tetradecanoylphorbol-13-acetate (TPA)-induced membrane ruffling in KB cells. *Mol. Cell. Biol.* 14:2247-2456.

16. Nobes, C. D., and A. Hall. 1999. Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J. Cell. Biol.* 144:1235-1244.

17. O'Connor, K. L., L. M. Shaw, and A. M. Mercurio. 1998. Release of cAMP gating by the α6β4 integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. *J. Cell Biol.* 143:1749-1760.

18. Rabinovitz, I., and A. M. Mercurio. 1997. The integrin α6β4 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.

19. Rabinovitz, I., A. Toker, and A. M. Mercurio. 1999. Protein kinase C-dependent mobilization of the α6β4 integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. *J. Cell Biol.* 146:1147-1159.

20. 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 J.* 18:578-585.

21. Schoenwaelder, S. M., and K. Burridge. 1999. Bidirectional signaling between the cytoskeleton and integrins. *Curr. Opin. Cell Biol.* 11:275-286.

22. Shaw, L. M., I. Rabinovitz, H. H.-F. Wang, A. Toker, and A. M. Mercurio. 1997. Activation of phosphoinositide 3-OH kinase by the α6β4 integrin promotes carcinoma invasion. *Cell.* 91:949-960.

23. 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.
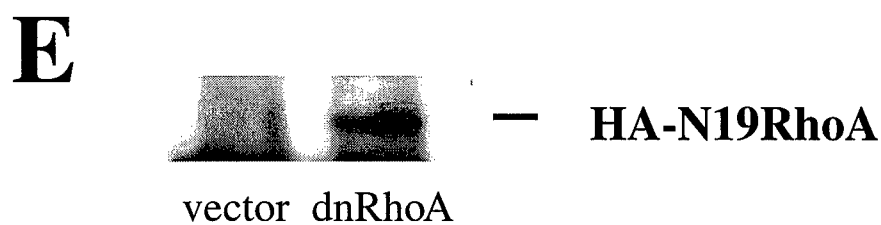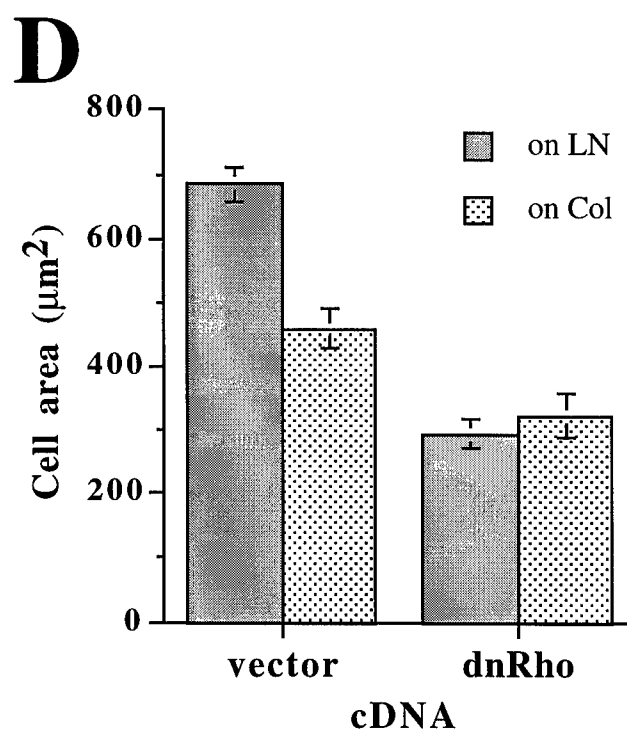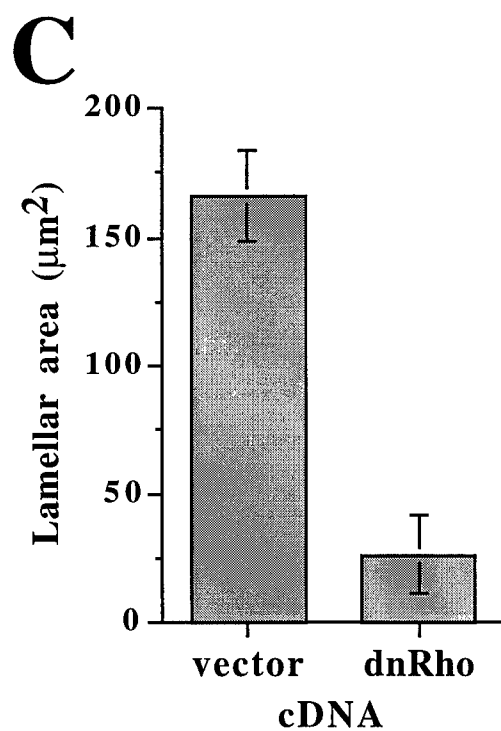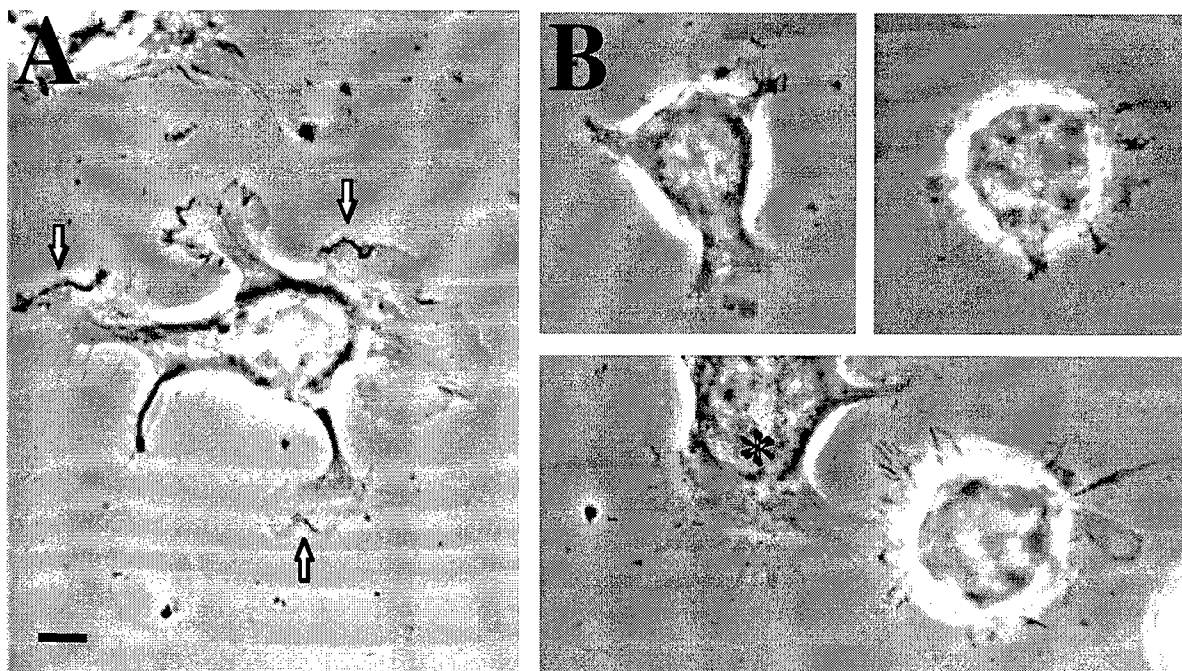
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Figure 1. Dominant negative RhoA-inhibits membrane ruffling and lamellae formation in clone A cells in response to laminin-1. Clone A cells were transfected with a green fluorescent protein (GFP) construct and either a control vector or a vector encoding N19RhoA as described in Materials and Methods. Cells were plated onto laminin coated coverslips for 40 minutes, fixed and assessed by phase contrast microscopy. (A, B) Phase contrast microscopy of vector control (A) or N19RhoA (B, 3-panels) transfected cells. Note large lamellae and membrane ruffles (arrows) in control cells (open arrow; A) but not in cells that express N19RhoA (B). Asterisk in the lower panel of (B) marks a non-transfected cell. Bar represents 10μm. (C) Quantitative analysis of lamellar area were obtained by digital imaging. Lamellae are defined as broad, flat cellular protrusions rich in F-actin and devoid of membrane-bound vesicles. (D) Quantitative analysis of total area covered by cells transfected with either vector control or N19RhoA when plated on laminin-1 (dark gray bars) or collagen I (light gray bars). Bars represent mean area ± standard error in which n > 20 (C, D). (E) Transfected cells were extracted with RIPA buffer, immunoprecipitated for HA tag and immunoblotted for RhoA. A representative blot is shown.
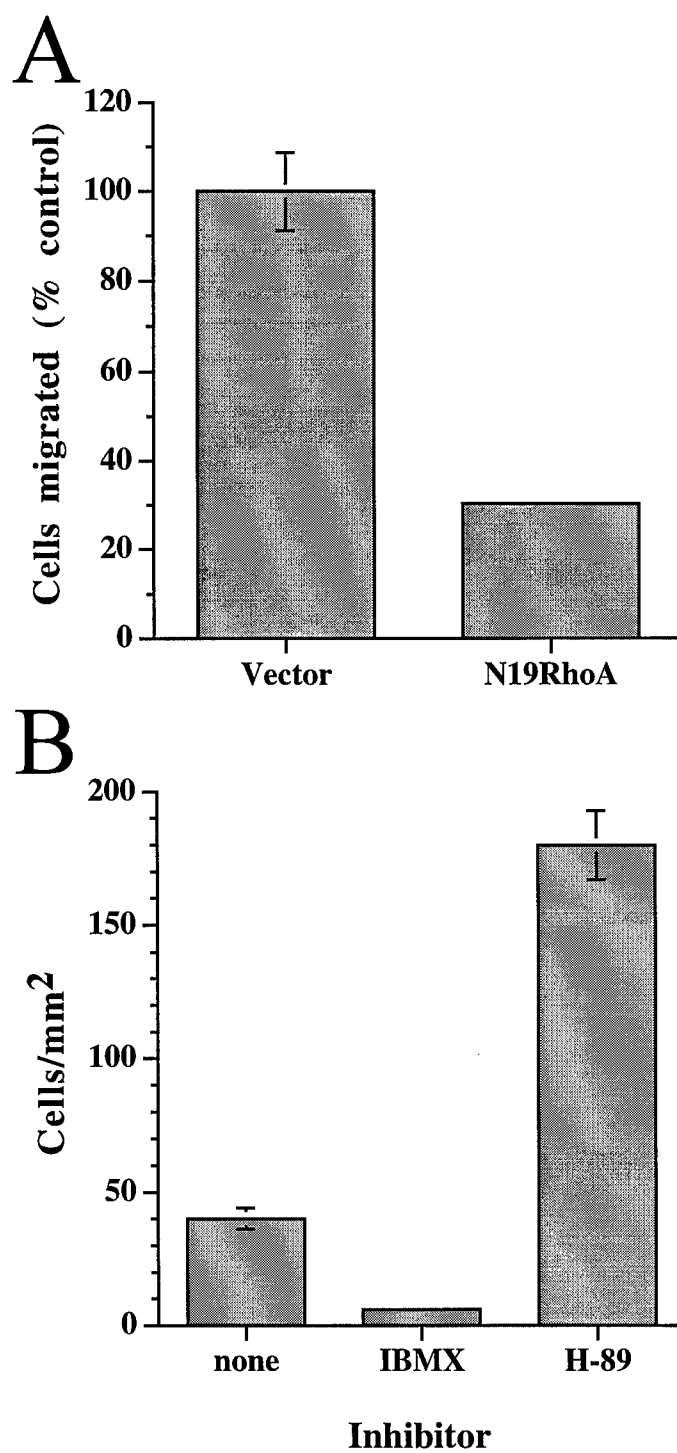
Figure 2. Effects of dominant negative RhoA (N19RhoA) and cAMP metabolism on laminin-1 stimulated migration. (A) Clone A cells that had been co-transfected with a β-gal cDNA and either N19RhoA or control vector were assayed for migration on laminin-1 as described in the Materials and Methods. After 5 hours, cells that did not migrate were removed from the upper chamber and the cells that had migrated to the lower surface of the membrane were stained for β-gal. Migration was scored as the total number of cells migrated compared to the vector only control. Transfection rates were comparable. (B) Clone A cells were left untreated or treated with 1mM IBMX or 15μM H-89 for 15 minutes and then assayed for laminin-1 mediated migration as described in (A). After 5 hours, cells that had migrated to the lower chamber were stained with crystal violet and quantified. Migration rates were reported as the number of cells migrated per mm^2. Bars (A, B) represent mean ± standard deviation from triplicate determinations.

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Figure 3. Engagement of the α6β4 integrin by either laminin-1 or antibody-mediated clustering activates RhoA. (A, B) Clone A cells were plated on either collagen or laminin for 30 min or pretreated with 1mM IBMX for 15 min and then plated on laminin for 30 min. Cells extracts were assayed for Rhotekin binding activity as described in the Materials and Methods. (C, D) Cells were either left in suspension (sus) or clustered with either β1 or β4 specific antibodies for 30 min as described in the Materials and Methods. Cells extracts were assessed for RhoA activity by RBD binding. For these experiments, the total RhoA bound to the RBD (top panels in A, C) was normalized to the RhoA content of cell extracts (bottom panels in A, C). (A, C) Representative immunoblots from these experiments are shown. (B, D) Quantitative analysis of the results obtained by densitometry is provided. Bars represent mean of four separate experiments ± standard error.

Figure 4. Laminin-1, but not collagen-I, promotes the colocalization of RhoA and β1 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 minutes, fixed and stained for both β1 integrin and RhoA using indirect immunofluorescence as described in the Materials and Methods. To assess the impact of cAMP signaling on recruitment of RhoA to the plasma membrane, cells were pretreated with 1mM IBMX (C) or 15μM H-89 (D) for 15 min prior to plating cells on laminin-1. Images were captured digitally approximately 1μm from the basal surface using a BioRad confocal microscope. Red color represents RhoA; Green, β1 integrin; Yellow, β1 and RhoA colocalization. (E, 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 represent 10μm.
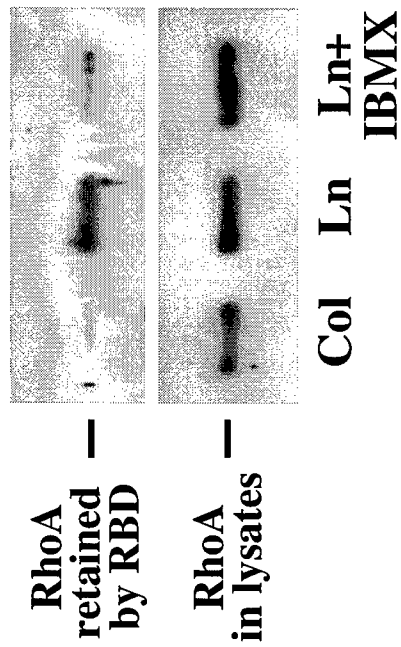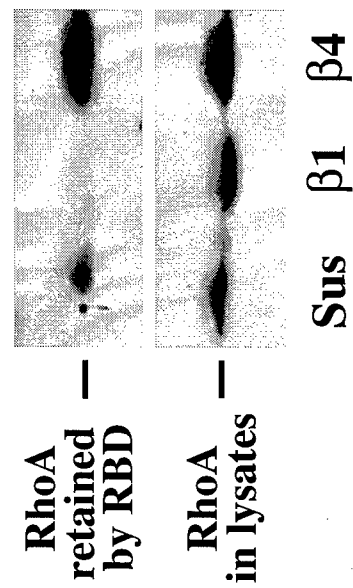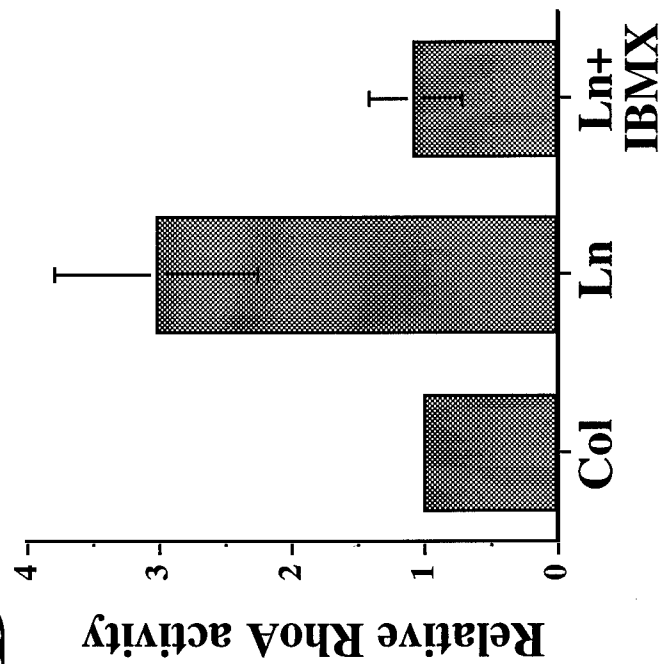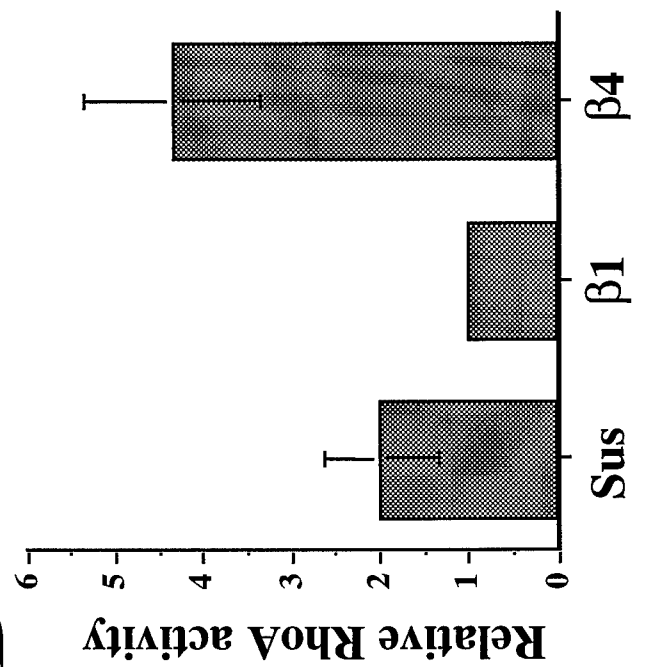
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Figure 1

C — Lamellar area (μm^2)

D — Cell area (μm^2), on LN, on Col

E — HA-N19RhoA
vector dnRhoA

A

B

Figure 3

Figure 4

p53 inhibits α6β4 integrin survival signaling by promoting the caspase 3-dependent cleavage of AKT/PKB

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Key Words: p53, integrin, AKT/PKB, survival, caspase

Nonstandard Abbreviations: dnAKT, dominant negative AKT
dnp53, dominant negative p53
GFP, green fluorescent protein
HA, hemagluttinin
Tsp53, temperature sensitive p53

Abstract

Although the interaction of matrix proteins with integrins is known to initiate signaling pathways that are essential for cell survival, a role for tumor suppressors in the regulation of these pathways has not been established. We demonstrate here that p53 can inhibit the survival function of integrins by inducing the caspase-dependent cleavage and inactivation of the serine/threonine kinase AKT/PKB. Specifically, we show that the α6β4 integrin promotes the survival of p53-deficient carcinoma cells by activating AKT/PKB. In contrast, this integrin does not activate AKT/PKB in carcinoma cells that express wild-type p53 and it actually stimulates their apoptosis, in agreement with our previous findings (Bachelder et al. 1999. JBC 274:20733-20737). Interestingly, we observed reduced levels of AKT/PKB protein following antibody clustering of α6β4 in carcinoma cells that express wild-type p53. In contrast, α6β4 clustering did not reduce the level of AKT/PKB in carcinoma cells that lack functional p53. The involvement of caspase 3 in AKT/PKB regulation was indicated by the ability of Z-DEVD-FMK, a caspase 3 inhibitor, to block the α6β4-associated reduction in AKT/PKB levels *in vivo*, and by the ability of recombinant caspase 3 to promote the cleavage of AKT/PKB *in vitro*. In addition, the ability of α6β4 to activate AKT/PKB can be restored in p53-wild-type carcinoma cells by inhibiting caspase 3 activity. These studies demonstrate that the p53 tumor suppressor can inhibit integrin-associated survival signaling pathways.

Primary epithelial (28) and endothelial (47) cells are prone to anoikis, a form of programmed cell death, when grown in the absence of growth factors and extracellular matrix proteins. This default apoptotic pathway is thought to be important in preventing cell growth at inappropriate anatomical sites. Survival signaling pathways associated with both growth factor receptors and cell adhesion molecules are important in protecting cells from anoikis. For example, growth factors such as EGF, PDGF and insulin can promote the survival of serum-starved epithelial cells (48, 62, 64). Similarly, the binding of integrins such as $\alpha v \beta 3$ (73), $\alpha 5 \beta 1$ (83) and $\alpha 6 \beta 1$ (26, 37, 78) to the appropriate extracellular matrix protein can inhibit anoikis. These survival signals have been attributed to the ability of integrins to activate numerous molecules including focal adhesion kinase (FAK) (29), integrin-linked kinase (60), AKT/PKB (41), and bcl-2 (73, 83). In addition, integrin survival functions have been associated with their ability to inhibit the activity of p53 (38, 73) and Rb (17) tumor suppressors, respectively. Tumor cells acquire a partial resistance to anoikis as a result of their transformation, which is thought to activate select survival signaling pathways in these cells constitutively (28). For this reason, the identification of molecules that can inhibit survival signaling is crucial for developing strategies aimed at blocking tumor cell growth.

The $\alpha 6 \beta 4$ integrin, a receptor for the laminin family of extracellular matrix proteins, plays an important role in diverse cellular activities. In addition to serving an important structural role in the assembly of hemidesmosomes in epithelial cells (8, 34), $\alpha 6 \beta 4$ promotes carcinoma cell migration and invasion (11, 56, 59, 67, 77) in a PI3-kinase dependent manner (67). The $\beta 4$ subunit of this integrin, which contains a cytoplasmic tail of approximately 1000 amino acids (35, 39, 40), has been shown to be crucial in the ability of this integrin to activate numerous signaling molecules, including PI-3-kinase (67), Shc (46), Ras (46), Jnk (46), p21[WAF1/CIP1] (13) and p53 (4). The diverse activities of this integrin are exemplified by its ability to promote both the survival of keratinocytes (19), as

well as the apoptosis of a number of carcinoma cell lines (4, 13, 42, 74). These apparently contradictory functions likely reflect the activation of distinct signaling pathways by this integrin in different cell types, as well as the influence of other signaling pathways on α6β4 function.

In the present studies, we define opposing signaling pathways that are activated by the α6β4 integrin that promote either carcinoma cell survival or apoptosis, depending on whether these cells express wild-type or functionally inactive mutants of p53. Specifically, we show that α6β4 can promote the AKT/PKB-dependent survival of p53-deficient carcinoma cells. This activity contrasts, however, with the ability of α6β4 to stimulate the caspase-dependent cleavage and inactivation of AKT/PKB in p53 wild-type carcinoma cells. The ability of wild-type p53 to inhibit α6β4-associated survival signals suggests that the p53 status of an α6β4-expressing carcinoma cell influences its growth potential.

Materials and Methods

Cells: The RKO colon carcinoma cell line was obtained from M. Brattain (University of Texas, San Antonio), MDA-MB-435 breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository (Georgetown University).

The cloning of the human β4 cDNA, the construction of the β4 cytoplasmic domain deletion mutant (β4-Δcyt), and their insertions into the pRc/CMV (β4) and pcDNA3 (β4-Δcyt) eukaryotic expression vectors respectively have been described (13). RKO/β4Δcyt clone 3E1, RKO/β4 clone D4 (RKO/β4 clone 1), RKO/β4 clone A7 (RKO/β4 clone 2), MDA-MB-435/β4–Δcyt clone 3C12, MDA-MB-435/β4 clone 5B3 (MDA-MB-435/β4 clone 1), and MDA-MB-435/β4 clone 3A7 (MDA/β4 clone 2) were selected for analysis based on their expression of similar surface levels of α6β4 and α6β4–Δcyt, as we have previously demonstrated (4, 13, 67).

Dominant negative p53-expressing RKO/β4-Δcyt and RKO/β4 subclones were obtained by co-transfecting RKO/β4–Δcyt clone 3E1 and RKO/β4 clone D4 with plasmids expressing the puromycin resistance gene (50) and a dominant negative p53 (dnp53) construct (kindly provided by M. Oren, Weizmann Institute for Science, Israel) that encodes for a carboxy-terminal domain of p53 that can heterodimerize with endogenous p53 and inhibit its transcriptional activity. Dnp53-expressing subclones were obtained and those subclones expressing high levels of dnp53 were selected by FACS using the Pab122 mAb (Boehringer-Mannhein), which recognizes a conserved, denaturation stable epitope in dnp53. In addition, RKO/β4 and RKO/β4–Δcyt cells were transfected with the puromycin resistance gene plasmid alone to obtain puromycin-resistant mock transfectants. All assays were performed using cell maintained below passage 10.

Stable transfectants of MDA/β4 clone 3A7 that expressed temperature sensitve p53 were obtained by co-transfecting this cell line with plasmids expressing the puromycin resistance gene (1 μg) (50) and a plasmid expressing a temperature sensitive mutant of

human p53 (tsp53-4 μg) that assumes a functional conformation at 32°C, but not at 37°C (82) using the Lipofectamine reagent (Gibco). After growing these transfectants in complete medium for two days, stable transfectants were selected by culturing these cells in puromycin-containing medium (2 μg/mL) for an additional 18 days. These bulk transfectants were expanded and tsp53 expression was confirmed by showing increased p53 levels in tsp53 transfectants relative to mock transfectants by immunoblotting with a goat anti-human p53, followed by HRP-conjugated donkey anti-goat IgG. All assays were performed on cells maintained below passage 5.

dnAKT/PKB-expressing MDA-MB-435/mock and MDA-MB-435/β4 transient transfectants were generated by cotransfecting these cell lines using the Lipofectamine reagent (Gibco) with a plasmid encoding for green fluorescence protein (pEGFP-1, Clonetech- 1 μg) and a dnAKT/PKB construct that contains inactivating mutations in the catalytic domain of AKT/PKB (4 μg) (20, 24, 70).

Antibodies: The following antibodies were used: 439-9B, a rat monoclonal antibody specific for the β4 integrin subunit (25), control rat IgG (Sigma); Pab122, a polyclonal rabbit serum specific for p53 (Boehringer-Mannheim), goat anti-human p53, rabbit polyclonal anti-AKT/PKB raised against a peptide corresponding to mouse AKT/PKB residues 466-479 (New England Biolabs), rabbit polyclonal anti-AKT/PKB phoshoserine 473 (New England Biolabs), rabbit anti-actin (Sigma), and mouse anti-Hemagluttinin (Boehringer Mannheim). Goat anti-mouse IgG and goat anti-rat IgG secondary antibodies, as well as HRP-conjugates of these antibodies, were obtained from Jackson Immunoresearch. HRP-conjugated donkey anti-goat IgG was obtained from BioSource.

Apoptosis Assays: To induce apoptosis in the RKO and MDA-MB-435 transfectants, the cells were plated in complete medium for eight hours in tissue culture wells (12 well plate; 2.5×10^5 cells/well) that had been coated overnight at 4°C with poly-L-lysine (Sigma- 2 mL of 25 μg/mL stock) and blocked with 1% BSA. After 8 hours, this medium was replaced with serum-free culture medium containing 1% BSA. After 15 hours at

37°C, adherent and suspension cells were harvested, combined, and the level of apoptosis in these cells was assessed as described below.

For annexin V stains, cells were washed once with serum-containing medium, once with PBS, once with annexin V-FITC buffer (10mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), and incubated for 15 minutes at room temperature with annexin V-FITC (Bender MedSystems) at a final concentration of 2.5 μg/mL in annexin V buffer. After washing once with annexin V buffer, the samples were resuspended in the same buffer and analyzed by flow cytometry. Immediately before analysis, propidium iodide was added to a final concentration of 5 μg/mL to distinguish apoptotic from necrotic cells, and five- thousand cells were analyzed for each sample.

For ApopTag reactions, cells were harvested as described above, fixed in 1% paraformaldehyde for 15 minutes on ice and washed twice with PBS. The samples were then resuspended in 1 mL ice-cold 70% ethanol and stored at -20°C overnight. After centrifugation at 2500 rpm for 15 minutes, cells were washed two times in PBS before performing ApopTag reactions (Oncor) according to the manufacturer's recommendations. These samples were analyzed by flow cytometry.

For *in situ* analysis of apoptosis in cells transfected transiently with the green fluorescence protein (GFP)-expressing vector pEGFP-1 (Clonetech) and dnAKT/PKB, the transfected cells were stained with annexin V-PE (Pharmingen) according to the manufacturer's directions, and plated on coverslips. The percentage of GFP-positive cells that was annexin V-PE positive was determined by fluorescence microscopy. A total of at least 80 GFP-positive cells from at least 10 microscopic fields were analyzed for each data point.

Analysis of AKT/PKB Expression and Activity: To assess the expression of endogenous AKT/PKB protein, cells were incubated with either rat Ig or 439-9B as described above in the presence of either DMSO (1:500), a caspase 3 inhibitor (Z-DEVD-FMK; Calbiochem-4 μg/mL), or a caspase 8 inhibitor (Z-IETD-FMK; Calbiochem- 4

μg/mL). After washing with PBS, the cells were plated in serum-free medium containing 1% BSA in wells of a 12- well plate that had been coated with anti-rat Ig (13.5 μg/mL) and blocked for one hour at 37°C with 1% BSA-containing medium. After a one hour stimulation, adherent and suspension cells were harvested and extracted with 'AKT/PKB lysis buffer' (20 mM Tris, pH 7.4, 0.14 M NaCl, 1% NP40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL pepstatin, and 50 ug/mL leupeptin, 1 mM sodium orthovanadate). After removing cellular debris by centrifugation at 12000 x g for 10 min, equivalent amounts of total cell protein from these extracts were resolved by SDS-PAGE (8%) and transferred to nitrocellulose. The blots were then probed with a rabbit anti-AKT/PKB antiserum, followed by HRP-conjugated goat anti-rabbit Ig, and the immunoreactive bands were visualized by enhanced chemiluminescence. These blots were also probed with a rabbit antiserum specific for actin to confirm the loading of equivalent amounts of protein. Relative AKT/PKB and actin expression levels were assessed by densitometry using IP Lab Spectrum software (Scanlytics, Vienna, VA).

To determine the level of serine 473-phosphorylated AKT/PKB, cells were transfected transiently using the Lipofectamine reagent (Gibco) with an HA-tagged AKT/PKB cDNA (provided by A. Toker). Twenty hours after transfection, these cells were harvested by trypsinization and subjected to antibody-mediated integrin clustering. Specifically, cells were incubated on ice for 30 min with either control rat IgG or 439-9B at a concentration of 10 μg/mL. After washing with PBS, the cells were plated in serum-free medium containing 1% BSA onto wells of a 60 mM tissue culture dish that had been coated at 4°C with anti-rat Ig (13.5 μg/mL) and blocked for one hour at 37°C in 1% BSA-containing medium. After one hour, adherent and suspension cells were harvested, and washed twice with PBS. Proteins from these cells were extracted with 'AKT/PKB lysis buffer' (see above). After removing cellular debris by centrifugation at 12000 x g for 10 min at 4°C, equivalent amounts of total cellular protein were precleared with a 1:1 mixture of protein A and protein G sepharose for 1 hour at 4°C. Immunoprecipitations were then

performed for one hour on these precleared lysates using an HA-specific monoclonal antibody (1 μg-Boehringer Mannheim) and protein A/protein G sepharose beads. Proteins from these immunoprecipitates were subjected to reducing SDS-PAGE (8%), transferred to nitrocellulose, and probed with an AKT/PKB phosphoserine 473-specific rabbit antiserum (New England Biolabs) followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. Phospho-AKT/PKB was then detected on these blots by chemiluminescence (Pierce). These samples were also probed with rabbit anti-AKT/PKB. The relative intensity of Phosphoserine AKT/PKB and AKT/PKB bands was assessed by densitometry, as described above.

Analysis of AKT/PKB Proteolysis: Baculovirus-expressed AKT/PKB (0.5 μg-kindly provided by Alex Toker) was incubated with either active recombinant caspase 8 (2 μg- Calbiochem) or active recombinant caspase 3 (2 μg-Calbiochem) at 37°C for one hour in a final volume of 10 μl. Subsequently, the reaction mixtures were divided into two aliquots and resolved by SDS-PAGE (8%). The gels were silver stained using the GelCode SilverSNAP Stain Kit (Pierce), or transferred to nitrocellulose and probed with a rabbit AKT/PKB antiserum as described above.

RESULTS

The α6β4 integrin promotes the survival of p53-deficient, but not p53-wild-type carcinoma cells: For our initial experiments, we used stable β4 transfectants of two α6β4-deficient carcinoma cell lines that differ in their p53 status: RKO colon carcinoma cells, which express wild-type p53 (54), and MDA-MB-435 breast carcinoma cells, which express a mutant, inactive form of p53 (45). We also used RKO and MDA-MB-435 cells that expressed a cytoplasmic domain deletion mutant of α6β4 (RKO/β4-Δcyt; MDA/β4-Δcyt) that is signaling deficient. The characterization of these cells has been described previously (13, 67).

To explore the potential influence of α6β4 expression on the survival of serum-starved carcinoma cells deprived of matrix attachment, the α6β4 and α6β4-Δcyt-expressing RKO and MDA-MB-435 subclones were plated on poly-L-lysine in serum-free medium. The level of apoptosis in these populations was then determined either by staining with annexin V-FITC to detect cells in the early stages of apoptosis or by performing terminal deoxynucleotidyl transferase (TdT) end labeling reactions (Apoptag) to detect DNA fragmentation (Fig. 1). In addition, we assessed the the viability of these serum-deprived cells by measuring the cellular uptake of propidium iodide (Table 1). The ability of α6β4 to promote the survival of these cells was determined by subtracting the percent apoptotic α6β4-expressing cells from the percent apoptotic α6β4–Δcyt-expressing cells. The expression of α6β4 in MDA-MB-435 cells significantly increased the survival of these cells relative to MDA-MB-435 cells expressing α6β4-Δcyt, as assessed by annexin V-FITC staining (Fig. 1), ApopTag staining (Fig. 1), and propidium iodide uptake (Table 1). In contrast, the expression of α6β4 in RKO cells did not increase the survival of these cells relative to either the mock (Table 1) or RKO/β4-Δcyt transfectants (Fig. 1). In fact, we observed a higher level of apoptosis and cell death in serum-starved RKO/β4 as compared to RKO/β4–Δcyt cells, in agreement with our previous demonstration that α6β4 can promote apoptosis in wild-type p53 carcinoma cells (4).

Based on the fact that RKO and MDA-MB-435 cells differ in their p53 status, we reasoned that the ability of α6β4 to promote cell survival may be inhibited by p53. This hypothesis was examined by investigating the effect of α6β4 expression on the survival of RKO cells in which p53 activity had been inhibited by expression of a dominant negative p53 (dnp53) construct. Indeed, α6β4 expression promoted the survival of serum-starved, dnp53-expressing RKO cells as determined by ApopTag and annexin V-FITC staining (Fig. 1). These results demonstrate that p53 can suppress the survival signaling mediated by α6β4 in serum-starved carcinoma cells.

α6β4-mediated survival in p53-deficient carcinoma cells is inhibited by dominant negative AKT/PKB: Given the importance of the AKT/PKB kinase in numerous survival signaling pathways (1, 7, 14, 15, 20, 32, 71), we investigated whether the survival function of α6β4 in serum-starved, p53-deficient carcinoma cells was AKT/PKB-dependent. The MDA-MB-435/β4-transfected clones, as well as the parental cells, were co-transfected with plasmids encoding for green fluorescent protein (GFP) and a Hemagglutinin (HA)-tagged, kinase-deficient AKT/PKB mutant that acts as a dominant negative construct (dnAKT/PKB) (20, 24, 70). Expression of this dnAKT/PKB construct was confirmed by immunoblotting extracts from these transfected cells with an HA-specific monoclonal antibody (data not shown). After 15 hours of serum starvation, the level of apoptosis in GFP-positive cells was assessed by annexin V-PE staining. As shown in Fig. 2, MDA-MB-435/β4 clones demonstrated signficantly less apoptosis than parental MDA-MB-435 cells in agreement with the data shown in Table 1. Importantly, dnAKT/PKB expression inhibited this α6β4 survival function in each of the two MDA-MB-435 /β4 clones examined, but it did not alter the level of apoptosis in parental MDA-MB-435 cells.

p53 inhibits the activation of AKT/PKB by α6β4: In order to understand the mechanism by which p53 inhibits α6β4-mediated survival, we investigated the possibility

that p53 alters the ability of this integrin to activate AKT/PKB. Initially, we examined whether the antibody-mediated clustering of α6β4 in MDA-MB-435 resulted in the phosphorylation of AKT/PKB on serine-473, an event that has been shown to correlate with AKT/PKB activation (2). MDA-MB-435/β4 subclones were transfected with an HA-tagged AKT/PKB construct. These cells were then incubated with either a control rat IgG or the β4-specific antibody 439-9B and plated in the absence of serum on secondary antibody-coated tissue culture wells for one hour. HA immunoprecipitations were performed on extracts from these cells and the levels of serine-phosphorylated AKT/PKB were assessed by blotting these immunoprecipitates with an antiserum specific for AKT/PKB molecules phosphorylated on serine residue 473. As shown in Fig. 3A, the antibody-mediated clustering of α6β4 stimulated an increase in the level of serine-phosphorylated AKT/PKB in each of two MDA-MB-435/β4 subclones relative to control cells (2.1-fold increase, β4 clone 1; 5.5-fold increase, β4 clone 2). This α6β4-induced increase in AKT/PKB serine phosphorylation was dependent on α6β4 signaling based on the inability of α6β4-Δcyt clustering to increase the level of serine 473-phosphorylated AKT/PKB in MDA-MB-435/β4-Δcyt subclones (data not shown).

To investigate the influence of p53 on the activation of AKT/PKB by α6β4, we explored whether α6β4 clustering induced the phosphorylation of AKT/PKB on serine residue 473 in MDA-MB-435/β4 that had been reconstituted with functional p53. Specifically, MDA-MB-435/β4 cells were transfected with a temperature-sensitive mutant of human p53 (tsp53) that assumes a functional conformation at 32°C, but not at 37°C (82). This construct has been used extensively to study the influence of p53 on signaling pathways involved in cell growth and apoptosis (43, 57). Stable transfectants of these cells were selected, and tsp53 expression was confirmed by immunoblotting (data not shown). Tsp53 and mock-transfected cells were then transfected transiently with HA-AKT/PKB. After incubating these cells with either rat IgG or 439-9B, they were plated on secondary antibody-coated wells and subjected to a 32°C incubation to stimulate p53 activity, followed

by a 37°C incubation to activate AKT/PKB. HA immunoprecipitations were performed on extracts from these cells, and these immunoprecipitates were subjected to immunoblotting with phosphoserine 473 AKT/PKB-specific rabbit antiserum. As shown in Fig. 3B, the clustering of α6β4 significantly increased the level of phosphoserine 473-AKT/PKB in mock-transfected MDA/β4 cells (7.9-fold increase), but not in tsp53-expressing MDA/β4 cells (1.2-fold increase). The importance of p53 in the inhibition of the α6β4-associated activation of AKT/PKB was indicated by the finding that α6β4 clustering increased the level of phosphoserine 473 AKT/PKB in MDA/β4+tsp53 transfectants that had been incubated at 37°C, the nonpermissive temperature for this tsp53 construct (data not shown).

The ability of p53 to suppress the α6β4-mediated activation of AKT/PKB was explored further in RKO carcinoma cells, which express wild-type p53. In agreement with the results obtained in MDA/β4 cells that had been reconstituted with functional p53, the clustering of α6β4 in two independent RKO/β4 subclones did not result in increased amounts of serine phosphorylated AKT/PKB (Figs. 3C and data not shown). Importantly, the expression of dnp53 in RKO/β4 cells restored the ability of α6β4 to activate AKT/PKB, as evidenced by an increase in phosphoserine 473-AKT/PKB immunoreactivity in RKO/β4+dnp53 cells that had been subjected to antibody-mediated α6β4 clustering (8.6-fold increase), as described above (Fig. 3C). The ability of α6β4 to stimulate AKT/PKB activity in RKO/β4 +dnp53 cells but not in RKO/β4 cells was confirmed by performing *in vitro* kinase assays using Histone H2B as substrate (data not shown). As a control for specificity, we also demonstrated that the clustering of α6β4 on dnp53-expressing RKO/β4-Δcyt cells did not stimulate AKT/PKB activity (data not shown).

α6β4 stimulation induces the caspase 3-dependent cleavage of AKT/PKB in a p53-dependent manner: To define the mechanism by which p53 inhibits the ability of α6β4 to activate AKT/PKB, we investigated whether p53 alters AKT/PKB expression levels in response to α6β4 clustering. RKO/β4 and RKO/β4+dnp53-expressing cells were

incubated with either rat Ig or 439-9B and stimulated on secondary antibody-coated wells for one hour. The amount of total AKT/PKB in equivalent amounts of total protein from these lysates was then assessed by immunoblotting. Importantly, the antibody-mediated clustering of the $\alpha6\beta4$ integrin on each of two RKO/β4 subclones resulted in a significant reduction in the total level of AKT/PKB in these cells (Fig. 4A). In contrast, AKT/PKB levels were not reduced in dnp53-expressing RKO/β4 cells (Fig. 4B) or in MDA-MB-435/β4 subclones (data not shown) following the antibody-mediated clustering of $\alpha6\beta4$. We also observed decreased levels of HA-AKT/PKB protein in HA-AKT/PKB-transfected RKO/β4 cells, but not in HA-AKT/PKB-transfected RKO/β4+dnp53 cells upon the antibody-mediated clustering of $\alpha6\beta4$ (data not shown).

Based on the reported ability of caspases to cleave signaling molecules that promote cell survival (12, 21, 66), we hypothesized that $\alpha6\beta4$ may promote the caspase-dependent cleavage of AKT/PKB in wild-type p53-expressing carcinoma cells. Initially, we explored the importance of caspase 3 activity, which has been shown to play a crucial role in p53-dependent apoptotic pathways (30), in the $\alpha6\beta4$-associated reduction of AKT/PKB expression levels. In agreement with the data shown in Fig. 4, the clustering of $\alpha6\beta4$ in control RKO/β4 cells significantly reduced the level of AKT/PKB in these carcinoma cells (Fig. 5). However, RKO/β4 cells that had been pretreated with Z-DEVD-FMK, a cell-permeable caspase 3 inhibitor, did not exhibit decreased levels of AKT/PKB in response to $\alpha6\beta4$ clustering (Fig. 5). In contrast, we detected a decreased amount of AKT/PKB following the clustering of $\alpha6\beta4$ in RKO/β4 cells that had been pretreated with Z-IETD-FMK, a cell permeable caspase 8 inhibitor (Fig. 5). Importantly, no effect of these inhibitors on AKT/PKB levels was observed upon the clustering of $\alpha6\beta4$ on RKO/$\alpha6\beta4$-Δcyt cells (data not shown).

The ability of the caspase 3 inhibitor to restore normal AKT/PKB levels suggested that AKT/PKB is cleaved by caspase 3 upon the clustering of $\alpha6\beta4$ in carcinoma cells expressing wild-type p53. To establish the caspase 3-mediated cleavage of AKT/PKB

more rigorously, we investigated whether a recombinant form of this cysteine protease could cleave AKT/PKB *in vitro*. Proteins in these reactions were resolved by SDS-PAGE and detected by silver staining. The results obtained revealed that the incubation of baculovirus-expressed AKT/PKB (Mr, 60kD) with recombinant caspase 3 resulted in the formation of an AKT/PKB cleavage product (Mr, 49 kD) (Fig. 6). In contrast, we did not detect an AKT/PKB cleavage product following the incubation of baculovirus AKT/PKB with recombinant caspase 8 (Fig. 6). This AKT/PKB cleavage product was also detected by immunoblotting with an antiserum specific for the COOH-terminus of AKT/PKB, suggesting that caspase 3 cleaves AKT/PKB at its amino terminus (data not shown).

Finally, to demonstrate that the caspase 3-dependent cleavage of AKT/PKB was responsible for the p53 inhibition of AKT/PKB activity in RKO/β4 cells, we explored the effects of a caspase 3 inhibitor on the ability of α6β4 to activate AKT/PKB. HA-AKT/PKB-transfected RKO/β4 cells were subjected to antibody-mediated α6β4 clustering in the presence of either DMSO or the caspase 3 inhibitor Z-DEVD-FMK. HA immunoprecipitates from extracts from these cells were then subjected to immunoblotting with the phosphoserine 473 AKT/PKB-specific rabbit antiserum. As shown in Figure 7, the pretreatment of RKO/β4 cells with Z-DEVD-FMK restored the ability of α6β4 to stimulate the phosphorylation of AKT/PKB in these cells. These results demonstrate that α6β4 stimulates the caspase 3-dependent cleavage and inactivation of AKT/PKB in p53-wild-type, but not in p53-deficient carcinoma cells.

Discussion

The binding of extracellular matrix proteins to integrins initiates survival signals that inhibit anoikis, a form of apoptosis induced upon the detachment of cells from extracellular matrix (28, 47). In the current studies, we show that the $\alpha6\beta4$ integrin suppresses anoikis exclusively in carcinoma cells that lack functional p53. Furthermore, we demonstrate that this $\alpha6\beta4$-associated survival function depends on the ability of this integrin to activate the serine/threonine kinase AKT/PKB in p53-deficient cells. Finally, we provide evidence that p53 inhibits the $\alpha6\beta4$-mediated activation of AKT/PKB by promoting the caspase 3-dependent cleavage of this kinase. Collectively, our findings establish that p53 can inhibit an integrin-associated survival function, a phenomenon that has important implications for tumor cell growth.

Our results suggest that the $\alpha6\beta4$ integrin can enhance the survival of carcinoma cells in an AKT/PKB-dependent manner. Although previous studies have shown that cell attachment to matrix proteins promotes the survival of primary epithelial cells (26, 41), $\alpha6\beta4$ is the first specific integrin to be implicated in the delivery of AKT/PKB-dependent survival signals to carcinoma cells. The importance of AKT/PKB in $\alpha6\beta4$ survival signaling was indicated in our studies by the ability of a dnAKT/PKB construct containing inactivating mutations in the catalytic domain to inhibit the survival effect of $\alpha6\beta4$ in serum-starved MDA-MB-435 cells. Although this dn/ has been used extensively to implicate AKT/PKB in survival pathways, it is possible that it associates with phosphoinositide-dependent kinases and inhibits their activity. However, our observation that the expression of a constitutively active AKT/PKB in MDA-MBA-435 enhances their survival (data not shown) strongly suggests that $\alpha6\beta4$ expression promotes the survival of these cells by activating AKT/PKB.

Our demonstration that p53 can inhibit AKT/PKB kinase activity is of interest in light of the recent finding that the PTEN tumor suppressor can also inhibit cell growth by inhibiting AKT/PKB in a manner that is dependent on its lipid phosphatase activity (16, 53,

61, 72, 75). Together, our current findings on p53 and the previously described activities of PTEN highlight the impact of tumor suppressors on integrin-mediated functions. Moreover, our demonstration that p53 inhibits α6β4 survival signaling by promoting the caspase-dependent cleavage of AKT/PKB provides a mechanistic link between tumor suppressor function and the regulation of integrin signaling, similar to the phosphatase activities of PTEN. Although previous studies have demonstrated that caspases can be activated by p53 in both cell-free systems (18) as well as in response to DNA damage (30, 81), our findings suggest that caspases can also be activated by an integrin in a p53-dependent manner. Indeed, it will be informative to determine if other activators of p53 such as DNA damage (44, 69) can promote the caspase-dependent cleavage of AKT/PKB.

The finding that AKT/PKB activity can be regulated by caspase 3 substantiates the hypothesis that caspases play an important role in many forms of apoptosis based on their ability to cleave signaling molecules that influence cell survival. For example, caspases have been shown to cleave and inactivate an inhibitor of caspase-activated deoxyribonuclease (CAD). Importantly, the cleavage of this inhibitor results in the activation of CAD, which is the enzyme responsible for the DNA fragmentation that is characteristic of apoptosis (21, 66). Caspase 3 has also been shown to cleave bcl-2, resulting in an inhibition of its anti-apoptotic function (12). While AKT/PKB has been suggested to be a target of caspase activity based on the reduced levels of this kinase observed in T cells in response to fas stimulation (79), our results extend this finding by establishing definitively that AKT/PKB is cleaved by caspase 3. More importantly, we provide evidence that this cleavage event results in the inhibition of AKT/PKB kinase activity, and implicate this event in the inhibition of α6β4 integrin survival signaling.

It is important to consider the mechanism by which the α6β4-induced, caspase-dependent cleavage of AKT/PKB inhibits its kinase activity. We detected an AKT/PKB fragment (Mr, 49 kD) following the *in vitro* incubation of AKT/PKB with recombinant caspase 3. This fragment was recognized by a rabbit antiserum raised against a peptide

corresponding to the extreme COOH-terminal amino acids of the molecule, suggesting that caspase 3 cleaves AKT/PKB at its amino terminus. Interestingly, the pleckstrin homology (PH) domain, which resides in the NH2-terminus of AKT/PKB, is important in both the translocation of this kinase to the membrane and its subsequent activation (3, 27). It is possible that the caspase 3-dependent cleavage of AKT/PKB prevents the membrane translocation of this kinase, thus preventing its activation. However, we were unable to identify an AKT/PKB fragment *in vivo* following the clustering of α6β4, despite our detection of reduced AKT/PKB levels under these conditions. This result suggests that following the initial cleavage of AKT/PKB by caspase 3, this kinase is subjected to further cleavage by other caspases, as has been shown for ICAD (76). Moreover, our inability to detect AKT/PKB fragments *in vivo* following the clustering of α6β4 suggests that AKT/PKB cannnot be detected by immunoblotting following its cleavage by multiple caspases. The ability of a caspase 3 inhibitor to restore both normal AKT/PKB levels as well as the α6β4-mediated activation of AKT/PKB suggests that the degradation of AKT/PKB observed *in vivo* is dependent on the initial cleavage of this kinase by caspase 3.

In contrast to our finding that p53-dependent, caspase 3 activity inhibits AKT/PKB, other studies have concluded that constitutively active AKT/PKB can delay p53-dependent apoptosis (65), inhibit caspases (10), and block caspase-dependent forms of apoptosis (6, 33). The demonstrated ability of AKT/PKB to inhibit p53 and caspase activity in these studies may relate to the kinetics of AKT/PKB activation. Specifically, the rapid stimulation of AKT/PKB may impede p53 or caspase activation. In contrast, the ability of α6β4 clustering to promote the caspase 3-dependent inactivation of AKT/PKB in p53-wild-type carcinoma cells may relate to the fact that α6β4 signaling stimulates caspase activity prior to AKT/PKB activity in these cells. Alternatively, it is possible that the ability of caspase 3 to cleave AKT/PKB has not observed in previous studies because insufficient amounts of endogenous caspase activity were present to inhibit the activity of exogenously

introduced, active AKT/PKB. Nonetheless, these results suggest that an intimate crosstalk exists between AKT/PKB and caspases that contributes to the regulation of cell survival.

We have previously demonstrated that the α6β4 integrin activates p53 function (4). The current studies describe an important consequence of this α6β4 activity, namely the inhibition of AKT/PKB activity and its associated cell survival function. Similar to previous results from our laboratory (4, 59, 67) and others (42, 74), the current studies demonstrate that the survival function of α6β4 is ligand-independent in β4-transfected, p53-deficient carcinoma cells. This ligand-independent survival function may be attributable to the ability of the β4 cytoplasmic domain to self-associate (63).

In addition to demonstrating that p53 inhibits α6β4-mediated survival, we observed that α6β4 increases the level of apoptosis observed in serum-starved p53-wild-type carcinoma cells. This result suggests that the apoptotic signaling pathway activated by α6β4 can augment the apoptotic signaling initiated by serum deprivation. Although p53 has been implicated in the apoptosis induced in endothelial cells upon their detachment from matrix (38), others have reported that epithelial cell anoikis is p53-independent (9). In agreement with the results of the latter study, we observed apoptosis in p53-deficient cells including MDA-MB-435 cells and dnp53-expressing RKO cells upon their detachment from matrix. These results indicate that carcinoma cells are subject to a p53-independent form of anoikis. In combination with our previous observation that α6β4 apoptotic signaling requires p53 activity (4), our findings suggest that the p53-independent apoptosis of carcinoma cells that occurs in response to matrix detachment can be enhanced by p53-dependent, α6β4 apoptotic signaling.

The current studies may explain why the α6β4 integrin has been implicated in the apoptosis of some cells and the survival of others. Specifically, α6β4 has been shown to induce growth arrest and apoptosis in several carcinoma cell lines (13, 42, 74) as well as in endothelial cells (49). However, this integrin has also been shown to promote the proliferation (46, 51) and survival (19) of keratinocytes. These apparently contradictory

functions of α6β4 may relate to the fact that the functions of α6β4 are cell type-specific. The current studies establish that the p53 tumor suppressor is one critical signaling molecule that may influence α6β4 function in different cell types because this integrin promotes apoptosis only in wild-type p53-expressing cells and survival only in p53-deficient cells. Interestingly, the reported ability of α6β4 to promote keratinocyte survival (19) may relate to the reported deficiency of p53 activity in these cells (55).

One implication of our findings is that the α6β4 integrin is similar to a number of oncogenes that promote cell proliferation in some settings and cell death in others. The recent observation that oncogenes can deliver such death signals has led to their seemingly contradictory categorization as tumor suppressors in select environments. For example, although the stimulation of c-myc and E2F normally promotes cell proliferation, the activation of these "oncogenes" induces apoptosis in the presence of secondary stress signals such as p53 expression, serum starvation or hypoxia (23, 36, 58, 68, 80). The ability of these stress signals to stimulate oncogene-dependent apoptosis is thought to be important in eliminating tumor cells that escape normal proliferation checkpoints as a result of oncogene expression. Similarly, the α6β4 integrin, which promotes the survival of p53-deficient cells, could also be classified loosely as a tumor suppressor based on its apoptotic function in carcinoma cells that express wild-type p53. The current studies demonstrate that, similar to the activity of oncogenes, integrin function and signaling can be profoundly influenced by physiological stimuli that activate other signaling pathways in a cell.

In summary, we have described the ability of the α6β4 integrin to promote the survival of p53-mutant, but not p53-wild-type carcinoma cells. This ability of p53 to influence integrin-mediated functions so markedly derives from its ability to activate the caspase 3-dependent cleavage of AKT/PKB. The fact that AKT/PKB overexpression has been suggested to contribute to the transformed phenotype of tumor cells (5) suggests that the introduction of the α6β4 integrin into p53-wild type tumors may inhibit their growth by inducing the cleavage of this transforming protein. The ability of α6β4 to induce the p53-

dependent cleavage of AKT/PKB also suggests that the acquisition of inactivating mutations in either p53 or caspase 3 will provide a selective growth advantage for carcinoma cells by stimulating α6β4-mediated AKT/PKB-dependent survival signaling. Moreover, given our previous demonstration that α6β4 promotes carcinoma cell migration and invasion (11, 56, 59, 67), we suggest that carcinoma cells that express α6β4 and mutant forms of p53 or caspase 3 will have a distinct advantage in their ability to disseminate and survive as metastatic lesions.

Acknowledgments

Figure Legends

Figure 1. p53 inhibits α6β4-mediated survival. MDA-MB-435, RKO, and RKO+dnp53 cells that expressed either α6β4 (β4) or α6β4-Δcyt (β4-Δcyt) were plated on poly-L-lysine-coated tissue culture wells and cultured in the absence of serum. After 15 hours, the cells were harvested, subjected to either ApopTag reactions (A) or annexin V-FITC staining (B), and analyzed by flow cytometry. A survival effect of α6β4 was quantified by subtracting the percentage of α6β4-expressing cells that were positive for either Apoptag (A) or annexin V-FITC (B) staining from the percentage of α6β4-Δcyt-expressing cells that were positive for these markers. This value was plotted on the bar graphs shown in (A) and (B), with positive values indicating that the specified β4 clone exhibits increased survival relative to the relevant β4-Δcyt subclone, and negative values indicating an increased apoptosis of the indicated clone relative to the appropriate β4-Δcyt clone. The data in (A) represent the means (+/- SEM) from three independent experiments. Similar results to those shown in (B) were observed in three separate trials.

Figure 2. Expression of a dominant negative AKT/PKB inhibits α6β4-mediated survival. Parental (neo) and α6β4-expressing (β4) MDA-MB-435 cells were transfected with either a GFP-expressing plasmid (mock) or both a GFP and a dnAKT/PKB-expressing construct (dnAKT/PKB), plated on poly-L-lysine, and cultured for 15 hours in the absence of serum. Apoptosis in these cells was then assessed by annexin V-PE staining. The data are reported as the percentage of GFP-positive cells that were stained by annexin V-PE. Similar results were observed in two additional experiments.

Figure 3. p53 inhibits the ability of α6β4 to induce AKT/PKB phosphorylation in carcinoma cells. MDA/β4, MDA/β4+tsp53, RKO/β4, and RKO/β4 + dnp53 cells were transfected transiently with a hemagluttinin (HA)-tagged AKT/PKB. These transfectants were incubated with the indicated primary antibodies, washed, and plated in the absence of

serum on secondary antibody-coated tissue culture wells. HA-AKT/PKB-transfected MDA/β4 (A), RKO/β4 (C), and RKO/β4 + dnp53 (C) cells were stimulated for one hour at 37°C. Alternatively, mock and tsp53-transfected MDA/β4 cells (B) were stimulated for one hour at 32°C to activate tsp53, followed by an additional hour at 37°C to activate AKT/PKB. Immunoprecipitations were performed with an HA-specific monoclonal antibody on equal amounts of total extracted protein. The immunoprecipitates were resolved by SDS-PAGE (8%), transferred to nitrocellulose, and probed with a phosphoserine 473 AKT/PKB-specific rabbit antiserum (New England Biolabs), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. Phosphoserine-specific AKT/PKB bands were detected by chemiluminescence, and are noted by arrows.

Figure 4. Clustering of the α6β4 integrin reduces AKT/PKB protein levels in p53-wild type but not in p53-deficient carcinoma cells. RKO/β4 (A&B) and RKO/β4+dnp53 (B)-expressing cells were incubated with either rat Ig or 439-9B and plated on secondary antibody-coated wells for one hour in the absence of serum. Equivalent amounts of total protein from lysates from these cells were resolved by SDS-PAGE (8%), transferred to nitrocellulose, and probed with an AKT/PKB-specific rabbit antiserum (New England Biolabs) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. These blots were also probed with an actin-specific rabbit antiserum (Sigma) to confirm the loading of equivalent amounts of protein. The AKT/PKB and actin bands were detected by enhanced chemiluminescence, and are indicated by arrows. These bands were then quantified by densitometry. α6β4 clustering decreased AKT/PKB levels in RKO/β4 subclone (1.7-fold decrease-β4 clone 1; 1.9-fold decrease- β4 clone 2), but not in RKO/β4 + dnp53 cells. Similar results were observed in four additional trials.

Figure 5. A caspase 3 inhibitor blocks α6β4-associated reductions in AKT/PKB protein levels. RKO/β4 cells were incubated with either rat Ig or 439-9B in the presence of DMSO

(1:500), a caspase 3 inhibitor (Z-DEVD-FMK; 4 μg/mL), or a caspase 8 inhibitor (Z-IETD-FMK; 4 μg/mL). These cells were then washed with PBS and plated onto secondary antibody-coated wells in the presence of the same drugs for one hour in serum-free medium. Equivalent amounts of total protein were resolved by SDS-PAGE (8%), transferred to nitrocellulose, and probed with an AKT/PKB-specific rabbit antiserum (New England Biolabs) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. AKT/PKB was detected by enhanced chemiluminescence and quantified by densitometry. The antibody-mediated clustering of α6β4 decreased the level of AKT/PKB in DMSO-treated cells (2.0-fold decrease- β4 clone 1; 1.9-fold decrease- β4 clone 2), as well as in cells pretreated with a caspase 8 inhibitor (1.9-fold decrease). In contrast, the pretreatment of these cells with a caspase 3 inhibitor partially restored AKT/PKB levels in RKO/β4 cells subjected to α6β4 clustering (1.1 fold decrease, β4 clone 1; 1.1-fold decrease- β4 clone 2). By probing these blots with an actin-specific rabbit antiserum (Sigma), we confirmed that equivalent amounts of actin were present in each lane (data not shown). Similar results were observed in three experiments.

Figure 6. AKT/PKB is cleaved by recombinant caspase 3 *in vitro*. Baculovirus-expressed AKT/PKB (0.5 μg) was incubated either alone, with recombinant caspase 3 (2 μg) or with recombinant caspase 8 (2 μg) for one hour at 37°C. Proteins in these reactions were resolved by SDS-PAGE (8%) and subjected to silver staining. AKT/PKB and its cleavage product are indicated by arrows. Similar results were observed in three trials.

Figure 7. A caspase 3 inhibitor restores the ability of α6β4 to induce AKT/PKB phosphorylation. HA-AKT/PKB-transfected RKO/β4 cells were incubated with either rat Ig or 439-9B in the presence of DMSO (1:500) or a caspase 3 inhibitor (Z-DEVD-FMK; 4μg/mL). After washing with PBS, these cells were plated on secondary antibody-coated wells in serum-free medium containing the indicated drugs for one hour. HA

immunoprecipitations were performed on equivalent amounts of total extracted protein from these samples. These immunoprecipitates were resolved by SDS-PAGE (8%), transferred to nitrocellulose, and probed with rabbit antiserum specific for phosphoserine 473-AKT/PKB, followed by horseradish peroxidase-conjugated goat anti-rabbit Ig. Phosphoserine 473-AKT/PKB was then detected by enhanced chemiluminescence, and is indicated by an arrow. Total AKT/PKB levels were also assessed by stripping these membranes and probing with an AKT/PKB-specific rabbit antiserum (data not shown). Relative activity was assessed by determining the ratio of serine phosphorylated AKT/PKB to that of total AKT/PKB for each sample (Relative AKT activity: Lane 1=1.0, lane 2=1.3, Lane 3=1.1, Lane 4=3.1). Similar results were observed in three experiments.

Table I. Influence of α6β4 Integrin on the Viability of
RKO and MDA-MB-435 Cells

Clone	% Propidium Iodide Positive Cells
MDA/Mock	21
β4 Clone 1	13
β4 Clone 2	9
RKO/Mock	32
β4 Clone 1	49
β4 Clone 2	47

Mock-transfected and β4-transfected MDA-MB-435 and RKO cells were plated on poly-L-lysine (25 μg/mL) in the absence of serum for 15 hours, harvested, and incubated with propidium iodide (PI). The percentage of PI-positive cells was then assessed by flow cytometry. Similar results were observed in four independent experiments.

References

1. Ahmed, N. N., H. L. Grimes, A. Bellacosa, T. O. Chan, and P. N. Tsichlis. 1997. Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. *Proceedings of the National Academy of Sciences of the United States of America.* 94:3627-32.

2. Alessi, D. R., M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen, and B. A. Hemmings. 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* 15:6541-6551.

3. Andjelkovic, M., D. R. Alessi, R. Meier, A. Fernandez, N. J. Lamb, M. Frech, P. Cron, P. Cohen, J. M. Lucocq, and B. A. Hemmings. 1997. Role of translocation in the activation and function of protein kinase B. *Journal of Biological Chemistry.* 272:31515-24.

4. Bachelder, R. E., A. Marchetti, R. Falcioni, S. Soddu, and A. M. Mercurio. 1999. Activation of p53 function in carcinoma cells by the a6b4 integrin. *J.B.C.* 274:20733-20737.

5. Bellacosa, A., D. de Feo, A. K. Godwin, D. W. Bell, J. Q. Cheng, D. A. Altomare, M. Wan, L. Dubeau, G. Scambia, V. Masciullo, and et al. 1995. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *International Journal of Cancer.* 64:280-5.

6. Berra, E., M. T. Diaz-Meco, and J. Moscat. 1998. The activation of p38 and apoptosis by the inhibition of Erk is antagonized by the phosphoinositide 3-kinase/Akt pathway [published erratum appears in J Biol Chem 1998 Jun 26;273(26):16630]. *Journal of Biological Chemistry.* 273:10792-7.

7. Blume-Jensen, P., R. Janknecht, and T. Hunter. 1998. The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. *Current Biology.* 8:779-82.

8. Borradori, L., and A. Sonnenberg. 1996. Hemidesmosomes: roles in adhesion, signaling and human diseases. [Review] [74 refs]. *Current Opinion in Cell Biology.* 8:647-56.

9. Boudreau, N., C. J. Sympson, Z. Werb, and M. J. Bissell. 1995. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science.* 267:891-3.

10. Cardone, M. H., N. Roy, H. R. Stennicke, G. S. Salvesen, T. F. Franke, E. Stanbridge, S. Frisch, and J. C. Reed. 1998. Regulation of cell death protease caspase-9 by phosphorylation. *Science.* 282:1318-21.

11. Chao, C., Lotz, M.M., Clark, A.C., and Mercurio, A.M. 1996. A function for the integrin alpha beta4 in the invasive properties of colorectal carcinoma cells. *Cancer. Res.* 56:4811-4819.

12. Cheng, E. H., D. G. Kirsch, R. J. Clem, R. Ravi, M. B. Kastan, A. Bedi, K. Ueno, and J. M. Hardwick. 1997. Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science.* 278:1966-8.

13. Clarke, A. S., Lotz, M.M., Chao, C.C., and Mercurio, A.M. 1995. Activaton of the p21 pathway of growth arrest and apoptosis by the β4 integrin cytoplasmic domain. *J. Biol. Chem.* 270:22673-22676.

14. Crowder, R. J., and R. S. Freeman. 1998. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. *Journal of Neuroscience.* 18:2933-43.

15. Datta, S. R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, and M. E. Greenberg. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell.* 91:231-41.

16. Davies, M. A., D. Koul, H. Dhesi, R. Berman, T. J. McDonnell, D. McConkey, W. K. Yung, and P. A. Steck. 1999. Regulation of Akt/PKB activity, cellular growth, and apoptosis in prostate carcinoma cells by MMAC/PTEN. *Cancer Research.* 59:2551-6.

17. Day, M. L., R. G. Foster, K. C. Day, X. Zhao, P. Humphrey, P. Swanson, A. A. Postigo, S. H. Zhang, and D. C. Dean. 1997. Cell anchorage regulates apoptosis through the retinoblastoma tumor suppressor/E2F pathway. *Journal of Biological Chemistry*. 272:8125-8.

18. Ding, H. F., G. McGill, S. Rowan, C. Schmaltz, A. Shimamura, and D. E. Fisher. 1998. Oncogene-dependent regulation of caspase activation by p53 protein in a cell-free system. *Journal of Biological Chemistry*. 273:28378-83.

19. Dowling, J., Yu, Q., Fuchs, E. 1996. b4 integrin is required for hemidesmosome formation, cell adhesion and cell survival. *J. Cell Biol.* 134:559-572.

20. Dudek, H., S. R. Datta, T. F. Franke, M. J. Birnbaum, R. Yao, G. M. Cooper, R. A. Segal, D. R. Kaplan, and M. E. Greenberg. 1997. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science*. 275:661-666.

21. Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD [see comments] [published erratum appears in Nature 1998 May 28;393(6683):396]. *Nature*. 391:43-50.

22. Enari, M., R. V. Talanian, W. W. Wong, and S. Nagata. 1996. Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature*. 380:723-6.

23. Evan, G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, L. Z. Penn, and D. C. Hancock. 1992. Induction of apoptosis in fibroblasts by c-myc protein. *Cell*. 69:119-28.

24. Eves, E. M., W. Xiong, A. Bellacosa, S. G. Kennedy, P. N. Tsichlis, M. R. Rosner, and N. Hay. 1998. Akt, a target of phosphatidylinositol 3-kinase, inhibits apoptosis in a differentiating neuronal cell line. *Molecular & Cellular Biology*. 18:2143-52.

25. Falcioni, R., A. Sacchi, J. Resau, and S. J. Kennel. 1998. Monoclonal antibody to human carcinoma protein complex: quantitation in normal and tumor tissue. *Cancer Res.* 48:816-821.

26. Farrelly, N., Y. J. Lee, J. Oliver, C. Dine, and C. H. Streuli. 1999. Extracellular matrix regulates apoptosis in mammary epithelium through a control on insulin signaling. *J. Cell Biol.* 144:1337-1347.

27. Franke, T. F., S. I. Yang, T. O. Chan, K. Datta, A. Kazlauskas, D. K. Morrison, D. R. Kaplan, and P. N. Tsichlis. 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell.* 81:727-36.

28. Frisch, S. M., and H. Francis. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. *Journal of Cell Biology.* 124:619-26.

29. Frisch, S. M., K. Vuori, E. Ruoslahti, and P. Y. Chan-Hui. 1996. Control of adhesion-dependent cell survival by focal adhesion kinase. *Journal of Cell Biology.* 134:793-9.

30. Fuchs, E. J., K. A. McKenna, and A. Bedi. 1997. p53-dependent DNA damage-induced apoptosis requires Fas/APO-1-independent activation of CPP32beta. *Cancer Research.* 57:2550-4.

31. Fujio, Y., K. Guo, T. Mano, Y. Mitsuuchi, J. R. Testa, and K. Walsh. 1999. Cell cycle withdrawal promotes myogenic induction of Akt, a positive modulator of myocyte survival. *Molecular & Cellular Biology.* 19:5073-82.

32. Gerber, H. P., A. McMurtrey, J. Kowalski, M. Yan, B. A. Keyt, V. Dixit, and N. Ferrara. 1998. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *Journal of Biological Chemistry.* 273:30336-43.

33. Gibson, S., S. Tu, R. Oyer, S. M. Anderson, and G. L. Johnson. 1999. Epidermal growth factor protects epithelial cells against Fas-induced apoptosis. Requirement for Akt activation. *Journal of Biological Chemistry.* 274:17612-8.

34. Green, K. J., and J. C. Jones. 1996. Desmosomes and hemidesmosomes: structure and function of molecular components. [Review] [81 refs]. *FASEB Journal.* 10:871-81.

35. Hemler, M. E., Crouse, C., and Sonnenberg, A. 1989. Association of the VLA alpha 6 subunit with a novel protein. A possible alternative to the common VLA beta1 subunit on certain cell lines. *J. Biol. Chem.* 264:6529-6535.

36. Hermeking, H., and D. Eick. 1994. Mediation of c-Myc-induced apoptosis by p53. *Science.* 265:2091-3.

37. Howlett, A. R., N. Bailey, C. Damsky, O. W. Petersen, and M. J. Bissell. 1995. Cellular growth and survival are mediated by beta 1 integrins in normal human breast epithelium but not in breast carcinoma. *Journal of Cell Science.* 108:1945-57.

38. Ilic, D., E. A. Almeida, D. D. Schlaepfer, P. Dazin, S. Aizawa, and C. H. Damsky. 1998. Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *Journal of Cell Biology.* 143:547-60.

39. Kajiji, S., Tamura, R.N., and Quaranta, V. 1989. A novel integrin (alpha E beta 4) from human epithelial cells suggests a fourth family of integrin adhesion receptors. *EMBO J.* 8:673-680.

40. Kennel, S. J., Foote, L.J., Falcioni, R., Sonnenberg, A., Stringer, C.J., Crouse, C., and Hemler, M.E. 1989. Analysis of the tumor-associated antigen TSP-180. Identity of alpha 6-beta 4 in the integrin superfamily. *J. Biol. Chem.* 264:15515-15521.

41. Khwaja, A., P. Rodriguez-Viciana, S. Wennstrom, P. H. Warne, and J. Downward. 1997. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO Journal.* 16:2783-93.

42. Kim, S. Y., Bachman, N.J., Nair, T.S., Goldsmith, S., Liebert, M., Grossman, H.B., Lomax, M.I., and Carey, T.E. 1997. Beta 4 integrin transfection of UM-UC-2 (human bladder carcinoma) cells: Stable expression of a spontaneous cytoplasmic truncation mutant with rapid loss of clones expressing intact beta 4. *Cancer Res.* 57:38-42.

43. Kobayashi, T., U. Consoli, M. Andreeff, H. Shiku, A. B. Deisseroth, and W. Zhang. 1995. Activation of p21WAF1/Cip1 expression by a temperature-sensitive mutant of human p53 does not lead to apoptosis. *Oncogene.* 11:2311-6.

44. Komarova, E. A., M. V. Chernov, R. Franks, K. Wang, G. Armin, C. R. Zelnick, D. M. Chin, S. S. Bacus, G. R. Stark, and A. V. Gudkov. 1997. Transgenic mice with p53-responsive lacZ: p53 activity varies dramatically during normal development and determines radiation and drug sensitivity in vivo. *EMBO Journal*. 16:1391-400.

45. Lesoon-Wood, L. A., W. H. Kim, H. K. Kleinman, B. D. Weintraub, and A. J. Mixson. 1995. Systemic gene therapy with p53 reduces growth and metastases of a malignant human breast cancer in nude mice. *Human Gene Therapy*. 6:395-405.

46. Mainiero, F., Murgia, C., Wary, K.K., Curatola, A.M., Pepe, A., Blumemberg, M., Westwick, J.K., Der, C., and Giancotti, F.G. 1997. The coupling of a6b4 integrin to the ras-MAP kinase pathways mediated by shc controls keratinocyte proliferation. *EMBO J.* 16:2365-2375.

47. Meredith, J. E., Jr., B. Fazeli, and M. A. Schwartz. 1993. The extracellular matrix as a cell survival factor. *Molecular Biology of the Cell*. 4:953-61.

48. Merlo, G. R., F. Basolo, L. Fiore, L. Duboc, and N. E. Hynes. 1995. p53-dependent and p53-independent activation of apoptosis in mammary epithelial cells reveals a survival function of EGF and insulin. *Journal of Cell Biology*. 128:1185-96.

49. Miao, J., S. Araki, K. Kaji, and H. Hayashi. 1997. Integrin $\beta4$ is involved in apoptotic signal transduction in endothelial cells. *Biochem. and Biophys. Res. Comm.* 233:182-186.

50. Morgenstern, J. P., and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucl. Acids Res.*:3587-3596.

51. Murgia, C., P. Blaikie, N. Kim, M. Dans, H. T. Petrie, and F. G. Giancotti. 1998. Cell cycle and adhesion defects in mice carrying a targeted deletion of the integrin beta4 cytoplasmic domain. *EMBO Journal*. 17:3940-51.

52. Muzio, M., A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J. D. Bretz, M. Zhang, R. Gentz, M. Mann, P. H. Krammer, M. E. Peter,

and V. M. Dixit. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell.* 85:817-27.

53. Myers, M. P., I. Pass, I. H. Batty, J. Van der Kaay, J. P. Stolarov, B. A. Hemmings, M. H. Wigler, C. P. Downes, and N. K. Tonks. 1998. The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proceedings of the National Academy of Sciences of the United States of America.* 95:13513-8.

54. Nagasawa, H., C. Y. Li, C. G. Maki, A. C. Imrich, and J. B. Little. 1995. Relationship between radiation-induced G1 phase arrest and p53 function in human tumor cells. *Cancer Research.* 55:1842-6.

55. Nigro, J. M., Aldape, K.D., Hess, S.M., and Tlsty, T.D. 1997. Cellular adhesion regulates p53 protein levels in primary human keratinocytes. *Cancer Res.* **57**:3635-3639.

56. O'Connor, K. L., L. M. Shaw, and A. M. Mercurio. 1998. Release of cAMP Gating by the a6b4 integrin stimulates lamellae formation and the chemotactic migration of carcinoma cells. *J. Cell Biol.* 143:1749-1760.

57. Owen-Schaub, L. B., W. Zhang, J. C. Cusack, L. S. Angelo, S. M. Santee, T. Fujiwara, J. A. Roth, A. B. Deisseroth, W. W. Zhang, E. Kruzel, and et al. 1995. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. *Molecular & Cellular Biology.* 15:3032-40.

58. Qin, X. Q., D. M. Livingston, W. G. Kaelin, Jr., and P. D. Adams. 1994. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proceedings of the National Academy of Sciences of the United States of America.* 91:10918-22.

59. Rabinovitz, I., and A. M. Mercurio. 1997. The integrin α6β4 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.

60. Radeva, G., T. Petrocelli, E. Behrend, C. Leung-Hagesteijn, J. Filmus, J. Slingerland, and S. Dedhar. 1997. Overexpression of the integrin-linked kinase promotes

anchorage-independent cell cycle progression. *Journal of Biological Chemistry.* 272:13937-44.

61. Ramaswamy, S., N. Nakamura, F. Vazquez, D. B. Batt, S. Perera, T. M. Roberts, and W. R. Sellers. 1999. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proceedings of the National Academy of Sciences of the United States of America.* 96:2110-5.

62. Rampalli, A. M., and P. S. Zelenka. 1995. Insulin regulates expression of c-fos and c-jun and suppresses apoptosis of lens epithelial cells. *Cell Growth & Differentiation.* 6:945-53.

63. Rezniczek, G.A., de Pereda, J.M., Relpert, M., and Aiche, G. 1998. Linking integrin α6β4–based adhesion to the intermediate filament cytoskeleton: direct interaction between the β4 subunit and plectin at multiple molecular sites. *J. Cell Biol.* **141**:2209-2225.

64. Rodeck, U., M. Jost, C. Kari, D. T. Shih, R. M. Lavker, D. L. Ewert, and P. J. Jensen. 1997. EGF-R dependent regulation of keratinocyte survival. *Journal of Cell Science.* 110:113-21.

65. Sabbatini, P., and F. McCormick. 1999. Phosphoinositide 3-OH Kinase (PI3K) and PKB/AKT Delay the Onset of p53-mediated, Transcriptionally Dependent Apoptosis. *J.B.C.* 274:24263-24269.
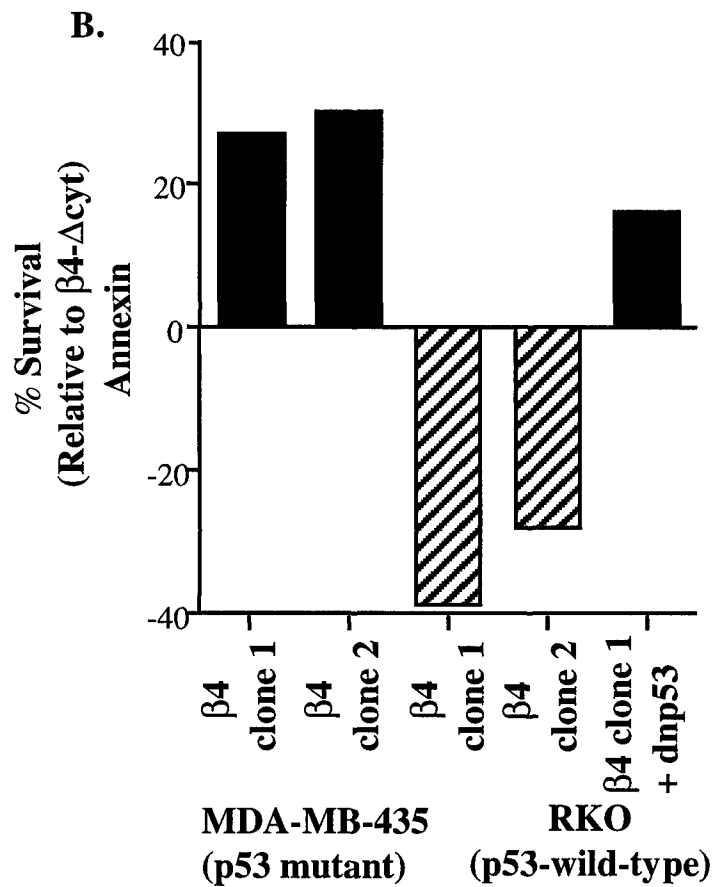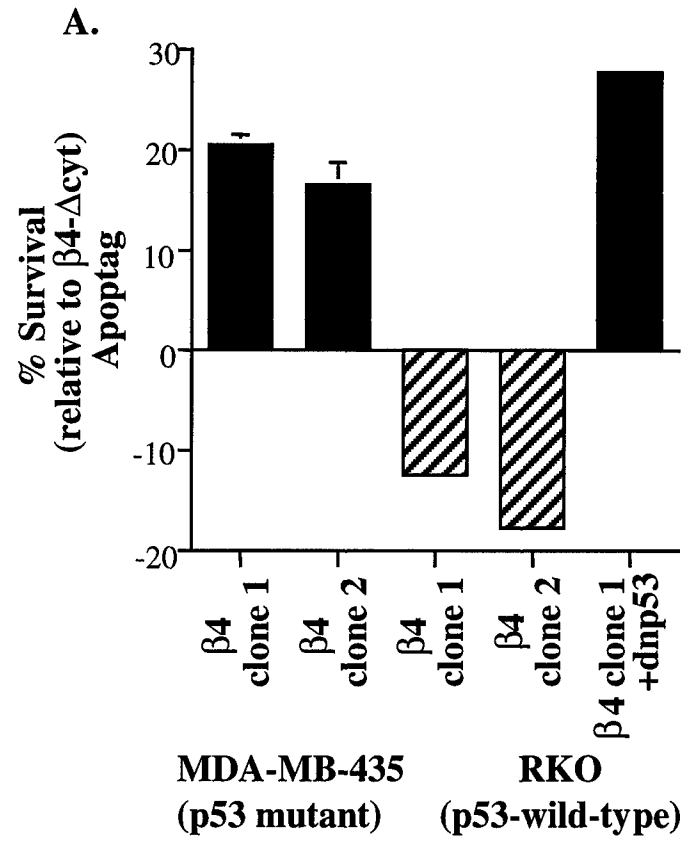
66. Sakahira, H., M. Enari, and S. Nagata. 1998. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis [see comments]. *Nature.* 391:96-9.

67. Shaw, L. M., Rabinovitz, I., Wang, H.F., Toker, A., and Mercurio, A.M. 1997. Activation of phosphoinositide 3-OH kinase by the a6b4 integrin promotes carcinoma invasion. *Cell.* 91:949-960.
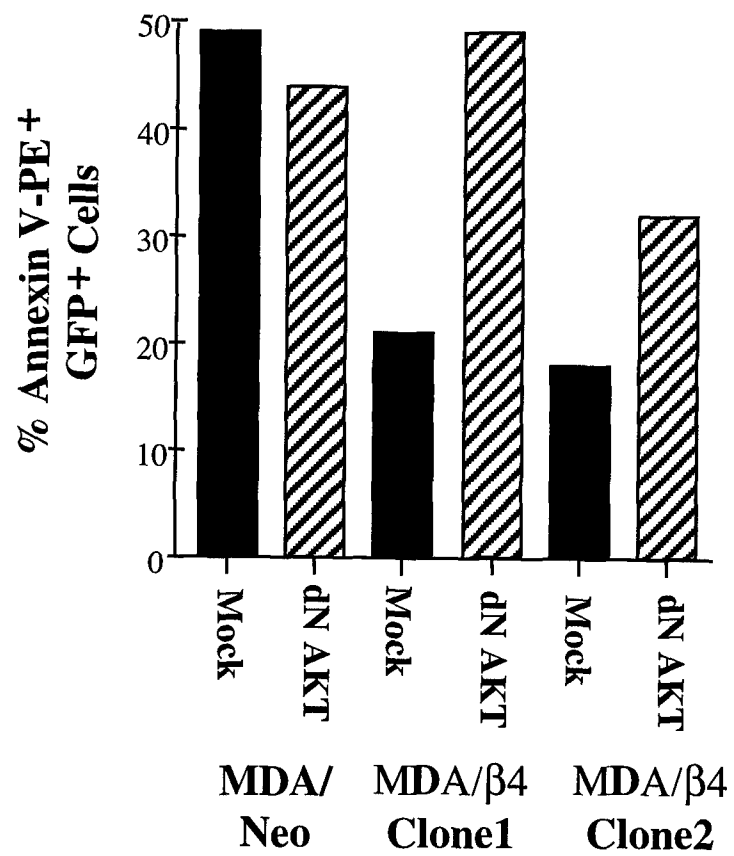
68. Shi, Y., J. M. Glynn, L. J. Guilbert, T. G. Cotter, R. P. Bissonnette, and D. R. Green. 1992. Role for c-myc in activation-induced apoptotic cell death in T cell hybridomas. *Science.* 257:212-4.
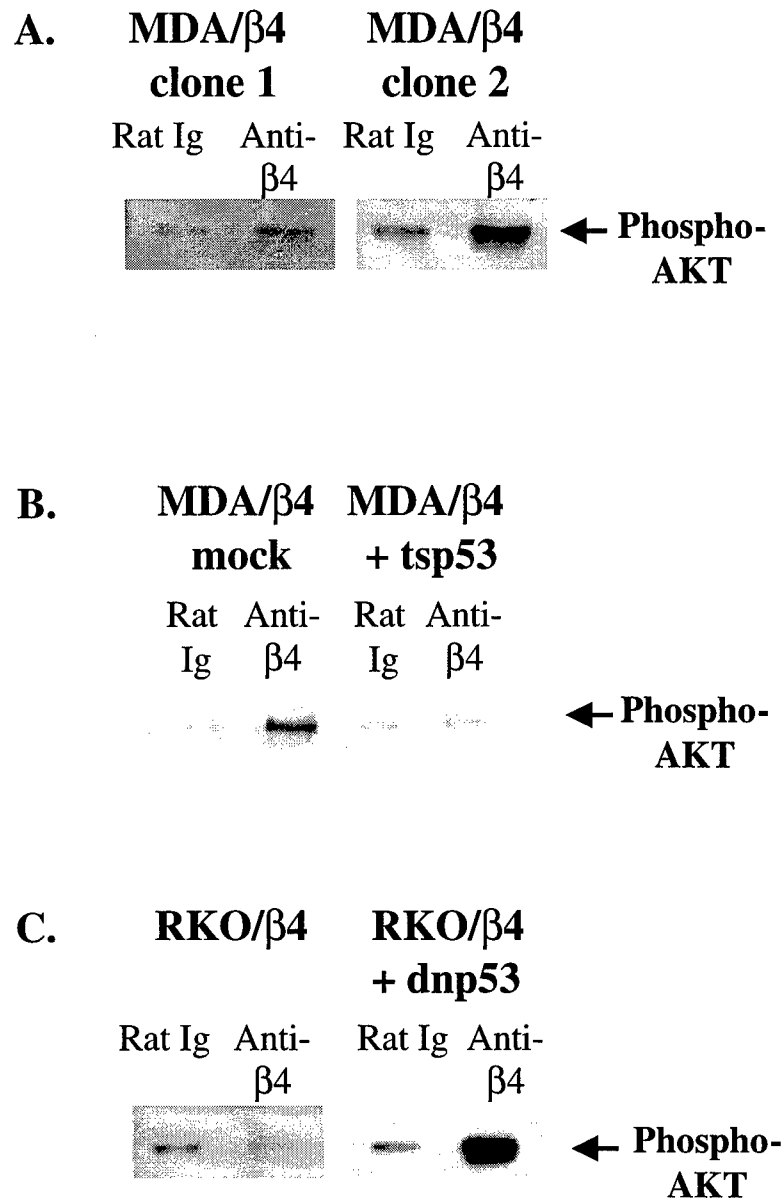
69. Siegel, J., M. Fritsche, S. Mai, G. Brandner, and R. D. Hess. 1995. Enhanced p53 activity and accumulation in response to DNA damage upon DNA transfection. *Oncogene.* 11:1363-70.

70. Skorski, T., A. Bellacosa, M. Nieborowska-Skorska, M. Majewski, R. Martinez, J. K. Choi, R. Trotta, P. Wlodarski, D. Perrotti, T. O. Chan, M. A. Wasik, P. N. Tsichlis, and B. Calabretta. 1997. Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway. *EMBO Journal.* 16:6151-61.

71. Songyang, Z., D. Baltimore, L. C. Cantley, D. R. Kaplan, and T. F. Franke. 1997. Interleukin 3-dependent survival by the Akt protein kinase. *Proceedings of the National Academy of Sciences of the United States of America.* 94:11345-50.

72. Stambolic, V., A. Suzuki, J. L. de la Pompa, G. M. Brothers, C. Mirtsos, T. Sasaki, J. Ruland, J. M. Penninger, D. P. Siderovski, and T. W. Mak. 1998. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell.* 95:29-39.

73. Stromblad, S., J. C. Becker, M. Yebra, P. C. Brooks, and D. A. Cheresh. 1996. Suppression of p53 activity and p21WAF1/CIP1 expression by vascular cell integrin alphaVbeta3 during angiogenesis. *Journal of Clinical Investigation.* 98:426-33.

74. Sun, H., Santoro, S.A., and Zutter, M.M. 1998. Downstream events in mammary gland morphogenesis mediated by reexpression of the alpha2beta1 integrin: the role of the alphaα6 and beta4 integrin subunits. *Cancer Res.* 58:2224-2233.

75. Sun, H., R. Lesche, D. M. Li, J. Liliental, H. Zhang, J. Gao, N. Gavrilova, B. Mueller, X. Liu, and H. Wu. 1999. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. *Proceedings of the National Academy of Sciences of the United States of America.* 96:6199-204.

76. Tang, D., and V. J. Kidd. 1998. Cleavage of DFF-45/ICAD by multiple caspases is essential for its function during apoptosis. *Journal of Biological Chemistry.* 273:28549-52.

77. Tozeren, A., Kleinman, H.K., Wu, S., Mercurio, A.M., and Byers, S.W. 1994. Integrin alpha 6 beta 4 mediates dynamic interactions with laminin. *J. Cell Science.* 107:3153-3163.

78. Wewer, U. M., Shaw, L.M., Albrechtsen, R., and Mercurio, A.M. 1997. The integrin alpha 6 beta 1 promotes the survival of metastatic human breast carcinoma cells in mice. *Am. J. Pathol.* 151:1191-1198.

79. Widmann, C., S. Gibson, and G. L. Johnson. 1998. Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals. *Journal of Biological Chemistry.* 273:7141-7.

80. Wu, X., and A. J. Levine. 1994. p53 and E2F-1 cooperate to mediate apoptosis. *Proceedings of the National Academy of Sciences of the United States of America.* 91:3602-6.

81. Yu, Y., and J. B. Little. 1998. p53 is involved in but not required for ionizing radiation-induced caspase-3 activation and apoptosis in human lymphoblast cell lines. *Cancer Research.* 58:4277-81.

82. Zhang, W., X. Y. Guo, G. Y. Hu, W. B. Liu, J. W. Shay, and A. B. Deisseroth. 1994. A temperature-sensitive mutant of human p53. *EMBO Journal.* 13:2535-44.

83. Zhang, Z., K. Vuori, J. C. Reed, and E. Ruoslahti. 1995. The alpha 5 beta 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proceedings of the National Academy of Sciences of the United States of America.* 92:6161-5.

A. (graph: % Survival (relative to β4-Δcyt) Apoptag vs. β4 clone 1, β4 clone 2, β4 clone 1, β4 clone 2, β4 clone 1 +dnp53; MDA-MB-435 (p53 mutant), RKO (p53-wild-type))

B. (graph: % Survival (Relative to β4-Δcyt) Annexin vs. β4 clone 1, β4 clone 2, β4 clone 1, β4 clone 2, β4 clone 1 + dnp53; MDA-MB-435 (p53 mutant), RKO (p53-wild-type))

A.

MDA/β4
clone 1

MDA/β4
clone 2

Rat Ig   Anti-   Rat Ig   Anti-
β4   β4

← Phospho-
AKT

B.

MDA/β4
mock

MDA/β4
+ tsp53

Rat   Anti-   Rat   Anti-
Ig   β4   Ig   β4

← Phospho-
AKT

C.

RKO/β4

RKO/β4
+ dnp53

Rat Ig   Anti-   Rat Ig   Anti-
β4   β4

← Phospho-
AKT

A.

RKO

β4   β4
Clone 1 Clone 2

← AKT

← Actin

Rat Anti- Rat Anti-
Ig   β4   Ig   β4

B.

RKO

β4   β4+
            dnp53

← AKT

← Actin

Rat Anti- Rat Anti-
Ig   β4   Ig   β4

RKO/β4 Clone 1   RKOβ4 Clone 2

DMSO   Casp 3   Casp 8   DMSO   Casp 3
          Inh   Inh   Inh

← AKT

Rat   Anti- Rat Anti- Rat Anti- Rat Anti- Rat Anti-
Ig   β4   Ig   β4   Ig   β4   Ig   β4   Ig   β4

AKT
(60 kDa)

AKT
Fragment
(49 kDa)

rCaspase   -   3   8

RKO/β4

DMSO   Caspase 3
Inhibitor

← Phospho-
AKT

Rat Anti- Rat Anti-
Ig   β4   Ig   β4

Release of cAMP Gating by the α6β4 Integrin Stimulates Lamellae Formation and the Chemotactic Migration of Invasive Carcinoma Cells

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Abstract. The α6β4 integrin promotes carcinoma invasion by its activation of a phosphoinositide 3-OH (PI3-K) signaling pathway (Shaw, L.M., I. Rabinovitz, H.H.-F. Wang, A. Toker, and A.M. Mercurio. *Cell.* 91: 949–960). We demonstrate here using MDA-MB-435 breast carcinoma cells that α6β4 stimulates chemotactic migration, a key component of invasion, but that it has no influence on haptotaxis. Stimulation of chemotaxis by α6β4 expression was observed in response to either lysophosphatidic acid (LPA) or fibroblast conditioned medium. Moreover, the LPA-dependent formation of lamellae in these cells is dependent upon α6β4 expression. Both lamellae formation and chemotactic migration are inhibited or "gated" by cAMP and our results reveal that a critical function of α6β4 is to suppress the intracellular cAMP concentration by increasing the activity of a rolipram-sensitive, cAMP-specific phosphodiesterase (PDE). This PDE activity is essential for lamellae formation, chemotactic migration and invasion based on data obtained with PDE inhibitors. Although PI3-K and cAMP-specific PDE activities are both required to promote lamellae formation and chemotactic migration, our data indicate that they are components of distinct signaling pathways. The essence of our findings is that α6β4 stimulates the chemotactic migration of carcinoma cells through its ability to influence key signaling events that underlie this critical component of carcinoma invasion.

Key words: integrin • migration • cyclic AMP • phosphodiesterase • cytoskeleton

CARCINOMA invasion is a complex process that involves directed migration and localized proteolysis (24). Although the mechanistic basis of invasion had been elusive, recent advances in molecular cell biology have facilitated a much more rigorous analysis of this important and critical component of cancer progression. In particular, insight into the function and regulation of cell adhesion receptors, as well as proteases, has fueled significant progress in our understanding of the invasive process. Studies aimed at defining specific signal transduction pathways that determine the behavior of invasive carcinoma cells are also contributing to an uncovering of the molecular basis of invasion.

Recent work by our group and others has implicated a key role for the α6β4 integrin in carcinoma invasion (3, 10, 32, 35, 40, 47). This integrin, which is a receptor for the laminins, is essential for the organization and maintenance of epithelial structure. In many epithelia, α6β4 mediates the formation of stable adhesive structures termed hemidesmosomes that link the intermediate filament cytoskeleton with the extracellular matrix (2, 12). The importance of this integrin in epithelial structure has been reinforced by the generation of β4-nullizygous mice that exhibit gross alterations in epithelial morphology and anchorage to the basement membrane (9, 46). In contrast to its function in normal epithelia, α6β4 can stimulate carcinoma migration and invasion through its ability to interact with the actin cytoskeleton and mediate the formation and stabilization of lamellae (32). This dynamic function of α6β4 in enhancing the migration of invasive carcinoma cells is quite distinct from its role in maintaining stable adhesive contacts in normal epithelia by associating with intermediate filaments. In fact, we have established that the ability of α6β4 to stimulate carcinoma migration and invasion depends upon its preferential activation of a phosphoinositide 3-OH kinase (PI3-K)[1]/Rac signaling pathway that we (40) and

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1. *Abbreviations used in this paper:* [cAMP]$_i$, intracellular cyclic AMP concentration; DIC, differential–interference contrast; Gi, inhibitory type G protein; IBMX, isobutylmethylxanthine; LPA, lysophosphatidic acid; PDE; phosphodiesterase; PI3-K, phosphoinositide 3-OH kinase.

others (18) have shown is necessary for invasion. In essence, our studies have defined an integrin-mediated mechanism of carcinoma invasion that involves the stimulation of carcinoma migration by the dynamic association of α6β4 with F-actin and the activation of a specific signaling pathway by this integrin.

Although we have established the involvement of α6β4 in the migration of invasive carcinoma cells, the nature of this migration has not been well defined. Moreover, signaling pathways distinct from PI3-K/Rac that are also regulated by α6β4 are likely to contribute to carcinoma migration. For these reasons, we sought to examine the migration mediated by α6β4 in more detail and to identify other signaling pathways regulated by this integrin that contribute to migration. The results obtained indicate that α6β4 stimulates the chemotactic migration of invasive carcinoma cells but that it has no influence on their haptotactic migration. Importantly, we demonstrate that the ability of α6β4 to suppress the intracellular cAMP concentration ($[cAMP]_i$) by activating a cAMP-specific phosphodiesterase (PDE) is essential for its enhancement of lamellae formation and chemotactic migration. Although PI3-K and cAMP-specific PDE activities are required for lamellae formation and chemotactic migration, we conclude that they are components of distinct signaling pathways.

Materials and Methods

Cell Culture and Antibodies

We used stable subclones of MDA-MB-435 human breast carcinoma cells that had been transfected with either the expression vector alone (mock transfectants), a full-length β4 cDNA (MDA/β4 transfectants), or a mutated β4 cDNA that lacked the entire cytoplasmic domain with the exception of four amino acids distal to the transmembrane sequence (MDA/β4-ΔCYT). The characterization of these transfectants has been described previously (38, 40). Both the β4 transfectants and the β4-ΔCYT transfectants expressed the α6β4 heterodimer on the cell surface as assessed by FACS® analysis and immunoprecipitation of surface-labeled extracts (40). The surface expression of α6β4 in these transfectants was comparable to the expression seen in other breast carcinoma cell lines that express this integrin endogenously such as MDA-MB-231 cells (Shaw, L.M., unpublished observation). All MDA-MB-435 cells were cultured in Dulbecco's modified Eagle's medium (DME) with 10% fetal calf serum plus 1% L-glutamine, 1% penicillin, and 1% streptomycin (GIBCO BRL, Gaithersburg, MD). Clone A cells, originally isolated from a human, poorly differentiated colon adenocarcinoma (7) and were cultured in RPMI 1640 medium containing 10% fetal calf serum plus 1% L-glutamine, 1% penicillin, and 1% streptomycin.

NIH-3T3 cells were cultured in DME containing 10% newborn calf serum plus 1% L-glutamine, 1% penicillin, and 1% streptomycin. NIH-3T3 conditioned medium was prepared from normal culture medium incubated with cells for 2 d before harvest with cellular debris removed by centrifugation.

The following function blocking, integrin-specific monoclonal antibodies (mAb) were used: mAb 13 (mouse anti-β1; S. Akiyama, National Institutes of Health, Research Triangle Park, NC), G0H3 (rat anti-α6; Immunotech, Westbrook, ME) and 2B7 (mouse anti-α6, prepared by our laboratory [39]). Non-specific mouse IgG was purchased from Sigma Chemical Co. (St. Louis, MO).

Migration and Invasion Assays

Cells were harvested using trypsin, rinsed three times with serum-free DME containing 250 µg/ml heat-inactivated BSA (DME/BSA), and then resuspended in DME/BSA. For migration assays, the lower surface of the membrane in each Transwell chamber (6.5-mm-diam, 8 µm pore size; Costar, Cambridge, MA) was coated for 30 min with either 15 µg/ml lami-

nin-1 purified from Englebreth-Holm-Swarm tumor (19), 15 µg/ml collagen I (Vitrogen®; Collagen Biomaterials, Palo Alto, CA), or NIH-3T3 conditioned medium. For chemotaxis assays, either NIH-3T3 conditioned medium or lysophosphatidic acid (LPA) was added to the lower chamber. For haptotaxis assays, DME/BSA was added to the lower chamber. Cells (5×10^4) suspended in DME/BSA were added to the upper chamber. After incubating for 4 h at 37°C, nonmigrating 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 with 100% methanol and stained with 0.2% (wt/vol) crystal violet in 2% ethanol. Migration was quantified by counting cells per square millimeter using bright-field optics. For antibody inhibition experiments, cells were incubated with 20 µg/ml of antibody for 30 min and then added to the Transwell chambers. The effects of pertussis toxin, IBMX (Calbiochem-Novabiochem, La Jolla, CA), forskolin, and rolipram (Sigma Chemical Co.) on migration were assessed by preincubating the cells with these reagents for 30 min before assay and including them in the assay medium at the concentrations noted in the figure legends.

Invasion assays were performed as described previously (40). In brief, 10 µg of Matrigel (Collaborative Research, Bedford, MA) was diluted with cold water and dried onto each Transwell filter. The Matrigel was reconstituted with DME for 1 h before its use in the assays. Cells were prepared as above and then added to the upper chamber of each well. NIH-3T3 conditioned medium was added to the lower chamber. Cells were allowed to invade for 4 h and then cells that had invaded were stained and quantified as described above.

cAMP Assays

Culture dishes (35-mm) were coated overnight with 20 µg/ml of collagen I in PBS and then blocked with serum-free RPMI containing 250 µg/ml BSA (RPMI/BSA). Cells (1.5×10^6) were then plated for 2 h and harvested by quickly removing the medium and extracting them directly with 80% (vol/vol) ethanol. Cell extracts were collected, cleared by centrifugation in a microcentrifuge for 10 min, dried in a SpeedVac (Savant Instruments, Farmingdale, NY) for 1.5 h, and then resuspended in 50 mM phosphate buffer, pH 6.2. The intracellular cAMP concentration was quantified using a cAMP enzyme-linked immunoabsorption assay (cAMP EIA; Cayman Biochemicals, Ann Arbor, MI) following the manufacturer's recommendation using nonacetylated cAMP as a standard and acetylcholine esterase-linked cAMP as a competitor. Values were corrected for cell number as determined from replicate plates. In some experiments, either 50 µM forskolin alone or forskolin plus 1 mM IBMX was added to cells 15 min before harvesting. To determine the cAMP content of cells under normal culture conditions, cells were plated in 35-mm dishes in DME plus 10% FCS, incubated for 18 h, and then processed as described above.

Phosphodiesterase Assays

cAMP PDE assays were performed according to the protocol of Sette et al. (37). In brief, cells were plated onto collagen I coated dishes as described for cAMP assays. Cells were then scraped from the dishes in a hypotonic lysis buffer (PDE lysis buffer: 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2 mM EGTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 4 µg/ml aprotinin, and 2 mM PMSF) and then sonicated. Cellular debris was removed by centrifugation and the supernatants were assayed immediately for PDE activity. PDE activity of cell extracts (2–4 µg protein) was assayed in cAMP PDE assay buffer (20 mM Tris-HCl, pH 8.0, 10 mM MgCl$_2$, 1.25 mM β-mercaptoethanol, 0.14 mg/ml BSA, 1 µM cAMP, and 0.2 µCi [³H] cAMP) for 10 min at 34°C. The reaction was stopped by adding 40 mM Tris, pH 7.5, containing 10 mM EDTA and heating for 2 min at 100°C. The reaction products were then digested with 50 µg Crotalus atrox snake venom (Sigma Chemical Co.) for 30 min at 34°C, separated from substrate using SpinZyme acidic alumina devices (Pierce Chemical Co., Rockford, IL), and quantified using a scintillation counter (Wallace, Gaithersburg, MD). Values were corrected for protein content and are reported as pmol cAMP hydrolyzed/min per milligram of protein. Protein content of cell extracts was determined using the Bio-Rad protein reagent (Hercules, CA) with BSA as a protein standard. Where noted, cells were incubated either for 30 min with 50 µM forskolin or 100 nM wortmannin or for 5 min with 100 nM LPA before harvest. For rolipram inhibition of PDE activity, 100 nM rolipram was added to cell extracts for 5 min before assaying for PDE activity.

Assessment of Lamellae Formation

Glass coverslips were coated overnight at 4°C with 20 μg/ml collagen I or laminin-1 and then blocked with BSA (0.25% in RPMI). The MDA-MB-435 transfectants were trypsinized and rinsed as described above, and then plated onto the coverslips for 2 h. As noted, cells were then treated with either 1 mM IBMX or 0.2% DMSO for 30 min. Subsequently, the cells were either treated with 100 nM LPA for 5 min or left untreated and then fixed for 10 min with 4% paraformaldehyde containing 10 mM Pipes, pH 6.8, 2 mM EGTA, 2 mM MgCl₂, 7% sucrose, and 100mM KCl. The coverslips were rinsed three times with PBS and mounted in glycerol. For the analysis of clone A cells, the cells were treated with IBMX or DMSO for 30 min, plated on laminin-1-coated coverslips, incubated for 45 min at 37°C, and then fixed. Clone A cells were then rinsed three times with PBS and incubated with blocking solution containing 1% BSA/5% normal donkey serum for 30 min. Cells were incubated with 20 μg/ml TRITC-labeled phalloidin in blocking solution for 30 min. Cells were rinsed four times with PBS over 30 min and then mounted in glycerol containing 1× PBS, pH 8.5, and 0.1% propylgallate. All cells were imaged with a Nikon Diaphot 300 inverted microscope (Tokyo, Japan) using either Nomarski differential–interference contrast (DIC) or phase–contrast optics. Images were captured with a charge-coupled device camera (Dage-MTI, Michigan City, IN), a frame grabber (Scion, Frederick, MD) and a 7600 Power Macintosh computer (Apple Computer, Cupertino, CA). Images were analyzed and lamellar area quantified using IPLab Spectrum image analysis software (Signal Analytics, Vienna, VA) using the criteria for defining lamellae used previously by our group (32). Lamellae were defined as broad, flat cellular protrusions rich in F-actin and devoid of membrane-bound vesicles. The lamellar area of each cell was determined using both phase contrast optics and FITC-phalloidin staining.

Analysis of PDE Expression

To determine the relative expression of PDE in the cells used in this study, cell extracts (40 μg of protein) were resolved by SDS-PAGE (8%), transferred to nitrocellulose, and then immunoblotted with PDE4-specific antibodies provided by M. Conti (Stanford University, Stanford, CA) (15). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and visualized using SuperSignal chemiluminescent substrate (Pierce Chemical Co.).

Analysis of PI3-K Activation

The activation of PI3-K by the integrin α6β4 was assessed as described previously (40). In brief, cells were trypsinized and rinsed as above, resuspended in RPMI/BSA at a concentration of 2×10^6 cells/ml and incubated for 30 min with integrin-specific antibodies or in buffer alone. Either IBMX (1 mM), forskolin (50 μM) or DMSO (0.2%) was added for 10 min before plating the cells onto tissue culture dishes coated with goat anti–rat IgG Ab. After incubation for 30 min at 37°C in the presence of IBMX, forskolin, or DMSO, the cells were washed twice with cold PBS and solubilized at 4°C for 10 min with 20 mM Tris, pH 7.4, 137 mM NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 2 mM PMSF, and 5 μg/ml of aprotinin, pepstatin, and leupeptin. Equivalent amounts of protein from each extract were incubated for 3 h at 4°C with the antiphosphotyrosine mAb, 4G10 (Upstate Biotechnology, Lake Placid, NY) and protein A–Sepharose (Pharmacia Biotech, Piscataway, NJ). The Sepharose beads were washed twice with lysis buffer then twice with 10 mM Hepes, pH 7.0, and 0.1 mM EGTA. Beads were then resuspended with kinase buffer plus 100 μM ATP, 25 μM MgCl₂, 10 μCi [γ-^{32}P]ATP, and 10 μl sonicated brain lipids and incubated for 10 min at room temperature. The reaction was stopped using 60 μl 2N HCl and 160 μl chloroform/methanol (1:1). Lipids were resolved using potassium oxalate-coated thin layer chromatography plates.

Results

Expression of the α6β4 Integrin in MDA-MB-435 Cells Enhances Their Chemotactic Migration

The possibility that expression of the α6β4 integrin influenced the rate of either haptotactic or chemotactic migration was assessed. For this purpose, stable transfectants of MDA-MB-435 cells were used that expressed either the α6β4 integrin (MDA/β4) or a deletion mutant of α6β4 (MDA/β4-ΔCYT) that retains only four amino acids of the β4 cytoplasmic domain, immediately proximal to the
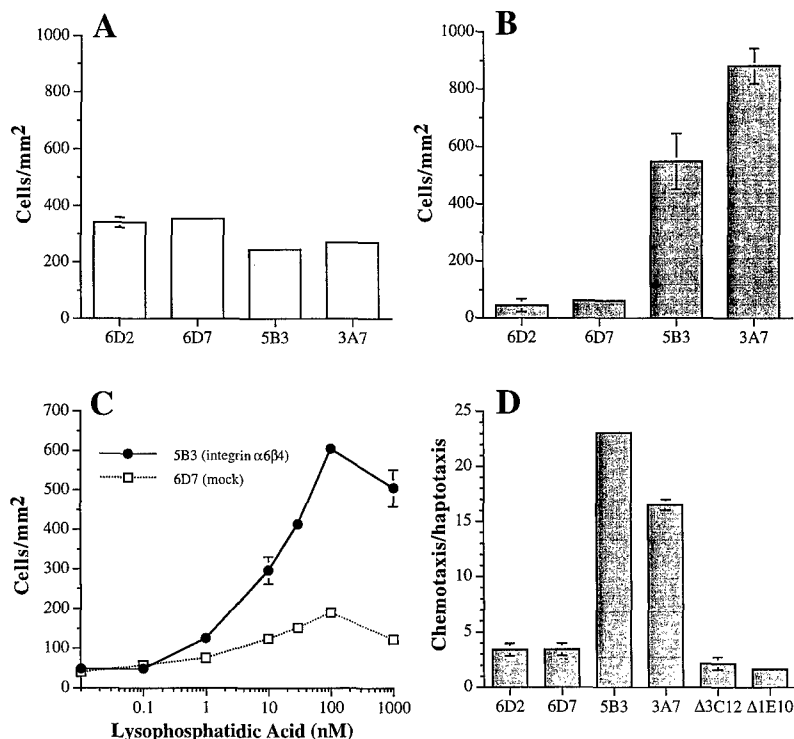
Figure 1. Expression of the α6β4 integrin in MDA-MB-435 carcinoma cells stimulates chemotaxis but not haptotaxis. The migration of the MDA/β4 (*5B3* and *3A7*) MDA/β4-ΔCYT (Δ3C12, Δ1E10), and MDA/mock (*6D2* and *6D7*) transfectants toward laminin-1 (haptotaxis; *A*), 3T3 conditioned medium (chemotaxis; *B*), or LPA (chemotaxis; *C* and *D*) was assessed using a modified Boyden chamber. The lower surfaces of Transwell membranes were coated with either laminin-1 (*A*), conditioned medium (*B*), or collagen I (*C* and *D*), and then either BSA (*A*) 3T3 conditioned medium (*B*) or LPA (*C* and *D*) was added to the lower chambers. Cells (10^5 [*A* and *B*] or 5×10^4 [*C* and *D*]) were placed in the upper chambers. After 4 h at 37°C, cells that did not migrate were removed from the upper chamber with a cotton swab and cells on the opposite side of the membrane were fixed, stained, and quantified manually as described in the Materials and Methods. (*A*) Haptotaxis toward laminin-1; (*B*) chemotaxis toward NIH-3T3 conditioned medium; (*C*) dose response of MDA-MB-435 subclones 5B3 (β4 transfected; *solid circles*) and 6D7 (mock transfected; *open squares*) chemotaxis toward LPA; (*D*) Chemotaxis toward 100 nM LPA. Data are reported as fold increases over haptotactic migration on collagen I in the absence of LPA. Data (all panels) are shown as mean ± standard deviation from triplicate determinations.

transmembrane domain (40). As shown in Fig. 1 *A*, subclones of the MDA/β4 transfectants (*5B3* and *3A7*) exhibited a rate of haptotactic migration toward laminin-1 that was slightly lower than the rate observed for subclones of the mock transfectants (*6D7* and *6D2*). In marked contrast, expression of α6β4 induced a substantial increase in the rate of chemotaxis of these cells towards conditioned medium from NIH-3T3 cells (Fig. 1 *B*). The rate of chemotaxis of the MDA/β4 transfectants (*5B3* and *3A7*) was 15–20-fold greater than that of the mock transfectants (*6D7* and *6D2*) over a 4-h time period. These data indicate that expression of α6β4 potentiates chemotactic migration of MDA-MB-435 cells without substantially altering their rate of haptotaxis.

To identify specific factors that could cooperate with α6β4 to promote chemotaxis of MDA-MB-435 cells, we tested several growth factors known to have chemotactic potential including epidermal growth factor, basic fibroblast growth factor, hepatocyte growth factor/scatter factor, insulin-like growth factor type I, transforming growth factor α and β, platelet-derived growth factor (AA and BB), somatostatin, thrombin, and LPA. Of these factors, only LPA was able to mimic the chemotactic effects of NIH-3T3 cell conditioned medium on the MDA-MB-435 transfectants (Fig. 1 *C* and data not shown). LPA stimulated the chemotaxis of MDA-MB-435 cells in a dose dependent manner with maximal stimulation observed at 100 nM. Of note, LPA stimulation of chemotaxis was five- to sevenfold greater in the MDA/β4 transfectants than in the mock transfectants. Subclones of the MDA/β4-ΔCYT transfectants (Δ3C12 and Δ1E10) exhibited a rate of chemotaxis that was similar to the mock transfectants (Fig. 1 *D*), indicating that the β4 cytoplasmic domain is critical for mediating the increased chemotaxis seen in the MDA/β4 transfectants.

The increased chemotaxis observed for the MDA/β4 transfectants in response to LPA was evident on both collagen I (Fig. 1, *C* and *D*) and laminin-1 (data not shown), indicating that α6β4-enhanced migration is independent of the matrix protein used for traction. This possibility was examined further by preincubating the MDA/β4 transfectants with function-blocking mAbs before their use in the chemotaxis assays. As shown in Fig. 2 *A*, inhibition of α6 integrin function with the mAb 2B7 did not block the chemotaxis of the MDA/β4 transfectants on collagen I towards LPA. However, this mAb inhibited the haptotaxis of MDA-MB-435 cells toward a laminin-1 gradient (Fig. 2 *B*), a process that is dependent on the α6β1 integrin (38). Chemotaxis toward LPA was inhibited completely, however, by preincubating the cells with the β1 integrin-specific mAb 13 (Fig. 2 *A*). Collectively, these data indicate that the stimulation of chemotaxis by expression of α6β4 can be independent of the adhesive functions of α6β4, and that the adhesive interactions required for α6β4-enhanced chemotaxis on collagen I are mediated through β1 integrins.

Expression of the α6β4 Integrin Is Required for the Formation of Lamellae in Response to LPA

Chemotactic migration frequently involves the formation of broad sheets of polymerized actin at the leading edge of
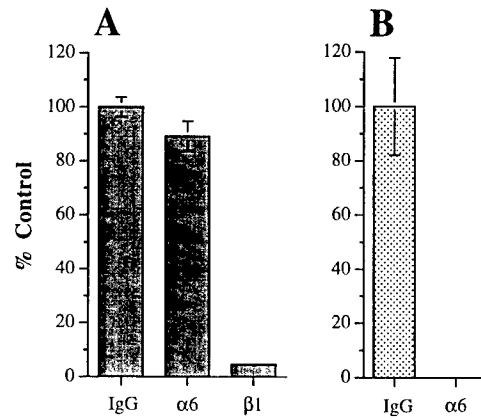

Figure 2. Inhibition of α6β4-stimulated migration by integrin-specific antibodies. MDA/β4 (5B3; *A, gray bars*) or mock transfectants (*6D7; B, stippled bars*) were incubated with the indicated function blocking mAbs for 30 min before their use in a chemotaxis assay using 100 nM LPA on collagen I (*A*) or a haptotaxis assay on laminin-1–coated wells (*B*) as described in Fig. 1. Nonspecific mouse IgG was used as a negative control. Data are reported as the percentage of migration observed for the IgG control ± standard deviation from triplicate determinations.

the cell termed lamellae (27). To determine if expression of the α6β4 integrin influenced the formation of such motile structures, we analyzed the morphology of the MDA-MB-435 transfectants plated on collagen I (Fig. 3). Prominent lamellae were not evident in the mock transfectants and treatment with 100 nM LPA did not stimulate a significant increase in lamellar area (Fig. 3, *C* and *D*). The MDA/β4 transfectants exhibited a similar morphology to that of the mock transfectants when plated on collagen I (Fig. 3, compare *A* with *C*) or laminin-1 (data not shown). Within minutes after LPA treatment, however, the MDA/β4 transfectants formed large, ruffling lamellae (Fig. 3 *C*). Quantification of these cells by digital image analysis indicated that LPA stimulated a dramatic increase in the lamellar area of the two subclones of the MDA/β4 transfectants (Fig. 3 *D*). In contrast, no increase in the lamellar area of the mock transfectants in response to LPA was detected by this analysis (Fig. 3 *D*).

Pharmacological Evidence for the Involvement of cAMP in Chemotaxis

LPA is a bioactive phospholipid that can mediate its effects on cells through a receptor linked to heterotrimeric G proteins, including inhibitory type G (Gi) proteins (29). To assess the possible involvement of a Gi protein in α6β4-enhanced chemotaxis, we used pertussis toxin, which inactivates heterotrimeric Gi-proteins by ADP ribosylation (31). The LPA-stimulated chemotaxis of both the MDA/β4 and mock transfectants was inhibited by pertussis toxin with maximal inhibition observed at 100 ng/ml (data not shown). These data suggested that the α6β4 integrin enhances chemotaxis that is mediated through pertussis toxin-sensitive, Gi-linked receptors. Gi proteins are known to inhibit certain classes of adenyl cyclases and thus limit cAMP production (45). For this reason, we analyzed the impact of stimulating cAMP production on chemotaxis
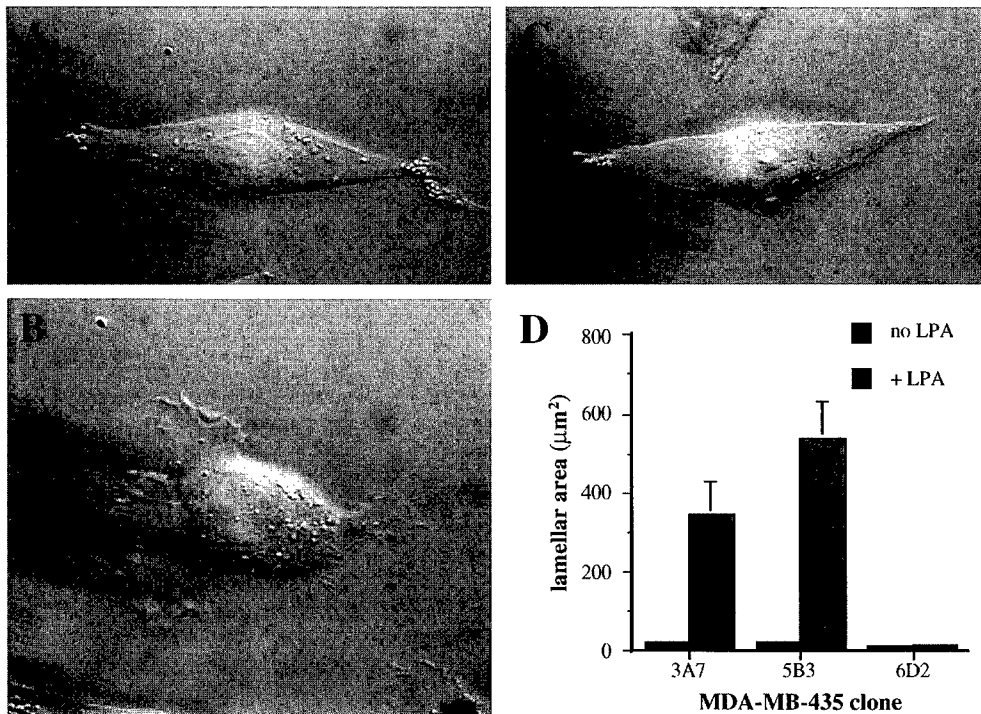
Figure 3. The α6β4 integrin is required for the LPA-dependent formation of lamellae in MDA-MB-435 cells. MDA/β4 (*A* and *B*) and mock transfectants (*C*) were plated onto coverslips that had been coated with 20 μg/ml collagen I. Cells were allowed to adhere for 2 h at 37°C and then treated with LPA for 5 min. (*B* and *C*) or left untreated (*A*). The cells were visualized using Nomarski DIC optics. Note the large lamellae that are formed in response to LPA stimulation of the MDA/β4 transfectants. (*D*) The effect of LPA on lamellar area was quantified using IPLab Spectrum imaging software. Data are shown as mean lamellar area ± standard error in which $n > 20$. Bar, 10 μm.

using forskolin. Although forskolin inhibited LPA-stimulated chemotaxis, the MDA/β4 and mock transfectants differed significantly in their response to this activator of adenyl cyclases. LPA-stimulated chemotaxis of the mock transfectants was inhibited to basal levels by 50 μM forskolin (Fig. 4 *A*). At this concentration of forskolin, the inhibition of chemotaxis of the MDA/β4 transfectants was only 50% and higher concentrations of forskolin (100 μM) did not abrogate chemotaxis of these cells (Fig. 4 *A*). Interestingly, treatment of the MDA/β4 or mock transfectants with forskolin did not inhibit haptotactic migration on laminin-1 (Fig. 4 *B*). These data indicate that a cAMP-sensitive pathway plays a key role in LPA-stimulated chemotaxis of MDA-MB-435 cells and they suggest that the α6β4 integrin may regulate this pathway.

Expression of the α6β4 Integrin in MDA-MB-435 Cells Influences cAMP Metabolism

To determine if α6β4 expression influences the [cAMP]$_i$, the [cAMP]$_i$ was determined in extracts obtained from subconfluent cultures of MDA/mock, β4, and β4-ΔCYT transfectants using a cAMP enzyme-linked immunoabsorption assay. As shown in Fig. 5 *A*, the MDA/β4 transfectants had a 30% lower [cAMP]$_i$ (2.7 pmol cAMP per 10^6 cells) than either the mock (3.7 pmol cAMP per 10^6 cells) or β4-ΔCYT transfectants (3.8 pmol cAMP per 10^6 cells). This difference was statistically significant ($P < 0.001$). Of note, neither clustering of α6β4 using the 2B7 mAb and an appropriate secondary Ab nor LPA treatment reduced cAMP levels further (data not shown).

The observation that the MDA/β4 transfectants were more resistant to forskolin inhibition of chemotaxis than the mock transfectants (Fig. 4) suggested that these two populations of cells differ in their ability to metabolize the

cAMP generated in response to forskolin stimulation. This possibility was examined by determining the [cAMP]$_i$ in forskolin-treated cells. As shown in Fig. 5 *B*, the MDA/β4 transfectants exhibited a 30% lower [cAMP]$_i$ than the mock transfectants when plated on collagen I. With forskolin stimulation, a 2.5-fold greater accumulation of cAMP was observed in the mock transfectants (6.6 pmol per 10^6 cells) compared with the β4 transfectants (2.6 pmol per 10^6 cells). When the forskolin-treated cells were also treated with the PDE inhibitor, IBMX, to prevent breakdown of cAMP, the MDA/β4 transfectants exhibited a [cAMP]$_i$ comparable to the mock transfectants (120 ± 11 versus 104 ± 18 pmol per 10^6 cells, respectively; Fig. 5 *C*). Together, these data suggest that expression of α6β4 integrin suppresses the [cAMP]$_i$ by increasing PDE activity.

To establish more directly that expression of the α6β4 integrin can regulate cAMP-dependent PDE activity, the activity of this enzyme was assayed in cell extracts obtained from the MDA/mock and β4 transfectants. As shown in Fig. 6 *A*, the MDA/β4 transfectants exhibited a significantly higher rate of PDE activity than the mock transfected cells. Moreover, the PDE activity of the MDA/β4 transfectants was markedly increased (51% for 5B3 and 45% for 3A7) in response to forskolin stimulation compared with the mock transfectants (29% for 6D7; Fig. 6 *A*). The difference in PDE activity between the MDA/β4 and mock transfectants was eliminated by rolipram, a type IV PDE-specific (PDE 4) inhibitor (Fig. 6 *B*). These data indicate that a cAMP-dependent PDE 4 activity is influenced by α6β4 expression in MDA-MB-435 cells. Also, this activity is likely responsible for the observed decrease in [cAMP]$_i$ and the resistance to forskolin-mediated inhibition of LPA chemotaxis observed in the MDA/β4 transfectants.

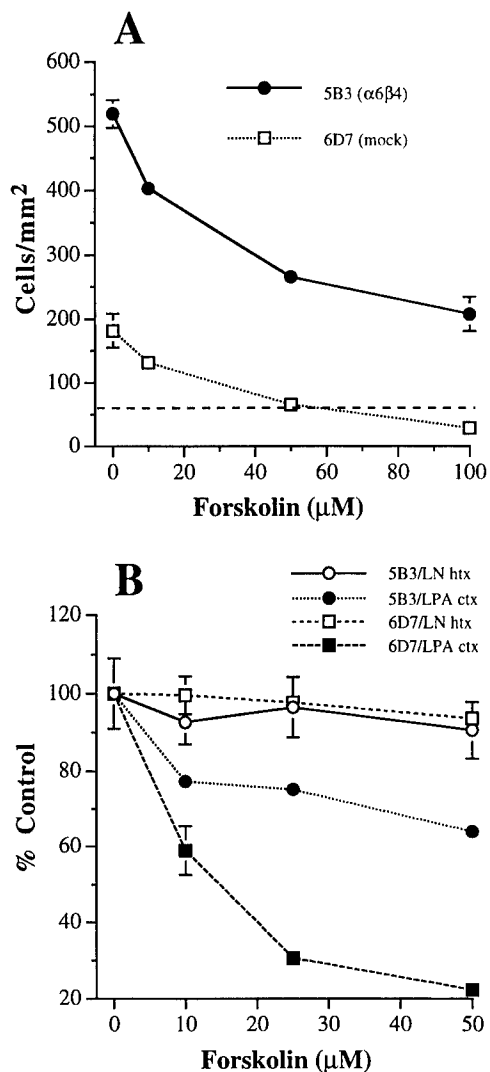To examine the possibility that the MDA/β4 and mock

Figure 4. Forskolin stimulation of adenyl cyclase inhibits LPA-mediated chemotaxis differentially in the MDA/β4 and mock transfectants. (*A*) MDA/β4 transfectants (*5B3; solid circles*) or mock transfectants (*6D7; open squares*) were treated with the indicated concentration of forskolin for 30 min before their addition to the upper wells of the Transwell chambers. Cells were assayed for LPA-mediated chemotaxis on collagen I as described in Fig. 1. The dashed line depicts the basal level of migration of both subclones in the absence of LPA. (*B*) In a separate experiment, the same cells were treated with forskolin for 30 min before assaying for LPA chemotaxis (*solid symbols*) or laminin haptotaxis (*open symbols*). Data are reported as the percent migration of cells not treated with forskolin ± standard deviation of triplicate determinations.

Figure 5. Intracellular cAMP content of the MDA-MB-435 transfectants. The MDA/β4 (*3A7, 5B3*), MDA/β4-ΔCYT (*Δ3C12, Δ1E10*) and MDA/mock transfectants (*6D2, 6D7*) were plated in DME containing 10% FCS. After 18 h, cells were harvested and cAMP content was measured using a cAMP EIA protocol as described in Materials and Methods. Data shown represent the mean of 10 sample determinations ± standard error. The difference in the [cAMP]$_i$ between the MDA/β4 and the mock transfectants is significant ($P < 0.001$; *asterisk*), but the difference between the mock and the β4-ΔCYT transfectants is not significant ($P = 0.2$). (*B* and *C*) Differential effects of forskolin stimulation on the [cAMP]$_i$ in the MDA/β4 and mock transfectants. The [cAMP]$_i$ was assayed in the 5B3 (*solid bars*) and 6D7 (*stippled bars*) clones plated on collagen I and treated for 15 min with either 50 μM forskolin (*B*) or forskolin and 1 mM IBMX (*C*). Note that the MDA/β4 transfectants (*5B3*) are more resistant to a forskolin-stimulated increase in [cAMP]$_i$ than the mock transfectants (*6D7*). The inhibition of PDE activity with IBMX shown in *C* reveals that α6β4 expression results in an increase in PDE activity and not a decrease in cAMP synthesis. Data shown are the mean values ± standard error obtained from multiple experiments.

transfectants differed in their level of PDE expression, we assessed PDE 4 expression in these cells using antibodies specific for the various PDE 4 variants (15). The predominant PDE 4 variant expressed in MDA-MB-435 cells is PDE 4B based on results obtained with antibodies specific for PDE 4A, 4B and 4D (data not shown). Importantly, the expression of PDE 4B did not differ significantly between the MDA/β4 and mock transfectants (Fig. 6 *C*). These data indicate that the increased PDE activity observed in the MDA/β4 transfectants is not the result of increased PDE expression.
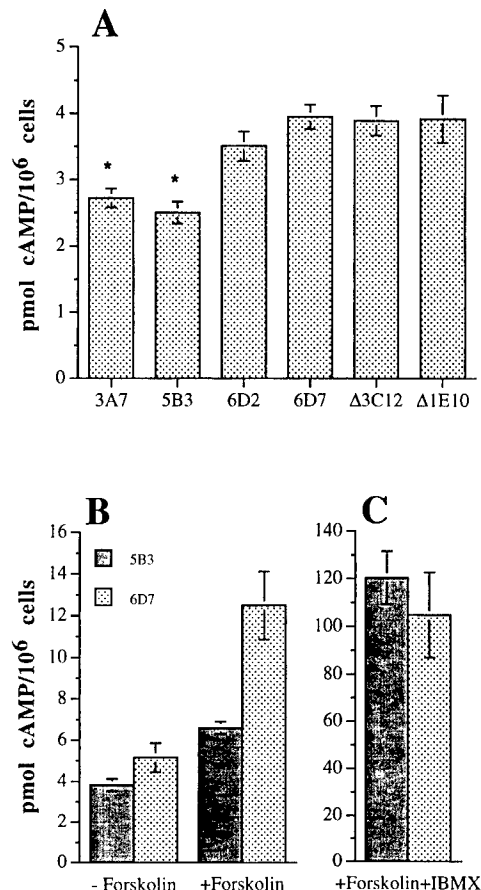
PDE Activity Is Necessary for Chemotaxis, Invasion, and Lamellae Formation

The importance of PDE for chemotactic migration was examined by treating the MDA/mock and β4 transfectants with IBMX before their use in the chemotaxis assay. As shown in Fig. 7 *A*, IBMX inhibited LPA-stimulated chemotaxis with maximal inhibition observed at 1 mM. Similar results were obtained with the cAMP-specific PDE inhibi-
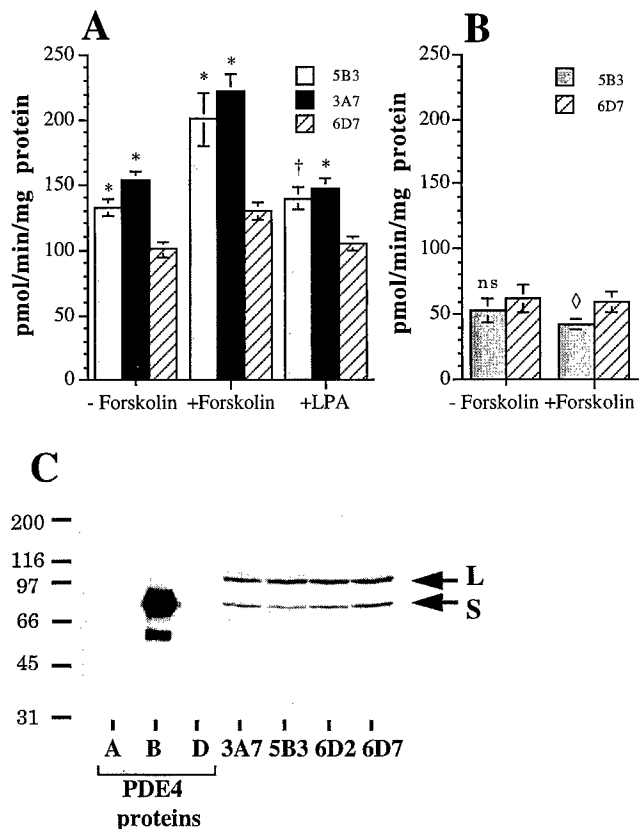
Figure 6. Assay of cAMP-specific PDE activity. (*A*) MDA/β4 (*3A7* and *5B3*) or mock transfectants (*6D7*) plated on collagen I were treated with 50 μM forskolin or 100 nM LPA as noted. Cells were harvested and the cytosolic fraction was assayed for PDE activity as described in Materials and Methods. The PDE activity of the MDA/β4 transfectants was compared with the MDA/mock transfectants for statistical significance: *, $P < 0.002$; †, $P < 0.01$. (*B*) Extracts from cells treated as in *A* were incubated with 100 μM rolipram before assaying for PDE activity to determine how much of the activity in *A* constitutes cAMP-specific PDE (*PDE 4*). Data shown are mean ± standard error of four separate determinations (*A* and *B*). *ns*, not significant; ◊, $P = 0.02$. (*C*) Relative expression of PDE 4B in the MDA-MB-435 transfectants. Extracts (40 μg protein) obtained from the MDA/β4 (*3A7* and *5B3*) and mock (*6D2* and *6D7*) transfectants, as well as purified PDE 4 proteins (short form of variants *A*, *B*, and *D*; 10 ng each; provided by M. Conti) were resolved by SDS-PAGE and immunoblotted with a PDE 4B-specific Ab. *Arrows*, long and short forms of PDE 4B.

Figure 7. cAMP specific-PDE activity is required for the chemotactic migration and invasion of MDA-MB-435 cells. The MDA/β4 (*5B3*; *squares*) or mock transfectants (*6D7*; *circles*) were treated with varying concentrations (*A*) or 1 mM (*B*) IBMX for 30 min before their use in either an LPA chemotaxis assay (*A*) or a Matrigel chemoinvasion assay (*B*). Data shown represent mean values ± standard deviation of triplicate determinations.

tor, rolipram (data not shown). We also examined the involvement of PDE in carcinoma invasion by treating cells with IBMX before their use in a standard Matrigel invasion assay. A substantial inhibition of invasion was observed in the presence of IBMX in comparison to the solvent control (Fig. 7 *B*).

The necessity of cAMP-specific PDE activity in the formation of lamellae was also assessed. IBMX had no effect on the morphology of the MDA/β4 transfectants in the absence of LPA (Fig. 8, compare *A* with *B*). However, IBMX-treated cells were unable to form the large, ruffling lamellae in response to LPA stimulation in comparison to untreated cells (Fig. 8, compare *C* with *D*). Quantitative
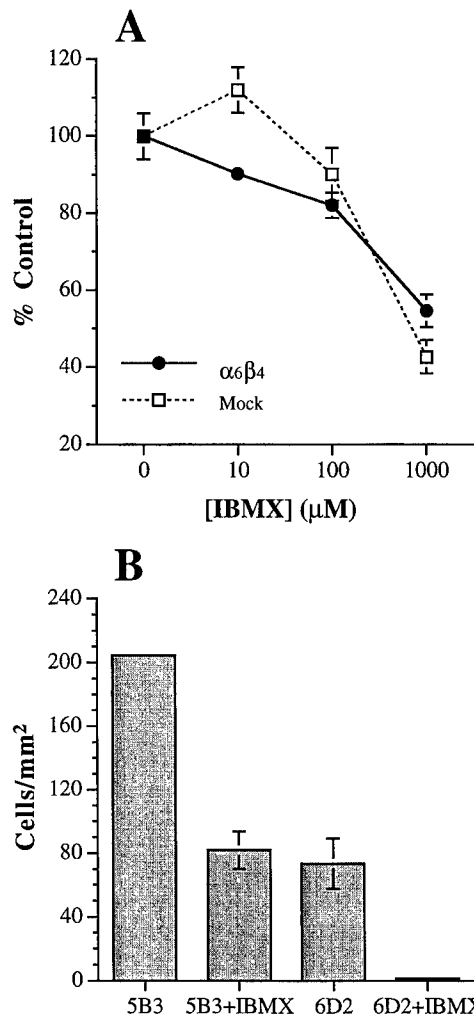
analysis of these cell populations revealed that inhibition of PDE activity resulted in an approximate fourfold reduction in the lamellar area of LPA-stimulated MDA/β4 transfectants (Fig. 8 *E*).

Recently, we reported that α6β4 is necessary for the formation and stabilization of lamellae in clone A colon carcinoma cells plated on laminin-1 (32). If PDE activity is needed for lamellae formation as indicated by the above results, IBMX should inhibit the formation of lamellae in clone A cells. To test this possibility, clone A cells were treated with IBMX or a solvent control and then plated onto laminin-1 for 45 min. The control cells formed large fan-shaped lamellae enriched in F-actin when plated on laminin-1 (Fig. 9, *A* and *B*) as we reported previously (32). In contrast, IBMX-treated cells formed small, immature lamellae with a marked reduction in F-actin content (Fig. 9, *C* and *D*). Quantitative analysis of these images re-
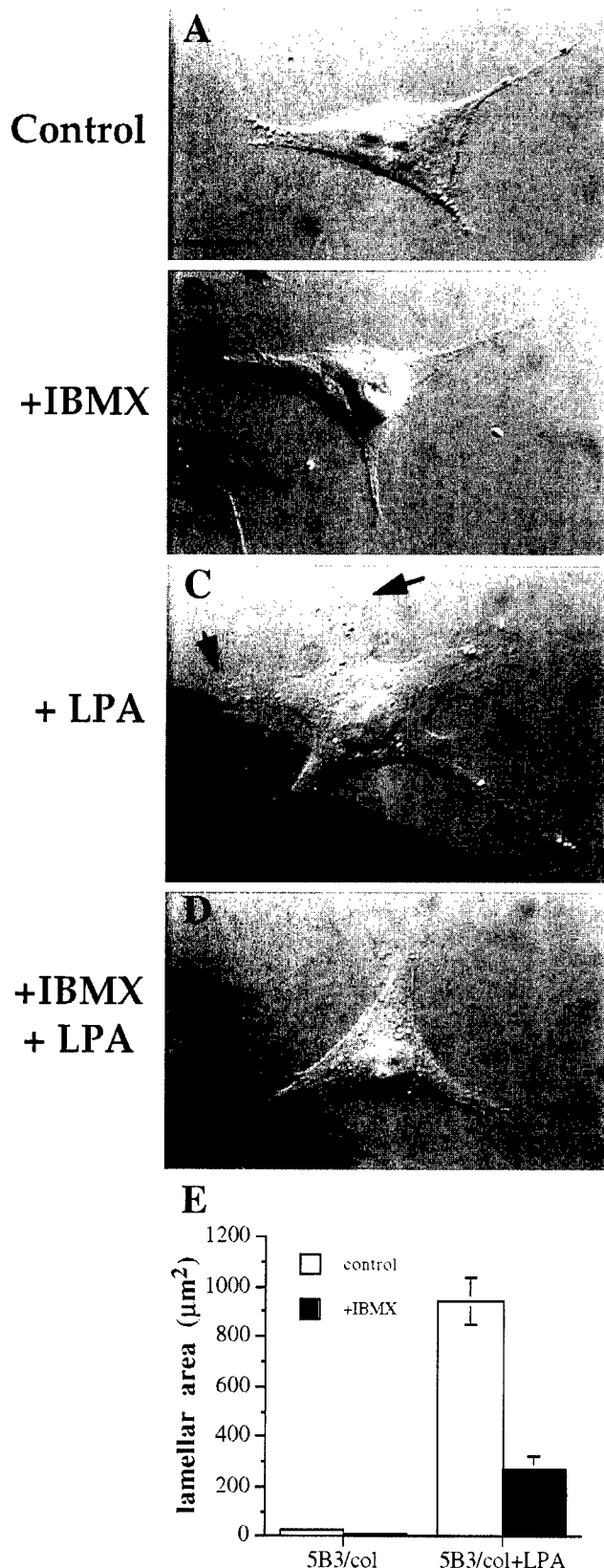
Control

+IBMX

+ LPA

+IBMX
+ LPA

E

Figure 8. cAMP specific-PDE activity is required for LPA-dependent formation of lamellae in the MDA/β4 transfectants. The MDA/β4 transfectants (*5B3*) were plated on collagen I–coated coverslips. After 2 h, the cells were either left untreated (*A* and *C*) or treated with 1mM IBMX (*B* and *D*) for 30 min. Subsequently, the cells were either left untreated (*A* and *B*) or treated

vealed that IBMX reduced the total lamellar area of clone A cells on laminin-1 by ∼75% (629 ± 74 μm² for control versus 164 ± 24μm² with IBMX). Interestingly, inhibition of PDE activity had no effect on the attachment or spreading of clone A cells on laminin-1 (Fig. 9).

cAMP Metabolism and PI3-K Signaling Are Not Directly Linked in MDA-MB-435 Cells

A possible relationship between cAMP metabolism and PI3-K signaling is of interest given our recent finding that α6β4 stimulates the preferential activation of PI3-K and that this activity is required for invasion and the formation of lamellae (40). To determine if PI3-K activity is required for the cAMP-specific PDE activity we observed in the MDA/β4 transfectants, these cells were incubated in the presence of wortmannin, a specific inhibitor of PI3-K, before extraction and assay of PDE activity. As shown in Fig. 10 *A*, wortmannin had no effect on PDE activity in these cells and it did not inhibit the marked induction of PDE activity that we had observed in response to forskolin stimulation. The possibility also existed that cAMP influences the α6β4-mediated activation of PI3-K. To address this issue, we used the α6-specific mAb G0H3 to cluster α6 integrins on the MDA/β4 transfectants in the presence of the PDE inhibitor IBMX and forskolin. As shown in Fig. 10 *B*, mAb-mediated clustering of α6β4 in MDA/β4 transfectants activated PI3-K markedly compared with cells maintained in suspension, in agreement with our previous results (40). However, treatment of MDA/β4 transfectants with either IBMX or forskolin did not inhibit α6β4-mediated activation of PI3-K (Fig. 10 *B*). In fact, no inhibition of PI3-K was observed when both of these inhibitors were used in combination, a treatment that increases the [cAMP]$_i$ from 4 to 120 pmoles per 10⁶ cells (Fig. 5).

Discussion

Recently, we established that the α6β4 integrin promotes carcinoma invasion (3, 32, 40). In the current study, we extend this observation by demonstrating that a major function of α6β4 is to stimulate the chemotactic migration of carcinoma cells, a function that is essential for invasion. This function is consistent with our previous finding that α6β4 is involved in the formation and stabilization of lamellae and filopodia (32). Importantly, the data presented here also provide evidence that α6β4 stimulates chemotaxis and lamellae formation by regulating the [cAMP]$_i$ by a mechanism that involves activation of a rolipram-sensitive, cAMP-specific PDE. Our finding that elevated [cAMP]$_i$ inhibits the formation of lamellae, chemotactic migration, and invasion is in agreement with recent studies indicating that cAMP can function to inhibit or "gate" specific signaling pathways (14, 16, 23). Further-

with 100 nM LPA for 5 min (*C* and *D*). The cells were then fixed and visualized using Nomarski DIC optics. (*E*) The effect of LPA and IBMX on lamellar area was quantified using IPLab Spectrum imaging software. Bars represent mean lamellar area ± standard error in which *n* > 20. Of note, IBMX inhibited the LPA-dependent formation of lamellae by 70%.
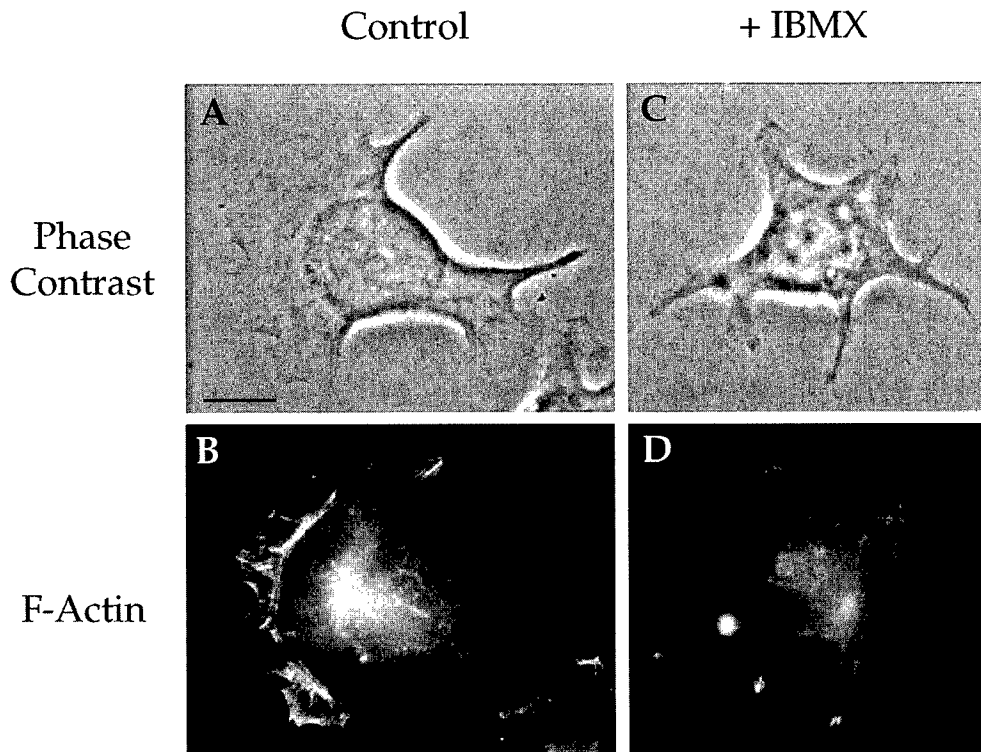
Phase
Contrast

F-Actin

Figure 9. Lamellae forma-
tion in clone A colon carci-
noma cells requires PDE
activity. Clone A colon carci-
noma cells were either treated
with solvent alone (*A* and *B*)
or 1 mM IBMX in solvent (*C*
and *D*) and then plated on
laminin-1–coated coverslips.
After 45 min the cells were
fixed and stained for F-actin
using TRITC-phalloidin. (*A*
and *C*) Phase–contrast im-
ages; (*B* and *D*) fluorescence
images. Bar, 10 μm.

more, we show that the cAMP-mediated gate does not in-
fluence haptotaxis thus providing additional evidence that
the signaling events governing chemotaxis and haptotaxis
differ (1, 20). Collectively, our results strengthen the hy-
pothesis that α6β4 promotes carcinoma invasion through
its ability to regulate signaling pathways required for mi-
gration. They also indicate that cAMP metabolism is likely
to be an important factor in the regulation of carcinoma
invasion and progression.

Although integrins can regulate a number of signaling
pathways (4), their ability to influence cAMP metabolism
has not been studied extensively. An earlier study, how-
ever, did provide evidence that the simultaneous engage-
ment of β2 integrins and tumor necrosis factor (TNF)
receptors decreases the [cAMP]$_i$ in neutrophils (30). Inter-
estingly, the reduction in [cAMP]$_i$ observed in response to
β2 integrin and TNF receptor engagement in neutrophils
is similar to the level of [cAMP]$_i$ suppression that we ob-
served in response to α6β4 expression (~30%). This level
of suppression of the total [cAMP]$_i$ is quite impressive
given that localized gradients of [cAMP]$_i$ are probably re-
quired to facilitate chemotactic migration, as well as for
other cell functions that are gated by cAMP. For example,
localized gradients of cAMP have been implicated in regu-
lating the direction of growth cone turning (41). It is also
important to note that we observed an inverse correlation
between the [cAMP]$_i$ and the rate of chemotaxis (compare
values in Fig. 4 *A* with Fig. 5 *B*). This observation rein-
forces the functional significance of α6β4 suppression of
the [cAMP]$_i$.

A novel aspect of our study is the finding that integrins,
and α6β4 in particular, can regulate the activity of a roli-
pram-sensitive, cAMP-specific PDE. This family of PDEs

is defined as type IV PDE (PDE 4) and consists of a num-
ber of structural variants (6). Because all of these variants
hydrolyze cAMP with a K$_m$ comparable to the [cAMP]$_i$, it
is thought that tissue-specific expression and the state of
activity of these variants are the major determinants of
their responsiveness to extracellular stimuli (6). Indeed,
the major focus of work in this area has been hormonal
regulation of PDE activity. Regulation of PDE activity can
occur rapidly in response to hormone stimulation through
a mechanism that involves PKA-dependent phosphoryla-
tion of the enzyme (36, 37). In addition, long-term, hor-
monal stimulation can actually increase *de novo* synthesis
of the cAMP-specific PDEs (6, 43). The data we obtained
suggest that expression of α6β4 does not increase the ex-
pression of PDE 4B, a predominant PDE variant ex-
pressed by MDA-MB-435 cells. For this reason, regulation
of PDE 4 activity by α6β4 expression may occur through a
mechanism that involves PDE phosphorylation. Another
possibility that has been proposed recently is that the sub-
cellular localization of the cAMP-specific PDEs influences
their function and activation (17). The possibility that
α6β4 increases the association of PDE 4 with either the
plasma membrane or cytoskeleton is certainly attractive
and could account, at least in part, for its ability to influ-
ence cAMP metabolism. Interestingly, LPA stimulation
by itself had no effect on either PDE activity or the
[cAMP]$_i$ in MDA-MB-435 cells. This observation rein-
forces our hypothesis that a major function of α6β4 is to
release cAMP gating of LPA-stimulated chemotaxis.

In previous studies, we established that an important
function of α6β4 in invasive carcinoma cells is its ability to
stimulate the formation of lamellae (32). This function of
α6β4 is highlighted by the observation in the present study

A

B

PI-3-P→

PI-3,4-P₂→

PI-3,4,5-P₃→

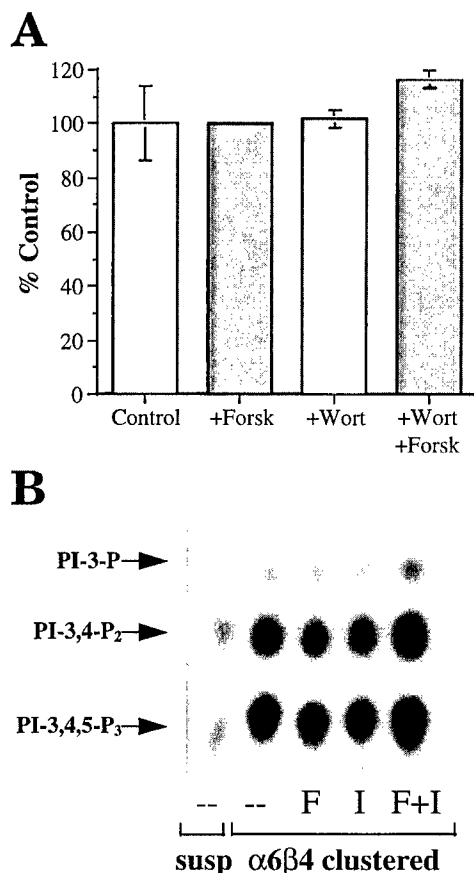-- -- F I F+I

susp α6β4 clustered

Figure 10. (*A*) Evaluation of PI3-K involvement in PDE activity. The MDA/β4 transfectants were incubated for 30 min in the presence of either forskolin or wortmannin, or both in combination, before assay of PDE activity as described in Materials and Methods. (*B*) Evaluation of the cAMP regulation of PI3-K activity. The MDA/β4 and mock transfectants were incubated in suspension with either forskolin or IBMX or both for 10 min. Subsequently, these cells were either maintained in suspension or incubated with a β4 integrin-specific antibody and allowed to adhere to anti-mouse IgG-coated plates or laminin-1–coated plates for 30 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with the anti-phosphotyrosine mAb 4G10 and protein A–Sepharose for 3 h. After washing, the beads were resuspended in kinase buffer and incubated for 10 min at room temperature. The phosphorylated lipids were resolved by thin layer chromatography. *Arrows*, position of the D3-phosphoinositides.

that LPA was able to induce significant lamellae formation only in MDA-MB-435 cells that expressed α6β4 (Fig. 3). Importantly, our finding that PDE activity is necessary for lamellae formation promoted by α6β4 expression implies that a localized suppression of the [cAMP]ᵢ plays an important role in controlling the signaling and cytoskeletal events that are required for lamellae formation. This hypothesis agrees with studies that have shown an inhibitory effect of cAMP on the organization of the actin cytoskeleton (11, 13, 21, 22). Moreover, the formation of lamellae is a dynamic process that is linked to the mechanism of cell migration. Therefore, it is likely that temporal fluxes in the [cAMP]ᵢ regulated by α6β4 contribute to the chemotactic migration of carcinoma cells.

Of particular relevance to our work, Butcher and colleagues (23) reported that cAMP is a negative regulator of leukocyte migration signaled through the classical chemoattractants. In this model, cAMP impedes or gates RhoA-mediated leukocyte integrin activation and adhesion. Our results support their conclusion that cAMP inhibits chemotactic migration. Importantly, we also provide evidence for an integrin-mediated mechanism of regulating the [cAMP]ᵢ to facilitate migration. Our findings support the work of the Butcher group because LPA is a potent activator of Rho (29) and, in fact, we have observed that the expression of a dominant-negative Rho can inhibit both LPA-stimulated chemotactic migration and invasion of MDA-MB-435 cells (O'Connor, K.L., and A.M. Mercurio, unpublished observation). Thus, a possibility worth investigating is that LPA-mediated activation of Rho is gated by cAMP and that α6β4 releases cAMP gating by increasing cAMP-specific PDE activity and thereby enhances Rho activation. A likely target of Rho activation is the actin cytoskeleton (44), which is consistent with the reported effects of cAMP on the cytoskeleton (11, 13, 21, 22), as well as our demonstration that cAMP inhibits lamellae formation. Although Rho has been linked to stress fiber formation and not lamellae formation in fibroblasts (25), much less is known about Rho function in epithelial-derived cells. In fact, our preliminary data suggest that LPA-induction of lamellae formation in the MDA/β4 transfectants is inhibited by expression of a dominant-negative Rho. Other integrins, especially the β1 integrins that mediate the adhesive interactions required for chemotactic migration, are another potential target of Rho (25, 34). It is worth noting in this context that expression of α6β4 has been shown to alter the function of collagen I–binding integrins in breast carcinoma cells (42).

The ability of α6β4 to promote lamellae formation and carcinoma invasion is dependent upon its preferential activation of a PI3-K and Rac signaling pathway (40). Our current finding that the release of cAMP gating by α6β4 is also required for these events raised the issue of a possible link between cAMP and the PI3-K/Rac pathway. Such a link was suggested, for example, by the finding that the interleukin-2 dependent activation of PI3-K is inhibited by cAMP (28). In our experiments, however, pharmacological stimulation of cAMP levels had no effect on the ability of α6β4 to activate PI3-K even under conditions in which the [cAMP]ᵢ increased 30-fold over basal levels. Our data also indicate that PI3-K probably does not function upstream of cAMP-specific PDE because wortmannin did not inhibit the activity of this enzyme. We conclude from these findings that PI3-K and cAMP-specific phosphodiesterase function in tandem to promote lamellae formation and chemotactic migration but they are components of distinct signaling pathways.

An interesting finding in the present study is that the ability of α6β4 to stimulate chemotactic migration and suppress the [cAMP]ᵢ can be independent of the adhesive function of this integrin. Although the laminins are the only known matrix ligands for α6β4 (26), expression of this integrin also stimulated chemotactic migration on a collagen I matrix and this migration was not inhibited by an α6-function blocking mAb. The possibility that the effects of α6β4 expression on migration result from a de-

crease of $\alpha6\beta1$ expression is discounted by the fact that expression of the $\alpha6\beta4$ ΔCYT integrin had no effect on either chemotaxis, cAMP levels or PDE activity even though expression of this mutant integrin eliminates $\alpha6\beta1$ expression in these cells (38). The observation that the ability of $\alpha6\beta4$ to promote chemotactic migration can be independent of its adhesive function is in agreement with several recent studies by our group and others that have revealed 'ligand-independent' functions for the $\alpha6$ integrins in carcinoma cells (3, 5, 8). Insight into the possible mechanism of this phenomenon was provided by a recent study that demonstrated self-association of the $\beta4$ cytoplasmic domains, a process that could initiate intracellular signaling events independently of ligand binding (33). One important implication of these findings is that the ability of $\alpha6\beta4$ to influence cAMP metabolism and stimulate the chemotactic migration of carcinoma cells need not be limited to sites of contact with laminin-containing matrices. This possibility is supported by the numerous studies that have implicated $\alpha6\beta4$ as a major determinant of carcinoma invasion and progression (10, 35, 47).

In summary, we have demonstrated that the $\alpha6\beta4$ integrin can stimulate lamellae formation and chemotactic migration of invasive carcinoma cells by increasing the activity of a rolipram-sensitive cAMP specific-PDE and lowering the $[cAMP]_i$. This cAMP specific-PDE functions in tandem with a PI3-K/Rac pathway, that is also regulated by $\alpha6\beta4$, and is required for carcinoma invasion and lamellae formation. The essence of our findings is that the $\alpha6\beta4$ integrin stimulates the chemotactic migration of carcinoma cells through its ability to influence key signaling events that underlie this critical component of carcinoma invasion.

References

1. Aznavoorian, S., M.L. Stracke, J. Parsons, J. McClanahan, and L.A. Liotta. 1996. Integrin $\alpha v\beta3$ mediates chemotactic and haptotactic motility in human melanoma cells through different signaling pathways. *J. Biol. Chem.* 271:3247–3254.

2. Borradori, L., and A. Sonnenberg. 1996. Hemidesmosomes—roles in adhesion, signaling and human disease. *Curr. Opin. Cell Biol.* 8:647–656.

3. Chao, C., M.M. Lotz, A.C. Clarke, and A.M. Mercurio. 1996. A function for the integrin $\alpha6\beta4$ in the invasive properties of colorectal carcinoma cells. *Cancer Res.* 56:4811–4819.

4. Clark, E.A., and J.S. Brugge. 1995. Integrins and signal transduction pathways: The road taken. *Science.* 268:233–239.

5. Clarke, A.S., M.M. Lotz, C. Chao, and A.M. Mercurio. 1995. Activation of the p21 pathway of growth arrest and apoptosis by the β_4 integrin cytoplasmic domain. *J. Biol. Chem.* 270:22672–22676.

6. Conti, M., G. Nemoz, C. Sette, and E. Vicini. 1995. Recent progress in understanding the hormonal regulation of phosphodiesterases. *Endocrine Rev.* 16:370–389.

7. Dexter, D.L., J.A. Barbosa, and P. Calabresi. 1979. N,N-dimethylformamide-induced alterations of cell culture characteristics and loss of tumorigenicity in cultured human colon carcinoma cells. *Cancer Res.* 39:1020–1025.

8. Domanico, S.Z., A.J. Pelletier, W.L. Havran, and V. Quaranta. 1997. Integrin $\alpha6A\beta1$ induces CD81-dependent cell motility without engaging the extracellular matrix migation substrate. *Mol. Biol. Cell.* 8:2253–2265.

9. Dowling, J., Q.C. Yu, and E. Fuchs. 1996. $\beta4$ integrin is required for hemidesmosome formation, cell adhesion and cell survival. *J. Cell Biol.* 134:559–572.

10. Falcioni, R., V. Turchi, P. Vittulo, G. Navarra, F. Ficari, F. Cavaliere, A. Sacchi, and R. Mariani-Costantini. 1994. Integrin $\beta4$ expression in colorectal cancer. *Int. J. Oncology.* 5:573–578.

11. Glass II, W.F., and J.I. Kreisberg. 1993. Regulation of integrin-mediated adhesion at focal contacts by cyclic AMP. *J. Cell. Physiol.* 157:296–306.

12. Green, K.J., and J.C.R. Jones. 1996. Desmosomes and hemidesmosomes—structure and function of molecular components. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 10:871–881.

13. Han, J.-D., and C.S. Rubin. 1996. Regulation of cytoskeletal organization and paxillin dephosphorylation by cAMP. *J. Biol. Chem.* 271:29211–29215.

14. Harvath, L., J.D. Robbins, A.A. Russell, and K.B. Seamon. 1991. cAMP and human neutrophil chemotaxis. Elevation of cAMP differentially affects chemotactic responsiveness. *J. Immunol.* 146:244–332.

15. Iona, S., M. Cuomo, T. Bushnik, F. Naro, C. Sette, M. Hess, E.R. Shelton, and M. Conti. 1998. Characterization of the rolipram-sensitive, cyclic AMP-specific phosphodiesterases: identification and differential expression of immunologically distinct forms in the rat brain. *Mol. Pharm.* 53:23–32.

16. Iyengar, R. 1996. Gating by cyclic AMP: expanded role for an old signaling pathway. *Science.* 271:461–463.

17. Jin, S.-J.C., T. Bushnik, L. Lan, and M. Conti. 1998. Subcellular localization of rolipram-sensitive, cAMP-specific phospodiesterases. *J. Biol. Chem.* 273:19672–19678.

18. 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.

19. Kleinman, H.K., M.L. McGarvey, J.T. Hassell, V.L. Star, F.B. Cannon, G.W. Laurie, and G.R. Martin. 1986. Basement membrane protein complexes with biological activity. *Biochemistry.* 25:312–318.

20. Klemke, R.L., S. Cai, A.L. Giannini, P.J. Gallageher, P. deLanerolle, and D.A. Cheresh. 1997. Regulation of cell motility by mitogen-activated protein kinase. *J. Cell Biol.* 137:481–492.

21. Lampugnani, M.G., M. Giorgi, M. Gaboli, E. Dejana, and P.C. Marchisio. 1990. Endothelial cell motility, integrin receptor clustering, and microfilament organization are inhibited by agents that increase intracellular cAMP. *Lab. Invest.* 63:521–531.

22. Lang, P., F. Gesbert, M. Delespine-Carmagnat, R. Stancou, M. Pouchelet, 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.

23. 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.

24. Liotta, L.A., and W.G. Stetler-Stevenson. 1991. Tumor invasion and metastasis: an imbalance of positive and negative regulation. *Cancer Res.* 51:5054s–5059s.

25. Mackay, D.J.G., and A. Hall. 1998. Rho GTPases. *J. Biol. Chem.* 273:20685–20688.

26. Mercurio, A.M. 1995. Laminin receptors: achieving specificity through cooperation. *Trends Cell Biol.* 5:419–423.

27. Mitchison, T.J., and L.P. Cramer. 1996. Actin-based cell motility and cell locomotion. *Cell.* 84:371–379.

28. Monfar, M., K.P. Lemon, T.C. Grammer, L. Cheatham, J. Chung, C.J. Vlahos, and J. Blenis. 1995. Activation of pp70/85 S6 kinases in interleukin-2-responsive lymphoid cells is mediated by phosphatidylinositol 3-kinase and inhibited by cyclic AMP. *Mol. Cell. Biol.* 15:326–337.

29. Moolenaar, W.H., O. Kranenburg, F.R. Postma, and G.C.M. Zondag. 1997. Lysophosphatidic acid: G-protein signalling and cellular responses. *Curr. Opin. Cell Biol.* 9:168–173.

30. Nathan, C., and E. Sanchez. 1990. Tumor necrosis factor and CD11/CD18 ($\beta2$) integrins act synergistically to lower cAMP in human neutrophils. *J. Cell Biol.* 111:2171–2181.

31. Neer, E.J. 1995. Heterotrimeric G proteins: organizers of transmembrane signals. *Cell.* 80:249–257.

32. Rabinovitz, I., and A.M. Mercurio. 1997. The integrin $\alpha6\beta4$ functions in carcinoma cell migration on laminin-1 by mediating the formation and stabilization of actin-containin motility structures. *J. Cell Biol.* 139:1873–1884.

33. Rezniczek, G.A., J.M. dePereda, S. Reipert, and G. Wiche. 1998. Linking integrin $\alpha6\beta4$-based cell adhesion to the intermediate filament cytoskeleton: direct interaction between the $\beta4$ subunit and plectin at multiple molecular sites. *J. Cell Biol.* 141:209–225.

34. Ridley, A.J., and A. Hall. 1992. The small GTP-binding protein rho regu-

lates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell.* 70:389–399.

35. Serini, G., L. Trusolino, E. Saggiorato, O. Cremona, M. Derossi, A. Angeli, F. Orlandi, and P.C. Marchisio. 1996. Changes in integrin and E-cadherin expression in neoplasatic verus normal thyroid tissue. *J. Natl. Cancer Inst.* 88:442–449.

36. Sette, C., and M. Conti. 1996. Phosphorylation and activation of a cAMP-specific phosphodiesterase by the cAMP-dependent protein kinase. *J. Biol. Chem.* 271:16526–16534.

37. Sette, C., S. Iona, and M. Conti. 1994. The short-term activation of a Rolipram-sensitive, cAMP-specific phophodiesterase by thyroid-stimulating hormone in thyroid FRTL-5 cells is mediated by a cAMP-dependent phosphorylation. *J. Biol. Chem.* 268:9245–9252.

38. Shaw, L.M., C. Chao, U.M. Wewer, and A.M. Mercurio. 1996. Function of the integrin α6β1 in metastatic breast carcinoma cells assessed by expression of a dominant-negative receptor. *Cancer Res.* 56:959–963.

39. Shaw, L.M., M.M. Lotz, and A.M. Mercurio. 1993. Inside-out integrin signaling in macrophages. *J. Biol. Chem.* 268:11401–11408.

40. Shaw, L.M., I. Rabinovitz, H.H.-F. Wang, A. Toker, and A.M. Mercurio. 1997. Activation of phosphoinositide 3-OH kinase by the α6β4 integrin promotes carcinoma invasion. *Cell.* 91:949–960.

41. Song, H.-J., G.-L. Ming, and M.-M. Poo. 1997. cAMP-induced switching in turning direction of nerve growth cones. *Nature.* 388:275–279.

42. Sun, H., S.A. Santoro, and M.M. Zutter. 1998. Downstream events in mammary gland morphogenesis mediated by repression of the α2β1 integrin: the role of the α6 and β4 integrin subunits. *Cancer Res.* 58:2224–2233.

43. Swinnen, J.V., K.E. Tsikalas, and M. Conti. 1991. Properties and hormonal regulation of two structurally related cAMP phosphodiesterases from the rat Sertoli cell. *J. Biol. Chem.* 266:18370–18377.

44. Tapon, N., and A. Hall. 1998. Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. *Curr. Opin. Cell Biol.* 9:86–92.

45. Taussig, R., and A.G. Gilman. 1995. Mammalian membrane-bound adenylyl cyclases. *J. Biol. Chem.* 270:1–4.

46. Vanderneut, R., P. Krimpenfort, J. Calafat, C.M. Niessen, and A. Sonnenberg. 1996. Epithelial detachment due to absence of hemidesmosomes in integrin β4 null mice. *Nat. Genet.* 13:366–369.

47. VanWaes, C., K.F. Kozarsky, A.B. Warren, L. Kidd, D. Paugh, M. Liebert, and T.E. Carey. 1991. The A9 antigen associated with aggressive human squamous carcinoma is structurally and functionally similar to the newly defined integrin α6β4. *Cancer Res.* 51:2395–2402.