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13. ABSTRACT (Maximum 200 Words) The work funded by this DOD grant has provided a mechanistic basis for the involvement of the $\alpha 6$ integrins in breast carcinoma progression. Our mechanistic studies substantiate several more clinical studies that have indicated the involvement of these integrins in breast cancer. The key findings are that the $\alpha 6$ integrins are necessary for the survival of metastatic breast carcinoma cells and that they also promote the invasion of these cells. In addition, considerable progress has been made in understanding the signaling mechanisms that underlie the involvement of these integrins in migration and invasion. Future work in this area will be multi-faceted. At the basic science level, more work is needed to define the signaling pathways regulated by these integrins in breast carcinoma cells, as well as the functional association of these integrins with growth factor receptors that have been implicated in breast cancer, especially the erbB family. In addition, the hypotheses that have been generated by our work need to be validated using transgenic models of breast cancer. From a clinical perspective, the potential use of the $\alpha 6$ integrins and associated molecules as prognostic markers for breast cancer and as potential therapeutic targets needs to be exploited.				
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Introduction: One of the major advances in cancer research made during the last several years has been the realization that integrin adhesion receptors play a major role in regulating tumor behavior. Specific tumor-associated functions that are regulated by integrins include adhesion and migration, differentiation, growth and apoptosis, and angiogenesis (1-5). This array of diverse but important functions reflects the fact that integrins are critical for the function and maintenance of the normal epithelial progenitors of all carcinomas. The importance of integrin-mediated interactions in normal epithelial biology has been particularly well-demonstrated for the mammary epithelium (6). Such studies have identified the laminins, a family of extracellular matrix proteins, as key players in determining the function of both normal and transformed mammary epithelia. For these reasons, integrin laminin receptors are prime candidates for investigating the role of cell adhesion events in breast carcinoma (7).

Our work, as well as the work from several other labs, has established the role of the $\alpha 6$ integrins, $\alpha 6\beta 1$ and $\alpha 6\beta 4$, as laminin receptors in many different cell types (1, 8-10). The potential importance of the $\alpha 6$ integrins in breast carcinoma was substantiated by the recent report that high expression of $\alpha 6$ integrins is correlated with reduced survival of breast cancer patients (11). In addition, other studies have suggested the involvement of the $\alpha 6$ integrins in carcinoma progression (12,13). Given these observations, this proposal was designed to elucidate the *functional* roles of the $\alpha 6$ integrins in human breast carcinoma. We are pleased to inform you in this final report that all of the proposed objectives have been accomplished and that the studies funded by this grant have resulted in many, high quality publications.

Body:

The $\alpha 6\beta 1$ integrin is required for the growth and survival of MDA-MB-435 breast carcinoma cells *in vivo*.

One of the Specific Aims of this grant was to 'Assess the functional significance of the $\alpha 6\beta 1$ integrin in the tumorigenic and metastatic behavior of breast carcinoma cells'. To examine the contribution of the $\alpha 6\beta 1$ integrin to breast carcinoma progression, we developed a dominant-negative technique for 'knocking-out' the expression of $\alpha 6\beta 1$ in MDA-MB-435 cells, a human breast carcinoma cell line that is highly metastatic in athymic mice. A mutant $\beta 4$ subunit that lacked the entire cytoplasmic domain was expressed at high levels in this cell line. $\beta 4$ dimerizes only with $\alpha 6$, and not other integrin α subunits, and therefore it selectively depleted $\alpha 6\beta 1$ surface expression by forming the $\alpha 6\beta 4$ - Δ CYT heterodimer. We found that elimination of $\alpha 6\beta 1$ expression inhibited the ability of these cells to mediate specific *in vitro* functions associated with metastatic spread such as laminin adhesion and migration (*Shaw, L.M., C. Chao, U.M. Wewer, and A.M. Mercurio. 1996. Function of the integrin $\alpha 6\beta 1$ in metastatic breast carcinoma cells assessed by expression of a dominant negative receptor. Cancer Res., 56:959-963.*). Subclones of these transfectants were isolated by FACS sorting. Mock (transfected with the vector alone) and $\beta 4$ - Δ CYT transfectant subclones were chosen that expressed similar levels of all integrin subunits. These subclones also adhered to other matrix proteins such as fibronectin, vitronectin, and collagen I to the same extent.

In collaboration with Ulla Wewer in Copenhagen, Denmark, we examined the *in vivo* behavior of MDA-MB-435 breast carcinoma cells that lack $\alpha 6 \beta 1$ expression. Mock and $\alpha 6 \beta 4$ - Δ CYT transfectant subclones were injected into the mammary fat pad of female athymic mice. After several weeks, the mice were sacrificed and the extent of tumor growth, invasion, and metastasis was analyzed. Distant metastasis was examined by sectioning the lungs and livers and counting metastatic foci. Although metastatic foci were not present in the lungs of the $\beta 4$ - Δ CYT transfectant mice, small foci of apoptotic cells were observed. These data suggest that the $\beta 4$ - Δ CYT transfectants were able to metastasize to the lungs but were not capable of surviving in this environment. In addition, the mock and $\beta 4$ - Δ CYT transfectants invaded through the abdominal wall into the peritoneal cavity in a similar percentage of mice. Taken together, these data indicate that the loss of $\alpha 6 \beta 1$ expression does not compromise the invasive potential of these breast carcinoma cells, a hypothesis that was predicted before these experiments were carried out. Rather, the results from the *in vivo* studies demonstrate that the $\alpha 6 \beta 1$ integrin functions in the growth and survival of breast carcinomas. *These data were published in Amer. J. Pathology 151:1191-1198 (1997).*

The $\alpha 6 \beta 4$ integrin promotes breast carcinoma invasion: We demonstrated that the $\alpha 6 \beta 4$ integrin promotes carcinoma invasion through a preferential and localized targeting of phosphoinositide-3 OH kinase (PI 3-K) activity. Stable expression of $\alpha 6 \beta 4$ increased carcinoma invasion in a PI 3-K-dependent manner and transient expression of a constitutively active PI 3-K increased invasion in the absence of $\alpha 6 \beta 4$. Ligation of $\alpha 6 \beta 4$ stimulated significantly more PI 3-K activity than ligation of $\beta 1$ integrins, establishing specificity among integrins for PI 3-K activation. $\alpha 6 \beta 4$ -regulated PI 3-K activity was required for the formation of lamellae, dynamic sites of motility, in carcinoma cells. The small G-protein Rac is required downstream of PI 3-K for invasion. These studies define a mechanism by which the $\alpha 6 \beta 4$ integrin promotes carcinoma invasion and invoke a novel function for PI 3-K signaling. *These data were published in Cell 91:949-960 (1997).*

The mechanism by which the $\alpha 6 \beta 4$ integrin promotes breast carcinoma invasion involves its ability to stimulate chemotaxis and regulate cAMP metabolism: The $\alpha 6 \beta 4$ integrin promotes carcinoma invasion by its activation of a phosphoinositide 3-OH (PI3-K) signaling pathway (Shaw et al., Cell 91:949-960). We demonstrate here using MDA-MB-435 breast carcinoma cells that $\alpha 6 \beta 4$ stimulates chemotactic migration, a key component of invasion, but that it has no influence on haptotaxis. Stimulation of chemotaxis by $\alpha 6 \beta 4$ 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 $\alpha 6 \beta 4$ expression. Both lamellae formation and chemotactic migration are inhibited or 'gated' by cAMP and our results reveal that a critical function of $\alpha 6 \beta 4$ 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 $\alpha 6 \beta 4$ stimulates the chemotactic migration of carcinoma cells through its ability to influence key signaling events that underlie this critical component of carcinoma invasion. *These data were published in the Journal of Cell Biology 143:1749-1760 (1998).*

The mechanism by which the $\alpha 6 \beta 4$ integrin promotes breast carcinoma migration involves its ability to regulate the Rho GTPase: Carcinoma cells develop fan-shaped lamellae and exhibit random migration when plated on laminin, processes that depend on the ligation of the $\alpha 6 \beta 4$ integrin. Here, we report that expression of a dominant negative RhoA (N19RhoA) in clone A cells inhibited $\alpha 6 \beta 4$ -dependent membrane ruffling, lamellae formation and migration. In contrast, expression of a dominant negative Rac (N17Rac1) had no effect on these processes. Using the Rhotekin binding assay to assess RhoA activation, we observed that engagement of $\alpha 6 \beta 4$ by either antibody-mediated clustering or laminin attachment resulted in a 2-3 fold increase in RhoA activation compared to cells maintained in suspension or plated on collagen. Antibody-mediated clustering of $\beta 1$ integrins, however, actually suppressed Rho A activation. The $\alpha 6 \beta 4$ -mediated interaction of clone A cells with laminin promoted the translocation of RhoA from the cytosol to membrane ruffles at the edges of lamellae and promoted its colocalization with $\beta 1$ integrins, as assessed by immunofluorescence microscopy. In addition, RhoA translocation was blocked by inhibiting phosphodiesterase activity and enhanced by inhibiting the activity of cAMP-dependent protein kinase 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. *These data were published in the Journal of Cell Biology 148:253-258 (2000).*

The mechanism by which the $\alpha 6 \beta 4$ integrin promotes breast carcinoma migration also requires the activity of protein kinase A (PKA): Members of the Rho family of small GTPases, such as Rho and Rac, are required for actin cytoskeletal reorganization during the migration of carcinoma cells. Phosphodiesterases (PDEs) are necessary for this migration because they alleviate cAMP/Protein kinase A (PKA)-mediated inhibition of RhoA (O'Connor et al, *J. Cell Biol.* 143:1749-1760; O'Connor et al, *J. Cell Biol.* 148:253-258). In this study, we report that migration of breast and squamous carcinoma cells toward either LPA or EGF involves not only PDE activity but also the cooperative signaling from PKA. Furthermore, we demonstrate that Rac1 activation in response to chemoattractant or $\beta 1$ integrin clustering is regulated by PKA and that Rac1 is required for this migration. Also, we find that $\beta 1$ integrin signaling stimulates the rapid and transient activation of PKA. A novel implication of these findings is that carcinoma cell migration is controlled by cAMP-dependent, as

well as cAMP-inhibitory signaling mechanisms. *These data will be published in the Journal of Biological Chemistry (in press).*

KEY RESEARCH ACCOMPLISHMENTS

- Established the involvement of the $\alpha 6 \beta 1$ integrin in the survival of metastatic breast carcinoma cells
- Defined the ability of the $\alpha 6 \beta 4$ integrin to promote the invasion of breast carcinoma cells
- Established the involvement of PI3-Kinase in breast carcinoma invasion
- Determined that the $\alpha 6 \beta 4$ integrin contributes to the chemotactic component of invasion
- Established the requirement for cAMP metabolism in the chemotaxis of breast carcinoma cells
- Defined the regulation of phosphodiesterases by the $\alpha 6 \beta 4$ integrin
- Established the involvement of the RhoA and Rac GTPases in breast carcinoma migration
- Established the involvement of protein kinase A in breast carcinoma migration
- Defined the role of protein kinase A in the regulation of the Rac GTPase

REPORTABLE OUTCOMES

Manuscripts: The following manuscripts resulted from this DOD grant:

Wewer, UM, Shaw, LM, Albrechtsen, R and AM Mercurio. 1997. The integrin $\alpha 6 \beta 1$ promotes the survival of human breast carcinoma cells in mice. *American J. Pathology*, 151:1191-1198.

Shaw, LM, Rabinovitz, I, Wang, H, and AM Mercurio. 1997. Activation of phosphoinositide 3OH kinase by the $\alpha 6 \beta 4$ integrin promotes carcinoma invasion. *Cell*, 91:949-960.

Rabinovitz, I and AM Mercurio. 1997. The integrin $\alpha 6 \beta 4$ functions in carcinoma cell migration on laminin-1 by mediating the formation and stabilization of actin-containing motility structures. *J. Cell Biology*, 139:1873-1884.

O'Connor KL, Shaw LM and AM Mercurio. 1998. Release of cAMP gating by the $\alpha 6 \beta 4$ integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. *J. Cell Biology*, 143:1749-1760.

O'Connor, KL, Nguyen, B-K. and AM Mercurio. 2000. RhoA function in lamellae formation and migration is regulated by the $\alpha 6 \beta 4$ integrin and cAMP metabolism. *J. Cell Biology*, 148:253-258.

Mercurio, A.M. and I. Rabinovitz. 2001. Towards a molecular understanding of tumor invasion- lessons from the $\alpha 6 \beta 4$ integrin. *Seminars in Cancer Biology* 11:129-141.

Mercurio, A.M., Bachelder, R.E., Rabinovitz, I., O'Connor, K.L., and T.T. Tani. 2001. The metastatic odyssey. The integrin connection. *Surgical Oncology Clinics of North America*. 10:313-328.

Mercurio, A.M., Bachelder, R.E., Chung, J., Rabinovitz, I., O'Connor, K.L., and T.T. Tani.. 2001. Integrin Signaling and Breast Cancer Progression. *J. Mammary Gland Biology and Neoplasia*. 6:299-309.

Mercurio, A.M. I. Rabinovitz and L.M. Shaw. 2001. The $\alpha 6 \beta 4$ integrin and epithelial cell migration. *Current Opinion in Cell Biology*, 13:541-545.

O'Connor, K.E. and A.M. Mercurio. Integrin-mediated stimulation of protein kinase A regulates Rac and promotes the chemotactic migration of carcinoma cells. *J. Biological Chemistry*, In Press.

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The work supported by this DOD grant resulted in the funding of an NIH R01 Grant entitled: "Mechanisms of Breast Carcinoma Survival"
Principal Investigator: Arthur M. Mercurio Agency:NIH/NCI
Type: RO1 (CA89209-01) Period: Feb 1, 2001-Jan 31, 20006

Career: During the tenure of this DOD grant, the PI was appointed Director of the Division of Cancer Biology and Angiogenesis, Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical Center.

Honors:

During the tenure of this DOD grant, the PI was appointed an Honorary Professor of Signal Transduction and Tumor Progression at the University of Copenhagen, Denmark.

CONCLUSIONS:

The work funded by this DOD grant has provided a mechanistic basis for the involvement of the $\alpha 6$ integrins in breast carcinoma progression. Our mechanistic studies substantiate several more clinical studies that have indicated the involvement of these integrins in breast cancer. Future work in this area will be multi-faceted. At the basic science level, more work is needed to define the signaling pathways regulated by these integrins in breast carcinoma cells, as well as the functional association of these integrins with growth factor receptors that have been implicated in breast cancer, especially the erbB family. In addition, the hypotheses that have been generated by our work need to be validated using transgenic models of breast cancer. In this direction, work from Dr. William Muller has already established the importance of PI3-K in a mouse model of breast cancer progression (14). From a clinical perspective, the potential use of the $\alpha 6$ integrins and associated molecules as prognostic markers for breast cancer and as potential therapeutic targets needs to be exploited.

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APPENDIX:

The following manuscripts are provided in this Appendix. These manuscripts resulted from this DOD grant and they contain the data described in this report. In addition, several review articles are included that summarize our findings and place them within the context of the field.

Wewer, UM, Shaw, LM, Albrechtsen, R and AM Mercurio. 1997. The integrin $\alpha 6 \beta 1$ promotes the survival of human breast carcinoma cells in mice. *American J. Pathology*, 151:1191-1198.

Shaw, LM, Rabinovitz, I, Wang, H, and AM Mercurio. 1997. Activation of phosphoinositide 3OH kinase by the $\alpha 6 \beta 4$ integrin promotes carcinoma invasion. *Cell*, 91:949-960.

Rabinovitz, I and AM Mercurio. 1997. The integrin $\alpha 6 \beta 4$ functions in carcinoma cell migration on laminin-1 by mediating the formation and stabilization of actin-containing motility structures. *J. Cell Biology*, 139:1873-1884.

O'Connor KL, Shaw LM and AM Mercurio. 1998. Release of cAMP gating by the $\alpha 6 \beta 4$ integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. *J. Cell Biology*, 143:1749-1760.

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Mercurio, A.M., Bachelder, R.E., Rabinovitz, I., O'Connor, K.L., and T.T. Tani. 2001. The metastatic odyssey. The integrin connection. *Surgical Oncology Clinics of North America*. 10:313-328.

Mercurio, A.M., Bachelder, R.E., Chung, J., Rabinovitz, I., O'Connor, K.L., and T.T. Tani. 2001. Integrin Signaling and Breast Cancer Progression. *J. Mammary Gland Biology and Neoplasia*. 6:299-309.

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O'Connor, K.E. and A.M. Mercurio. 2001. Integrin-mediated stimulation of protein kinase A regulates Rac and promotes the chemotactic migration of carcinoma cells. *J. Biological Chemistry*, In Press.

Short Communication

The Integrin $\alpha 6 \beta 1$ Promotes the Survival of Metastatic Human Breast Carcinoma Cells in Mice

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Reidar Albrechtsen,* and Arthur M. Mercurio[†]

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The role of the integrin $\alpha 6 \beta 1$ in breast carcinoma progression was studied by targeted elimination of this integrin in MDA-MB-435 cells, a human breast carcinoma cell line that is highly metastatic in athymic mice. The strategy used is based on the finding that expression of a cytoplasmic domain deletion mutant of the $\beta 4$ -integrin subunit ($\beta 4\Delta$ CYT) in MDA-MB-435 cells eliminates formation of the $\alpha 6 \beta 1$ heterodimer. MDA-MB-435 cells that lacked $\alpha 6 \beta 1$ expression ($\beta 4\Delta$ CYT transfectants) formed tumors in athymic mice that were suppressed in their growth and that exhibited a significant increase in apoptosis in comparison to the control tumors. Unlike the control MDA-MB-435 cells, the $\beta 4\Delta$ CYT transfectants were unable to establish metastatic foci in the lungs. Also, the control transfectants grew substantially better than the $\beta 4\Delta$ CYT transfectants in the liver after intrahepatic injection because of extensive apoptosis in the $\beta 4\Delta$ CYT transfectants. These data suggest that a major function of the $\alpha 6 \beta 1$ integrin in breast carcinoma is to facilitate tumorigenesis and promote tumor cell survival in distant organs. (*Am J Pathol* 1997, 151:1191-1198)

Cell adhesion receptors including integrins are likely to play critical roles in the metastatic progression of breast and other carcinomas.¹⁻⁴ The contribution of such receptors to metastasis probably involves their adhesive functions as well as their ability to stimulate signaling pathways that influence tumor behavior. It is important, therefore, not only to establish the involvement of specific integrins in progression but also to understand how these integrins actually contribute to the metastatic process. In this direction, we have been interested in assessing the involvement of the $\alpha 6 \beta 1$ integrin in breast carcinoma

progression. This integrin functions as an adhesion receptor for the laminin family of basement membrane proteins⁵ as well as for at least one member of the disintegrin family of surface receptors.⁶ The expression of this integrin in normal breast tissue, benign lesions, and neoplastic disease has been studied by several groups.⁷⁻¹³ Although no consensus has emerged from these studies with respect to disease stage and $\alpha 6 \beta 1$ expression, one study of particular interest observed that expression of $\alpha 6$ integrins in women with breast cancer correlates with reduced survival times.¹³ In fact, $\alpha 6$ -integrin expression was found to be superior in predicting reduced survival than other known factors alone, including the estrogen receptor.¹³

To examine the hypothesis that the $\alpha 6 \beta 1$ integrin functions in breast carcinoma progression more rigorously, we developed a technique for eliminating its expression in MDA-MB-435 cells,¹⁴ a metastatic human breast carcinoma cell line.¹⁵ MDA-MB-435 cells use the $\alpha 6 \beta 1$ integrin as their only laminin-1 receptor, and they do not express the $\alpha 6 \beta 4$ integrin laminin receptor.¹⁴ The technique used is based on our observation that expression of a cytoplasmic domain deletion mutant of the $\beta 4$ -integrin subunit in MDA-MB-435 cells eliminates formation of the $\alpha 6 \beta 1$ heterodimer. Targeted removal of the $\alpha 6 \beta 1$ integrin in these breast carcinoma cells inhibited their ability to mediate specific *in vitro* functions associated with tumor spread such as adhesion and migration through laminin-containing matrices.¹⁴ In the present study, we assessed the involvement of the $\alpha 6 \beta 1$ integrin in spontaneous metastasis using these MDA-MB-435 transfectants. The data presented suggest that a major function of the

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$\alpha 6 \beta 1$ integrin in breast carcinoma is to facilitate tumorigenesis and promote tumor cell survival in distant organs.

Materials and Methods

Analysis of MDA-MB-435 Transfectants

Subclones of MDA-MB-435 cells that had been transfected with either the pcDNA3 vector alone (control transfectants) or with the $\beta 4$ -integrin cytoplasmic domain deletion mutant in this vector ($\beta 4$ - Δ CYT transfectants) were generated as described previously.¹⁴ The surface expression of integrin subunits on the control and $\beta 4$ - Δ CYT transfectants was assessed by flow cytometry. For this purpose, aliquots of cells (5×10^5) were incubated for 45 to 60 minutes at room temperature with RPMI 1640 medium containing 25 mmol/L Hepes (RPMI-H) and 0.2% bovine serum albumin (RH/BSA) and the following integrin-specific Abs: HP2B6 ($\alpha 1$, Immunotech, Westbrook, ME); IIE10 ($\alpha 2$, provided by Martin Hemler); IVA5 ($\alpha 3$, provided by Martin Hemler); SAM1 ($\alpha 5$, Immunotech); 2B7 ($\alpha 6$, prepared in our laboratory); AMF7 (αv); mAb 13 ($\beta 1$, provided by Stephen Akiyama); A9 ($\beta 4$, provided by Thomas Carey); and mouse IgG (Sigma, St. Louis, MO). The cells were washed two times with RH/BSA and then incubated with goat F(ab')₂ anti-mouse IgG coupled to fluorescein (Tago, Camarillo, CA) for 45 to 60 minutes at room temperature. After washing two times with RH/BSA, the cells were resuspended in the same buffer and analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ).

In Vivo Assays

For injection, the cells were harvested, equilibrated in complete growth medium for 1 hour in suspension at 37°C, rinsed in phosphate-buffered saline, and resuspended at a concentration of 10^8 cells/ml. Female *nu/nu* NMRI mice or META mice (6 to 8 weeks old) (Bomholtgard Breeding and Research Center, Ry, Denmark) were injected with the cells (10^7 per inoculum) into the mammary fat pad region or subcutaneously using a 26-gauge needle. For serial transplantation, small pieces of viable tumor tissue were inoculated subcutaneously in the flank of the thoracic region of the NMRI mice. The mice were maintained in specific pathogen free conditions in sterile laminar flow benches and fed sterile water and food *ad libitum*. Mice were killed by cervical dislocation before the overgrowth of primary tumors or peritoneal tumor growth with ascites fluid, or after 8 to 9 weeks of observation. Upon autopsy, primary tumors were dissected, and the tumor weight was determined. The abdominal cavity was inspected for the presence of macroscopic tumor growth, and tumor tissue and liver specimens were removed. Similarly, the thoracic cavity was inspected, and the lungs and mediastinum were removed. Tissue specimens were fixed in formalin before staining with the ApopTag reagent (Oncor, Gaithersburg, MD) or in ethanol/acetic acid and processed for H&E histological ex-

amination using standard techniques. Tissue specimens were also frozen in liquid nitrogen and stored at -70°C until use for immunohistochemistry with an anti-human p53 protein-specific mAb (Dako, Denmark).

In Situ Growth and Apoptosis Assays

The mitotic index (MI) was determined by calculating the percentage of definite mitotic figures in approximately 1000 viable cells within each tumor, and the apoptotic index (AI) was determined by calculating the percentage of apoptotic cells contained within 2000 viable cells within each tumor. Apoptotic cells were identified at the light microscope level by the presence of hyperchromatic compact or fragmented nuclei in single cells within areas of viable tumor cells that were devoid of neutrophils and distant from areas of necrosis. Cells identified by these features also stained positively with the ApopTag reagent in contrast to surrounding viable cells. Intestinal epithelium, which contains apoptotic cells in the villi, served as an internal positive control for these analyses.

Liver Colonization Assay

The liver colonization assay used is based on the procedure described in a study by Kuo et al.¹⁶ Briefly, mice were anesthetized and an incision was made through the upper abdominal wall and peritoneum. The liver was exposed and injected with either cultured tumor cells ($2.5 \times 10^6/50 \mu\text{l}$) or phosphate-buffered saline for the sham-operated animals using a 26-gauge needle. Mice were killed 4, 11, and 28 days after the injection and autopsied. Histological examination and *in situ* growth and apoptosis assays were performed as described above.

Results

Characterization of the MDA-MB-435 Transfectants

Subclones of MDA-MB-435 cells that had been transfected with either the pcDNA3 vector alone (control transfectants) or with the $\beta 4$ -integrin cytoplasmic domain deletion mutant in this vector ($\beta 4$ - Δ CYT transfectants) were used in these studies. The control transfectant subclones expressed equivalent levels of $\alpha 6$, and the $\beta 4$ - Δ CYT transfectant subclones expressed equivalent levels of both $\alpha 6$ and $\beta 4$. Other integrin subunits ($\alpha 1$, $\alpha 2$, $\alpha 5$, αv , and $\beta 1$) were expressed at equivalent levels although the $\alpha 3$ subunit was expressed at variable levels on both the mock and $\beta 4$ - Δ CYT transfectant subclones (Table 1). However, this variability did not correlate with tumorigenesis or metastasis. All of the $\beta 4$ - Δ CYT subclones adhered to collagen I, fibronectin, and vitronectin to the same extent as the control transfectants (data not shown). The only observed difference was that the $\beta 4$ - Δ CYT subclones did not adhere well to laminin-1.¹⁴

Table 1. Surface Expression of Integrin Subunits in Mock and $\beta 4$ - Δ CYT Transfectants of MDA-MB-435 Cells

Transfectants	IgG	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 5$	$\alpha 6$	αv	$\beta 1$	$\beta 4$
Mock	4.9	4.4	36.7	29.9	24.5	29.6	20.4	62.5	4.9
$\beta 4$ - Δ CYT	4.9	4.3	40.5	47.3	35.6	22.8	29.8	68.1	23.2

Representative subclones of the mock (B1) and $\beta 4$ - Δ CYT (1E10) transfectants were analyzed by flow cytometry using mAbs specific for the indicated integrin subunits (see Materials and Methods). The data shown represent the mean fluorescence intensity for each integrin-specific mAb as well as for a nonspecific IgG control.

The Integrin $\alpha 6 \beta 1$ Promotes the Tumorigenic Properties of MDA-MB-435 Cells

Subclones of the control and $\beta 4$ - Δ CYT transfectants were injected into the mammary fat pads in the abdominal region of *nu/nu* NMRI mice. All subclones of the control transfectants grew rapidly and formed large primary tumors (mean tumor weight 2.0 ± 0.13 g) within 7 to 8 weeks (Table 2). Although the $\beta 4$ - Δ CYT subclones were capable of primary tumor growth, these tumors were significantly smaller (mean tumor weight 0.3 ± 0.03 g) than those observed for the control subclones (Table 2). Insight into the size difference between these two groups of primary tumors was obtained by calculating their mitotic and apoptotic indices. Tumors formed by the control transfectants had a higher MI/AI ratio (2.2) than did the $\beta 4$ - Δ CYT tumors (0.34) (Table 3). These data indicate that the $\beta 4$ - Δ CYT tumors were suppressed in their growth and exhibited a significant increase in apoptosis in comparison with the control tumors.

The differences in tumor weight observed for the control and $\beta 4$ - Δ CYT transfectants is not dependent on the environment of the mammary fat pad because such differences were also observed after subcutaneous injection. Specifically, the mean tumor weight observed for the control transfectants ($n = 20$) was 1.8 ± 0.27 g and for the $\beta 4$ - Δ CYT transfectants ($n = 20$) was 0.7 ± 0.11 g at

8 weeks after subcutaneous injection. These data were reinforced by serial subcutaneous transplantation of tumor tissue. The mean tumor weight for tumors that grew from transplantation of control transfectant tumors ($n = 19$) was 2.9 ± 0.35 g and the mean tumor weight for tumors that grew from the $\beta 4$ - Δ CYT transfectant tumors ($n = 15$) was 0.2 ± 0.03 g.

Growth differences between the control and $\beta 4$ - Δ CYT transfectants were not apparent *in vitro*. Specifically, the mean doubling time ($\pm$ SEM) for the control transfectant subclones grown on tissue culture plastic in the presence of 5% fetal calf serum was 37.5 ± 2.0 hours. The $\beta 4$ - Δ CYT subclones exhibited a mean doubling time of 35.6 ± 1.3 hours. Although serum deprivation increased these doubling times, the control and $\beta 4$ - Δ CYT subclones did not differ in their ability to survive and proliferate under this condition. Also, there was no evidence of growth suppression of the $\beta 4$ - Δ CYT transfectants when the subclones were grown in three-dimensional cultures of Matrigel in either 10% serum or in the absence of serum (data not shown).

Loss of $\alpha 6 \beta 1$ expression did not affect the ability of MDA-MB-435 cells to invade from the mammary fat pads in the abdominal region into the peritoneal cavity (Table 2; Figure 1A). Approximately one-third of mice injected in the mammary fat pad with either the control transfectant subclones or $\beta 4$ - Δ CYT transfectant sub-

Table 2. Effect of Targeted Elimination of the $\alpha 6 \beta 1$ Integrin on the Growth, Peritoneal Spread, and Lung Metastasis of MDA-MB-435 Breast Carcinoma Cells in *nu/nu* NMRI Mice

MDA-MB-435 Transfectants	Number Mice	Time weeks	Tumor weight (g)	Lung Metastasis	
				No mice	%
Control, pcDNA3					
B1, G2 with peritoneal spread	19	6	1.4 ± 0.22	17	90
B1, G2 without peritoneal spread	34	7.3	2.4 ± 0.13	28	82
$\beta 4$ - Δ CYT					
1A2, 1E10, 3C12, 3E3 with peritoneal spread	25	7.8	0.4 ± 0.08	2	8
1A2, 1E10, 3C12, 3E3 without peritoneal spread	50	8.5	0.3 ± 0.03	0	0
Total, Control	53	6.8	2.0 ± 0.13	45	85
Total, $\beta 4$ - Δ CYT	75	8.2	0.3 ± 0.03	2	3

Tumors derived from the $\beta 4$ - Δ CYT transfectants ($\alpha 6 \beta 1$ negative) were significantly smaller than tumors derived from the control transfectants. Tumor weight in grams (g) is presented as the mean value (means $\pm$ SE). $P < 0.0001$ using the Mann-Whitney test. The same number of mice injected with the control and $\beta 4$ - Δ CYT transfectants exhibited peritoneal spread (36 and 33% of the mice, respectively). This difference is not statistically significant by the chi-square test ($P = 0.54$). The ability of the $\beta 4$ - Δ CYT transfectants to form lung metastases was dramatically reduced (3% of total mice injected) compared with the control transfectants (85% of total mice injected). This difference is highly statistically significant using the chi-square test ($P < 0.000001$). In all experiments, several subclones of each transfectant were tested as indicated and no statistically significant differences among them were observed.

Table 3. *In Situ* Growth Analysis of Primary Tumors in MDA-MB-435 Breast Carcinoma Cells in NMRI Mice

MDA-MB-435 Transfectants	Number of Mice	MI (%) Mitosis	AI (%) Apoptosis	MI/AI Ratio
pcDNA3	10	1.05 ± 0.05	0.48 ± 0.10	2.2
$\beta 4$ - Δ CYT	10	0.48 ± 0.11	1.14 ± 0.10	0.34

The mitotic index (MI) and apoptotic index (AI) obtained from the primary tumors formed by the control and $\beta 4$ - Δ CYT transfectants. The MI of the control tumors is significantly higher than that of the $\beta 4$ - Δ CYT tumors ($P < 0.01$ using the Mann-Whitney test). In contrast, the AI of the $\beta 4$ - Δ CYT tumors is significantly higher than that of the control tumors ($P < 0.001$ using the Mann-Whitney test). The MI/AI ratio derived from the control tumors (2.2) is significantly higher than that derived from the $\beta 4$ - Δ CYT tumors (0.34) ($P < 0.002$ using the Mann-Whitney test). The data are presented as mean index (%) and standard error of the mean (Means $\pm$ SE).

clones exhibited extensive tumor growth in the peritoneal cavity, although the control transfectants grew better than the $\beta 4$ - Δ CYT transfectants based on visual observation. Both populations of transfectants grew as single cells and clusters in the peritoneal cavity. Tumor nodules were dispersed throughout the peritoneal cavity. In particular, they were seen in omental tissue and surrounding the gastric sac, intestines, spleen, pancreas, and hepatic portal tract. Invasion of tumor cells into the pancreas and blood vessels was also evident (Figure 1B). The $\beta 4$ - Δ CYT transfectants that grew in the peritoneal cavity were not revertants because they stained positively with $\beta 4$ integrin-specific mAbs (data not shown).

The Integrin $\alpha 6 \beta 1$ is Linked to the Survival of Metastatic MDA-MB-435 Cells

The most dramatic effect observed was that expression of $\alpha 6 \beta 1$ appears to be essential for the establish-

ment of lung metastases by MDA-MB-435 cells (Table 2; Figure 2). Of the 53 NMRI mice injected with the mock transfectant subclones, 45 developed foci of tumor cells in their lungs. The formation of these lung metastases was not dependent upon peritoneal tumor growth because 82% of the mice that did not exhibit such growth developed these metastases. In marked contrast to the mock transfectants, only 2 of 75 mice injected with the $\beta 4$ - Δ CYT transfectant subclones had lung metastases. However, the metastatic foci seen in these two mice stained positively with a mAb specific for the human p53 protein indicating that some of the $\beta 4$ - Δ CYT transfectants had the capacity to reach the lungs (Figure 2D). Additional evidence to support the conclusion that $\alpha 6 \beta 1$ is essential for the formation of metastases by MDA-MB-435 cells was obtained using the META strain of mice that is highly prone to metastasis formation. The results obtained from injection of these mice were similar to those obtained with the NMRI mice (data not shown).

Although there were very few metastatic foci in the lungs of mice that had been injected with the $\beta 4$ - Δ CYT transfectant subclones, microscopic analysis revealed the presence of mononuclear infiltrates in some of these lungs that were located around small blood vessels (Figure 2B). These infiltrates were comprised primarily of macrophages, lymphocytes, and a few single cells that were apoptotic by morphological criteria and because they stained positively with the ApopTag reagent. Because such infiltrates and apoptotic cells were not observed in the parenchyma of control transfectants with metastases, a reasonable suggestion is that some of the apoptotic cells were $\beta 4$ - Δ CYT transfectants that had spread to the lungs but were unable to survive and establish metastatic colonies.

The observations on the behavior of the MDA-MB-435 transfectants in the lungs raised the possibility that the

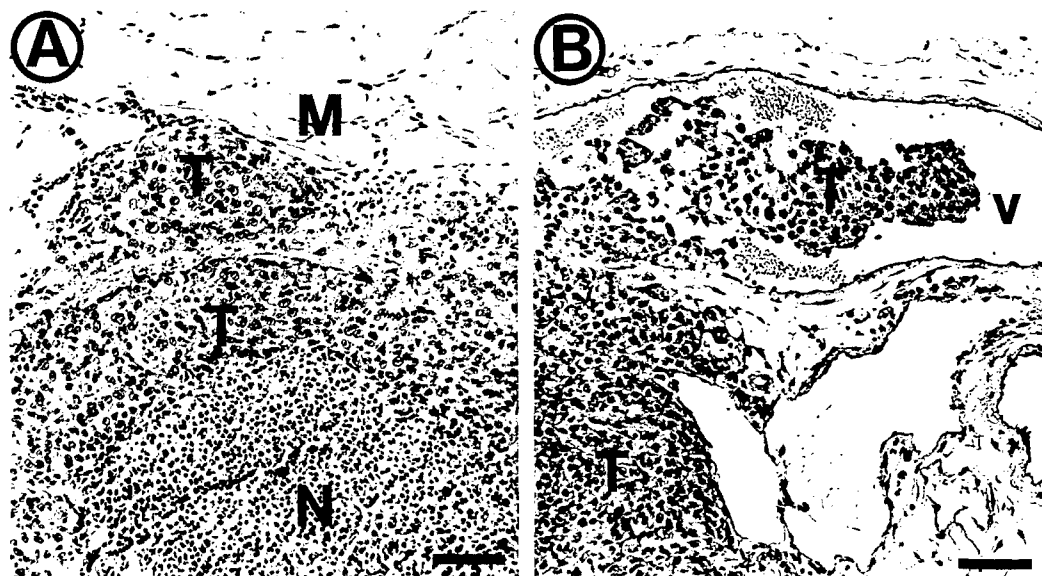


Figure 1. Invasion of the MDA-MB-435 $\beta 4$ - Δ CYT transfectants. A: Paraffin section of a primary tumor in the abdominal region. Tumor cells (T) are present in striated musculature (M) indicating that invasion from the mammary fat pad had occurred. An area of tumor necrosis (N) is also evident. B: Paraffin section of a peritoneal tumor showing tumor cells (T) within a blood vessel (V). Sections were stained with H&E. Scale bar = 90 and 83 μ m, respectively.

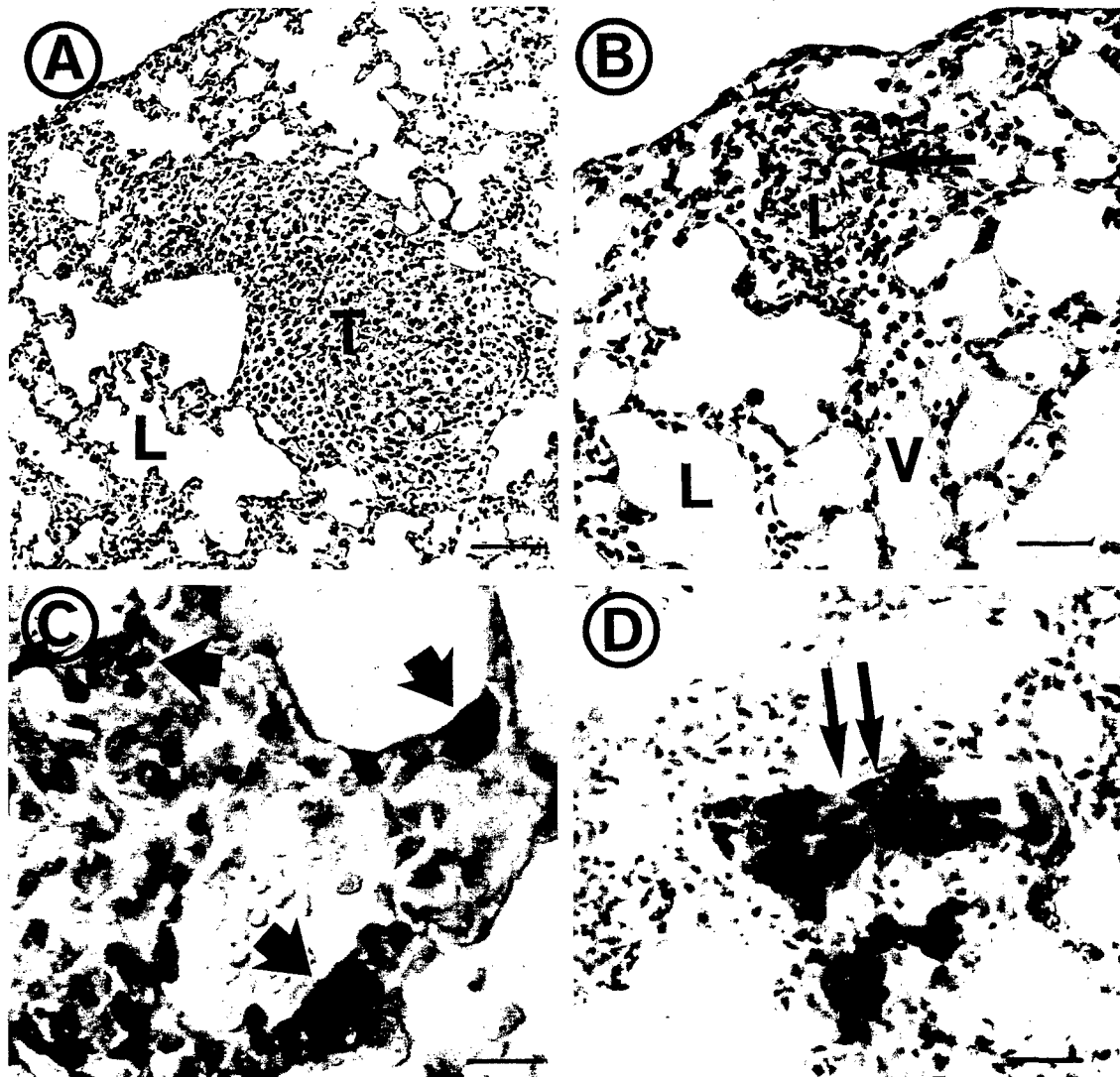


Figure 2. Suppression of lung metastasis by targeted elimination of the $\alpha 6 \beta 1$ integrin in MDA-MB-435 breast carcinoma cells. **A:** Lung metastases are seen in mice injected subcutaneously with the control transfectants but not in the lungs of mice injected with the $\beta 4$ - Δ CYT transfectants (**B**) with the exception of two mice (Table 2). However, perivascular, granuloma-like infiltrates comprised of macrophages, lymphocytes, and apoptotic cells are evident in some of the lungs of the $\beta 4$ - Δ CYT transfectant-injected mice. The apoptotic cells in these infiltrates were distributed as single cells in areas that were free of necrosis and devoid of neutrophils. These cells also stained positively with ApopTag (**C**). These infiltrates were seen in 20 of the 75 mice injected with the $\beta 4$ - Δ CYT transfectants. In these 20 mice, a total of 60 such foci were seen. In the control mice, only 3 foci were evident in the 53 mice analyzed. The difference in the number of foci between the control and $\beta 4$ - Δ CYT injected mice is highly statistically significant by the chi-square test ($P < 0.001$). **D:** Positive immunostaining of tumor cells with a mAb to human p53 in one of the two cases in which lung metastases were seen in the $\beta 4$ - Δ CYT transfectants. This positive staining provides evidence that at least some of the $\beta 4$ - Δ CYT transfectants have the capacity to enter the lung parenchyma. All photomicrographs shown are paraffin sections. **A** and **B** are stained with H&E, **C** is stained with ApopTag with a light hematoxylin counterstaining. T, tumor cells; L, lung parenchyma; V, blood vessel; I, mononuclear inflammatory infiltrate. Arrows indicate an apoptotic cell, arrowheads indicate apoptotic cells that are ApopTag positive, and double arrows indicate p53 immunopositive cells with pleomorphic nuclei. Scale bars = 50, 25, 12.5, and 25 μ m, respectively.

$\alpha 6 \beta 1$ integrin is required for their ability to survive in and colonize distant organs. This possibility was strengthened by examining the behavior of control and $\beta 4$ - Δ CYT transfectant subclones that had been injected directly into the liver.¹⁶ Although the liver is not a major site of spontaneous metastasis for MDA-MB-435 cells (Ref. 15; data not shown), intrahepatic injections provided a direct assay for assessing the ability of the $\beta 4$ - Δ CYT transfectants to grow, survive, and form colonies in a distant organ. Both the control and the $\beta 4$ - Δ CYT transfectant subclones were capable of growth in the liver after intrahepatic injection. However, the control transfectants grew

substantially better than the $\beta 4$ - Δ CYT transfectants (Figure 3). Most likely, this difference in growth can be attributed to extensive apoptosis in the $\beta 4$ - Δ CYT transfectants because the tumor masses that arose from these cells had a much higher apoptotic index (3.4 ± 0.41) than did the tumor masses that arose from the control transfectants (0.73 ± 0.14), and they stained positively with the ApopTag reagent (Figure 3). Taken together with the lung results, these data indicate that the inability of the $\beta 4$ - Δ CYT transfectants to develop distant metastases probably reflects their inability to survive after they have extravasated into potential metastatic sites.

A.

MDA-MB-435 Transfectants	No. Mice	Extent of Tumor Growth	MI (%) Mitosis	AI (%) Apoptosis	MI/AI Ratio
pcDNA3	10	Heavy (+++)	0.97 ± 0.23	0.73 ± 0.14	1.4
$\beta 4$ - Δ CYT	10	Low (+)	0.95 ± 0.13	3.4 ± 0.10	0.28

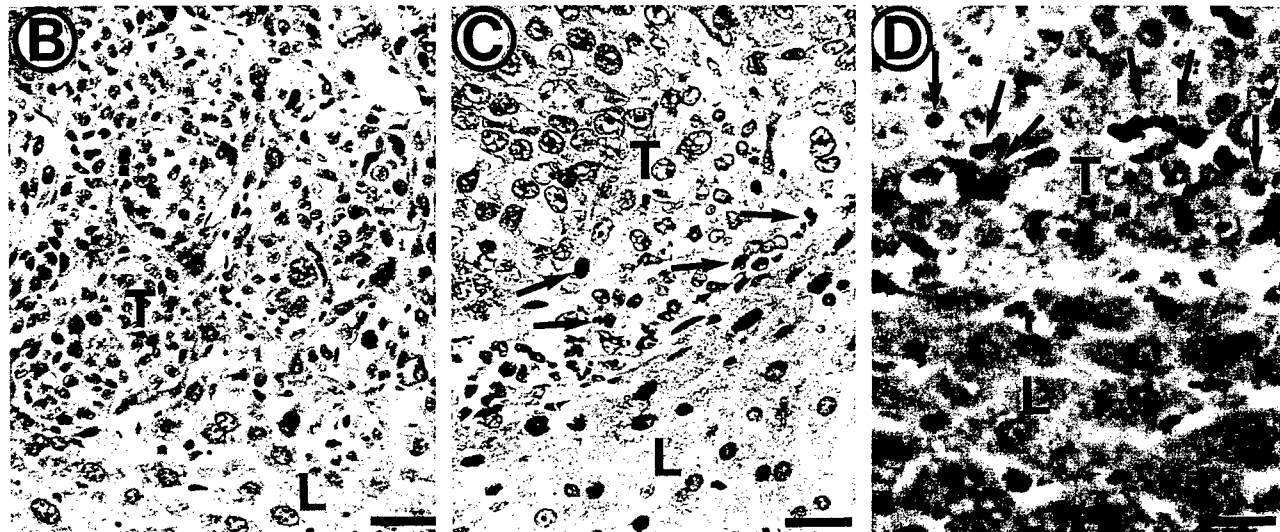


Figure 3. Liver colonization by the MDA-MB-435 transfectants. A: Control transfectants and $\beta 4$ - Δ CYT transfectants were injected directly into the liver parenchyma. Both cell types exhibited tumor growth in the liver, but the size of the tumors was markedly different. Notably, the apoptotic index (AI) of the $\beta 4$ - Δ CYT tumors was significantly higher than that of the control tumors ($P < 0.0002$ using the Mann-Whitney test). The MI/AI ratio of the control tumors (1.4) is significantly higher than that of the $\beta 4$ - Δ CYT tumors (0.28) ($P < 0.03$ using the Mann-Whitney test). The data are presented as mean index (%) and standard error of the mean. B to D: Morphological analysis of tumor nodules in the liver. B: H&E staining of a paraffin section of a control tumor. C: H&E staining of paraffin section of a $\beta 4$ - Δ CYT tumor. The zone of the tumor closest to the liver parenchyma exhibited numerous tumor cells undergoing apoptosis as evidenced by morphological criteria and by ApopTag staining as shown in D. T is tumor, L is liver. Arrows point to apoptotic cells. B and C, scale bars = 50 μ m, and D, scale bar = 25 μ m.

Discussion

The data presented here argue for an essential role of the integrin $\alpha 6 \beta 1$ in breast carcinoma progression. Specifically, we observed that targeted elimination of this integrin in MDA-MB-435 cells reduced the size of primary tumors formed by these cells and diminished evidence of metastatic foci in the lungs of these mice. Both of these effects appear to derive from a decrease in growth and an increase in apoptosis that occurs in the absence of $\alpha 6 \beta 1$ expression. Our findings provide insight into the mechanism of breast cancer progression and they also highlight the importance of integrin-mediated events in this disease process. From a clinical perspective, these data provide a functional explanation for the finding that a strong correlation exists between $\alpha 6$ integrin expression in pathological specimens obtained from breast cancer patients and the mortality of these patients.¹³

The technique we used to eliminate $\alpha 6 \beta 1$ expression in MDA-MB-435 cells involved expression of a cytoplasmic domain truncation of the $\beta 4$ integrin subunit ($\beta 4$ - Δ CYT). These cells do not express the $\alpha 6 \beta 4$ inte-

grin and expression of this truncated $\beta 4$ subunit promotes the formation of the $\alpha 6 \beta 4$ - Δ CYT heterodimer on the cell surface at the expense of $\alpha 6 \beta 1$ because $\alpha 6$ associates preferentially with the $\beta 4$ subunit in comparison to the $\beta 1$ integrin subunit.¹⁷ Two concerns could be raised by the use of this technique and the interpretation of the data we obtained: 1) expression of $\beta 4$ - Δ CYT could alter expression of other integrins on the cell surface, and the altered expression of these integrins could contribute to the differences in tumor metastasis and survival we observed; and 2) the $\alpha 6 \beta 4$ - Δ CYT heterodimer could retain signaling properties that affect tumor behavior *in vivo*. We have addressed both of these issues. As stated, the surface expression pattern of other known integrin subunits was not altered significantly by expression of $\beta 4$ - Δ CYT. This observation was substantiated by the fact that the adhesion of MDA-MB-435 cells to matrix proteins other than laminin-1 was not influenced by expression of $\beta 4$ - Δ CYT. Although we noted variations in $\alpha 3 \beta 1$ integrin expression among the MDA-MB-435 subclones used in

this study, the levels of $\alpha 3 \beta 1$ expression did not correlate with either tumorigenesis or survival of these subclones *in vivo*. With respect to the signaling properties of $\alpha 6 \beta 4$ - Δ CYT, several observations suggest that it is a nonfunctional receptor. We observed previously that this truncated integrin is unable to stimulate laminin adhesion or invasion through Matrigel, which are two functions we have identified for the $\alpha 6 \beta 4$ integrin.¹⁸ Also, ligation of $\alpha 6 \beta 4$ - Δ CYT failed to induce any detectable increase in tyrosine phosphorylation in contrast to ligation of $\alpha 6 \beta 1$ (data not shown).

Our conclusion that the $\alpha 6 \beta 1$ integrin is involved in spontaneous metastasis is supported by previous findings that have implicated a role for this integrin in models of experimental metastasis. In these particular studies, the ability of melanoma and fibrosarcoma cells to colonize the lungs after tail vein injection was assessed.^{19,20} Using such assays, it was observed that melanoma metastasis could be inhibited by pretreatment of the cells with an $\alpha 6$ -specific mAb,¹⁹ and fibrosarcoma metastasis could be blocked using a ribozyme that abrogated expression of the $\alpha 6$ subunit.²⁰ Although these studies did not address the mechanism by which $\alpha 6 \beta 1$ contributes to the metastatic process, they do support the data we obtained. Moreover, other studies have also suggested a link between $\alpha 6 \beta 1$ expression and the progression of fibrosarcomas,²¹ as well as prostate carcinoma.²² It will be extremely interesting and informative to determine if $\alpha 6 \beta 1$ also functions to promote survival in these cancers.

The involvement of integrins and the signaling pathways they modulate in controlling cell survival has become an explosive area of research.²³⁻²⁸ The mechanism by which the $\alpha 6 \beta 1$ integrin promotes breast carcinoma growth and survival may differ somewhat from recent findings in this field, however, because this integrin does not appear to be essential for the growth and survival of MDA-MB-435 cells *in vitro* even in serum-free conditions. We suggest that MDA-MB-435 cells that express $\alpha 6 \beta 1$ can respond *in vivo* to either a specific growth factor, matrix protein, or other cell type to promote their survival. Indeed, a role for $\alpha 6 \beta 1$ in promoting survival is supported by the reports that this integrin may be a costimulator of thymocyte²⁹ and melanoma³⁰ proliferation. Clearly, understanding how the $\alpha 6 \beta 1$ integrin promotes MDA-MB-435 survival *in vivo* and establishing the involvement of specific signaling pathways in this mechanism is an area of investigation that should provide considerable insight into both breast carcinoma progression and integrin signaling.

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The Integrin $\alpha 6 \beta 4$ Functions in Carcinoma Cell Migration on Laminin-1 by Mediating the Formation and Stabilization of Actin-containing Motility Structures

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Abstract. Functional studies on the $\alpha 6 \beta 4$ integrin have focused primarily on its role in the organization of hemidesmosomes, stable adhesive structures that associate with the intermediate filament cytoskeleton. In this study, we examined the function of the $\alpha 6 \beta 4$ integrin in clone A cells, a colon carcinoma cell line that expresses $\alpha 6 \beta 4$ but no $\alpha 6 \beta 1$ integrin and exhibits dynamic adhesion and motility on laminin-1. Time-lapse videomicroscopy of clone A cells on laminin-1 revealed that their migration is characterized by filopodial extension and stabilization followed by lamellae that extend in the direction of stabilized filopodia. A function-blocking mAb specific for the $\alpha 6 \beta 4$ integrin inhibited clone A migration on laminin-1. This mAb also inhibited filopodial formation and stabilization and lamella formation. Indirect immunofluorescence microscopy revealed that the $\alpha 6 \beta 4$ integrin is localized as discrete clusters in filopodia, lamellae, and retraction fibers. Although $\beta 1$ integrins were also localized in the same structures, a spatial separation of these two integrin

populations was evident. In filopodia and lamellae, a striking colocalization of the $\alpha 6 \beta 4$ integrin and F-actin was seen. An association between $\alpha 6 \beta 4$ and F-actin is supported by the fact that $\alpha 6 \beta 4$ integrin and actin were released from clone A cells by treatment with the F-actin-severing protein gelsolin and that $\alpha 6 \beta 4$ immunostaining at the marginal edges of clone A cells on laminin-1 was resistant to solubilization with Triton X-100. Cytokeratins were not observed in filopodia and lamellipodia. Moreover, $\alpha 6 \beta 4$ was extracted from these marginal edges with a Tween-40/deoxycholate buffer that solubilizes the actin cytoskeleton but not cytokeratins. Three other carcinoma cell lines (MIP-101, CCL-228, and MDA-MB-231) exhibited $\alpha 6 \beta 4$ colocalized with actin in filopodia and lamellae. Formation of lamellae in these cells was inhibited with an $\alpha 6$ -specific antibody. Together, these results indicate that the $\alpha 6 \beta 4$ integrin functions in carcinoma migration on laminin-1 through its ability to promote the formation and stabilization of actin-containing motility structures.

THE integrin $\alpha 6 \beta 4$, a receptor for the laminins, is essential for the organization and maintenance of epithelial structure (11, 57). In many epithelia, this integrin mediates the formation of stable adhesive structures termed hemidesmosomes that link the intermediate filament cytoskeleton with the extracellular matrix (3, 16). Indeed, the ability of $\alpha 6 \beta 4$ to associate with intermediate filaments distinguishes it from other integrins that interact primarily with the actin cytoskeleton (21). The importance of this integrin in epithelial structure has been reinforced by the recent generation of $\beta 4$ -knockout mice that exhibit gross alterations in epithelial morphology and loss of anchorage to the basement membrane (11, 57).

Although the $\alpha 6 \beta 4$ integrin is also expressed in many

carcinomas, its biological functions in these epithelial-derived tumors have not been well studied (43). We and others have argued that $\alpha 6 \beta 4$ may be associated with the process of carcinoma invasion (6, 12, 26, 43, 45). Initially, this argument was based on immunohistochemical data that correlated $\alpha 6 \beta 4$ expression and localization with invasive carcinoma (12, 45, 56). More recently, we demonstrated that ectopic expression of $\alpha 6 \beta 4$ in $\beta 4$ -deficient colon carcinoma cells significantly increased the rate at which these cells invaded laminin matrices (6). Such data that associate $\alpha 6 \beta 4$ with carcinoma invasion, however, are not consistent with the established role for this integrin in the formation of stable and rigid adhesive structures and maintenance of cell polarity in normal epithelial cells because invasive carcinoma cells are characterized by their dynamic interactions with extracellular matrices and their rapid rate of migration, as well as a loss of polarity (24). A priori, these dynamic functions of carcinoma cells would be impeded by the presence of $\alpha 6 \beta 4$ -containing hemides-

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mosomes. In fact, hemidesmosomes are not commonly observed in invasive carcinoma cells, although $\alpha 6 \beta 4$ expression often persists (for review see reference 43). The hypothesis can be derived from these observations that $\alpha 6 \beta 4$ is associated with different functions in invasive carcinoma cells than in normal epithelial cells. Moreover, this functional difference may derive from the interaction of $\alpha 6 \beta 4$ in carcinoma cells with cytoskeletal proteins and other molecules that do not interact with this integrin in normal cells.

In this study, we investigated the function and cytoskeletal associations of $\alpha 6 \beta 4$ in invasive colon carcinoma cells that migrate on laminin-1 matrices. The data obtained implicate $\alpha 6 \beta 4$ in cell migration, and they demonstrate that this integrin is localized in cell structures associated with motility, namely filopodia and lamellae. Importantly, we also show that $\alpha 6 \beta 4$ associates with the actin cytoskeleton in filopodia and lamellae and that it participates in their formation and stabilization.

Materials and Methods

Cells and Antibodies

The clone A cell line was originally isolated from a human, poorly differentiated colon adenocarcinoma (10), and its *in vitro* properties and repertoire of integrin receptors have been described previously (8, 32, 34, 54). The CCL-228 colon carcinoma and the MDA-MB-231 breast carcinoma cell lines were obtained from American Type Culture Collection (Rockville, MD). The MIP-101 colon carcinoma cell line has been described previously (8). Cells were grown in RPMI 1640 medium containing 10 mM Hepes, penicillin (50 U/ μ l), streptomycin (50 μ g/ml), and 10% FBS.

The following mAbs were used in this study: mouse mAb 2B7 (integrin $\alpha 6$) prepared in our laboratory (46); rat mAb GoH3 (integrin $\alpha 6$) from Immunotech (Westbrook, ME); mouse mAb K20 (integrin $\beta 1$); mouse mAb MC-13 (integrin $\beta 1$) provided by Steven Akiyama (National Institutes of Health, Bethesda MD) (1); mouse mAb A9 (integrin $\beta 4$) provided by Thomas Carey (University of Michigan, Ann Arbor, MI); mouse anti-pan-cytokeratin (a mixture of antibodies that recognizes cytokeratins 1, 4, 5, 6, 8, 10, 13, and 19) from Sigma Chemical Co. (St. Louis, MO).

Cell Migration Assays

To assay the migration of clone A cells, bacteriological dishes were coated with 10–100 μ g of laminin-1 prepared from the EHS sarcoma as described (27) or collagen type I (Collaborative Research, Waltham, MA) for 2 h at room temperature and then blocked with PBS containing 1% BSA for 1 h. Clone A cells in exponential growth were removed from culture dishes and resuspended in serum-free RPMI 1640 medium containing 10 mM Hepes and 0.1% BSA. The cells were then plated at low density (1×10^4 /cm²) on the matrix-coated dishes and allowed to adhere for 30 min in a humidified atmosphere with 5% CO₂ at 37°C. In some experiments, integrin-specific antibodies (2B7 or MC-13, 10 μ g/ml) or a nonspecific mouse IgG control (10 μ g/ml) were added to the cells either before the cells were plated or after the cells had adhered for the 30 min. The dishes were then sealed with parafilm and placed on a microscope stage heated to 37°C. For image analysis, an inverted microscope (model Diaphot 300; Nikon, Inc., Melville, NY) with phase contrast optics was used. This microscope was connected to a CCD camera (Dage-MTI, Michigan City, IN), a framegrabber (Scion, Frederick, MD), and a 7600 Power Macintosh computer (Cupertino, CA) to capture the images. Images were collected for 1 h and analyzed with IPLab Spectrum image analysis software.

Migration speed was determined by following cell centroid displacements as a function of time for 1 h at intervals of 15 min. For each individual experiment, 30–40 cells were analyzed. A frame-by-frame analysis of filopodia at intervals of 1 min for 1 h was used to differentiate filopodia from retraction fibers and to monitor the formation and stabilization of filopodia. Lamellar area was determined by tracing their contour and quantifying the area digitally.

Indirect Immunofluorescence Microscopy

Clone A cells were plated on matrix-coated dishes as described above and incubated for 1 h in a humidified atmosphere with 5% CO₂ at 37°C. The cells were then fixed for 20 min at room temperature with a buffer containing 4% paraformaldehyde, 100 mM KCl, 300 mM sucrose, 2 mM EGTA, 2 mM MgCl₂, and 10 mM Pipes at pH 6.8. Based on a previously described extraction protocol (4, 13), the cells in some experiments were extracted for 1 min at 4°C before fixation with either a “membrane” buffer containing 0.5% Triton X-100, 100 mM KCl, 300 mM sucrose, 10 mM EGTA, 2 mM MgCl₂, 1 mM PMSF, and 10 mM Pipes at pH 6.8 or for 5 min at room temperature with a “cytoskeletal” buffer containing 1% Tween-40, 0.5% sodium deoxycholate, 10 mM NaCl, 2 mM MgCl₂, 1 mM PMSF, and 20 mM Tris-HCl, pH 7.4, and then fixed for 20 min in the paraformaldehyde buffer. Subsequently, the fixed cells were rinsed with PBS and incubated with a blocking solution that contained 1% albumin and 5% donkey serum in PBS for 30 min. Primary antibodies (GoH3, 1:50; K20, 1:50; pan-cytokeratin, 1:200) and/or FITC phalloidin (20 μ g/ml) in blocking solution were immunoreacted separately or in combination with the cells for 30 min. The cells were rinsed three times and either a fluorescein-conjugated donkey anti-mouse or a rhodamine-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Labs, West Grove, PA) in blocking buffer (1:150) were used separately or in combination to stain the cells for 30 min. Cells were rinsed with PBS and mounted in a mixture (9:1) of glycerol and PBS, pH 8.5, containing 0.1% propylgallate. The dishes were cut into slides and examined by confocal microscopy (model LSM; Carl Zeiss, Inc., Thornwood, NY).

Actin-severing Experiments

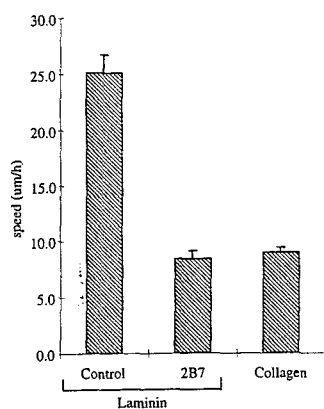
Clone A cells (2×10^6) suspended in RPMI-H with 0.1% albumin were plated on laminin-1-coated dishes and incubated for 1 h at 37°C. The following steps were done at 4°C. The medium was removed and a membrane buffer (see above) was added for 30 s and removed by aspiration. A “low calcium” buffer (25 μ M CaCl₂, 100 mM KCl, 300 mM sucrose, 10 mM EGTA, 2 mM MgCl₂, leupeptin [10 μ g/ml], aprotinin [1 μ g/ml], pepstatin [5 μ g/ml], and 10 mM Pipes, pH 6.8) was used to remove the membrane buffer by washing the cells four times with gentle rocking. Subsequently, the low calcium buffer containing 200 nM gelsolin (kindly provided by Dr. Paul Janmey, Harvard Medical School, Boston, MA) and 50 μ g/ml of GC-globulin (Calbiochem, La Jolla, CA) was added to the cells and incubated for 30 min. Control cells were treated with the low calcium buffer alone. An equal volume of membrane buffer was added to the cells for 30 s to terminate the reaction. The buffer was removed and collected in microfuge tubes, centrifuged at 12,000 rpm for 10 min, and immunoprecipitated with the 2B7 antibody. The immune complexes were resolved by SDS-PAGE and immunoblotted with an anti- $\beta 4$ integrin polyclonal antibody elicited against the last 20 amino acids of the $\beta 4$ cytoplasmic tail.

Results

The Integrin $\alpha 6 \beta 4$ Functions in the Migration of Clone A Cells on Laminin-1 Matrices

Clone A cells were chosen to investigate the possible function of the $\alpha 6 \beta 4$ integrin in the migration of carcinoma cells for several reasons. These cells, which were derived from a poorly differentiated colon carcinoma, are invasive both *in vitro* (8) and *in vivo* (53). They adhere avidly to laminin-1 (34) and, in fact, can mediate dynamic adhesion on laminin-1 under laminar flow conditions (54). Clone A cells are also advantageous because they express the $\alpha 6 \beta 4$ but not the $\alpha 6 \beta 1$ integrin laminin receptor (32, 34), enabling the study of the $\alpha 6 \beta 4$ integrin in the absence of $\alpha 6 \beta 1$ function and the use of $\alpha 6$ -specific reagents to target the $\alpha 6 \beta 4$ integrin specifically. Clone A cells also employ a $\beta 1$ integrin ($\alpha 2 \beta 1$) as a laminin-1 receptor and for this reason are useful for studying functional differences between $\beta 1$ and $\beta 4$ integrin laminin receptors (32, 34).

The migration of clone A cells was examined by time-



(i.e., displacement of the cell centroid as a function of time) obtained from the analysis of 30–40 cells for each experimental condition is reported in this bar graph. Error bars represent SEM.

lapse videomicroscopy in serum-free conditions. Clone A cells exhibit random migration (mean rate = 25 $\mu\text{M}/\text{h}$) when plated on laminin-1. As shown in Fig. 1, this rate of migration on laminin-1 is approximately threefold greater than on an equivalent concentration of collagen type I, even though these cells adhere equally well to both matrices (34). Varying the concentration of collagen type I in these assays did not induce migration, suggesting a specific role for laminin in stimulating clone A migration (data not shown).

The $\alpha 6 \beta 4$ integrin functions in migration on laminin-1 based on the finding that treatment of clone A cells with an $\alpha 6$ -specific mAb (2B7) (46) inhibited this migration significantly (66%) (Fig. 1), but it did not detach the cells from laminin-1 (Fig. 2). In contrast, the cells “rounded up”

and detached after exposure to mAb 13, a $\beta 1$ -specific mAb (1) (data not shown). These mAb inhibition data suggest different functions for the $\alpha 6 \beta 4$ integrin and $\beta 1$ integrins in mediating the dynamic interactions of clone A cells with laminin-1.

Photomicrographs of clone A cells on laminin-1 demonstrate the morphological changes induced by inhibiting the $\alpha 6 \beta 4$ integrin. Clone A cells exhibited a fan-shaped appearance on laminin-1 with prominent lamellae and numerous filopodia (Fig. 2, A and B). Treatment of these cells with 2B7 antibody either before or after plating on laminin-1 had a modest effect on inhibiting cell spreading, but it markedly inhibited the formation of lamellae (Fig. 2 C). In contrast to laminin-1, clone A cells plated on collagen type I were well spread but did not exhibit prominent lamellae (Fig. 2 D).

The Integrin $\alpha 6 \beta 4$ Participates in the Dynamic Formation of Actin-based Motility Structures: Filopodia and Lamellae

We analyzed the sequence of events by which filopodia and lamellae lead to cell translation on laminin-1 using time-lapse videomicroscopy to understand how the $\alpha 6 \beta 4$ integrin contributes to migration. Subsequent to attachment and spreading on laminin-1, numerous filopodia protruded from clone A cells, as evidenced by the sequence of three cells shown in Fig. 3. Only characteristic threadlike structures that actively protruded from the cells were considered to be filopodia. At later times, filopodia also protruded from the lamellae that had formed in these cells. Video analysis revealed that nascent filopodia exhibited a wide range of motion until they either retracted or were stabilized by anchoring to the laminin-1 substratum. Stabilization was detected when the filopodia anchored to lami-

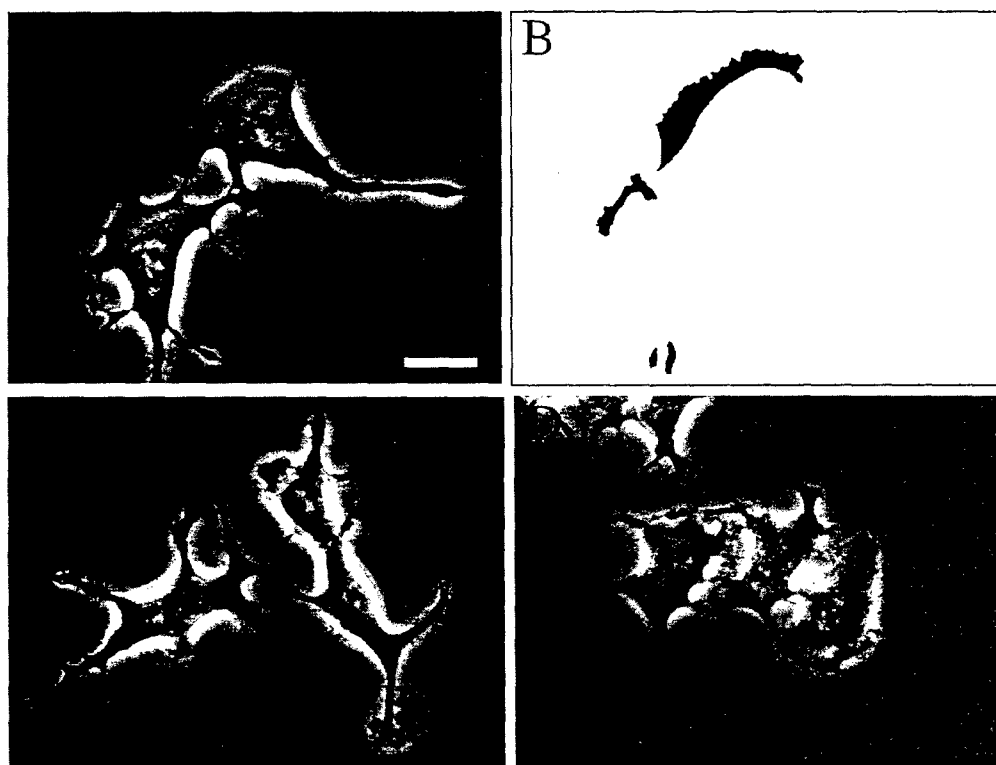


Figure 2. The motile morphology of clone A cells on laminin-1 is dependent on the integrin $\alpha 6 \beta 4$. Cells were plated on either laminin-1-coated (A and C) or collagen I-coated (D) dishes and incubated at 37°C for 30 min before the addition of either (A) control mouse IgG (10 $\mu\text{g}/\text{ml}$) or (C) 2B7 mAb (10 $\mu\text{g}/\text{ml}$). After 30 min, the cells were photographed using phase contrast optics. Note the presence of prominent fan-shaped lamellae in cells on laminin-1 (A) and their digitally traced area in B. The $\alpha 6$ -specific mAb 2B7 inhibits formation of these lamellae (C). Prominent lamellae are not seen when clone A cells are plated on collagen I (D). Bar, 20 μm .

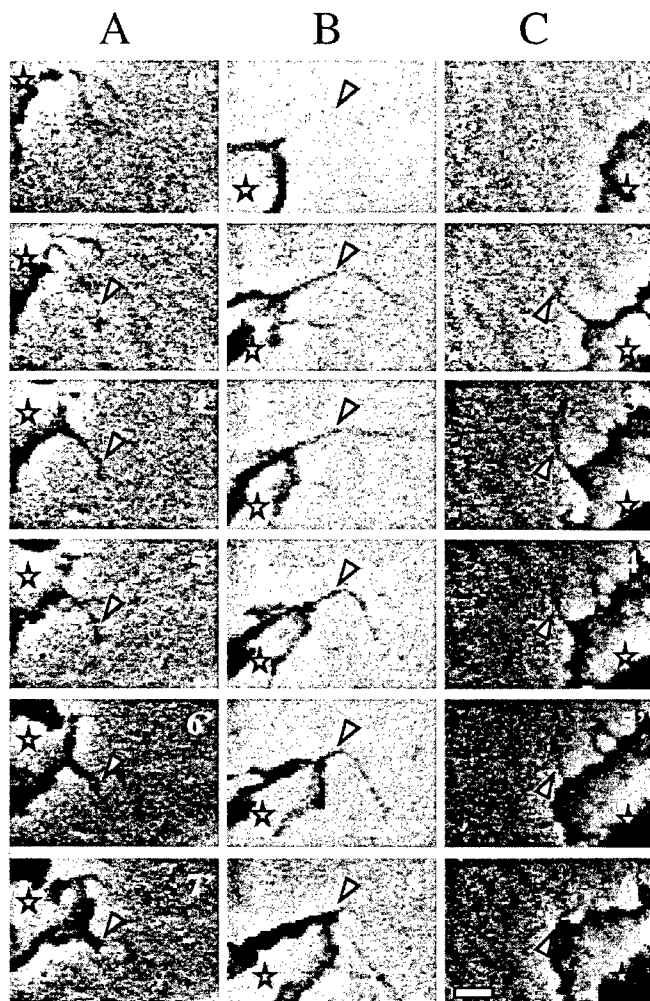


Figure 3. Dynamics of filopodia and lamellae in clone A cells on laminin-1. Cells plated on laminin-1 were analyzed by time-lapse videomicroscopy. Each column shown (A–C) represents a sequence of frames recorded at the specified times from different cells migrating on laminin-1. Arrowheads indicate points at which the filopodia stabilize on the laminin-1 matrix. Asterisks denote the protruding lamella. In A, the filopodium shown stabilized at 2 min and formed an angle at the point indicated by the arrowhead. Note that only the anchoring point is attached because both proximal and distal segments shift positions at later times, during which the lamella extended following the direction of the filopodium. In B and C, the filopodia shown stabilized at 4 and 2 min, respectively. In contrast to the filopodium shown in A, the entire segment of these filopodia proximal to the vertex of the angle became immobilized and the lamellae extended in the direction of these stabilized filopodia. Bar, 5 μ m.

nin-1 at one or more points along their length, a process that restricted their movement and prevented their retraction. When stabilization occurred at a point proximal to the tip of the filopodium, the filopodium usually continued to extend or move freely distal to the attachment point forming a conspicuous angle, the vertex being the anchoring point (Fig. 3, A–C). The formation of such angles was observed in 80% of the stabilized filopodia examined.

The extension of a lamella occurred frequently after the stabilization of a filopodium (Fig. 3, A–C). Such lamellae extruded from the roots of stabilized filopodia and fol-

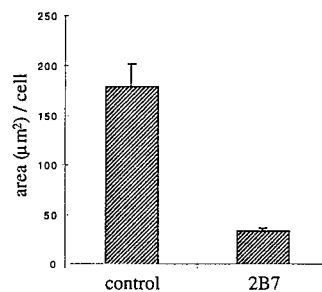


Figure 4. Formation of lamellae in clone A cells on laminin-1 requires the $\alpha 6 \beta 4$ integrin. Clone A cells were plated on laminin-1-coated dishes and incubated for 30 min at 37°C before the addition of 2B7 (10 μ g/ml) or a control IgG. The cells were photographed after 1 h, and their lamellar area (μm^2 /cell) was determined by digital image analysis (see example in Fig. 2 B). 50 cells were analyzed for each condition. Error bar represents SEM.

lowed the direction of these filopodia (Fig. 3, A–C). Subsequently, the cell bodies translocated in the direction of well-developed lamellae, as described previously (2). Thus, the stabilization of filopodia appeared to be a major determinant of the direction of cell migration. The time that clone A cells moved in a specific direction on laminin-1 was relatively brief (<1 h) because of more active lamellae at other sites on the cell and the collapse of the initial lamellae. This frequent change in direction gave the appearance of a random walk.

We studied the influence of the $\alpha 6 \beta 4$ integrin on clone A cells by inhibiting the action of this integrin with the 2B7 mAb and assessing the qualitative and quantitative effects of this inhibition on lamellar dynamics. The formation of lamellae was markedly inhibited (80%) within 60 min of adding the 2B7 mAb to motile cells (Fig. 4). However, the cells inhibited with 2B7 remained well spread (Fig. 2), suggesting a specific function for the $\alpha 6 \beta 4$ integrin in the formation of lamellae and not in cell attachment or spreading. Similarly, quantification of higher magnification video images revealed that 2B7 inhibited filopodial formation significantly and that the filopodia

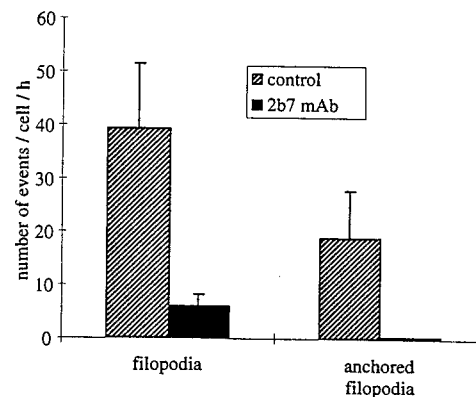


Figure 5. Inhibition of the integrin $\alpha 6 \beta 4$ reduces the formation and stabilization of filopodia in clone A cells on laminin-1. Clone A cells were plated on laminin-1-coated dishes and incubated for 30 min at 37°C before the addition of either 2B7 (10 μ g/ml) or a control IgG (10 μ g/ml) and analysis by time-lapse videomicroscopy. For each condition, five cells were monitored for 1 h at a frequency of one frame per minute and each frame was analyzed for the active formation of filopodia. A filopodium was considered stabilized if it remained immobile for several frames. The data shown represent the number of filopodia that either formed or stabilized/cell/hour. Error bar represents SEM.

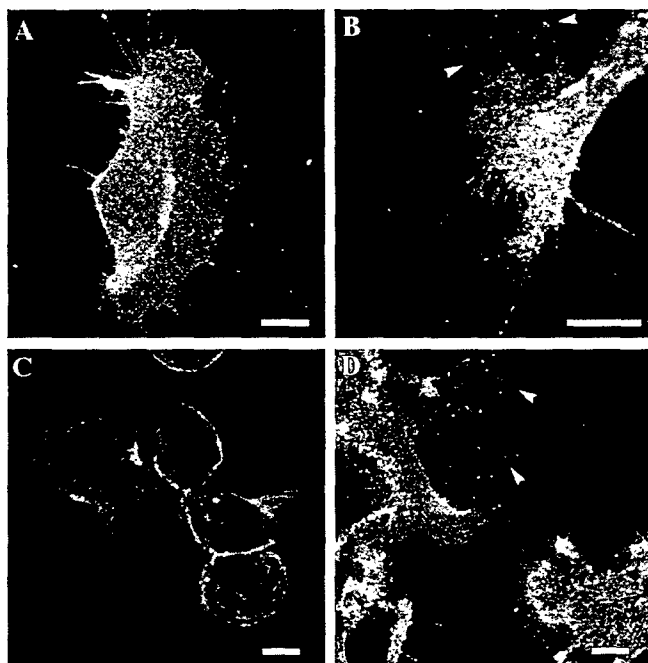


Figure 6. The integrin $\alpha 6 \beta 4$ is localized in lamellae and filopodia in clone A cells on laminin-1. Cells plated on laminin-1 (A, B, and D) or collagen I (C) were fixed and processed for immunofluorescence using the rat GoH3 (anti- $\alpha 6$) mAb followed by a rhodamine-conjugated anti-rat antibody as described in the Materials and Methods section. The confocal images shown represent optical sections of the ventral surface. (A) Note the presence of $\alpha 6 \beta 4$ staining on the lamellae and in the filopodia at the leading edge (right side of the cell), as well as in retraction fibers at the trailing edge (left side of cell). (B) A higher magnification demonstrates the clustered appearance of $\alpha 6 \beta 4$ integrin in the filopodia and its presence at points of filopodial angling. (D) Fibers that are positive for $\alpha 6 \beta 4$ staining (arrowheads) are apparently left behind by the advancing cell on the left. (C) Clone A cells plated on collagen I exhibit only a diffuse pattern of $\alpha 6 \beta 4$ staining on their ventral surface. Bars, 10 μ m.

that did form in the presence of this inhibitory antibody stabilized much less frequently. Specifically, 48% of filopodia stabilized in control cells compared with 3% in 2B7-treated cells (Fig. 5). In contrast to these effects of 2B7 on clone A cells, the addition of mAb 13, a $\beta 1$ integrin-specific mAb, caused the cells to round-up and subsequently detach as mentioned above.

The Integrin $\alpha 6 \beta 4$ Is Localized in Filopodia and Lamellae in Areas Distinct from $\beta 1$ Integrins

To gain insight into how the $\alpha 6 \beta 4$ integrin influences the formation of filopodia and lamellae, we analyzed the spatial distribution of this integrin by indirect immunofluorescence microscopy using GoH3, an $\alpha 6$ -specific mAb (48). Identical immunostaining results were obtained with A9, a $\beta 4$ -specific mAb (data not shown).

The $\alpha 6 \beta 4$ integrin was distributed throughout the cell body, as well as in the lamellae and filopodia of clone A cells plated on laminin-1. In the lamellae, it exhibited a fine grainy pattern of staining (Fig. 6, A and B). In the filopodia themselves, $\alpha 6 \beta 4$ staining was localized in discrete clusters that were distributed throughout the shaft

(Fig. 6 B). At points of filopodial angling, a concentration of $\alpha 6 \beta 4$ staining was usually observed (Figs. 6 B and 7, C and D). In contrast to cells plated on laminin-1, clone A cells plated on collagen type I displayed a diffuse distribution of $\alpha 6 \beta 4$ staining with few cluster formations evident on the ventral surface of the cells (Fig. 6 C).

The $\alpha 6 \beta 4$ integrin was expressed intensely in characteristic retraction fibers of clone A cells on laminin-1 (Figs. 6 A and 8 A). Retraction fibers were identified by their unique appearance at the trailing edges of fan-shaped cells (Fig. 2). In these fan-shaped cells, there was a clear gradient of $\alpha 6 \beta 4$ towards the rear that peaked in intensity in retraction fibers (Fig. 6 A). In addition, some retraction fibers that were enriched in $\alpha 6 \beta 4$ expression were observed detached from cells, most likely remnants of migrating cells (Fig. 6 D).

Because $\beta 1$ integrin function is also critical in the interaction of clone A cells with laminin-1 (32, 34, 54), we compared the distribution of $\beta 1$ integrin with that of the $\alpha 6 \beta 4$ integrin by double immunostaining using GoH3 and a $\beta 1$ -specific mAb, K20. Although $\beta 1$ integrin was also localized in filopodia and lamellae, a striking difference in the localization of $\beta 4$ and $\beta 1$ integrin staining was evident in these structures (Fig. 7, A–D). In filopodia, discrete clusters or patches of $\beta 1$ and $\alpha 6 \beta 4$ integrin staining were apparent. As noted above, $\alpha 6 \beta 4$ staining was observed frequently at points of filopodial angling (Fig. 7, C and D). There were also some regions of overlapping staining, most consistently at the tips of filopodia (Fig. 7, C and D). The staining pattern of the $\alpha 6 \beta 4$ and $\beta 1$ integrins was segregated in the lamellae as well, except at those points where the root of a filopodium projected into the lamella (Fig. 7 B). In the characteristic retraction fibers of clone A cells on laminin-1, the double immunostaining revealed that $\alpha 6 \beta 4$ expression predominated over $\beta 1$ integrin expression (Fig. 8, A and B).

Association of $\alpha 6 \beta 4$ with the Actin Cytoskeleton in Motile Structures

Our observations that the $\alpha 6 \beta 4$ integrin functions in cell migration and that it is localized in actin-containing structures such as filopodia and lamellae suggest that it does interact with the actin cytoskeleton in clone A cells. However, the majority of studies on the $\alpha 6 \beta 4$ integrin and the cytoskeleton have focused on its association with intermediate filaments, especially in hemidesmosomes (5, 11, 15, 22, 49, 51). Very little is known, in fact, about possible interactions of $\alpha 6 \beta 4$ with the actin cytoskeleton. For this reason, we assessed the association of the $\alpha 6 \beta 4$ integrin with both the actin cytoskeleton and cytokeratins in clone A cells plated on laminin-1 by indirect immunofluorescence microscopy using the GoH3 mAb and either phalloidin or a pan-cytokeratin antibody.

Visualization of clone A cells that had been stained with the GoH3 mAb and phalloidin revealed a striking colocalization of the $\alpha 6 \beta 4$ integrin and F-actin in filopodia and at the edges of the lamellae (Fig. 9, A–D). In contrast, cytokeratins were excluded from filopodia and the edges of lamellae in clone A cells plated on laminin-1 based on the staining pattern observed with the pan-cytokeratin antibody (Fig. 10 A). Cytokeratin staining was concentrated

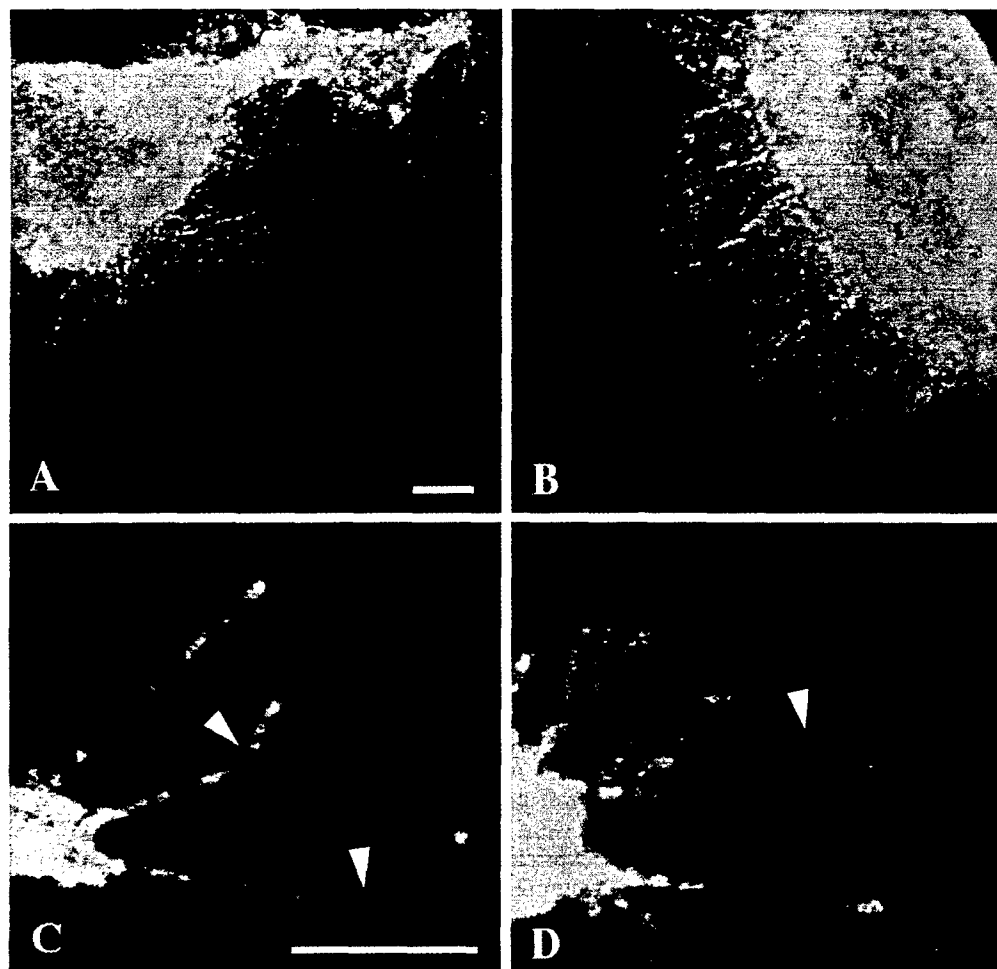


Figure 7. Distinct localization of $\alpha 6 \beta 4$ and $\beta 1$ integrins in the filopodia and lamellae of clone A cells on laminin-1. Clone A cells were plated on laminin-1 for 1 h at 37°C and processed for double immunofluorescence as described using rat GoH3 mAb and the mouse K-20 mAb followed by a combination of a TRITC-conjugated anti-rat antibody and an FITC-conjugated anti-mouse antibody that do not cross-react. The ventral surface of the cells was analyzed by confocal microscopy. Red, GoH3 mAb; green, K-20 mAb; yellow, colocalization. (A) Several filopodia and retraction fibers show a segregated distribution of $\alpha 6 \beta 4$ and $\beta 1$ staining. (B) In lamellae, $\alpha 6 \beta 4$ staining is largely segregated from $\beta 1$ staining except in the streak-shaped areas where filopodia project into the lamella. (C and D) Higher magnification images of filopodia showing the spatial segregation of $\alpha 6 \beta 4$ and $\beta 1$ staining and the presence of $\alpha 6 \beta 4$ in the angles of filopodia (arrowheads). Bar, 5 μ m.

largely in the cell body and proximal portions of lamellae. Costaining with GoH3 did not indicate any significant association of the $\alpha 6 \beta 4$ integrin and cytokeratins in filopodia or at the edges of lamellae (Fig. 10 A).

To study the interaction of the $\alpha 6 \beta 4$ integrin with the cytoskeleton in more detail, we used an *in situ* extraction scheme that solubilizes proteins to an extent that correlates with their cytoskeletal associations (4, 13). Clone A cells adherent to laminin-1 were extracted with either a 0.5% Triton X-100 buffer that removes most of the soluble protein and phospholipid but not the actin and intermediate filament cytoskeletons, or a two-detergent buffer (1.0% Tween-40/0.5% deoxycholate) that removes the bulk of the actin cytoskeleton but not intermediate filaments and associated proteins (4, 13). Subsequent to extraction, the cells were fixed and costained with integrin-specific mAbs (GoH3 or K20) and cytoskeletal-specific reagents (phalloidin or pan-cytokeratin mAbs). Extraction of clone A cells with the Triton X-100 buffer revealed that the $\alpha 6 \beta 4$ and F-actin colocalization observed in unextracted cells is preserved in clusters at proximal sites in filopodia, as well as at the roots of filopodia that project into the lamellae (Fig. 11, A and B). Several of these colocalization sites were also the origins of actin filament bundles (Fig. 11, A and B). In contrast, $\alpha 6 \beta 4$ did not colocalize with cytokeratins in filopodia and distal sites of many of the lamellae either in unextracted cells (Fig. 10 A) or after the Triton X-100 buffer extraction (Fig. 10 B). These re-

sults suggest that $\alpha 6 \beta 4$ is retained at the cell edges because of its association with actin and not with cytokeratins. In agreement with this possibility, these marginal areas of actin-associated $\alpha 6 \beta 4$ integrin were removed by the Tween/deoxycholate buffer. As shown in Fig. 10 C, the only GoH3-positive staining that remained after this buffer was the staining that colocalized with cytokeratins at the base of lamellae, the lamellae themselves having been largely removed by the Tween/deoxycholate buffer. F-actin was extracted by the Tween/deoxycholate buffer, as indicated by a lack of phalloidin staining (Fig. 11 D). Interestingly, most of the $\beta 1$ integrin staining was removed from cells on laminin-1 by the Triton X-100 buffer, suggesting a weaker interaction of $\beta 1$ integrins with the cytoskeleton than with the $\alpha 6 \beta 4$ integrin in these cells (Fig. 11 C). Also, most of the $\alpha 6 \beta 4$ staining was extracted by the Triton X-100 buffer in cells plated on collagen I, indicating that the association of $\alpha 6 \beta 4$ with actin is dependent on laminin-1 (data not shown). Together, these results reinforce the hypothesis that the retention of $\alpha 6 \beta 4$ in filopodia and edges of lamellae of clone A cells on laminin-1 is mediated by the actin cytoskeleton. In these motile structures, the only apparent association of $\alpha 6 \beta 4$ with cytokeratins occurs at the base of the lamellae.

The presence of $\alpha 6 \beta 4$ at the origin sites of actin filament bundles described above prompted us to examine the possibility of a functional relationship. The actin bundles were usually organized as multiple cables that ran parallel to the

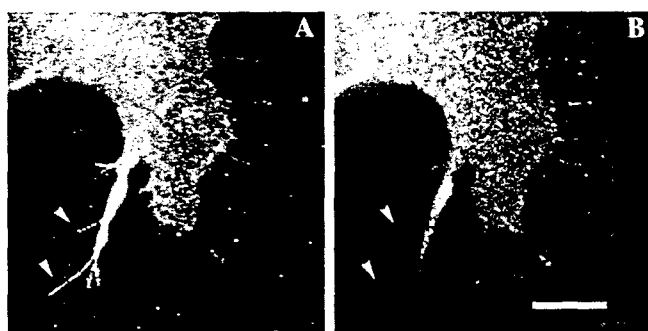


Figure 8. Enhanced localization of the $\alpha 6 \beta 4$ integrin in retraction fibers of clone A cells on laminin-1. Cells plated on laminin-1 for 1 h at 37°C were processed for double immunofluorescence as described using the rat GoH3 mAb and mouse K-20 mAb followed by a combination of a TRITC-conjugated anti-rat antibody and an FITC-conjugated anti-mouse antibody that do not cross-react. The ventral surface of the cells was analyzed by confocal microscopy. (A) GoH3 mAb. (B) K-20 mAb. Note the presence of $\alpha 6 \beta 4$ staining in retraction fibers (A, arrowheads) at the trailing edge of the cells. Staining of $\beta 1$ integrin is absent in these fibers (B). Bar, 10 μ m.

margins of lamellae and were most commonly observed in fan-shaped cells (Fig. 9 D). The $\alpha 6 \beta 4$ integrin colocalized with F-actin at the termini of several of these parallel actin bundles, which were in continuity with filopodia, but it was not localized along the bundles themselves (Fig. 9, C and D; Fig. 11). These actin bundles were not organized into polygonal arrays traversing the nuclear area, such as those that are characteristic of fibroblasts. Interestingly, however, clone A cells on collagen type I did exhibit such a polygonal array of filament bundles (Fig. 9 E). The parallel actin bundles were no longer evident when cells plated on laminin-1 were treated with the 2B7 antibody, suggesting that the $\alpha 6 \beta 4$ integrin is critical for their formation (Fig. 9 F).

Biochemical Evidence for an Association of the $\alpha 6 \beta 4$ Integrin with Actin Filaments

The data presented above strongly support an association of the $\alpha 6 \beta 4$ integrin with actin filaments in clone A cells. To obtain biochemical evidence for this association, we used the actin-severing protein gelsolin to assess whether severing actin filaments would liberate $\alpha 6 \beta 4$. Clone A cells adherent to laminin-1 were extracted with the 0.5% Triton X-100 buffer to remove soluble proteins and then treated with gelsolin. The proteins liberated from extracted cells treated with gelsolin, as well as from extracted cells treated with the buffer alone, were analyzed for the presence of $\alpha 6 \beta 4$ by immunoprecipitation with 2B7 and subsequent immunoblotting with a polyclonal antibody specific for the $\beta 4$ cytoplasmic domain. As shown in Fig. 12, the 200-kD $\beta 4$ subunit was liberated from gelsolin-treated cells but not from cells treated with buffer alone. Moreover, the material obtained from the gelsolin-treated cells but not the control cells was enriched in actin (Fig. 12).

Functional Association of the $\alpha 6 \beta 4$ Integrin with Filopodia and Lamellae in Other Carcinoma Cells

We explored the possibility that the functional and topographical properties of the $\alpha 6 \beta 4$ integrin observed in clone

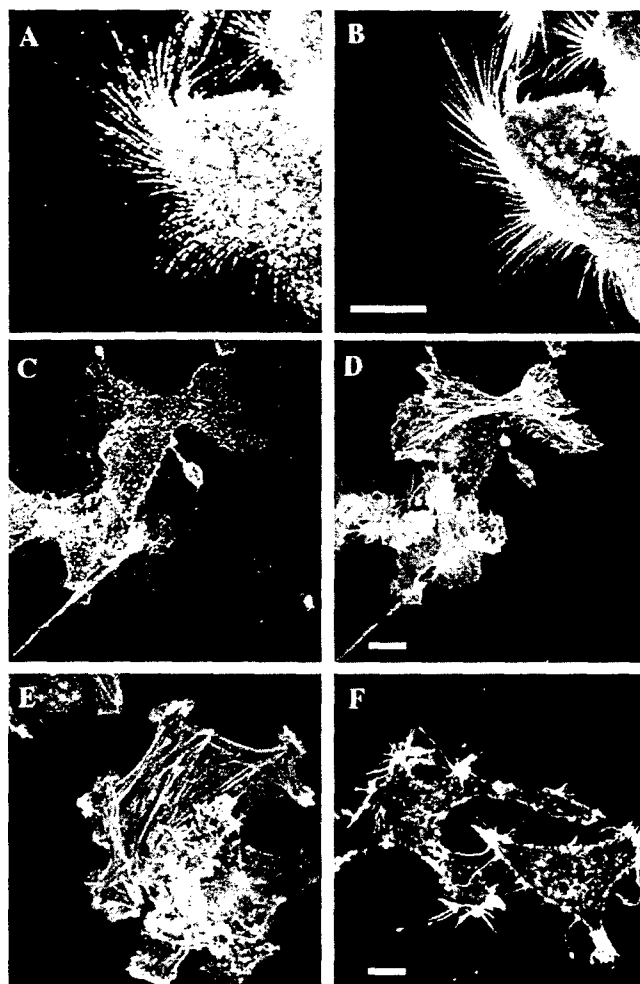


Figure 9. $\alpha 6 \beta 4$ integrin colocalizes with F-actin in filopodia of clone A cells on laminin-1. Cells plated on either laminin-1 (A–D) or collagen I (E) at 37°C for 1 h were processed for double immunofluorescence as described using the rat GoH3 mAb followed by a rhodamine-conjugated anti-rat antibody and FITC-conjugated phalloidin. The confocal images shown represent optical sections of the ventral surface of the cells. (A and C) GoH3. (B, D, E, and F) Phalloidin. A and B demonstrate colocalization of $\alpha 6 \beta 4$ and F-actin in a group of filopodia. D shows the formation of actin cables on the top lamella that project into filopodia. These filopodia are enriched in $\alpha 6 \beta 4$ (C). E shows the presence of polygonal actin cables in clone A cells plated on collagen I. In F, the cells were incubated with 2B7 antibody for 30 min before fixation. Note the disappearance of actin cables (remaining protrusions are presumably retraction fibers, see text). Bars, 10 μ m.

A cells could be extended to other carcinoma cells that express $\alpha 6 \beta 4$. The CCL-228 and MIP-101 colon carcinoma cells have been shown previously to express the $\alpha 6$ integrin subunit exclusively associated with $\beta 4$ (19), while the MDA-MB-231 breast carcinoma cells express primarily the $\alpha 6 \beta 4$ heterodimer (data not shown). These carcinoma cells were analyzed by indirect immunofluorescence using an anti- $\beta 4$ antibody (A9) and FITC-phalloidin. Although these carcinoma cells differed markedly in their morphology on laminin-1, all of them exhibited a fine grainy pattern of $\beta 4$ staining on their ventral surfaces (Fig. 13, A–E). More specifically, $\beta 4$ was localized in discrete clusters in filopodia, retraction fibers, and lamellae. A striking colo-

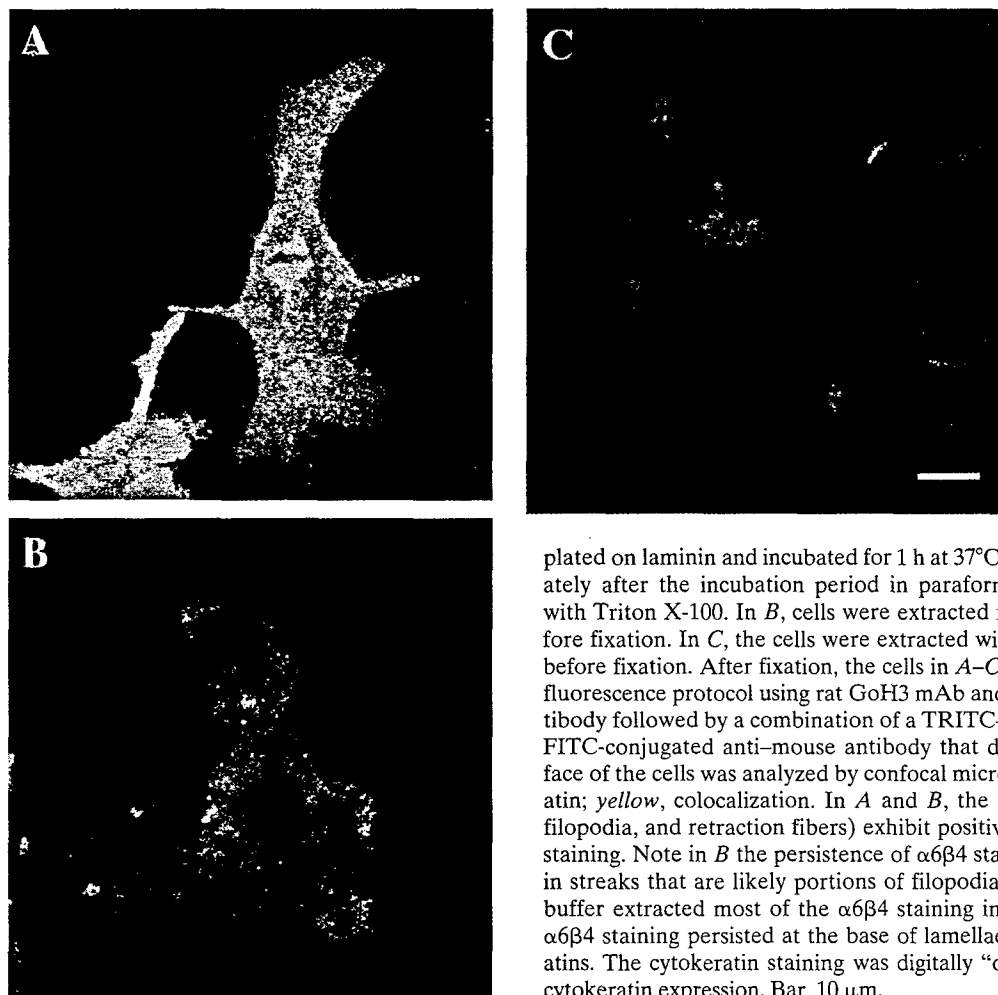


Figure 10. The integrin $\alpha 6 \beta 4$ localized at the marginal areas of clone A cells on laminin-1 does not colocalize with cytokeratins. Cells were

plated on laminin and incubated for 1 h at 37°C. In *A*, the cells were fixed immediately after the incubation period in paraformaldehyde and then permeabilized with Triton X-100. In *B*, cells were extracted first with a Triton X-100 buffer before fixation. In *C*, the cells were extracted with a Tween-40/deoxycholate buffer before fixation. After fixation, the cells in *A–C* were stained by a double immunofluorescence protocol using rat GoH3 mAb and a mouse pan-cytokeratin mAb antibody followed by a combination of a TRITC-conjugated anti-rat antibody and a FITC-conjugated anti-mouse antibody that do not cross-react. The ventral surface of the cells was analyzed by confocal microscopy. *Red*, GoH3; *green*, cytokeratin; *yellow*, colocalization. In *A* and *B*, the marginal areas (edges of lamellae, filopodia, and retraction fibers) exhibit positive $\alpha 6 \beta 4$ staining but no cytokeratin staining. Note in *B* the persistence of $\alpha 6 \beta 4$ staining in marginal clusters arranged in streaks that are likely portions of filopodia. In *C*, the Tween-40/deoxycholate buffer extracted most of the $\alpha 6 \beta 4$ staining in filopodia and lamellae. However, $\alpha 6 \beta 4$ staining persisted at the base of lamellae where it colocalized with cytokeratins. The cytokeratin staining was digitally “overexposed” to detect any possible cytokeratin expression. Bar, 10 μ m.

calization of $\beta 4$ and actin was seen in these structures similar to the results obtained with clone A cells. Interestingly, MIP-101 cells exhibited long filopodia with distinct “actin nodes” that were enriched in $\beta 4$ staining. (Fig. 13, *A* and *B*).

We also explored the function of the $\alpha 6 \beta 4$ integrin in the dynamic behavior of CCL-228 cells on laminin-1 using the function-blocking 2B7 antibody. As shown in Fig. 13 *F*, 2B7 markedly inhibited the formation of lamellae, but it did not affect cell attachment. Similar results were obtained with MIP-101 and MDA-MB-231 cells (data not shown). These data indicate that the interaction of $\alpha 6 \beta 4$ with actin-containing motility structures is a frequent phenomenon in carcinoma cells.

Discussion

Functional studies on the $\alpha 6 \beta 4$ integrin have focused primarily on its role in the organization of hemidesmosomes, stable adhesive structures that associate with the intermediate filament cytoskeleton (5, 15, 22, 49, 51). We report here that the $\alpha 6 \beta 4$ integrin can also function in the dynamic process of cell migration on laminin-1 based on data obtained from the analysis of clone A colon carcinoma and other carcinoma cells. Interestingly, our results demonstrate that this integrin participates in a specific aspect of the migration process, the formation and stabilization of

filopodia and lamellae. The functional involvement of the $\alpha 6 \beta 4$ integrin in the dynamics of these actin-containing motility structures is supported by our findings that $\alpha 6 \beta 4$ is localized in filopodia and edges of the lamellae and that it associates with actin and not with cytokeratins in these structures. These specific functions of $\alpha 6 \beta 4$ in the migration process of clone A cells are in contrast to a more general function for $\beta 1$ integrins, which are required for the adhesion and spreading of clone A cells on laminin-1.

The premise for this study was our interest in understanding how the $\alpha 6 \beta 4$ integrin contributes to carcinoma progression. Although several studies have linked this integrin to the invasive behavior of various carcinomas, mechanistic data to support a function for $\alpha 6 \beta 4$ in a dynamic process such as invasion have been lacking (43 and references therein). Our finding that $\alpha 6 \beta 4$ can participate in the formation and function of actin-containing motility structures in colon carcinoma cells clearly suggests an important role for this integrin in cell migration, a process that is critical for tumor invasion and metastasis. Indeed, it will be extremely informative to study the localization and cytoskeletal dynamics of $\alpha 6 \beta 4$ in other carcinoma cells in which it has been linked to invasion, such as thyroid and gastric carcinomas (45, 52). In this direction, it is worth mentioning that many aspects of epithelial wound healing, including a loss of cell polarity and an induction of cell migration, resemble the behavior of invasive carcinoma cells

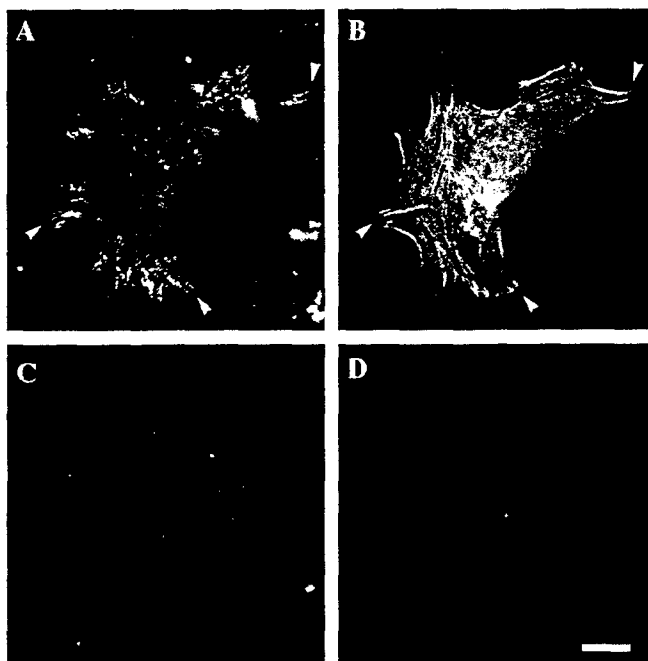


Figure 11. The integrin $\alpha 6 \beta 4$ is associated with F-actin. Cells were plated on laminin and incubated for 1 h at 37°C. In A–C, the cells were extracted first with a Triton X-100 buffer before fixation with paraformaldehyde. In D, the cells were extracted with a Tween-40/deoxycholate buffer before fixation. After fixation, the cells in A and B were double immunostained using rat GoH3 mAb followed by a rhodamine-conjugated anti-rat antibody (A) and FITC-conjugated phalloidin (B). In C, the cells were stained with mouse K-20 mAb antibody followed by an FITC-conjugated anti-mouse antibody. In D, the cells were stained with phalloidin. The ventral area of the cells was analyzed by confocal microscopy. Note in A and B the colocalization of $\alpha 6 \beta 4$ (A) and F-actin (B) at the roots of filopodia (arrowheads). Several of these colocalization areas are in continuity with actin cables (B). Bar, 10 μ m.

exemplified by the fibroblast-like morphology and active motility seen in clone A cells (37). There is some evidence in fact that $\alpha 6 \beta 4$ may participate in epithelial wound healing (9, 23, 29, 30, 35, 37), although the nature of this involvement has not been explored. In addition, EGF has been shown to increase the migration of 804G bladder cells by disrupting hemidesmosomes and possibly enabling $\alpha 6 \beta 4$ to participate in migration (36). Collectively, these findings raise the possibility of a general function for the $\alpha 6 \beta 4$ integrin in epithelial cell migration.

The videomicroscopy data presented indicate that filopodial formation and stabilization play a key role in the migration of clone A cells on laminin-1, reminiscent of earlier studies on cell locomotion by Albrecht-Buehler that ascribed exploratory and sensory capabilities to filopodia (2). Using fibroblasts plated on surfaces coated with gold, he observed that lamellipodia form when filopodia find and contact a gold-coated area and that these lamellipodia follow the direction of the stabilized filopodia. The importance of filopodial contacts in directing cell migration is underscored by the pioneer growth cone model in grasshoppers where a single filopodial contact can re-orient an entire growth cone (41). This sequence of events is consistent with the more recent transport-track model of mi-

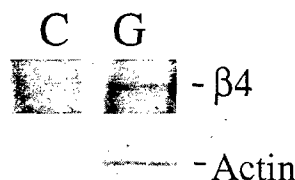


Figure 12. The actin-severing protein gelsolin releases $\alpha 6 \beta 4$ integrin from permeabilized clone A cells. Cells were plated on laminin-1 and incubated for 1 h at 37°C. After permeabilization with a Triton X-100 buffer, the cells were incubated with either gelsolin (G) or control buffer (C) for 30 min. The gelsolin-liberated fraction was immunoprecipitated with an $\alpha 6$ -specific antibody (2B7), subjected to SDS-PAGE, and immunoblotted with a $\beta 4$ -specific polyclonal antibody. An aliquot of the gelsolin-liberated fraction was subjected to SDS-PAGE and stained with Coomassie blue to detect the 43-kD actin band that was evident in the gelsolin-treated but not the control cells.

gration (39). This model implies that stabilized filopodia or any other polarized, actin filament bundle fixed onto a substrate provides the cell with a structure that can be used as a one-way transport track by myosin II motors. Such motors pull other cytoplasmic components bound to substratum-free actin filaments forward (39). This model has also been supported by the respreading of cells after mitosis. Such cells use retraction fibers to guide the spreading edge, and these retraction fibers contain polarized bundles of actin filaments anchored to the substrate at their tips. If the retraction fibers are mechanically detached from the substrate, a spreading edge is not extended (7, 38). These observations are mentioned because we observed a related sequence of events during the migration of clone A cells on laminin-1. The nascent lamellae formed in the direction of filopodia that had stabilized on laminin-1. Moreover, this stabilization is mediated by the $\alpha 6 \beta 4$ integrin because function-blocking antibodies substantially reduced the number of stabilized filopodia. Stabilization is probably a direct interaction of $\alpha 6 \beta 4$ with laminin-1 at the anchorage point, based on the observation that $\alpha 6 \beta 4$ is enriched at the angles of filopodia, sites that are clearly the anchoring points detected by videomicroscopy. This scenario implies that the stabilizing function of the $\alpha 6 \beta 4$ integrin is an early event in clone A motility on laminin-1.

One question that arises from our data is whether the role of the $\alpha 6 \beta 4$ integrin in filopodial formation and stabilization is unique. Clearly, many cell types that do not express this integrin can form filopodia and migrate on laminin-1. We propose that the $\alpha 6 \beta 4$ integrin enhances the process of filopodial extension and stabilization because of its distinctive adhesive properties on laminin-1. Previously, we demonstrated that the $\alpha 6 \beta 4$ integrin expressed by clone A cells has an extremely high adhesive strength for laminin-1 (50). Specifically, laminin-1 adhesion mediated by this integrin was able to resist shear forces up to 100 dynes/cm². The high adhesive strength of this integrin for laminin-1 supports its involvement in filopodial stabilization. Moreover, the fact that we observed $\alpha 6 \beta 4$ preferentially expressed in retraction fibers at the trailing edge of migrating cells, as well as in retraction fibers detached from migrating cells, supports the notion that $\alpha 6 \beta 4$ interacts avidly with laminin-1 and that this interaction, once formed, is not disrupted easily. A mechanism for releasing the adhesive strength of $\alpha 6 \beta 4$ at the rear of the cell must exist, however, because the bulk of this integrin remains

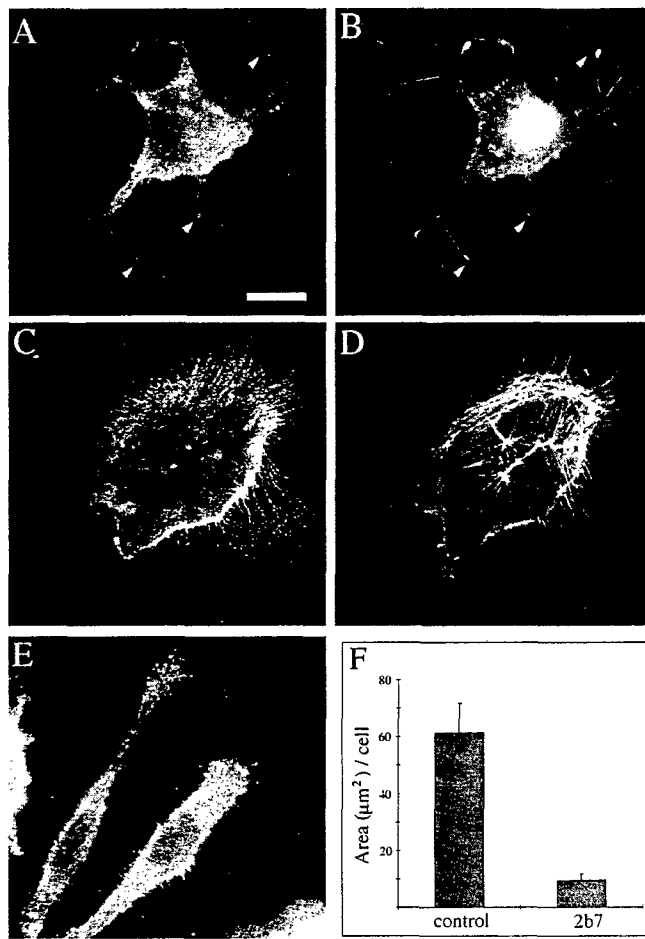


Figure 13. The $\alpha 6 \beta 4$ integrin is localized in actin-containing motility structures in other carcinoma cells. MIP-101 (A and B), MDA-MB-231 (C and D), and CCL-228 (E) carcinoma cells were analyzed by double immunostaining with the $\beta 4$ -specific A9 antibody (A, C, and E) and FITC-phalloidin (B and D). Note the concentration of $\alpha 6 \beta 4$ in the actin nodes present in the filopodia of MIP-101 cells (A and B, arrowheads) and the distribution of $\alpha 6 \beta 4$ in filopodia, retraction fibers, and lamellae of MDA-MB-231 and CCL-228 cells. (F) An $\alpha 6$ -specific antibody inhibits formation of lamellae in CCL-228 cells. Cells were plated on laminin-1 in the presence or absence of 2B7 for 1 h. The cells were photographed, and their lamellar area ($\mu\text{m}^2/\text{cell}$) was determined by digital image analysis. 50 cells were analyzed for each condition. Error bar represents SEM. Bar, 20 μm .

with the migrating cell. Indeed, the dynamics of integrins at the rear of migrating cells have been linked to the regulation of cell motility (31, 42).

The data provided here suggest an association of the $\alpha 6 \beta 4$ integrin with actin filaments and that this association is dependent on the adhesion of these cells to laminin-1. Specifically, the actin-severing protein gelsolin was able to liberate $\alpha 6 \beta 4$ from clone A cells along with actin. Also, the immunostaining studies revealed that both filopodia and the edges of lamellae contain $\alpha 6 \beta 4$ distributed in the form of discrete clusters that colocalized with F-actin. Filopodia and many of the margins of lamellae did not contain any cytokeratins detectable by immunofluorescence microscopy using a pan-cytokeratin antibody. An association between $\alpha 6 \beta 4$ and F-actin is also supported by

the fact that $\alpha 6 \beta 4$ immunostaining at the marginal edges of the cell was resistant to solubilization with Triton X-100. Moreover, $\alpha 6 \beta 4$ was extracted from these marginal edges with a Tween-40/deoxycholate buffer that has been shown to solubilize most of the actin cytoskeleton but not intermediate filaments (4, 13), providing additional evidence that $\alpha 6 \beta 4$ does not associate with cytokeratins in filopodia and the distal areas of lamellae. However, $\alpha 6 \beta 4$ appears to associate with cytokeratins at the base of the lamellae based on the colocalization data and the fact that the $\alpha 6 \beta 4$ immunostaining in these regions was resistant to the Tween-40/deoxycholate buffer. These observations suggest that a more stable $\alpha 6 \beta 4$ -mediated adhesion may occur at the base of the lamellae than in filopodia and the margins of the lamellae.

The association of the $\alpha 6 \beta 4$ integrin and actin filament bundles is intriguing and it could relate to the mechanism of clone A migration. A parallel arrangement of actin filament bundles was consistently found in fan-shaped lamellae of cells that moved at a higher rate and that persisted in one direction for longer times than cells that did not express such bundles. These parallel actin bundles are probably related to those described earlier as actin arcs that are preferentially seen in very motile cells, in contrast to stress fibers that organize orthogonally and are frequently seen in stationary cells (18, 25, 50). These arcs originate on the ventral margins of the cell and then traverse in parallel across the dorsal surface of the lamella. The integrin $\alpha 6 \beta 4$ appears to have a critical role in the formation of these actin filament bundles because they were disrupted by $\alpha 6$ -blocking antibodies. Moreover, $\alpha 6 \beta 4$ was associated with actin at the origins of these actin filament bundles based on the results obtained with the detergent extraction protocol. Additional studies on the involvement of these actin bundles in cell migration and the role of $\alpha 6 \beta 4$ in their function should be insightful.

The $\alpha 6 \beta 4$ integrin can participate in filopodial formation on laminin-1 based on the findings that function-blocking antibodies inhibited their formation. Although the mechanism by which $\alpha 6 \beta 4$ contributes to filopodial growth remains to be elucidated, an area that should be explored is the involvement of $\alpha 6 \beta 4$ in actin dynamics. One model of filopodial growth suggests that substrate-anchored proteins coupled with molecular motors at the base of filopodia regulate the rearward flow of actin, while those at the tip may regulate the polymerization of actin (47). Based on this model and our finding that $\alpha 6 \beta 4$ was found in clusters at the roots of filopodia in association with actin, one possibility is that $\alpha 6 \beta 4$ functions as a substrate-anchoring molecule that is involved in reducing the rearward flow of actin at the roots of filopodia. Interestingly, reduction of the rearward flow of actin has been shown to induce cell protrusions (33). Another likely possibility is that a signaling cascade initiated by ligation of $\alpha 6 \beta 4$ is linked to the mechanism of actin polymerization and filopodial growth. For example, the GTPase cdc42 (28, 40) has been implicated in filopodial formation in fibroblasts, and the possibility that $\alpha 6 \beta 4$ regulates the activity of such molecules is attractive.

An interesting aspect of this study is the apparent division of labor between the $\alpha 6 \beta 4$ and $\beta 1$ integrins in mediating clone A interactions with laminin-1. Previously, we

demonstrated that clone A cells use the $\alpha 6 \beta 4$ and $\alpha 2 \beta 1$ integrins to interact with laminin-1 (32, 34). The mAb inhibition data presented here extend these observations by demonstrating that a $\beta 1$ integrin, presumably $\alpha 2 \beta 1$, is essential for the adhesion and spreading of clone A cells on laminin-1 but that $\alpha 6 \beta 4$ is not required for these processes. As shown, the $\alpha 6 \beta 4$ integrin appears to be involved in a much more specific function in clone A cells, the formation and stabilization of filopodia and lamellae. Although it is difficult to assess the role of $\beta 1$ integrins in these events directly because function-blocking mAbs cause the cells to detach from laminin-1, it is likely that they are essential. This possibility is supported by our finding that both $\alpha 6 \beta 4$ and $\beta 1$ integrins are localized in filopodia and lamellae. A reasonable hypothesis based on these observations is that the $\alpha 6 \beta 4$ and $\alpha 2 \beta 1$ integrins mediate distinct signaling pathways in response to their ligation by laminin-1 and that both signaling pathways are required for the migration of clone A cells on laminin-1.

The cytoskeletal associations of the $\alpha 6 \beta 4$ integrin that we have characterized in clone A cells should be compared with recent studies on the molecular interactions between $\alpha 6 \beta 4$ and cytokeratins (16). There is now evidence that the hemidesmosomal proteins BPAG-2 (bullous pemphigoid antigen) and HD-1 interact with $\alpha 6 \beta 4$ (20, 44), and that HD-1 can provide the link between $\alpha 6 \beta 4$ and cytokeratins (55). Another hemidesmosomal protein, BPAG-1, has been shown to be essential in the attachment of cytokeratins to the hemidesmosome, although an interaction with $\alpha 6 \beta 4$ has not been demonstrated (17). Clone A cells express HD-1 but they do not express either of the BPAG proteins (data not shown), and they do not contain classical hemidesmosomes (32). In this regard, however, another type of hemidesmosome (type II), has been described in a mammary epithelial cell line that contains only $\alpha 6 \beta 4$ in association with HD-1 (55). Type II hemidesmosomes have also been observed in HT29-Fu cells, a colon carcinoma cell line that was reverted to a differentiated phenotype by fluorouracil treatment (14). An important difference, however, between these cells and clone A cells is that the distribution pattern of $\alpha 6 \beta 4$ in the mammary epithelial and HT29-Fu cells did not suggest an association with actin (14, 55). Although the molecular basis for these differences in the cytoskeletal associations of $\alpha 6 \beta 4$ are not known, an invasive carcinoma cell such as clone A may be an extremely useful cell type to define the molecules that link $\alpha 6 \beta 4$ to the actin cytoskeleton and the factors that promote this association. In clone A cells, HD-1 is probably not involved in $\alpha 6 \beta 4$ linkage with actin because preliminary studies indicate that HD-1 is not present in the filopodia and edges of the lamellae of these cells (Rabinovitz, I., unpublished results). Regardless of the mechanism, however, a shift from a stable interaction of $\alpha 6 \beta 4$ with cytokeratins to a more dynamic interaction with the actin cytoskeleton could have important implications for epithelial cell migration and tumor progression.

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Note Added in Proof. Since submission of this manuscript, we have shown that the mechanism by which the $\alpha 6 \beta 4$ integrin mediates the formation of lamellae involves activation of phosphoinositide 3-OH kinase (Shaw, L.M., I. Rabinovitz, H. Wang, A. Toker, and A.M. Mercurio. 1997. *Cell*. In press).

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Brief Report

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

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

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

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

Introduction

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

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

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

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

Materials and Methods

Cells and Antibodies

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

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

Microscopic Analyses

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

Migration Assays

The lower compartments of Transwell chambers (6.5-mm diam, 8 μ m pore size; Costar) were coated for 30 min with 15 μ g/ml laminin-1 diluted

in RPMI medium. RPMI/BSA was added to the lower chamber and cells (1×10^6) suspended in RPMI/BSA were added to the upper chamber. After incubating for 5 h at 37°C, cells were removed from the upper chamber with a cotton swab and cells that had migrated to the lower surface of the membrane were fixed, stained with crystal violet or for β -galactosidase (β -gal), and quantified as described previously (Shaw et al., 1997).

RhoA Activity

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

Results

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

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

Our observation that RhoA functions in lamellae for-

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

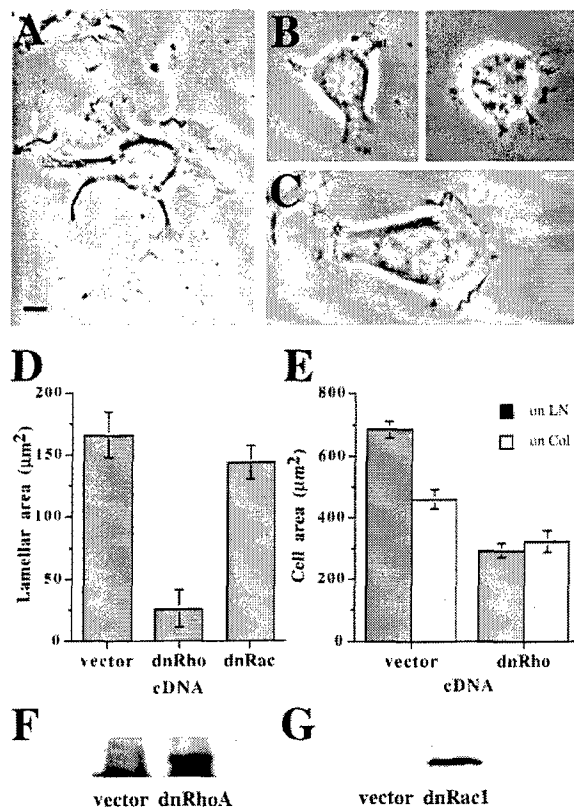


Figure 1. Dominant negative RhoA inhibits membrane ruffling and lamellae formation in clone A cells in response to laminin-1. Clone A cells were cotransfected with a GFP construct and either a control vector or a vector encoding N19RhoA or N17Rac as described in Materials and Methods. Cells were plated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

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

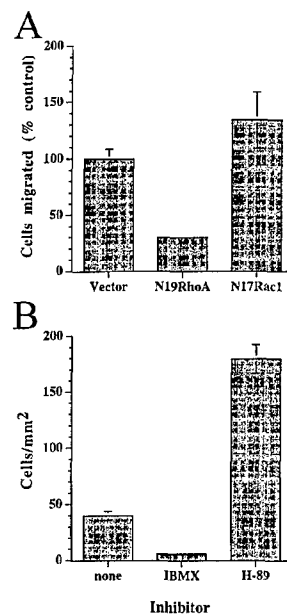


Figure 2. Effects of dominant negative RhoA (N19RhoA) and cAMP metabolism on laminin-1 stimulated migration. A, Clone A cells that had been cotransfected with a β -gal cDNA and either N19RhoA, N17Rac1, or control vector were assayed for migration on laminin-1 as described in Materials and Methods. Migration was scored as the relative number of β -gal staining cells migrated compared with the vector only control. Transfection rates were comparable. B, Clone A cells were left untreated or treated with 1 mM IBMX or 15 μ M H-89 for 15 minutes and then assayed for laminin-1 mediated migration as described in Materials and Methods. Migration rates were reported as the number of cells migrated per mm². Bars represent mean $\pm$ SD from triplicate determinations.

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

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

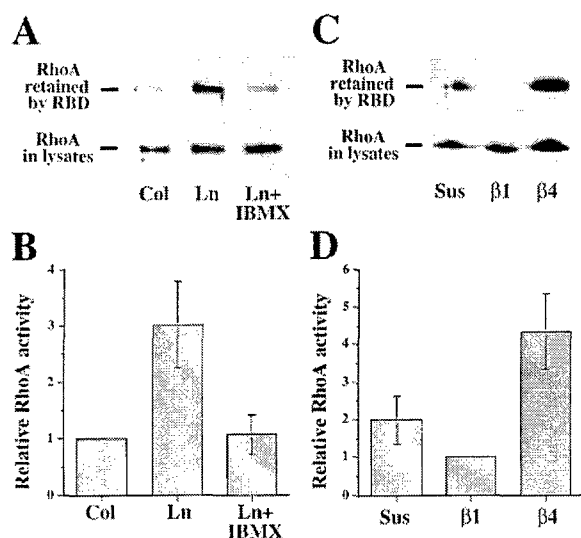


Figure 3. Engagement of the $\alpha 6 \beta 4$ integrin by either laminin-1 or antibody-mediated clustering activates RhoA. **A** and **B**, Clone A cells were plated on either collagen or laminin for 30 min or pretreated with 1 mM IBMX for 15 min and then plated on laminin for 30 min. Cell extracts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

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

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

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

Discussion

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

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

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

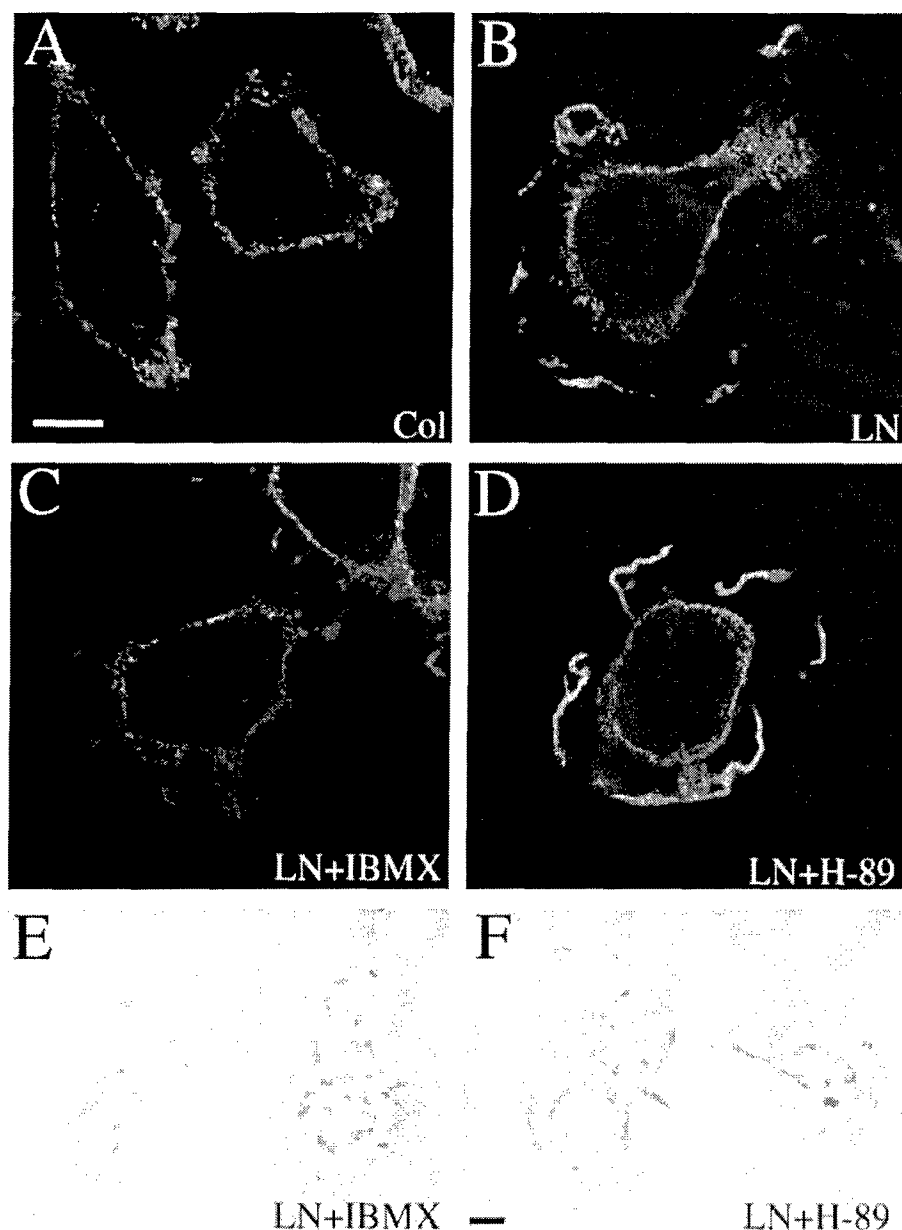


Figure 4. Laminin-1, but not collagen-I, promotes the colocalization of RhoA and $\beta 1$ integrin in membrane ruffles in a cAMP-sensitive manner. A–D, Clone A cells were plated on either collagen I (A) or laminin-1 (B–D) for 30 min, fixed, and stained for both $\beta 1$ integrin and RhoA using indirect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

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

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

Interestingly, expression of the dominant negative

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

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

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Towards a mechanistic understanding of tumor invasion—lessons from the $\alpha 6 \beta 4$ integrin

Arthur M. Mercurio* and Isaac Rabinovitz

This review explores the mechanistic basis of carcinoma migration and invasion by focusing on the contribution of integrins. Integrins are essential for invasion not only for their ability to mediate physical interactions with extracellular matrices, but also for their ability to regulate signaling pathways that control actin dynamics and cell movement, as well as for growth and survival. Our comments center on a unique member of the integrin family, the $\alpha 6 \beta 4$ integrin, which is a receptor for the laminin family of basement membrane components. Numerous studies have implicated this integrin in the invasion of solid tumors and have provided a rationale for studying the mechanistic basis of its contribution to the invasive process. Such studies have revealed novel insights into the mechanism of carcinoma invasion that involve both the dynamics of cell migration and signaling pathways that regulate this migration.

Key words: invasion / cancer / integrin / signaling / cytoskeleton

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Introduction

Overview

Invasion, or the penetration of tumor cells into adjacent tissues, is one of the hallmarks of malignant tumors.¹ In contrast to benign tumors that are encapsulated, invasive tumors have the potential to

metastasize because of their access to lymphatics and the vasculature. Invasion can also result in patient morbidity and mortality in the absence of metastasis. For these reasons, understanding the process of invasion is of obvious importance. The observations and insights of pathologists have taught us much about the nature of tumor invasion and have provided a foundation for more mechanistic studies.¹ We know, for example, that the breaching of the basement membrane by carcinoma cells is a defining event for malignant tumors, because it allows their 'escape' into the stroma and beyond. This observation spawned the 'three-step' model of invasion.² This model implied that tumor cells attach themselves to the basement membrane and then secrete proteases that degrade localized regions of the basement membrane, enabling their migration into stroma. The most significant contribution of this model was that it identified critical components of the invasive process that could be studied in more detail, such as adhesion and proteolysis. As a result, we know much, for example, about receptors on tumor cells that mediate interactions with basement membrane components and proteases that degrade these components. We also know that the invasive process is more complex than originally envisioned. A significant advance in this regard was the realization that highly invasive carcinoma cells often lose contact with each other and exhibit a mesenchymal or motile phenotype that is distinct from normal epithelial structure.^{3–5} This realization led to the hypothesis that an epithelial to mesenchymal transition is a major component of the invasive process. Subsequent studies on the loss of cadherin-mediated cell–cell adhesion in invasive carcinomas established a mechanistic basis for this epithelial to mesenchymal transition.^{6–9} In addition, the advent of molecular cell biology has provided a new prospectus on signaling molecules and cytoskeletal dynamics that could regulate invasion. Also, the realization that invading

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tumor cells must survive in foreign and often hostile environments has made the study of survival mechanisms an integral component of tumor progression. These advances are enabling us to approach the problem of invasion at a fundamental level.

Cell migration and invasion

The phrase 'cell migration and invasion' is used quite frequently these days with the implication that migration is an integral component of the invasive process (see also Kassis *et al.*, this issue). Most of the evidence to implicate cell migration in the invasion of primary carcinomas derives from inferences made from the pathological analysis of tumors. Such analyses have revealed that carcinomas can invade as cell sheets or as single cells, the latter morphology indicative of an epithelial to mesenchymal transition and associated with the most aggressive cancers.^{6,8,9} In addition, numerous experimental observations indicate that the translocation of tumor cells from one tissue compartment to another (e.g. epithelium to stroma) involves active cell movement.¹⁰⁻¹³ Thus, the assertion that cell migration is a key component of invasion is justified. Moreover, there is ample evidence to indicate that invasion is facilitated by localized proteolysis² and the deposition of novel ECM proteins by the tumor cells themselves.^{10,11,14}

The importance of cell migration in the invasive process warrants a rigorous analysis of the motility mechanisms used by tumor cells. Essentially, cell migration involves dynamic and coordinated interactions among ECM receptors, growth factor receptors and the actin cytoskeleton. More specifically, the engagement of ECM and growth factor receptors initiates signaling cascades that result in localized actin polymerization in the direction of cell movement coupled with the establishment of traction forces necessary for migration¹⁵⁻¹⁸ (see also Segall *et al.*, this issue). Progress in understanding this 'migratory' component of the invasive process has accelerated in recent years, especially due to its appeal to molecular cell biologists.

The purpose of this review is to explore the mechanistic basis of carcinoma migration and invasion by focusing on the contribution of integrins, a major family of ECM receptors. Indeed, integrins are essential for invasion not only for their ability to mediate physical interactions with ECMs, but also for their ability to regulate signaling pathways that control actin dynamics and cell movement, as well as for growth and survival.^{19,20} Integrins also contribute

to the function and localization of proteases involved in tumor invasion.²¹ Our comments will center on a unique member of the integrin family, the $\alpha 6 \beta 4$ integrin, which is a receptor for the laminin family of basement membrane components.^{22,23} Numerous studies have linked this integrin to the invasion of many solid tumors and have provided a rationale for studying the mechanistic basis of its contribution to the invasive process (Table 1 and reviewed in Reference 24). In fact, recent work by our group and others on the $\alpha 6 \beta 4$ integrin, that will be discussed here, have revealed novel insights into the mechanism of carcinoma invasion that involve both the mechanical aspects of cell migration and signaling pathways that regulate this migration.

The $\alpha 6 \beta 4$ integrin—A functionally diverse receptor

The $\beta 4$ integrin subunit associates exclusively with the $\alpha 6$ subunit to form the $\alpha 6 \beta 4$ heterodimer.²⁵ The $\alpha 6$ subunit also associates with the $\beta 1$ subunit to form $\alpha 6 \beta 1$, and both $\alpha 6 \beta 1$ and $\alpha 6 \beta 4$ are receptors for the laminins.²³ The distinguishing feature of the $\alpha 6 \beta 4$ integrin is the cytoplasmic domain of the $\beta 4$ subunit.²⁶⁻²⁸ The $\beta 4$ cytoplasmic domain is distinct both in size (approximately 1000 aa) and structure from any other integrin subunit. Two pairs of fibronectin type III repeats separated by a connecting segment characterize this domain. Although some progress has been made in identifying specific regions and motifs within this domain that mediate $\alpha 6 \beta 4$ -specific functions, this domain is largely 'terra incognita' and ripe for investigation. In this direction, the crystal structure of the first tandem pair of FN repeats was reported recently providing a level of insight that should facilitate structure-function studies.²⁹

From a functional perspective, it is well-established that $\alpha 6 \beta 4$ is essential for the organization and maintenance of epithelial architecture. In many epithelia, $\alpha 6 \beta 4$ mediates the formation of stable adhesive structures termed hemidesmosomes on the basal cell surface that link the intermediate filament cytoskeleton with laminins in the basement membrane.^{30,31} In addition to $\alpha 6 \beta 4$, the classical hemidesmosome contains at least three other known proteins: BPAG-1, BPAG-2 and HD1/plectin.³¹ A second type of hemidesmosome has been described that contains only $\alpha 6 \beta 4$ and HD1/plectin.³² Recent studies have provided evidence that $\alpha 6 \beta 4$ is linked to intermediate filaments through HD1/plectin, and that this interaction is critical for hemidesmosome formation.³³⁻³⁵ BPAG-1 has also been shown to link

Table 1. Evidence to support the hypothesis that expression of the $\alpha 6 \beta 4$ integrin is maintained in carcinoma and that expression of this integrin can be linked to tumor aggressiveness and patient survival. See text and Reference 24 for more details and critical discussions of the literature

Carcinoma	Integrin $\alpha 6 \beta 4$ Expression	Tumor Behavior/ Patient Prognosis	References
Squamous	Increased expression associated with progression	Expression linked to reduced survival	41,42
Breast	Expression maintained in tumors	Co-expression with laminin linked to poor prognosis	48
Thyroid	Neoexpression in tumors	Expression linked to tumor invasion	44
Bladder	Expression increased in tumors	Expression linked to reduced survival	102
Colorectal	Expression maintained in tumors	Expression correlated inversely with tumor differentiation	43
Gastric	Expression increased at the interface between tumor and stroma	ND	103

the hemidesmosome to intermediate filaments.³⁶ The importance of the $\alpha 6 \beta 4$ integrin in hemidesmosome function and epithelial architecture has been reinforced by the generation of $\beta 4$ -nullizygous mice.^{37,38} The most obvious defect in these mice is a loss of hemidesmosomes and detachment of the epidermis.

Although the $\beta 4$ integrin subunit was identified initially as a tumor-associated antigen (TS180) associated with metastasis,^{39,40} a role for $\alpha 6 \beta 4$ in mediating the process of carcinoma invasion was not anticipated given its widely known function of mediating rigid adhesive contacts in epithelia. In fact, a prevailing opinion only a few years ago was that $\alpha 6 \beta 4$ expression had to be suppressed to enable tumor cells to progress to invasive carcinoma.²⁴ This opinion was tempered, however, by reports that expression of $\alpha 6 \beta 4$ was maintained or even increased in several types of invasive and metastatic carcinomas and that $\alpha 6 \beta 4$ expression levels actually correlated with the progression of these carcinomas (Table 1). However, other studies indicated that expression was reduced in some carcinomas (reviewed in Reference 24). A common observation in many of these studies was that the localization of the $\alpha 6 \beta 4$ integrin in invasive and metastatic carcinoma cells is diffuse in comparison to its polarized, basal localization in normal epithelia. This altered localization of $\alpha 6 \beta 4$

could account for the apparent decrease in $\alpha 6 \beta 4$ expression reported for several tumors. Moreover, the loss of polarization of the $\alpha 6 \beta 4$ integrin could be related to the invasive phenotype of carcinomas. In murine squamous papillomas, for example, the loss of polarization and diffuse suprabasilar expression of $\alpha 6 \beta 4$ integrin has been associated with a high risk of malignant progression⁴¹ and in human head and neck carcinomas a similar phenotype has been related to poor prognosis.⁴² It is also interesting to note that studies that have examined $\beta 4$ integrin expression using more quantitative methods, such as immunoblots and Northern blots, have observed an increase in the expression of this subunit in some carcinomas in comparison to normal tissue.^{43,44}

Definitive evidence to implicate $\alpha 6 \beta 4$ in invasion was provided initially by the finding that exogenous expression of this integrin in $\beta 4$ -deficient colon and breast carcinoma cell lines dramatically increased their ability to invade Matrigel, a reconstituted basement membrane preparation.^{45,46} Subsequent studies by others substantiated the involvement of $\alpha 6 \beta 4$ in invasion.^{44,47-50} More recently, the interesting observation was made that androgen receptor expression in prostate carcinoma cells suppresses $\alpha 6 \beta 4$ expression and the invasive phenotype.⁵⁰ This observation suggests that the loss of androgen receptor expression, a common feature of aggressive

prostate tumors, stimulates $\alpha 6 \beta 4$ expression and $\alpha 6 \beta 4$ -mediated invasion.

The data described above provide evidence to implicate $\alpha 6 \beta 4$ in the invasive process, but they do not provide a mechanism to explain how an integrin, which associates with intermediate filaments and forms stable adhesive contacts, could promote the dynamic process of invasion. Indeed, the presence of $\alpha 6 \beta 4$ -containing hemidesmosomes would impede invasion. A significant breakthrough, therefore, was our finding that $\alpha 6 \beta 4$ can associate with F-actin and is localized at the leading edges of invasive carcinoma cells.⁵¹ Moreover, we demonstrated that $\alpha 6 \beta 4$ actually mediates the migration of such cells through its ability to associate with the actin cytoskeleton and promote the formation and stabilization of filopodia and lamellae.^{51,52} This finding implied that the function and cytoskeletal association of $\alpha 6 \beta 4$ in invasive carcinoma cells are distinct from its established role of anchoring epithelial cells to the basement membrane through its association with cytokeratins.

A second significant finding that provided a mechanistic basis for the involvement of $\alpha 6 \beta 4$ in invasion was that this integrin stimulated the activity of the enzyme phosphoinositide 3-OH kinase (PI3-K) in invasive carcinoma cells and that PI3-K is essential for invasion.⁴⁶ In fact, it now appears that the signaling properties of $\alpha 6 \beta 4$ may be distinct both quantitatively and qualitatively from those of other integrins and it is these signaling properties that contribute to its ability to promote carcinoma migration and invasion. Our findings on the mechanical and signaling properties of the $\alpha 6 \beta 4$ integrin will be used as focal points in this review to discuss the dynamics of carcinoma invasion.

Dynamics of carcinoma migration and invasion

Overview

Consider the dynamics involved in initiating the migration of polarized epithelial cells, which are anchored to the basement membrane by $\alpha 6 \beta 4$ -containing hemidesmosomes. Although this migration can involve sheets of epithelial cells as seen, for example, in epithelial wound healing,^{53,54} numerous studies have demonstrated that their migration and invasion are facilitated by a loss of cell-cell adhesion and a consequent epithelial to mesenchymal transition.⁶⁻⁹ In fact, many invasive carcinomas are characterized by loss of function mu-

tations in molecules that mediate cell-cell adhesion such as E-cadherin and the catenins.⁶ In addition to mutational inactivation, cell-cell adhesions can be disrupted by growth factors such as HGF and EGF/TGF α that induce the migration of epithelial cells.^{55,56} The importance of these cell adhesion molecules in regulating the invasive phenotype has led to their designation as 'suppressors of invasion'.

Another mechanical concern that has not been considered as extensively as the loss of cell-cell adhesion is the fact that hemidesmosomes anchor cells to the matrix tightly and their presence impedes migration and invasion. As mentioned above, this concern led to the hypothesis that the expression of $\alpha 6 \beta 4$ had to be suppressed in order for tumors to progress. We now know, however, that $\alpha 6 \beta 4$ expression is maintained, if not increased, in many invasive carcinomas, even though hemidesmosomes are not detected (Table 1). The finding that $\alpha 6 \beta 4$ is found in association with F-actin in lamellipodia, filopodia, and retraction fibers of invasive carcinoma cells is consistent with an altered localization of this integrin in tumor cells. Our work has also revealed that $\alpha 6 \beta 4$ can be mobilized from its association with intermediate filaments in hemidesmosomes and incorporated into F-actin structures such as lamellipodia in response to stimuli that induce migration (Figure 1 and Reference 57). In particular, we observed that stimulation of squamous-derived carcinoma cells with EGF, which stimulates their chemotactic migration, mobilizes $\alpha 6 \beta 4$ from its association with intermediate filaments, dissolves the hemidesmosome and enables $\alpha 6 \beta 4$ to be recruited into lamellipodia that are formed in response to EGF (Figure 1). The chemotactic response induced by EGF in this manner is dependent on the function of $\alpha 6 \beta 4$ when cells are plated on laminin. The mechanism by which EGF exerts these effects involves activation of PKC- α and the consequent phosphorylation of the $\beta 4$ cytoplasmic domain mainly on serine residues,⁵⁷ although it has also been reported that EGF stimulates the tyrosine phosphorylation of this subunit.⁵⁸ Although the causal significance of $\beta 4$ phosphorylation remains to be established, these findings provide an important link between the mobilization of $\alpha 6 \beta 4$ from hemidesmosomes and its involvement in chemotactic migration. It is also interesting to consider the possibility that the disruption of cell-cell adhesion and the mobilization of $\alpha 6 \beta 4$ integrin from hemidesmosomes are coordinated events that facilitate carcinoma migration and consequent invasion and that both of these events

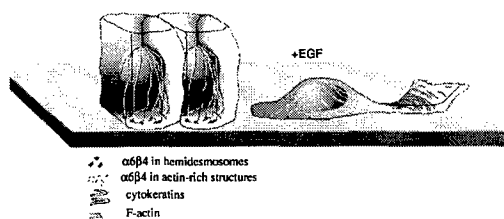


Figure 1. Growth factor mobilization of the $\alpha 6 \beta 4$ integrin from its association with cytokeratins in hemidesmosomes to F-actin in lamellae and lamellipodia. A chemotactic stimulus such as EGF can disassemble hemidesmosomes and promote the formation of $\alpha 6 \beta 4$ -containing lamellipodia and lamellae. These findings establish a mechanism for the dichotomy of $\alpha 6 \beta 4$ function in stably adherent and migrating epithelial-derived cells. An important implication of this model is that chemotactic factors can drive the migration of invasive carcinoma cells by mobilizing $\alpha 6 \beta 4$ and disassembling hemidesmosomes. For details, see text and Reference 57.

may be regulated by stimuli such as growth factors that induce migration.

An important issue that has yet to be resolved is the molecular nature of the association between $\alpha 6 \beta 4$ and F-actin. Most likely, this association is indirect because the $\beta 4$ cytoplasmic domain lacks a consensus actin-binding motif. One potential 'linker' molecule is HD1/plectin, which also connects the $\beta 4$ subunit to intermediate filaments (see above), because it contains an actin-binding site.⁵⁹ Although it has been reported that actin and $\beta 4$ compete for a similar site on HD1,⁶⁰ this consideration would not necessarily exclude HD1 as the connecting molecule. HD1 is a rod-like tetramer with binding sites for actin and $\beta 4$ on each end; thus, it could bind $\beta 4$ on one end and F-actin on the other.^{61,62} Regardless, a better understanding of the interaction between the $\alpha 6 \beta 4$ integrin and F-actin and the mechanisms that regulate this interaction will expand our knowledge of carcinoma migration and invasion.

Dynamics of migration

Let us now consider the dynamics of migration itself and the contribution of the $\alpha 6 \beta 4$ integrin to this process. The migration of many cells in culture involves actin polymerization that generates protrusions at the edge of the cell and the contraction of the actin network by associated myosin motors.¹⁵⁻¹⁸ The forces created by the actomyosin system are transmitted to the substrate, creating the necessary traction to produce forward movement that results in the net

translocation of the cell body and a subsequent detachment of the trailing edge of the cell. Integrins are essential for migration not only because they anchor cells to the substratum, but also because they mediate the dynamic formation of adhesive contacts that is necessary to generate traction.¹⁶⁻¹⁸ Moreover, integrin-mediated adhesion at the rear of a migrating cell must be disrupted to enable forward movement.⁶³ Our interest has been to understand the contribution of the $\alpha 6 \beta 4$ integrin to the migration process and to relate this involvement to invasion. Clearly, $\alpha 6 \beta 4$ is not essential for invasion. Carcinoma cells that lack expression of this integrin migrate and invade. However, expression of $\alpha 6 \beta 4$ appears to enhance migration and invasion quite significantly, and we have pursued the mechanisms involved.

One mechanism by which the $\alpha 6 \beta 4$ integrin enhances migration may be to anchor nascent filopodia to the ECM. We observed that many carcinoma cells protrude filopodial extensions that contain discrete clusters of $\alpha 6 \beta 4$ and that these filopodia anchor to a laminin substrate at these sites of $\alpha 6 \beta 4$ expression.⁵¹ This anchoring phenomenon is frequently followed by the extension of flat lamellae, and ultimately by the translocation of the cell body. The finding that this integrin exhibits a very high adhesive strength for laminin (100 dynes per cm^2),⁶⁴ supports a distinct role for $\alpha 6 \beta 4$ in anchoring filopodia to laminin matrices. Although in various cell models lamellipodia can extend without filopodia,⁶⁵ several studies have implicated filopodial anchoring as a means to direct cell movement.⁶⁶⁻⁶⁸ Stabilized filopodia can act as a traction track for myosin II motors to facilitate the formation of a spreading edge, similar to the way retraction fibers promote cell re-spreading after mitosis.^{15,68} This model of filopodial extension invokes the concept of molecular clutching, in which actin fibers that normally undergo retrograde flow are 'clutched' to the substrate by the engagement of an adhesion receptor that links actin to the matrix.¹⁵ Such clutching could result in the movement of the cell towards the point of attachment. The high adhesive strength of $\alpha 6 \beta 4$ for laminin would support its function as a clutch for cells migrating on laminin matrices.

The $\alpha 6 \beta 4$ integrin may also stimulate migration by contributing to the generation of traction by lamellae. It has become apparent from studies on fish keratocytes and fibroblasts that adhesion to the substrate in the lamellar area provides the necessary resistance to support the propulsive forces generated by the cytoskeleton to haul the rest of the cell.^{69,70} We would

argue that the presence of $\alpha 6 \beta 4$ in carcinoma cells would increase the stability of lamellae and, consequently, migration because this stability is dependent upon the adhesive strength of the integrins that mediate these adhesive contacts. In fact, we have shown that $\alpha 6 \beta 4$ is localized to the base of lamellae in carcinoma cells migrating on laminin.⁵¹ Moreover, the formation of actin bundles parallel to the lamella in such cells, which is considered to be crucial for the generation of locomotive traction in the keratocyte and fibroblast models, is dependent on $\alpha 6 \beta 4$.⁵¹ We have observed, in fact, that the $\alpha 6 \beta 4$ integrin can mediate traction under the lamella using a system that measures traction forces (unpublished data).

Dynamics of invasion

The foregoing commentary on the mechanical contribution of $\alpha 6 \beta 4$ to migration raises the issue of how this integrin actually functions in carcinoma invasion. Although a common assumption is that invasion involves transgression of the basement membrane by tumor cells and subsequent direct migration through stroma,² it is known that invasive tumors can secrete basement membrane components and assemble them into structures that range from seemingly intact to sparse patches of basement membrane.^{71,72} These tumor-produced basement membranes could facilitate invasion by providing a provisional matrix for the tumor cells to migrate over the stroma using laminin receptors such as $\alpha 6 \beta 4$. It is worth noting in this regard that the reorganization or remodeling of basement membranes and other ECMs to generate 'paths' for migration has been documented for several types of cells *in vitro*. For example, endothelial cells remodel reconstituted basement membranes into tubular paths through which they travel.⁷³ In this respect, we observed that colon carcinoma cells that express the $\alpha 6 \beta 4$ integrin remodel or 'compress' reconstituted basement membranes into very compact structures that surround the cells, and form paths connecting distant cell aggregates (unpublished data). The importance of $\alpha 6 \beta 4$ in this compression is evidenced by the fact that it is blocked by antibodies that inhibit the function of this integrin. Also, exogenous expression of $\alpha 6 \beta 4$ in $\beta 4$ -deficient breast carcinoma cells, which stimulates markedly their ability to invade,⁴⁶ correlates with a substantial increase in their ability to compress basement membranes (unpublished data). The ability of $\alpha 6 \beta 4$ to remodel basement membranes may derive from its ability to generate traction on such matrices.

This $\alpha 6 \beta 4$ -dependent remodeling is dependent on a functional actomyosin system and it appears that most of the compression occurs under the lamellae and not at the trailing edges (unpublished data).

One implication of the above observations is that $\alpha 6 \beta 4$ -dependent remodeling of basement membranes might be a mechanism by which carcinoma cells breach basement membranes. For example, this process could result in the compression of the basement membrane in discrete regions, a process that would deplete basement membrane components from surrounding regions and create 'gaps' through which carcinoma cells could escape (Figure 2). Even though the breaching of basement membranes has been attributed to protease action, it is likely from our studies on $\alpha 6 \beta 4$ that integrin-dependent remodeling contributes to this process. Interestingly, a correlation between remodeling activity and invasive capability has also been observed for melanoma cells, in which the most aggressive cells are more likely to remodel extracellular matrix.⁷⁴ Similar studies observed that highly invasive melanoma cells can remodel three-dimensional matrices, although partial matrix degradation was also observed.⁷⁵ Most likely, both remodeling activity and protease activity necessary are needed to maximize invasion.⁷⁶

Signaling of migration and invasion

Overview

The current literature abounds with studies on integrin-mediated regulation of signaling pathways that control cell growth, differentiation and survival, as well as cytoskeletal dynamics and cell migration. Moreover, it has become apparent that integrins often function in concert with specific growth factor receptors to execute these functions. A mechanistic understanding of invasion requires that integrins be identified that contribute to specific components of the invasive process and that the signaling pathways involved be elucidated. Such studies must incorporate the contribution of growth factor receptors, as well as those extracellular matrix components that interact with these integrins. Although multiple integrins and signaling pathways contribute to migration and invasion, our comments here will focus on the $\alpha 6 \beta 4$ integrin because of our interests and the fact that studies on this integrin have provided novel insights into the signaling of migration and invasion.

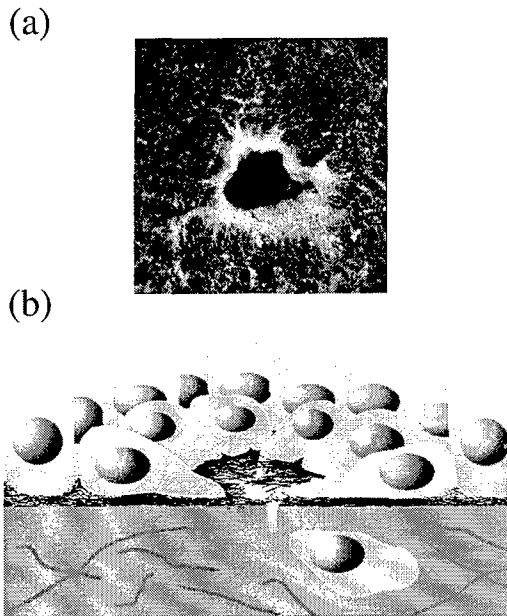


Figure 2. (a) Immunofluorescence image of a colon carcinoma cell plated on Matrigel for 4 h and stained with a laminin-specific Ab. Note that the cell (dark area in center) has compressed the Matrigel around itself. This compression is inhibited by Abs that block the function of the $\alpha 6 \beta 4$ integrin. (b) Schematic representation of tumor invasion by integrin-mediated remodeling of basement membrane. Compression or remodeling of the basement membrane in discrete regions, a process stimulated by the $\alpha 6 \beta 4$ integrin, would deplete basement membrane components from surrounding regions and create 'gaps' through which carcinoma cells could escape.

Central role of PI3-K

A link between $\alpha 6 \beta 4$ -stimulation of invasion and signal transduction was provided by our finding that this integrin activates phosphoinositide 3-OH kinase (PI3-K), a key signaling molecule, and that the activity of PI3-K is essential for invasion.⁴⁶ Other studies also established the importance of PI3-K in carcinoma invasion.⁷⁷ PI3-K phosphorylates phosphatidylinositol (PtdIns) lipids on the 3' position of the inositol ring resulting in an accumulation of PtdIns(3)P1, PtdIns(3,4)P2 and PtdIns(3,4,5)P3.⁷⁸ Accumulation of D3 phosphoinositides at the plasma membrane recruits secondary signaling molecules or effectors that mediate the diverse functions of PI3-K.⁷⁸ Given these considerations, it is important that the mechanism by which $\alpha 6 \beta 4$ activates PI3-

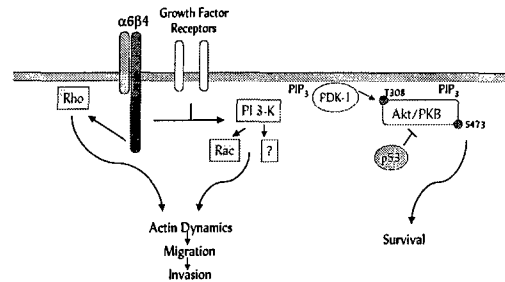


Figure 3. Signaling of carcinoma progression by the $\alpha 6 \beta 4$ integrin. Schematic representation of key signaling pathways regulated by the integrin that influence the migration and survival of carcinoma cells. For details, see text and References 46,91,92 and 98.

kinase and by which PI3-kinase promotes invasion be elucidated.

Interestingly, it appears that $\alpha 6 \beta 4$ is able to stimulate PI3-K activity to a higher level than other integrins, at least in carcinoma cell lines.⁴⁶ Although the mechanism by which $\alpha 6 \beta 4$ or any integrin activates PI3-K has not yet been established, recent studies have provided insight into this important problem. Falcioni and colleagues⁸⁰ reported that $\alpha 6 \beta 4$ associates with erbB2, a ligand orphan receptor of the EGFR family implicated in breast cancer progression,⁷⁹ on the surface of breast carcinoma cell lines. Subsequently, this group⁴⁹ made the important observation that both $\alpha 6 \beta 4$ and erbB2 are required for the activation of PI3-K and the stimulation of invasion using a 3T3 cell model system. The implication of this finding is that the cooperation of $\alpha 6 \beta 4$ integrin signaling with specific growth factor receptor signaling is required for PI3-K activation and consequent stimulation of invasion (Figure 3). The task ahead is to define the mechanism of this cooperative signaling. One hypothesis is that $\alpha 6 \beta 4$ potentiates growth factor receptor signaling by increasing the activation of a key signaling intermediate(s). Clearly, elucidation of this mechanism will be a significant advance not only because of the potential importance of $\alpha 6 \beta 4$ in invasion, but also because it will provide a paradigm for how integrin and growth factor receptors cooperate to drive the invasive process.

The mechanism by which activation of PI3-K promotes invasion is central to our understanding of carcinoma progression. One major function of this enzyme with respect to invasion appears to be its ability to regulate actin dynamics and, consequently, cell migration.^{46,77,81} For this reason, effectors of PI3-K

that have the potential to regulate actin dynamics, such as the Rho-family of GTPases, are prime targets for study.⁸² Several GTP exchange factors (GEFs) that stimulate the activity of these GTPases contain pleckstrin homology domains (PH) that bind the lipid products of PI3-K.⁸³ In fact, we reported that Rac is involved in carcinoma invasion and that it is activated by $\alpha 6 \beta 4$ /PI3-K signaling.⁴⁶ There is also evidence that cdc42, another GTPase that can be activated by PI3-K-dependent GEFs, is involved in invasion.⁷⁷ Interestingly, cdc42 can stimulate the actin nucleating activity of the Arp2/3 complex,⁸⁴⁻⁸⁷ providing a link between PI3-K signaling and actin dynamics. An emerging scenario, therefore, is that integrins and growth factor receptors 'signal' migration and invasion by regulating actin dynamics and that PI3-K is at the nexus of many of these signaling pathways (Figure 3).

The activation of PI3-K by $\alpha 6 \beta 4$ signaling has important implications not only for the migration of invasive carcinoma cells but also for their ability to survive outside the nurturing confines of the epithelium and in environments that are often pro-apoptotic such as the hypoxic areas of solid tumors.⁸⁸ In this regard, focus is on the Akt/PKB kinase, which is an effector of PI3-K^{89,90} and essential for the survival of most normal and tumor cells. PI3-K is essential for Akt/PKB activation because the D3 phosphoinositide products of PI3-K bind to its pleckstrin homology (PH) domain and recruit it to the cell membrane.^{78,90} These phosphoinositides also recruit another critical enzyme PI3-K-dependent kinase 1 (PDK1) to the membrane enabling it to phosphorylate Akt/PKB at threonine residue 308. This phosphorylation event induces Akt/PKB autophosphorylation at serine residue 473, which is the final step required for the stimulation of Akt/PKB kinase activity towards exogenous substrates (Figure 3 and Reference 91). Signaling through the $\alpha 6 \beta 4$ integrin activates Akt/PKB in carcinoma cell lines (Figure 3 and References 46 and 92). An implication of this finding is that $\alpha 6 \beta 4$ should also promote the survival of these cells. In fact, we observed that expression of $\alpha 6 \beta 4$ enhances the survival of breast carcinoma cells that have been deprived of both serum and matrix, conditions that normally promote detachment-induced apoptosis, also referred to as anoikis.⁹² Interestingly, however, this stimulation of AKT-mediated survival by $\alpha 6 \beta 4$ is apparent only in carcinoma cells that contain inactivating p53 mutations. In fact, the deprivation of wild type p53-expressing cells of both matrix and serum actually

triggers the caspase-3 mediated cleavage and inactivation of AKT and prevents $\alpha 6 \beta 4$ -mediated survival signaling.⁹² Thus, these findings demonstrate the importance of $\alpha 6 \beta 4$ signaling in promoting the AKT-dependent survival of carcinoma cells. This survival function could be particularly important in late-stage carcinomas, which exhibit a high frequency of p53 mutations and which are more prone to survive in conditions such as hypoxia.⁸⁸

Rho GTPase

The Rho GTPase is of particular interest with respect to the signaling of migration and invasion. Although most studies on Rho have illuminated its role in the formation of stress fibers and focal adhesions in fibroblasts,⁹³ it has become apparent that it also contributes to the dynamics of cell migration in epithelial cells and tumor cell invasion^{94,95} (see also Price and Collard, this issue). A recent study using gene chip technology identified RhoC, a Rho isoform, as a gene that is up-regulated in metastatic melanoma and confirmed its importance in tumor migration and invasion.⁹⁶ If Rho is important for tumor invasion, its mode of activation must be understood. Interestingly, $\beta 1$ integrins appear to be unable to activate Rho and may, in fact, suppress its activation both in fibroblasts⁹⁷ and carcinoma cells.⁹⁸ In contrast, we reported that the $\alpha 6 \beta 4$ integrin signals Rho activation in invasive carcinoma cells and that Rho is necessary for their ability to form lamellae and migrate (Figure 3 and Reference 98). Although the signaling pathway by which $\alpha 6 \beta 4$ activates Rho is not known, these observations provide evidence that the signaling properties of $\alpha 6 \beta 4$ are distinct from other integrins and that these signaling differences contribute to tumor migration and invasion. In other words, the argument can be made that expression of the $\alpha 6 \beta 4$ integrin in tumor cells facilitates Rho activation and enhances PI3-K activation, two key signaling molecules for invasion.

cAMP metabolism

The $\alpha 6 \beta 4$ integrin also contributes to another signaling pathway important for invasion, albeit a pathway that has not been studied as well as PI3-K or Rho GTPases. This pathway involves the metabolism of cAMP. Elevations in the intracellular cAMP concentration [cAMP]_i inhibit chemotactic migration.^{52,99} We observed that $\alpha 6 \beta 4$ can suppress the intracellular cAMP concentration by activating

phosphodiesterases, which degrade cAMP, and that phosphodiesterase activity is essential for the $\alpha 6 \beta 4$ -mediated enhancement of lamellae formation and chemotactic migration.⁵² Subsequent studies revealed that cAMP inhibits activation of the Rho GTPase in carcinoma cells,⁵² presumably by a mechanism that involves the PKA-dependent phosphorylation of Rho on Ser 188.^{100,101} Thus, it appears that $\alpha 6 \beta 4$ can influence Rho activation by direct signaling as described above or, indirectly, through the activation of PDEs.

Link between dynamics and signaling of invasion

The contribution of $\alpha 6 \beta 4$ signaling to invasion needs to be discussed within the context of the dynamics of migration and invasion highlighted in the first part of the review. Although the contribution of $\alpha 6 \beta 4$ to the mechanics of migration and invasion centers on its ability to engage laminins in the matrix, the signaling properties of this integrin probably facilitate these mechanics. Notably, the contractile forces that are converted to traction by $\alpha 6 \beta 4$ under lamellae could arise from $\alpha 6 \beta 4$ -mediated activation of Rho and the consequent Rho stimulation of actin myosin contraction. This $\alpha 6 \beta 4$ -mediated signaling of contraction may also be an important component of invasion that occurs by $\alpha 6 \beta 4$ -dependent basement membrane remodeling as we postulated above. In support of this possibility, we observed that inhibition of Rho kinase activity disrupts the $\alpha 6 \beta 4$ -mediated compression of Matrigel (unpublished data). Along the same lines, $\alpha 6 \beta 4$ stimulation of PI3-K and consequent changes in actin dynamics may contribute to the formation of filopodial and lamellar protrusions, processes that are also facilitated by the engagement of $\alpha 6 \beta 4$ with laminins in the matrix. Together, the studies on $\alpha 6 \beta 4$ exemplify the fruitfulness of integrating signaling studies with studies on the dynamics of migration and invasion.

Adhesion-independent signaling

Signaling through $\alpha 6 \beta 4$ may not always depend on the adhesive functions of this integrin. Several of our studies indicate that the expression of this integrin is sufficient to enhance migration and invasion, as well as lamellae formation, on non-laminin substrata such as collagen.^{46,52} Moreover, these functions are not blocked by mAbs that inhibit $\alpha 6 \beta 4$ adhesive interactions, an observation that discounts the possibility that the cells are adhering to laminins deposited on

the substrate by the tumor cells. Insight into the possible mechanism of this phenomenon was provided by a recent study that demonstrated self-association of the $\beta 4$ cytoplasmic domains,³³ a process that could initiate intracellular signaling events independently of ligand binding. The possibility that the signaling functions of $\alpha 6 \beta 4$ can occur independently of ligand binding has profound implications for tumor invasion, because it implies that the ability of $\alpha 6 \beta 4$ to stimulate those pathways is not limited to specific matrix environments.

Concluding comments

Studies on the $\alpha 6 \beta 4$ integrin have taught us much not only about the contribution of integrins to the invasive process but also about the nature of invasion itself. From the integrin perspective, an important lesson learned is that integrin function can differ markedly between normal cells and invasive tumor cells. Although the 'shift' in $\alpha 6 \beta 4$ function from mediating stable adhesive contacts in hemidesmosomes to promoting migration at the leading edges by associating with F-actin may be an extreme example, it is likely the functions of other integrins also differ in invasive cells. Another lesson learned from studies on the $\alpha 6 \beta 4$ integrin is the importance of integrin-growth factor receptor cooperation in the invasive process. Indeed, the functional cooperation between $\alpha 6 \beta 4$ and erbB2 to activate PI3-K and promote invasion is probably the 'tip of the iceberg' and other such cooperative interactions are likely to occur for $\alpha 6 \beta 4$, as well as for other integrins.

Although studies on $\alpha 6 \beta 4$ have shed light on the contribution of integrins to the invasive process, they have also revealed that $\alpha 6 \beta 4$ is distinct from other integrins in many aspects. As discussed, $\alpha 6 \beta 4$ is the only known integrin present in hemidesmosomes that can interact with intermediate filaments and be 'translocated' to F-actin structures at the leading edges of migrating cells (Figure 1). In addition, the ability of this integrin to activate signaling pathways important for invasion such as PI3-K and the Rho GTPase is distinct both quantitatively and qualitatively from other integrins (Figure 3). We propose that these distinct properties of $\alpha 6 \beta 4$ underlie its important contribution to the invasive process. In many respects, $\alpha 6 \beta 4$ exhibits properties more consistent with a growth factor receptor than an integrin. A final assessment of this integrin, however, awaits a detailed understanding of

the structure and function of the arcane cytoplasmic domain of the $\beta 4$ subunit.

Insight into the nature of tumor invasion has also been obtained from work on the $\alpha 6 \beta 4$ integrin. Most importantly, this work has revealed that remodeling or compression of basement membranes may be a mechanism to create openings and paths for tumor cell egress (Figure 2). Moreover, the mechanical and signaling properties of $\alpha 6 \beta 4$ could be an important component of this remodeling phenomenon. This mode of invasion has been relatively unexplored because the study of tumor invasion has focused on the contribution of proteases and proteolytic degradation of ECM. The contribution of proteases to invasion notwithstanding, a more comprehensive perspective that includes both ECM remodeling and proteolysis appears to be warranted. Indeed, invasion is likely to be a dynamic process that involves reciprocal interactions between the invading cells and the host environment.

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THE METASTATIC ODYSSEY

The Integrin Connection

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A seminal review published recently on the occasion of the millennium proposed that the myriad cellular alterations associated with cancer could be codified into six functional groups that define malignancy: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, limitless replicative potential, sustained angiogenesis, evasion of apoptosis and tissue invasion and metastasis.²⁴ From the perspective of cancer progression, or the sequence of events that enable tumor cells to become invasive and eventually metastatic, it is the latter three groups that are of paramount importance. The metastatic odyssey of a malignant carcinoma cell, for example, from its genesis in one of the major epithelia to a distal organ requires that this cell acquire a motile phenotype to invade through tissue and gain access to the vasculature and lymphatics. In addition, this cell will apoptose outside of the nurturing environment of its origin unless it adapts survival mechanisms. The survival of such cells depends, in part, on their ability to elicit an angiogenic response that is essential not only for their growth as primary tumors but also for their progression to metastasis.²³ Thus, the view that cancer progression mirrors Darwinian evolution is apt because progression selects for the survival of the fittest cells.^{24, 16}

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Although our knowledge of cancer progression is relatively naïve, recent advances in molecular cell biology are enabling a fundamental shift in the understanding of this complex process. In contrast to the descriptive, black-box view of metastasis that prevailed for much of the last century, it now appears feasible to define the progression to metastatic disease in terms of discrete, albeit complex, molecular events. The emerging view is that coordinated interactions among the extracellular environment (matrix proteins, growth factors, other cells), cell surface molecules (adhesion receptors, growth factor receptors, proteases), and intracellular molecules (signaling, cytoskeleton) are responsible for the metastatic phenotype. These interactions can be facilitated and sometimes circumvented by genetic alterations in oncogenes and tumor suppressor genes. The challenge ahead is to define these interactions for individual cancers and to assess the influence of cancer genes on their ability to impact specific components of the metastatic cascade.

Integrins, one class of adhesion receptors, are attractive targets for elucidating mechanisms involved in progression because of their central role in linking the extracellular environment to signaling pathways and the cytoskeleton.²⁵ This article highlights studies by the authors' groups and others on the contribution of a specific class of integrin receptors, the $\alpha 6$ integrins, to carcinoma progression. The purpose is to highlight the importance of these integrins to progression and also to illustrate how the study of one type of surface receptor can reveal much about the involvement of growth factor receptors, cytoskeletal proteins, and signaling molecules in progression. To accomplish this purpose, the authors focus comments on two critical components of carcinoma progression: invasion and survival.

CARCINOMA INVASION

Overview

Understanding the progression from carcinoma in situ to invasive and metastatic carcinoma is one of the most complex and challenging problems in the pathobiology of cancer. Because the biology of carcinoma is essentially an aberration of epithelial cell biology, insight into this problem has been obtained by comparing the properties of epithelial and carcinoma cells. Distinguishing features of epithelia are their polarized morphology, attachment to an underlying basement membrane, and presence of specialized cell-cell contacts.⁴⁶ The progression to invasive carcinoma has been shown to involve perturbations in these features resulting in the acquisition of a motile, mesenchymal phenotype. Notably, progression involves alterations in the expression and function of surface receptors that maintain the epithelial phenotype. Invasive carcinoma, for example, is characterized by a loss of function of cadherins, receptors that mediate cell-cell adhesion.⁴ Integrins, in contrast, are essential for normal epithelial function and for mediating dynamic processes associated with invasive carcinoma such as migration.^{25, 31}

Many studies have examined the possibility that carcinoma progression involves changes in the expression of specific integrins. To a large extent, these studies have used immunohistochemistry to assess integrin expression in normal epithelia and carcinoma. The existing literature encompasses all of the major types of carcinomas and most of the known integrins. A survey of this vast literature, however, leaves most readers confused and bewildered because of discrepancies among these studies and the realization that gross changes in integrin expression during progression are uncommon. A more constructive approach to this problem is to compare integrin function in epithelial and carcinoma cells. Most epithelial cells express several integrins on their basolateral surfaces including $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, $\alpha v\beta 3$ and $\alpha v\beta 6$.⁵¹ Presumably, all of these integrins contribute to the formation and maintenance of the epithelial phenotype. The $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins, in particular, are important for epithelial function because they anchor the epithelium to the basal lamina. Interestingly, it is these two integrins that appear to be critical for carcinoma invasion. The $\alpha 3\beta 1$ integrin has been shown to mediate cell motility and invasion in carcinomas and nonepithelial malignancies. The interaction between $\alpha 3\beta 1$ integrin and basement membrane protein laminin-5 has been shown to be important for cell motility in many experimental models.^{37, 63, 59, 19} The data on the contribution of the $\alpha 6\beta 4$ integrin to invasion are discussed later.

The $\alpha 6\beta 4$ Integrin and Carcinoma Invasion

In many epithelia, the $\alpha 6\beta 4$ integrin, which is a receptor for the laminins, mediates the formation of stable adhesive structures termed *hemidesmosomes* that link the intermediate filament cytoskeleton with specific laminins in the basement membrane.⁴¹ Expression of the $\alpha 6\beta 4$ integrin persists in many carcinoma cells that do not form stable adhesive contacts but rather exhibit the motile phenotype characteristic of invasive cancer. In fact, the $\beta 4$ subunit was identified initially as a tumor-associated antigen¹³ and numerous pathologic studies have correlated $\alpha 6\beta 4$ expression and localization with invasive carcinoma.^{13, 14, 20, 50, 64} Notable examples of the association between $\alpha 6\beta 4$ expression and carcinoma include the finding that this integrin is not expressed in the normal thyroid but induction of its expression correlates with the progression to invasive thyroid carcinoma.⁵⁰ Also, expression of the $\alpha 6\beta 4$ integrin is enhanced at the invading fronts of gastric carcinomas.⁵⁸ More recently, $\alpha 6\beta 4$ expression has been correlated with poor prognosis for breast cancer patients.⁵⁷ Such correlative studies have been substantiated by the findings that expression of $\alpha 6\beta 4$ in a $\beta 4$ -deficient colon and breast carcinoma cells dramatically increases the invasive potential of these cells (Fig. 1).^{9, 53, 56} Subsequent studies by the authors' group provided evidence that the $\alpha 6\beta 4$ integrin promotes invasion by stimulating lamellae formation and the chemotactic migration of carcinoma cells (see Fig. 1).⁴³ Collectively, the available data support an important contribution of the $\alpha 6\beta 4$ integrin to carcinoma invasion. More

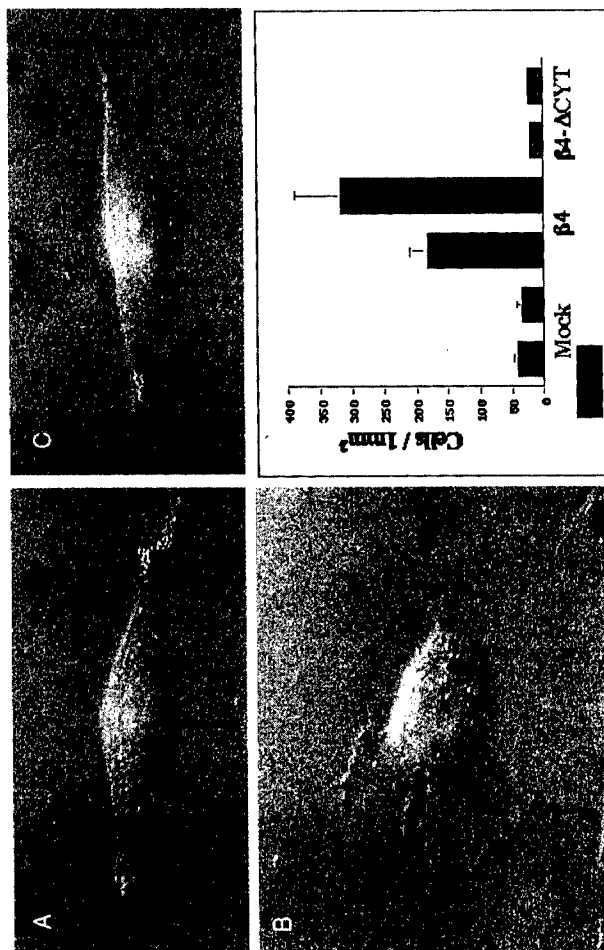


Figure 1. Cooperation between $\alpha 6 \beta 4$ integrin and growth factor signaling promotes lamellae formation and invasion. Human breast carcinoma cells (MDA-MB-435) that either lack expression of the $\alpha 6 \beta 4$ integrin (C) or express this integrin (A and B) were plated onto coverslips that had been coated with 20 μ g/mL collagen I. Cells were allowed to adhere for 2 hours at 37°C and then treated with lysophosphatidic acid (LPA) for 5 minutes (B and C) or left untreated (A). The cells were visualized using Nomarski DIC optics. Note the large lamella that is formed in response to LPA stimulation of cells that express $\alpha 6 \beta 4$ (B) but not in cells that lack expression of this integrin (C). Also, note that $\alpha 6 \beta 4$ expression does not result in lamellae formation in the absence of LPA stimulation (A). The influence of $\alpha 6 \beta 4$ expression on the chemoinvasion of MDA-MB-435 cells using a standard Matrigel assay (D). Note expression of a cytoplasmic domain deletion of the $\beta 4$ subunit ($\beta 4$ - Δ CYT) does not stimulate invasion.

data are needed, however, to substantiate this finding. In particular, the involvement of $\alpha 6 \beta 4$ in progression needs to be validated in transgenic models of cancer and more studies are needed to assess the prognostic significance of $\alpha 6 \beta 4$ expression in human carcinomas.

Cytoskeletal Interactions of the $\alpha 6 \beta 4$ Integrin

The involvement of the $\alpha 6 \beta 4$ integrin in invasion and migration conflicts with the established function for this integrin in mediating stable adhesive contacts in hemidesmosomes. In simple terms, migration is a dynamic process that requires the rapid formation and disassembly of adhesive contacts.³¹ The presence of $\alpha 6 \beta 4$ -containing hemidesmosomes would impede such dynamic events. A significant breakthrough, therefore, was the authors' finding that the $\alpha 6 \beta 4$ integrin can associate with F-actin and is localized at the leading edges of invasive carcinoma cells.⁴⁴ Moreover, the authors demonstrated that $\alpha 6 \beta 4$ actually mediates the migration of such cells through its ability to associate with the actin cytoskeleton and promote the formation and stabilization of filopodia and lamellae.⁴⁴ This finding implied that the function and cytoskeletal association of $\alpha 6 \beta 4$ in invasive carcinoma cells are distinct from its established role of anchoring epithelial cells to the basement membrane through its association with cytokeratins. The ability of $\alpha 6 \beta 4$ to associate with the actin cytoskeleton and promote migration does not appear to be limited to invasion. Studies on wound healing in intestinal epithelia, a process that involves the migration of cells at the wound edge, have revealed that $\alpha 6 \beta 4$ is localized in lamellipodia of such migrating cells and that it functions in their migration.^{32, 33}

The observations on $\alpha 6 \beta 4$ and migration raise two important issues: the mechanism by which hemidesmosomes are disrupted to enable $\alpha 6 \beta 4$ to interact with F-actin and the molecular characterization of the interaction between $\alpha 6 \beta 4$ and F-actin. The latter issue has yet to be explored but recent studies have yielded insight into the mechanism of $\alpha 6 \beta 4$ translocation to F-actin. Specifically, growth factors such as epidermal growth factor (EGF) can stimulate the chemotactic migration of carcinoma cells that form hemidesmosomes such as squamous-derived carcinoma cells.^{35, 45} The mechanism by which growth factors stimulate migration involves disruption of $\alpha 6 \beta 4$ -containing hemidesmosomes^{35, 45} a process that liberates such cells from rigid anchorage to the matrix and facilitates their motility. Using EGF as prototypic growth factor, we demonstrated that growth factor stimulation of squamous carcinoma cells redistributes $\alpha 6 \beta 4$ from hemidesmosomes to F-actin rich lamellipodia and membrane ruffles and that $\alpha 6 \beta 4$ is required for chemotactic migration in response to EGF (Fig. 2).⁴⁵ An analysis of the signaling events involved in this redistribution of $\alpha 6 \beta 4$ from hemidesmosomes to F-actin revealed an essential role for protein kinase C- α . In addition, EGF stimulates phosphorylation of the $\beta 4$ subunit coincident with $\alpha 6 \beta 4$ redistribution.⁴⁵ The causal role of $\beta 4$ phosphorylation in disrupting hemidesmosomes and $\alpha 6 \beta 4$ redistribution needs to

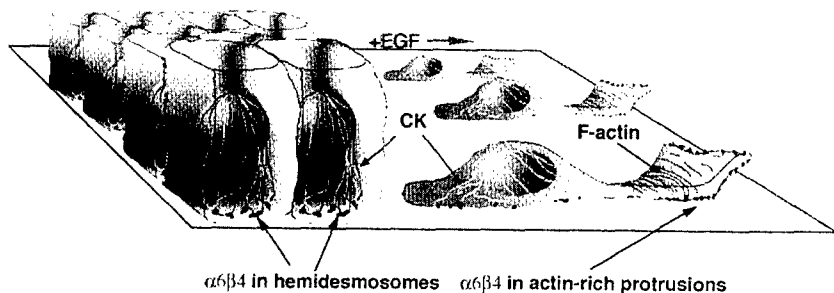


Figure 2. Growth factor mobilization of the $\alpha 6 \beta 4$ integrin from its association with cytokeratins (CK) in hemidesmosomes to F-actin in lamellae and lamellipodia. A chemotactic stimulus such as EGF can disassemble hemidesmosomes and promote the formation of $\alpha 6 \beta 4$ -containing lamellipodia and lamellae. These findings establish a mechanism for the dichotomy of $\alpha 6 \beta 4$ function in stably adherent and migrating epithelial-derived cells. An important implication of this model is that chemotactic factors can drive the migration of invasive carcinoma cells by mobilizing $\alpha 6 \beta 4$ and disassembling hemidesmosomes. For details see text and reference 45.

be established. Nonetheless, these findings demonstrate that a chemotactic stimulus will not only disassemble hemidesmosomes but also promote the formation of $\alpha 6 \beta 4$ -containing lamellipodia and membrane ruffles, thus establishing a mechanism for the dichotomy of $\alpha 6 \beta 4$ function in stably adherent and migrating epithelial-derived cells.

Signaling Properties of the $\alpha 6 \beta 4$ Integrin that Promote Invasion

The ability of integrins to regulate intracellular signaling pathways is well established.¹ The current literature abounds with studies on integrin-mediated regulation of signaling pathways that control cell growth, differentiation, survival, and cytoskeletal dynamics and cell migration. Moreover, it has become apparent that integrins often function in concert with specific growth factor receptors to execute these functions. A mechanistic understanding of invasion requires that integrins be identified that contribute to specific components of the invasive process and that the signaling pathways involved be elucidated. Such studies must incorporate the contribution of growth factor receptors, and those extracellular matrix components that interact with these integrins. Although a challenging task, considerable progress has been made in recent years, largely because of conceptual and technical advances in cell biology. The authors' comments here focus on the contribution of $\alpha 6 \beta 4$ integrin signaling to progression based on their interests and research. The reader, however, should bear in mind that the signaling pathways that are highlighted are regulated by other integrins and the contribution of these integrins to invasion needs to be considered.

A link between $\alpha 6 \beta 4$ -stimulation of invasion and signal transduction was provided by the authors' finding that this integrin activates phosphoinositide 3-OH kinase (PI3-K), a key signaling molecule, and that

the activity of PI3-K is essential for invasion.⁵³ Studies by Keely et al²⁸ also established the importance of PI3-K in carcinoma invasion. PI3-K phosphorylates phosphatidylinositol (PtdIns) lipids on the 3' position of the inositol ring resulting in an accumulation of PtdIns-3-P,³¹ PtdIns-3, 4-P₂,^{3, 42} and PtdIns-3, 4, 5-P₃.^{3, 4, 53, 61} Accumulation of D3 phosphoinositides at the plasma membrane recruits secondary signaling molecules or effectors that mediate the diverse functions of PI3-K.⁶¹ Given these considerations, it is important that the mechanism by which $\alpha 6 \beta 4$ activates PI3-kinase and by which PI3-kinase promotes invasion be elucidated.

Interestingly, it appears that $\alpha 6 \beta 4$ is able to stimulate PI3-K activity to a higher level than other integrins, at least in carcinoma cell lines.⁵³ Although the mechanism by which $\alpha 6 \beta 4$ or any integrin activates PI3-K has not been established, recent studies by Falcioni and colleagues have provided insight into this important problem. They reported that $\alpha 6 \beta 4$ associates with erbB2, a ligand orphan receptor implicated in breast cancer progression,³⁸ on the surface of breast carcinoma cell lines.¹² Subsequently, this group made the important observation that both $\alpha 6 \beta 4$ and erbB2 are required for the activation of PI3-K and the stimulation of invasion using a 3T3 cell model system.²¹ The implication of this finding is that the cooperation of $\alpha 6 \beta 4$ integrin signaling with specific growth factor receptor signaling is required for PI3-K activation and consequent stimulation of invasion (Fig. 3). The task ahead is to define the mechanism of this cooperative signaling.

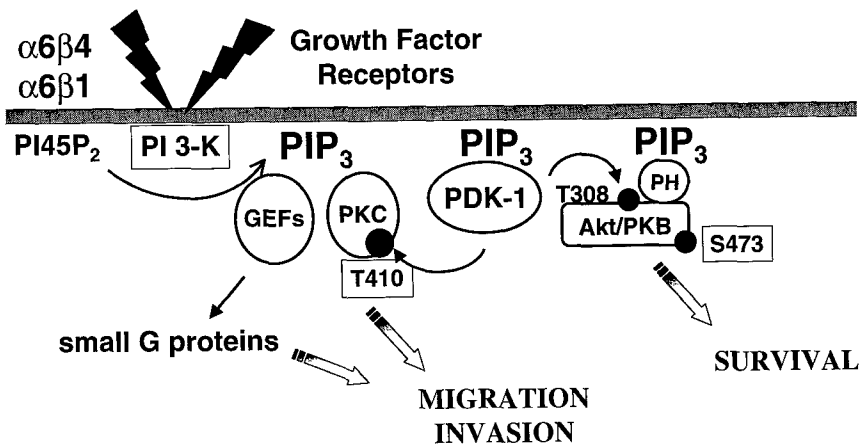


Figure 3. Proposed model for how the $\alpha 6$ integrins and growth factor receptors signal carcinoma progression. This model highlights the PI3-K signaling pathway. One implication of this model is that signaling from both the $\alpha 6$ integrins and growth factor receptors is necessary for optimal activation of PI3-K. The ability of PI3-K signaling to promote invasion is hypothesized to result from the PI3-K-dependent activation of small G proteins and specific protein kinase C (PKC) isoforms that regulate actin dynamics as discussed in. Also, the ability of the $\alpha 6$ integrins to maintain the survival of carcinoma cells in adverse conditions results from the PI3-K-dependent activation of the Akt/PKB kinase. (Data from references 2, 21, 53, 60, 62, and 66.)

One hypothesis is that $\alpha 6 \beta 4$ potentiates growth factor receptor signaling by increasing the activation of a key signaling intermediate. Clearly, elucidation of this mechanism is a significant advance because of the potential importance of $\alpha 6 \beta 4$ in invasion and also because it provides a paradigm for how integrin and growth factor receptors cooperate to drive the invasive process.

The mechanism by which activation of PI3-K promotes invasion is central to understanding of carcinoma progression. The major function of this enzyme with respect to invasion appears to be its ability to regulate the cytoskeleton and cell migration.^{53, 28} (Its role in survival is discussed later.) For this reason, the effectors of PI3-K that have the potential to regulate these functions are prime targets for study. In reality, however, little is known about the involvement of specific PI3-K effectors in invasion and how they contribute to the migration of these cells. The remarkable progress that has been made recently in understanding how signaling pathways, including the PI3-K pathway, regulate actin dynamics^{5, 48, 34} should accelerate studies on carcinoma migration and invasion and perhaps reveal differences in signaling between normal epithelial and carcinoma cells. Interesting observations have been made already in this direction. For example, the small G protein Rac, a PI3-K effector, appears to be involved in the migration and invasion of breast carcinoma cell lines.^{53, 28} In contrast, Rac inhibits the migration of normal epithelial cells by promoting the formation of cell-cell adhesions.⁴⁷ Another effector of PI3-K that is of paramount importance in cell survival and cancer progression is the Akt/PKB kinase (see later discussion). Although a role for this kinase in migration and invasion had been discounted^{53, 65} recent work on dictyostelium supports a role for Akt/PKB in migration.³⁶ It may be worthwhile, therefore, to re-evaluate the contribution of this kinase to carcinoma invasion.

The $\alpha 6 \beta 4$ integrin also regulates another signaling pathway, apparently distinct from PI3-K, that is important for the migration and invasion of carcinoma cells. This pathway involves the metabolism of cAMP. The $\alpha 6 \beta 4$ integrin, in concert with growth factor receptors, stimulates the formation of lamellae and the consequent chemotactic migration of carcinoma cells, processes that are inhibited by intracellular cAMP.⁴³ The authors observed that $\alpha 6 \beta 4$ can suppress the intracellular cAMP concentration by activating a cAMP-specific phosphodiesterase and that this activity is essential for the $\alpha 6 \beta 4$ -mediated enhancement of lamellae formation and chemotactic migration.⁴³ Subsequent studies revealed that cAMP inhibits activation of the Rho GTPase in carcinoma cells,⁴² similar to the regulation of Rho in leukocytes.³⁰ Moreover, these studies led to the important observation that $\alpha 6 \beta 4$ stimulates Rho activity in carcinoma cells and that Rho is essential for their migration and invasion. These results are of interest in light of other reports that both Rho and Rho kinase are important for tumor cell invasion.^{26, 67} Together, these findings suggest that $\alpha 6 \beta 4$ -mediated regulation of the Rho/Rho kinase pathway may be an important signaling component of carcinoma progression.

CARCINOMA SURVIVAL

Overview

Arguably, the ability of tumor cells to survive at sites different from their origin is the most important determinant of metastasis. If such cells can survive at distal sites, they have the opportunity to expand in number and form metastatic lesions. On the contrary, invasive and metastatic cells that are unable to survive present no threat to the patient. For this reason, an understanding of the mechanisms that enable the survival of metastatic cells is essential for an understanding of progression and also for the development of therapeutics.

Given the fact that carcinoma cells are essentially malignant epithelial cells, an understanding of survival mechanisms used by epithelial cells merits discussion. It has been known for some time that the attachment of primary epithelial cells to extracellular matrix (ECM) proteins is essential for their survival.¹⁸ More specifically, integrin-mediated interactions with ECM proteins initiate signals that sustain survival.⁴⁹ Survival is enhanced significantly by growth factor stimulation of attached cells providing evidence that integrins and growth factor receptors cooperate to promote survival.⁴⁹ The environment within the epithelium that supports survival, however, is progressively lost during malignant transformation, and especially as malignant cells become invasive and metastatic. Positional cues such as basement membrane anchoring and cell-cell adhesive contacts that provide survival signals in the normal epithelium are often absent in invasive and metastatic cancer.^{22, 6} Moreover, the environment encountered by invasive and metastatic cells is foreign and often proapoptotic. For example, one of the most formidable barriers to their survival is hypoxia.⁷ The oxygen tension within many solid tumors is substantially less than that in adjacent normal tissue, presumably because of poor vascularization.⁷ The conclusion can be drawn from these considerations that invasive and metastatic cells must acquire mechanisms that maintain their survival outside the confines of the epithelium. As mentioned previously, cancer progression is an evolutionary process that selects for cells that exhibit the capacity for survival, among other properties.^{16, 24} One important implication of this hypothesis is that those cells that do survive are the most aggressive and have the greatest propensity to metastasize. In other terms, a strong selective pressure promotes the growth of metastatic cells that have evolved mechanisms for surviving in hostile environments.

The ability of tumor cells to recruit blood vessels, a process referred to as the *angiogenic switch*, is a major mechanism for sustaining the growth and survival of tumor cells.²³ In fact, considerable evidence supports a direct link between angiogenesis and tumor progression.⁶⁹ Another mechanism of survival that has not been investigated as intensively as the angiogenic switch is the ability of tumor cells themselves to turn on signaling pathways that promote their survival. The hypoxic regions of many solid tumors, for example, are poorly vascularized yet some of the cells are able to survive and progress to metastatic disease⁷ as the possible result, for example, of

their secretion of growth factors that act in an autocrine manner to promote their survival.

The Central Role of PI3-K in Survival

Numerous studies have substantiated the importance of PI3-K and its effectors in promoting cell survival.^{61, 10} Moreover, it is worth noting that at least two of the genes implicated in cancer, Ras,⁵⁴ and PTEN,⁸ impact the PI3-K signaling pathway. The key effector molecule in this pathway appears to be Akt/PKB. PI3-K is essential for Akt/PKB activation because the D3 phosphoinositide products of PI3-K bind to its pleckstrin homology (PH) domain and recruit it to the cell membrane.^{61, 10} These phosphoinositides also recruit another critical enzyme, PI3-K-dependent kinase 1 (PDK1), to the membrane enabling it to phosphorylate Akt/PKB at threonine residue 308. This phosphorylation event induces Akt/PKB autophosphorylation at serine residue 473, which is the final step required for the stimulation of Akt/PKB kinase activity towards exogenous substrates.⁶²

The importance of Akt/PKB in cell survival has been associated with its ability to inactivate key apoptotic molecules to stimulate antiapoptotic signaling pathways (reviewed in reference 10). Specifically, Akt/PKB has been shown to phosphorylate and inhibit caspase 9 and the Bcl-2 family member, Bad; inhibit the activity of forkhead transcription factors, which play an essential role in Fas death receptor-induced apoptosis; and phosphorylate and activate transcription factors implicated in diverse survival signaling pathways, including NF kappa B and CREB. The stimulation of Akt/PKB activity is associated with growth factor receptor and integrin signaling and optimal Akt/PKB activation is achieved as a result of the cooperative signaling that occurs between these two types of receptors.¹⁵

Given the potential importance of Akt/PKB in the survival of primary²⁹ and transformed² epithelial cells, the hypothesis can be formulated that cancer progression selects for cells that have the capacity to sustain Akt/PKB activation. This hypothesis is supported, in fact, by compelling genetic data. The PTEN tumor suppressor is a lipid phosphatase that dephosphorylates PtdIns,^{3, 4, 53} a PI3-K product that is essential for Akt/PKB activation.⁸ Importantly, PTEN-deficient cells are resistant to numerous apoptotic stimuli and have constitutively elevated levels of Akt/PKB activity.⁵⁵ Moreover, numerous cancers contain mutations or deletions in PTEN, emphasizing the importance of Akt/PKB in tumor cell survival (reviewed in reference 8). In addition to PTEN, the Ras GTPase is activated by mutation in a significant fraction of human cancers⁵⁴ and activated Ras can sustain activation of the PI3-K-Akt/PKB pathway.^{29, 11} The importance of this activation is suggested by the finding that activated Ras can protect epithelial cells from the apoptosis that occurs when they are detached from matrix and deprived of integrin signals, a process referred to as *anoikis*.²⁹ One implication of this finding is that activated Ras can promote cell survival in the absence of integrin signaling, a process, for

example, that could enable tumor cells to survive in environments where integrin signaling is deficient because of the ECM composition.

Another mechanism that may contribute to the survival of carcinoma cells is Akt/PKB overexpression. Of the three Akt/PKB isoforms that have been described, Akt-2 has been shown to be upregulated in numerous cancers as a result of gene duplication.^{3, 27, 68} Moreover, there is some evidence that Akt-3 activity may be elevated in aggressive breast carcinomas and prostate cancer cell lines.⁴⁰ Although more data are needed to assess the impact of Akt/PKB overexpression on carcinoma survival, as well as the contribution of specific Akt/PKB isoforms, this clearly is an area of potential importance.

The probability that specific integrin and growth factor receptors contribute to carcinoma survival by stimulating Akt/PKB activity is high, based on the known ability of these receptors to activate Akt/PKB and their involvement in carcinoma progression. Moreover, this signaling mechanism could function together with genetic alterations in other signaling molecules, as well as Akt/PKB overexpression, to sustain elevated Akt/PKB activity in tumor cells existing in hostile environments as a means of maintaining their survival. The $\alpha 6$ integrins are obvious candidates for contributing to the Akt/PKB-mediated survival of carcinoma cells based on their ability to activate this kinase and their involvement in carcinoma progression.

The $\alpha 6$ Integrins and Carcinoma Survival

Studies, particularly on breast carcinoma, indicate an important contribution of the $\alpha 6$ integrins to carcinoma survival.^{17, 52, 66, 39} This function was foreshadowed by the finding that high expression of the $\alpha 6$ subunit in women with breast cancer correlated significantly with reduced survival times.¹⁷ In an analysis of 119 patients with invasive breast carcinoma, all of the patients with low or absent $\alpha 6$ expression survived while the mortality rate of the patients with a high level of $\alpha 6$ expression was 19%. Of note, 30 out of 34 of the patients that presented with distant metastases were highly positive for $\alpha 6$ expression. Although this study did not distinguish the relative contributions of the $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins, more recent work summarized later has implicated both of these integrins in breast carcinoma survival.

To define the contribution of the $\alpha 6\beta 1$ receptor to breast cancer more rigorously, the authors developed a genetic strategy for eliminating expression of this integrin in metastatic breast carcinoma cells.^{52, 66} When these $\alpha 6\beta 1$ -deficient cells were inoculated into the mammary fat pad of nude mice, primary tumor size was significantly diminished compared with the parental cells. Further analysis revealed that this reduction in tumor size resulted from the apoptosis of these $\alpha 6\beta 1$ -deficient cells.⁶⁶ More importantly, the $\alpha 6\beta 1$ -deficient cells did not form metastases in the lung, as did the parental cells, because of their inability to survive in this organ.⁶⁶ These data indicated that $\alpha 6\beta 1$ is essential for survival of breast carcinomas

in vivo. The mechanism by which the $\alpha 6 \beta 1$ integrin promotes survival in vivo is not known but a reasonable hypothesis based on other studies is that this integrin is needed to cooperate with specific growth factor receptors for optimal activation of Akt/PKB.

Recent work by the authors' group on $\alpha 6 \beta 4$ integrin signaling has also highlighted the importance of the $\alpha 6$ integrins in carcinoma survival and substantiated the involvement of Akt/PKB. This work derives from the finding, discussed previously, that $\alpha 6 \beta 4$ activates the PI3-K/Akt/PKB pathway in carcinoma cell lines.⁵³ An implication of this finding is that $\alpha 6 \beta 4$ should also promote the survival of these cells. In fact, we observed that expression of $\alpha 6 \beta 4$ enhances the survival of breast carcinoma cells that have been deprived of serum and matrix, conditions that normally promote detachment-induced apoptosis.² Interestingly, however, this stimulation of AKT-mediated survival by $\alpha 6 \beta 4$ is apparent only in carcinoma cells that contain inactivating p53 mutations. In fact, the deprivation of wild type p53-expressing cells of both matrix and serum actually triggers the caspase-3 mediated cleavage and inactivation of AKT and prevents $\alpha 6 \beta 4$ -mediated survival signaling.² Collectively, the authors' findings highlight the importance of $\alpha 6 \beta 4$ in promoting the AKT-dependent survival of breast carcinoma cells. This survival function could be particularly important in late-stage carcinomas, which exhibit a high frequency of p53 mutations and are more prone to survive in hypoxia.⁷

The observation that the p53 tumor suppressor can inhibit Akt/PKB kinase activity is of interest in light of the finding mentioned above 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.⁸ Together, the authors' findings on p53 and the previously described activities of PTEN highlight the impact of tumor suppressors on integrin-mediated functions. Moreover, the authors' demonstration that p53 inhibits $\alpha 6 \beta 4$ 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.

SUMMARY

Hopefully, this article has affirmed the potential contribution of one class of surface receptors to the progression of human carcinoma. Although more issues have been raised than have been resolved, a blueprint for future studies on the molecular cell biology of progression is emerging. First and foremost, progression appears to be a web of many strands. Surface receptors, the ECM, signaling molecules and the cytoskeleton are some of the major strands that form the web of progression. Studies on one strand inevitably lead to other strands as work on the $\alpha 6$ integrins has demonstrated. Another theme that is emerging is that the key functional components of progression such as invasion and survival may result from stimulation of a common signaling pathway. In this direction, the argument can be made

that the PI3-K pathway is a major determinant of progression because the effectors of PI3-K signaling regulate invasion and survival (see Fig. 3).

One direction for future work is to define further the contribution of effector molecules to specific aspects of progression. There is much to be learned, for example, from studies on the small G proteins and specific PKC isoforms with respect to cytoskeletal dynamics, migration, and invasion. Also, studies investigating how Akt/PKB isoforms are regulated, positively by integrin and growth factor receptor signaling and negatively by tumor suppressors, will clarify understanding of the mechanisms by which tumor cells survive in hostile environments that normally promote apoptosis. From the clinical perspective, the mechanistic understanding of progression that is emerging provides an array of targets for rational drug design.

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Integrin Laminin Receptors and Breast Carcinoma Progression

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This review explores the mechanistic basis of breast carcinoma progression by focusing on the contribution of integrins. Integrins are essential for progression not only for their ability to mediate physical interactions with extracellular matrices but also for their ability to regulate signaling pathways that control actin dynamics and cell movement, as well as for growth and survival. Our comments center on the $\alpha 6$ integrins ($\alpha 6 \beta 1$ and $\alpha 6 \beta 4$), which are receptors for the laminin family of basement membrane components. Numerous studies have implicated these integrins in breast cancer progression and have provided a rationale for studying the mechanistic basis of their contribution to aggressive disease. Recent work by our group and others on mechanisms of breast carcinoma invasion and survival that are influenced by the $\alpha 6$ integrins are discussed.

KEY WORDS: Integrin; laminin; breast carcinoma; invasion.

INTRODUCTION

Cancer progression, defined as the sequence of events that enable tumor cells to become invasive and eventually metastatic, is among the most challenging and important problems in cancer biology and cancer medicine (1, 2). Although progression is a complex, multi-factorial process, the salient features are relatively simple. The metastatic odyssey of a malignant breast carcinoma cell, for example, from its genesis in the mammary gland to a distal organ requires that this cell acquire a motile phenotype to invade through tissue and gain access to the vasculature and lymphatics. In addition, this cell will apoptose outside of the nurturing environment of the mammary gland unless it adapts survival mechanisms. Thus, a simplified per-

spective on progression focuses on the ability of tumor cells to invade and survive. Indeed, much of contemporary cancer biology is linked to these two key parameters of progression.

The biology of breast carcinoma, as well as other carcinomas, is essentially an aberration of epithelial cell biology. The distinguishing features of epithelia are their polarized morphology, attachment to an underlying basement membrane, presence of specialized cell-cell contacts, and their capacity for rapid self-renewal, differentiation and death (3). These features are manifested in the epithelial and myoepithelial cells of the mammary gland. The basement membrane, in particular, plays a central role in the biology of epithelia and carcinomas. This thin sheet of connective tissue, which is comprised primarily of collagen type IV, laminins, entactin (nidogen) and proteoglycans, separates the epithelium from underlying stroma (4). Interestingly, much of our knowledge on the importance of the basement membrane in epithelial biology derives from studies on the mammary gland [reviewed in (5, 6)]. Such seminal studies established that the adhesive interactions of mammary epithelial cells with basement membrane laminins are

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essential for their differentiation and survival. The importance of these laminin-mediated interactions has stimulated interest in the receptors on epithelial cells that mediate laminin interactions and on the intracellular signaling pathways that these receptors influence.

Basement membrane interactions are also an important component of carcinoma progression. In fact, the breaching of the basement membrane by carcinoma cells is a defining event for malignant tumors (7). In addition, some carcinoma cells synthesize and deposit basement membrane components, especially laminins, and receptor-mediated interactions with these components generate signals that facilitate their migration and sustain their survival (8, 9).

LAMININS AND THEIR RECEPTORS IN MAMMARY EPITHELIA AND BREAST CARCINOMAS

The laminins, a large family of heterotrimeric glycoproteins, are major components of basement membranes. To date, more than twelve distinct laminins have been identified [for comprehensive review see (4)]. Although the importance of the laminins to epithelial and carcinoma biology is established, there is much to be learned about the expression and function of specific laminins. This situation is particularly evident for laminin function in mammary epithelia and breast carcinomas. Most of the pioneering functional studies on mammary epithelia used laminin purified from a murine sarcoma or a reconstituted basement membrane preparation from this tumor termed Matrigel, which is comprised primarily of laminin-1 and type IV collagen. Studies using laminin-1 and Matrigel have provided valuable information on the importance of laminin in mammary gland biology (5, 6). In addition, much has been learned about breast carcinoma migration and survival, as well as receptors that mediate these processes, using these reagents [e.g. (10, 11)]. Future studies, however, need to characterize the expression and function of specific laminins in the normal breast and in breast carcinoma in more detail. Recent studies on laminin-5, a laminin that appears to be particularly important for epithelial migration, exemplify the direction of future work in this area (12, 13).

Numerous surface proteins function as receptors for the laminins, including members of the integrin family, dystroglycan, a receptor tyrosine phosphatase, heparan sulfates and various other surface proteins

(14, 4). Although integrins appear to be the 'preeminent' laminin receptors on most cells, including mammary epithelial and breast carcinoma cells, and they will be the focus in this review, the functional contribution of other laminin receptors to breast biology has not been studied extensively and warrants further investigation.

A recent review in this journal summarized integrin expression and function in mammary epithelia and breast carcinoma (15). As discussed in this review, the integrins expressed on the epithelial and myoepithelial cells of the breast include $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, $\alpha v\beta 3$, and $\alpha v\beta 6$ (15). Several of these integrins function as laminin receptors ($\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$). Although most of these integrins probably contribute to breast cancer progression, it is the $\alpha 6$ integrins ($\alpha 6\beta 1$ and $\alpha 6\beta 4$) that have captured the interest of many investigators because of their link to aggressive disease. This link was foreshadowed by the finding that high expression of the $\alpha 6$ subunit in women with breast cancer correlated significantly with reduced survival times (16). In an analysis of 119 patients with invasive breast carcinoma, all of the patients with low or absent $\alpha 6$ expression survived, while the mortality rate of the patients with a high level of $\alpha 6$ expression was 19%. Of note, 30 out of 34 of the patients that presented with distant metastases were highly positive for $\alpha 6$ expression. Although this study did not distinguish the relative contributions of the $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins, subsequent work has implicated both of these integrins in breast carcinoma progression. For example, co-expression of $\alpha 6\beta 4$ and laminin in breast tumors has been correlated with poor prognosis (17). Also, expression of the $\alpha 6\beta 1$ integrin has been linked to the survival and metastatic potential of human breast carcinoma cells (18, 19). Given the potential importance of the $\alpha 6$ integrins to breast cancer, the challenge ahead is to define the mechanisms by which these receptors promote progression.

BREAST CARCINOMA INVASION

Invasion, or the penetration of tumor cells into adjacent tissues, is one of the hallmarks of malignant tumors (7). In contrast to benign tumors that are encapsulated, invasive tumors have the potential to metastasize because of their access to lymphatics and the vasculature. Invasion can also result in patient morbidity and mortality in the absence of metastasis. For these reasons, understanding the process of

invasion is of obvious importance. The insightful observations of pathologists have taught us much about the nature of tumor invasion and have provided a foundation for more mechanistic studies (7). As mentioned above, breaching of the basement membrane by carcinoma cells is a defining event for malignant tumors because it facilitates their access to the vasculature and lymphatics. This observation spawned the 'three-step' model of invasion (20). This model implied that tumor cells attach themselves to the basement membrane and then secrete proteases that degrade localized regions of the basement membrane, enabling their migration into stroma. The most significant contribution of this model was that it identified critical components of the invasive process that could be studied in more detail, such as adhesion and proteolysis. As a result, we know much, for example, about receptors on tumor cells that mediate interactions with basement membrane components and proteases that degrade these components. We also know that the invasive process is more complex than envisioned originally. A significant advance in this regard was the realization that highly invasive carcinoma cells often lose contact with each other and exhibit a mesenchymal or motile phenotype that is distinct from normal epithelial structure (21–23). This realization led to the hypothesis that an epithelial to mesenchymal transition is a major component of the invasive process. Subsequent studies on the loss of cadherin-mediated cell-cell adhesion in invasive carcinomas established a mechanistic basis for this epithelial to mesenchymal transition (24–27). In addition, the advent of molecular cell biology has provided a new prospectus on signaling molecules and cytoskeletal dynamics that could regulate invasion. And, the realization that invading tumor cells must survive in foreign and often hostile environments has made the study of survival mechanisms an integral component of tumor progression. These advances are enabling us to approach the problem of invasion at a fundamental level.

The $\alpha 6 \beta 4$ Integrin and Breast Carcinoma Invasion

In many epithelia, the $\alpha 6 \beta 4$ integrin, which is a receptor for the laminins, mediates the formation of stable adhesive structures termed hemidesmosomes that link the intermediate filament cytoskeleton with specific laminins in the basement membrane (28). Although hemidesmosomes are most apparent in stratified epithelia, they also exist in breast epithelia but not in invasive breast carcinomas (29). Expression

of the $\alpha 6 \beta 4$ integrin, however, persists in many invasive cancers. In the breast, expression of $\alpha 6 \beta 4$ is maintained in ductal carcinoma *in situ*, invasive carcinoma and in metastatic lesions as assessed by *in situ* hybridization (unpublished data). Moreover, as mentioned above, $\alpha 6 \beta 4$ expression has been correlated with poor prognosis for breast cancer patients (17). Similar results have been reported for other solid tumors [reviewed in reference (30)]. Such studies have been substantiated by the findings that expression of $\alpha 6 \beta 4$ in a $\alpha 4$ -deficient breast carcinoma cells dramatically increases the invasive potential of these cells (Fig. 1) (10, 31, 32). Other studies by our group provided evidence that the $\alpha 6 \beta 4$ integrin promotes breast carcinoma invasion by stimulating lamellae formation and the chemotactic migration of breast carcinoma cells (Fig. 1) (33). Collectively, the available data support an important contribution of the $\alpha 6 \beta 4$ integrin to carcinoma invasion (10, 31, 32, 34). More data are needed, however, to substantiate this finding. In particular, the involvement of $\alpha 6 \beta 4$ in progression needs to be validated in transgenic models of breast cancer, and more studies are needed to validate the prognostic significance of $\alpha 6 \beta 4$ expression in human breast carcinomas.

Cytoskeletal Interactions of the $\alpha 6 \beta 4$ Integrin

The involvement of the $\alpha 6 \beta 4$ integrin in invasion and migration conflicts with the established function for this integrin in mediating stable adhesive contacts in hemidesmosomes. In simple terms, migration is a dynamic process that requires the rapid formation and disassembly of adhesive contacts (35). The presence of $\alpha 6 \beta 4$ -containing hemidesmosomes would impede such dynamic events. A significant breakthrough, therefore, was our finding that the $\alpha 6 \beta 4$ integrin can associate with F-actin and is localized at the leading edges of invasive carcinoma cells (36). Moreover, we demonstrated that $\alpha 6 \beta 4$ actually mediates the migration of such cells through its ability to associate with the actin cytoskeleton and promote the formation and stabilization of filopodia and lamellae (36). This finding implied that the function and cytoskeletal association of $\alpha 6 \beta 4$ in invasive carcinoma cells are distinct from its established role of anchoring epithelial cells to the basement membrane through its association with cytokeratins.

The observations on $\alpha 6 \beta 4$ and migration raise two important issues: the mechanism by which hemidesmosomes are disrupted to enable $\alpha 6 \beta 4$ to

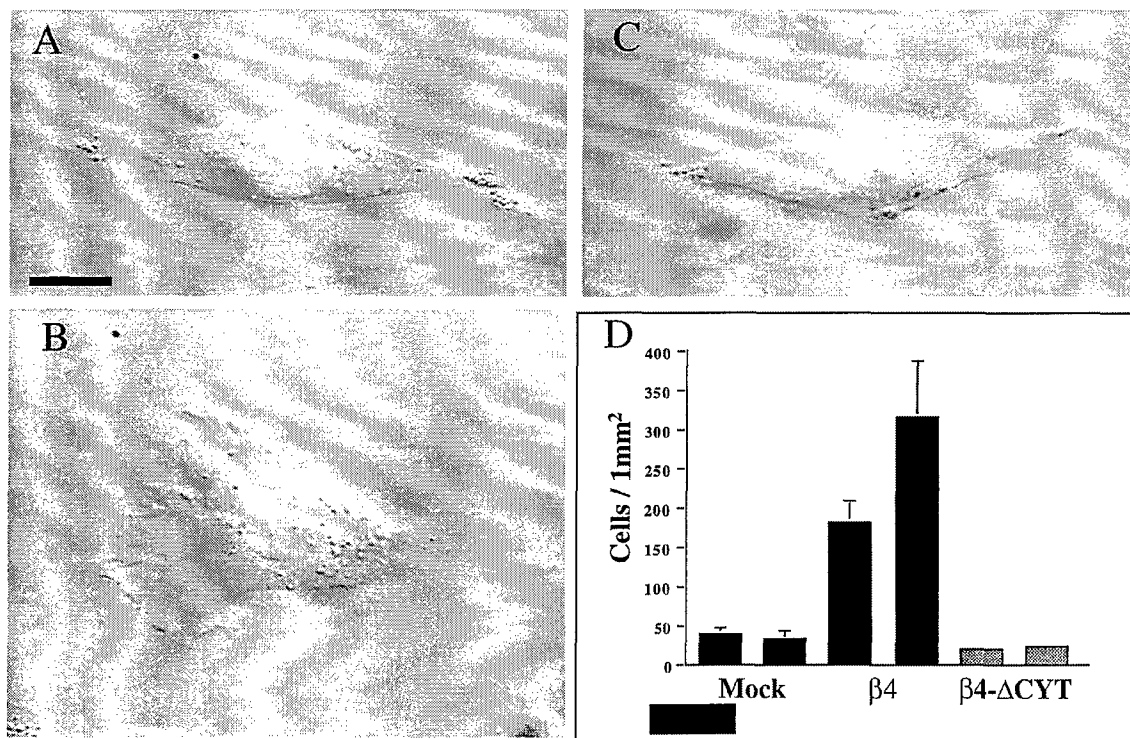


Fig. 1. Cooperation between $\alpha 6 \beta 4$ integrin and growth factor signaling promotes lamellae formation and invasion (33). Human breast carcinoma cells (MDA-MB-435) that either lack expression of the $\alpha 6 \beta 4$ integrin (C) or express this integrin (A, B) were plated onto coverslips that had been coated with 20 $\mu\text{g}/\text{ml}$ collagen I. Cells were allowed to adhere for 2 hrs at 37°C and then treated with LPA for 5 min. (B, C) or left untreated (A). The cells were visualized using Nomarski DIC optics. Note the large lamella that is formed in response to LPA stimulation of cells that express $\alpha 6 \beta 4$ (B) but not in cells that lack expression of this integrin (C). Also, note that $\alpha 6 \beta 4$ expression does not result in lamellae formation in the absence of LPA stimulation (A). The influence of $\alpha 6 \beta 4$ expression of the chemoinvasion of MDA-MB-435 cells using a standard Matrigel assay is shown in (D). Note expression of a cytoplasmic domain deletion of the $\beta 4$ subunit ($\beta 4\text{-}\Delta\text{CYT}$) does not stimulate invasion.

interact with F-actin and the molecular characterization of the interaction between $\alpha 6 \beta 4$ and F-actin. The latter issue has yet to be explored but recent studies have yielded insight into the mechanism of $\alpha 6 \beta 4$ translocation to F-actin. Specifically, growth factors such as EGF can stimulate the chemotactic migration of carcinoma cells that form hemidesmosomes such as squamous-derived carcinoma cells (37, 38). The mechanism by which growth factors stimulate migration involves disruption of $\alpha 6 \beta 4$ -containing hemidesmosomes (37, 38), a process that liberates such cells from rigid anchorage to the matrix and facilitates their motility. Using EGF as prototypic growth factor, we demonstrated that growth factor stimulation of squamous carcinoma cells redistributes $\alpha 6 \beta 4$ from hemidesmosomes to F-actin-rich lamellipodia

and membrane ruffles, and that $\alpha 6 \beta 4$ is required for chemotactic migration in response to EGF (Fig. 2) (38). An analysis of the signaling events involved in this re-distribution of $\alpha 6 \beta 4$ from hemidesmosomes to F-actin revealed an essential role for protein kinase C- α . In addition, EGF stimulates phosphorylation of the $\beta 4$ subunit coincident with $\alpha 6 \beta 4$ redistribution (38). The causal role of $\beta 4$ phosphorylation in disrupting hemidesmosomes and $\alpha 6 \beta 4$ redistribution needs to be established. Nonetheless, these findings demonstrate that a chemotactic stimulus will not only disassemble hemidesmosomes but also promote the formation of $\alpha 6 \beta 4$ -containing lamellipodia and membrane ruffles, thus establishing a mechanism for the dichotomy of $\alpha 6 \beta 4$ function in stably adherent and migrating epithelial-derived cells.

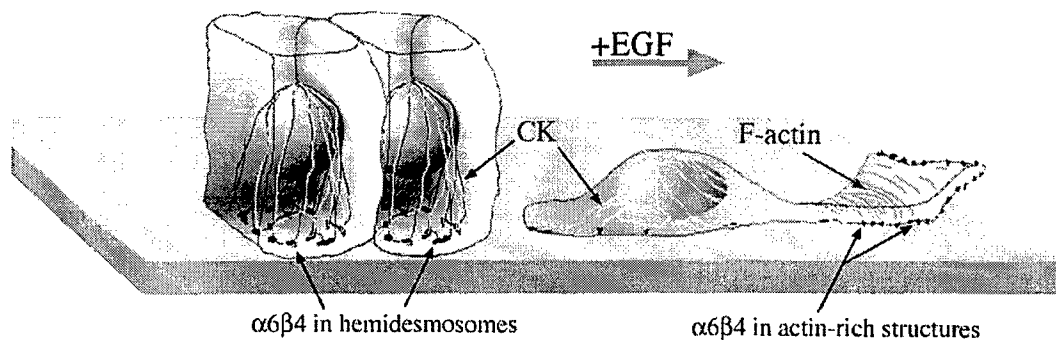


Fig. 2. Growth factor mobilization of the $\alpha 6 \beta 4$ integrin from its association with cytokeratins (CK) in hemidesmosomes to F-actin in lamellae and lamellipodia. A chemotactic stimulus such as EGF can disassemble hemidesmosomes and promote the formation of $\alpha 6 \beta 4$ -containing lamellipodia and lamellae. These findings establish a mechanism for the dichotomy of $\alpha 6 \beta 4$ function in stably adherent and migrating epithelial-derived cells. An important implication of this model is that chemotactic factors can drive the migration of invasive carcinoma cells by mobilizing $\alpha 6 \beta 4$ and disassembling hemidesmosomes.

Signaling Properties of the $\alpha 6 \beta 4$ Integrin that Promote Invasion

The ability of integrins to regulate intracellular signaling pathways has been established [reviewed in (39)]. The current literature abounds with studies on integrin-mediated regulation of signaling pathways that control cell growth, differentiation and survival, as well as cytoskeletal dynamics and cell migration. Moreover, it has become apparent that integrins often function in concert with specific growth factor receptors to execute these functions. A mechanistic understanding of invasion requires that integrins be identified that contribute to specific components of the invasive process and that the signaling pathways involved be elucidated. Such studies must incorporate the contribution of growth factor receptors, as well as those extracellular matrix components that interact with these integrins. Although a challenging task, considerable progress has been made in recent years, largely because of conceptual and technical advances in cell biology. Our comments here will focus on the contribution of $\alpha 6 \beta 4$ integrin signaling to progression based on our interests and research. However, the reader should bear in mind that the signaling pathways that we highlight are regulated by other integrins as well and the contribution of these integrins to invasion needs to be considered.

A link between $\alpha 6 \beta 4$ -stimulation of breast carcinoma invasion and signal transduction was provided by our finding that this integrin activates phosphoinositide 3-OH kinase (PI3-K), a key signaling molecule, and that the activity of PI3-K is essential

for invasion (10). Studies by Keely *et al.* (40) also established the importance of PI3-K in breast carcinoma invasion. PI3-K phosphorylates phosphatidylinositol (PtdIns) lipids on the 3' position of the inositol ring, resulting in an accumulation of PtdIns(3)P1, PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (41). Accumulation of D3 phosphoinositides at the plasma membrane recruits secondary signaling molecules or effectors that mediate the diverse functions of PI-3K (41). Given these considerations, it is important that the mechanism by which $\alpha 6 \beta 4$ activates PI3-kinase and by which PI3-kinase promotes invasion be elucidated.

Interestingly, it appears that $\alpha 6 \beta 4$ is able to stimulate PI3-K activity to a higher level than other integrins, at least in carcinoma cell lines (10). Although the mechanism by which $\alpha 6 \beta 4$ or any integrin activates PI3-K has not been established, recent studies by Falcioni and colleagues have provided insight into this important problem. They reported that $\alpha 6 \beta 4$ associates with erbB2, a receptor implicated in breast cancer progression (42), on the surface of breast carcinoma cell lines (43). Subsequently, this group made the important observation that both $\alpha 6 \beta 4$ and erbB2 are required for the activation of PI3-K and the stimulation of invasion using a 3T3 cell model system (44). The implication of this finding is that the cooperation of $\alpha 6 \beta 4$ integrin signaling with specific growth factor receptor signaling is required for PI3-K activation and consequent stimulation of invasion (Fig. 3). The task ahead is to define the mechanism of this cooperative signaling. One hypothesis is that $\alpha 6 \beta 4$ potentiates growth factor receptor signaling by increasing the activation of a key signaling intermediate(s). Clearly,

$\alpha 6$ Integrin Signaling of Breast Carcinoma Progression

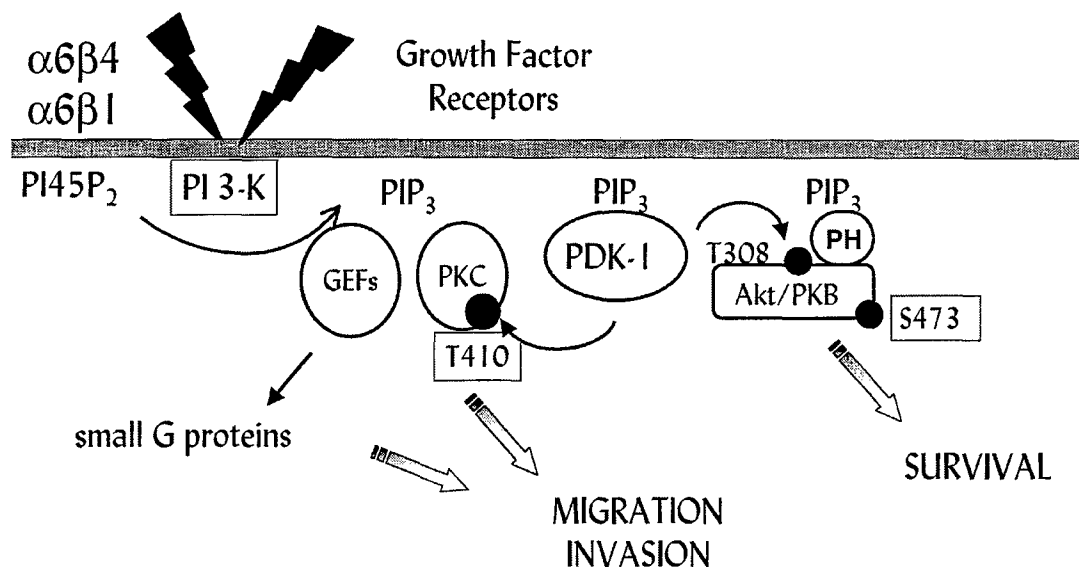


Fig. 3. Proposed model for how the $\alpha 6$ integrins signal breast carcinoma progression. This model highlights the PI3-K signaling pathway (10). One important aspect of this model is that signaling from both the $\alpha 6$ integrins and growth factor receptors is necessary for optimal activation of PI3-K as demonstrated in (44). However, the mechanism by which the $\alpha 6$ integrins and growth factor receptors cooperate to activate PI3-K remains to be elucidated. Most likely, this synergy occurs at the level of signaling molecules that function downstream of these surface receptors and upstream of PI3-K. The ability of PI3-K signaling to promote invasion is hypothesized to result from the PI3K-dependent activation of small G proteins and specific PKC isoforms that regulate actin dynamics as discussed in (74). Also, the ability of the $\alpha 6$ integrins to maintain the survival of carcinoma cells in adverse conditions (11, 18) results from the PI3-K dependent activation of the Akt/PKB kinase (65).

elucidation of this mechanism will be a significant advance not only because of the potential importance of $\alpha 6\beta 4$ in invasion but also because it will provide a paradigm for how integrin and growth factor receptors cooperate to drive the invasive process.

The mechanism by which activation of PI3-K promotes invasion is central to our understanding of carcinoma progression. The major function of this enzyme with respect to invasion appears to be its ability to regulate the cytoskeleton and cell migration (10, 40). Its role in survival will be discussed below. For this reason, the effectors of PI3-K that have the potential to regulate these functions are prime targets for study. In reality, however, little is known about the involvement of specific PI3-K effectors in invasion and how they contribute to the migration of these cells. The remarkable progress that has been made recently in understanding how signaling pathways, including the PI3-K pathway, regulate actin dynamics (45–47) should accelerate studies on carcinoma migration and

invasion and perhaps reveal differences in signaling between normal epithelial and carcinoma cells. Interesting observations have already been made in this direction. For example, the small G protein Rac, a PI3-K effector, appears to be involved in the migration and invasion of breast carcinoma cell lines (10, 40). In contrast, Rac inhibits the migration of normal epithelial cells by promoting the formation of cell-cell adhesions (48). Another effector of PI3-K that is of paramount importance in both cell survival and cancer progression is the Akt/PKB kinase (see below). Although a role for this kinase in migration and invasion had been discounted (10, 49), recent work on Dictyostelium support a role for Akt/PKB in migration (50). It may be worthwhile, therefore, to re-evaluate the contribution of this kinase to carcinoma invasion.

The $\alpha 6\beta 4$ integrin also regulates another signaling pathway, apparently distinct from PI3-K, that is important for the migration and invasion of carcinoma cells. This pathway involves the metabolism of

cAMP. The $\alpha 6 \beta 4$ integrin, in concert with growth factor receptors, stimulates the formation of lamellae and the consequent chemotactic migration of breast carcinoma cells, processes that are inhibited by intracellular cAMP (33). We observed that $\alpha 6 \beta 4$ can reduce the intracellular cAMP concentration by activating a cAMP-specific phosphodiesterase, and that this activity is essential for the $\alpha 6 \beta 4$ -mediated enhancement of lamellae formation and chemotactic migration (33). Subsequent studies revealed that cAMP inhibits activation of the Rho GTPase in carcinoma cells (51), similar to the regulation of Rho in leukocytes (52). Moreover, these studies led to the important observation that $\alpha 6 \beta 4$ stimulates Rho activity in carcinoma cells, and that Rho is essential for their migration and invasion. These results are of interest in light of other reports that both Rho and Rho kinase are important for tumor cell invasion (53, 54). Together, these findings suggest that $\alpha 6 \beta 4$ -mediated regulation of the Rho/Rho kinase pathway may be an important signaling component of carcinoma progression.

BREAST CARCINOMA SURVIVAL

Arguably, the ability of tumor cells to survive at sites different from their origin is the most important determinant of metastasis. If such cells can survive at distal sites, they have the opportunity to expand in number and form metastatic lesions. On the contrary, invasive and metastatic cells that are unable to survive present no threat to the patient. For this reason, an understanding of the mechanisms that enable the survival of metastatic cells is essential not only for an understanding of progression but also for the development of therapeutics.

Given the fact that carcinoma cells are essentially malignant epithelial cells, an understanding of survival mechanisms used by epithelial cells merits discussion. It has been known for some time that the attachment of primary epithelial cells to extracellular matrix (ECM) proteins is essential for their survival (55, 56). More specifically, integrin-mediated interactions with ECM proteins initiate signals that sustain survival (57). Survival is enhanced significantly by growth factor stimulation of attached cells, providing evidence that integrins and growth factor receptors cooperate to promote survival (57). The environment within the epithelium that supports survival, however, is progressively lost during malignant transformation, and especially as malignant cells become invasive and metastatic. Positional cues such as basement mem-

brane anchoring and cell-cell adhesive contacts that provide survival signals in the normal epithelium are often absent in invasive and metastatic cancer (5, 58). Moreover, the environment encountered by invasive and metastatic cells is foreign and often pro-apoptotic. For example, one of the most formidable barriers to their survival is hypoxia (reviewed in (59)). The oxygen tension within many solid tumors is substantially less than that in adjacent normal tissue, presumably because of poor vascularization (59). The conclusion can be drawn from these considerations that invasive and metastatic cells must acquire mechanisms that maintain their survival outside the confines of the epithelium. Indeed, cancer progression can be considered an evolutionary process that selects for cells that exhibit the capacity for survival, among other properties (1, 2). One important implication of this hypothesis is that those cells that do survive will be the most aggressive because they have the capacity to survive in inappropriate locations and form metastatic lesions. In other terms, a strong selective pressure promotes the growth of metastatic cells that have evolved mechanisms for surviving in hostile environments.

The ability of tumor cells to recruit blood vessels, a process referred to as the 'angiogenic switch' is a major mechanism for sustaining the growth and survival of tumor cells (60). In fact, considerable evidence supports a direct link between angiogenesis and tumor progression (61). Another mechanism of survival that has not been investigated as intensively as the angiogenic switch is the ability of tumor cells themselves to turn on signaling pathways that promote their survival. The hypoxic regions of many solid tumors, for example, are poorly vascularized, yet some of the cells are able to survive and progress to metastatic disease (reviewed in (59) as the possible result, for example, of their secretion of growth factors that act in an autocrine manner to promote their survival.

The Central Role of PI3-K in Survival

Numerous studies have substantiated the importance of PI3-K and its effectors in promoting cell survival (41, 62). Moreover, it is worth noting that at least two of the genes implicated in cancer, Ras (63) and PTEN (64), impact the PI3-K signaling pathway. The key effector molecule in this pathway appears to be Akt/PKB (Fig. 3). PI3-K is essential for Akt/PKB activation because the D3 phosphoinositide products of PI3-K bind to its pleckstrin homology (PH) domain and recruit it to the cell membrane (41, 62).

These phosphoinositides also recruit another critical enzyme PI3-K-dependent kinase 1 (PDK1) to the membrane enabling it to phosphorylate Akt/PKB at threonine residue 308 (Fig. 3). This phosphorylation event stimulates Akt/PKB autophosphorylation at serine residue 473, which is the final step required for the stimulation of Akt/PKB kinase activity towards exogenous substrates (65).

The importance of Akt/PKB in cell survival has been associated with both its ability to inactivate key apoptotic molecules as well as its ability to stimulate anti-apoptotic signaling pathways [reviewed in (62)]. Specifically, Akt/PKB has been shown to phosphorylate and inhibit Bad, a Bcl-2 family member, and inhibit the activity of forkhead transcription factors, which play an essential role in Fas death receptor-induced apoptosis. Akt/PKB also phosphorylates and activates transcription factors implicated in diverse survival signaling pathways, including NF kappa B and CREB. The stimulation of Akt/PKB activity is associated with both growth factor receptor and integrin signaling, and optimal Akt/PKB activation is achieved as a result of the cooperative signaling that occurs between these two types of receptors (56).

Given the potential importance of Akt/PKB in the survival of both primary (66) and transformed (11) epithelial cells, the hypothesis can be formulated that cancer progression selects for cells that have the capacity to sustain Akt/PKB activation. This hypothesis is supported, in fact, by compelling genetic data. The PTEN tumor suppressor is a lipid phosphatase that dephosphorylates PtdIns(3,4,5)P₃, a PI3-K product that is essential for Akt/PKB activation (64). Importantly, PTEN-deficient cells are resistant to numerous apoptotic stimuli and have constitutively elevated levels of Akt/PKB activity (67). Moreover, numerous cancers contain mutations or deletions in PTEN that could facilitate Akt/PKB activation [reviewed in reference (64)]. In addition to PTEN, the Ras GTPase is activated by mutation in a significant fraction of human cancers (63), and activated Ras can sustain activation of the PI3-K-Akt/PKB pathway (66, 68). The importance of this activation is suggested by finding that activated Ras can protect epithelial cells from the apoptosis that occurs when they are detached from matrix and deprived of integrin signals, a process referred to as anoikis (66). One implication of this finding is that activated Ras can promote cell survival in the absence of integrin signaling, a process, for example, that could enable tumor cells to survive in environments where integrin signaling is deficient because of the ECM composition.

Another mechanism that may contribute to the survival of carcinoma cells is Akt/PKB overexpression. Of the three Akt/PKB isoforms that have been described, Akt-2 has been shown to be upregulated in numerous cancers, including breast, as a result of gene duplication (69–71). Moreover, there is some evidence that Akt-3 activity may be elevated in aggressive breast carcinoma cancer cell lines (72). Although more data are needed to assess the impact of Akt/PKB overexpression on carcinoma survival, as well as the contribution of specific Akt/PKB isoforms, this clearly is an area of potential importance.

The probability that specific integrin and growth factor receptors contribute to carcinoma survival by stimulating Akt/PKB activity is high, based on the known ability of these receptors to activate Akt/PKB and their involvement in carcinoma progression. Moreover, this signaling mechanism could function together with genetic alterations in other signaling molecules, as well as Akt/PKB overexpression, to sustain elevated Akt/PKB activity in tumor cells existing in hostile environments as a means of maintaining their survival. The $\alpha 6$ integrins are obvious candidates for contributing to the Akt/PKB-mediated survival of carcinoma cells based on their ability to activate this kinase, as well as their involvement in carcinoma progression.

The $\alpha 6$ Integrins and Carcinoma Survival

Studies, particularly on breast carcinoma, indicate an important contribution of the $\alpha 6$ integrins to carcinoma survival (16, 18, 19, 73). As discussed above, this function was foreshadowed by the finding that high expression of the $\alpha 6$ subunit in women with breast cancer correlated significantly with reduced patient survival times (16).

To define the contribution of the $\alpha 6\beta 1$ receptor to breast cancer more rigorously, we developed a genetic strategy for eliminating expression of this integrin in metastatic breast carcinoma cells (18, 73). When these $\alpha 6\beta 1$ -deficient cells were inoculated into the mammary fat pad of nude mice, primary tumor size was significantly diminished compared to the parental cells. Further analysis revealed that this reduction in tumor size resulted from the apoptosis of these $\alpha 6\beta 1$ -deficient cells (18). More importantly, the $\alpha 6\beta 1$ -deficient cells did not form metastases in the lung, as did the parental cells, because of their inability to survive in this organ (18). These data indicated that $\alpha 6\beta 1$ is essential for survival of breast carcinomas

in vivo. The mechanism by which the $\alpha 6 \beta 1$ integrin promotes survival *in vivo* is not known but a reasonable hypothesis based on other studies is that this integrin is needed to cooperate with specific growth factor receptors for optimal activation of Akt/PKB.

Recent work by our group on $\alpha 6 \beta 4$ integrin signaling has also highlighted the importance of the $\alpha 6$ integrins in breast carcinoma survival and substantiated the involvement of Akt/PKB. This work derives from the finding, discussed above, that $\alpha 6 \beta 4$ activates the PI3-K/Akt/PKB pathway in carcinoma cell lines (10). An implication of this finding is that $\alpha 6 \beta 4$ should also promote the survival of these cells. In fact, we observed that expression of $\alpha 6 \beta 4$ enhances the survival of breast carcinoma cells that have been deprived of both serum and matrix, conditions that normally promote detachment-induced apoptosis (11). Interestingly, however, this stimulation of AKT-mediated survival by $\alpha 6 \beta 4$ is apparent only in carcinoma cells that contain inactivating p53 mutations. In fact, the deprivation of wild type p53-expressing cells of both matrix and serum actually triggers the caspase-3 mediated cleavage and inactivation of AKT and prevents $\alpha 6 \beta 4$ -mediated survival signaling (11). Collectively, our findings highlight the importance of $\alpha 6 \beta 4$ in promoting the AKT-dependent survival of breast carcinoma cells. This survival function could be particularly important in late-stage carcinomas, which exhibit a high frequency of p53 mutations and which are more prone to survive in hypoxia (59).

The observation that the p53 tumor suppressor can inhibit Akt/PKB kinase activity is of interest in light of the finding mentioned above 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 (64). Together, our 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 $\alpha 6 \beta 4$ 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.

SUMMARY OBSERVATIONS

This essay has affirmed the potential contribution of one class of surface receptors to the progression of breast carcinoma. Although more issues have been raised than have been resolved, a blueprint for

future studies on the molecular cell biology of progression is emerging. First and foremost, progression appears to be a web of many strands. Surface receptors, the ECM, signaling molecules and the cytoskeleton are some of the major 'strands' that form the web of progression. Studies on one 'strand' inevitably lead to other strands as our work on the $\alpha 6$ integrins has demonstrated. Another theme that is emerging is that the key functional components of progression such as invasion and survival may result from stimulation of a common signaling pathway. In this direction, the argument can be made that the PI3-K pathway is a major determinant of progression because the effectors of PI3-K signaling regulate both invasion and survival (Fig. 3).

One direction for future work is to define further the contribution of effector molecules to specific aspects of progression. There is much to be learned, for example, from studies on the small G proteins and specific PKC isoforms with respect to cytoskeletal dynamics, migration and invasion. Also, studies investigating how Akt/PKB isoforms are regulated, both positively by integrin and growth factor receptor signaling, as well as negatively by tumor suppressors, will clarify our understanding of the mechanisms by which breast tumor cells survive in hostile environments that normally promote apoptosis. From the clinical perspective, the mechanistic understanding of breast cancer progression that is emerging provides an array of targets for rational drug design.

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The $\alpha 6 \beta 4$ integrin and epithelial cell migration

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Although the involvement of $\alpha 6 \beta 4$, an integrin laminin receptor, in hemidesmosome organization has dominated the study of this integrin, recent studies are revealing novel functions for $\alpha 6 \beta 4$ in the migration of epithelial and carcinoma cells. The engagement of laminin by $\alpha 6 \beta 4$ can stabilize actin-rich protrusions and mediate traction forces necessary for cell movement. This integrin also has a significant impact on signaling molecules that stimulate migration and invasion, especially PI3-K and Rho GTPases. Activation of PI3-K by $\alpha 6 \beta 4$ enhances the formation of actin protrusions, and it may stimulate the function of other integrins, such as $\alpha 3 \beta 1$, that are also important for epithelial migration. Signaling through $\alpha 6 \beta 4$ may not always depend on the adhesive functions of this integrin, a possibility that has profound implications for migration and invasion because it implies that the ability of $\alpha 6 \beta 4$ to stimulate these processes is not limited to specific matrix environments.

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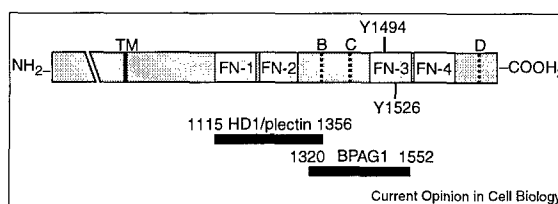
Abbreviations

EGF epidermal growth factor
IRS insulin receptor substrate
MAPK mitogen-activated protein kinase
PI3-K phosphatidylinositol 3-kinase
PKC protein kinase C

Introduction

The $\alpha 6 \beta 4$ integrin is a structural and functional anomaly among the integrin family of receptors. This integrin, which is expressed primarily on the basal surface of most epithelia and in a few other cell types, is defined as an adhesion receptor for most of the known laminins [1–3]. The distinguishing structural feature of $\alpha 6 \beta 4$ is the atypical cytoplasmic domain of the $\beta 4$ subunit (Figure 1). Two pairs of fibronectin type III repeats separated by a connecting segment characterize this domain, and it is distinct both in size (approximately 1000 amino acids) and structure from any other integrin subunit [4]. The existence of four structural variants of this domain ($\beta 4A$, $\beta 4B$, $\beta 4C$ and $\beta 4D$) increases its complexity but their functional significance is unknown [5]. Progress has been made in identifying specific regions and motifs within the $\beta 4$ cytoplasmic domain that mediate the cytoskeletal interactions and signaling properties of $\alpha 6 \beta 4$, but much remains to be learned.

Figure 1



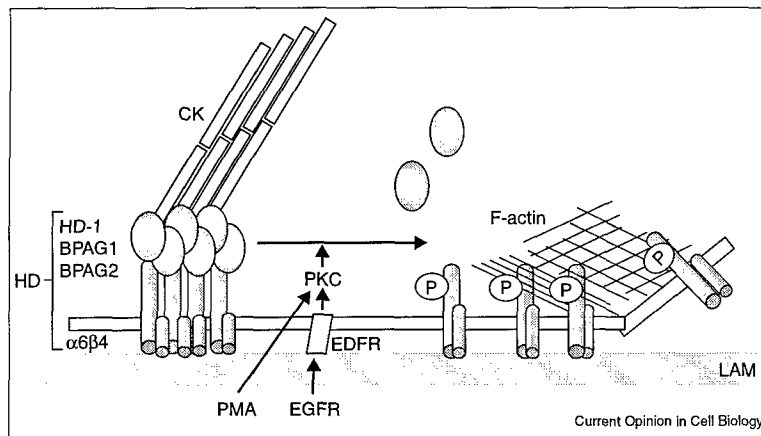
Schematic of the $\beta 4A$ integrin subunit. The $\beta 4$ cytoplasmic domain contains two sets of fibronectin Type III repeats (FN) separated by a connecting segment. Shown are the tyrosine residues that have been implicated in $\alpha 6 \beta 4$ -dependent activation of PI3-K (Y1494) and MAPK (Y1526). The black bars denote regions of the $\beta 4$ cytoplasmic domain that are essential for hemidesmosome formation. The segment encompassing amino acids 1115–1356 is required for HD1/plectin interactions and the segment spanning amino acids 1320–1552 is required for BPAG1 binding (see Figure 2). Dotted lines indicate the location of insertions or a deletion (carboxy-terminal dotted line) that result from differential splicing and that give rise to the three alternative isoforms of the $\beta 4A$ subunit; $\beta 4B$, $\beta 4C$, and $\beta 4D$. TM, transmembrane domain.

A primary function of $\alpha 6 \beta 4$, revealed definitively by studies on knockout and transgenic mice, is to maintain the integrity of epithelia, especially the epidermis [6–8]. In the absence of $\alpha 6 \beta 4$ expression, skin morphogenesis in mice appears normal [9] but the epidermis detaches in response to mechanical stress, a condition that results in death shortly after birth. In humans, mutations in the $\beta 4$ subunit also cause epidermal blistering [10]. This critical role for $\alpha 6 \beta 4$ derives from its ability to mediate the formation of stable and rigid adhesive structures termed hemidesmosomes on the basal cell surface that link the intermediate filament cytoskeleton with laminins in the basement membrane [11,12]. Such structures are most pronounced in stratified epithelia, but rudimentary forms exist in most epithelia.

Although the involvement of $\alpha 6 \beta 4$ in hemidesmosome organization and function has dominated the study of this integrin, recent studies are revealing novel functions for $\alpha 6 \beta 4$ in the migration of epithelial and epithelial-derived carcinoma cells. The laminins are important physiological substrates for the adhesion and migration of epithelial cells, an observation that underscores the significance of integrin laminin receptors in epithelial biology. The involvement of the $\alpha 3 \beta 1$ integrin laminin receptor in epithelial migration is established [13,14,15*,16*], but the contribution of $\alpha 6 \beta 4$ to migration, which derives largely from findings on epithelial wound healing and carcinoma invasion, is somewhat more complex.

Epithelial wounds such as those that occur in the skin and intestinal mucosa must heal quickly to preserve barrier function. Healing is mediated, in large part, by cell migration.

Figure 2



Mobilization of the $\alpha 6 \beta 4$ integrin from its association with cytokeratins in hemidesmosomes to F-actin in lamellipodia and lamellipodia. A chemotactic stimulus such as EGF or PMA can disassemble hemidesmosomes (HD) and promote the formation of $\alpha 6 \beta 4$ -containing lamellipodia and lamellipodia (LAM). This process is dependent on PKC- α and may involve phosphorylation (P) of the $\beta 4$ integrin. These findings might explain the dichotomy observed in $\alpha 6 \beta 4$ function in stably adherent and migrating epithelial-derived cells. An important implication of this model is that chemotactic factors can drive migration by mobilizing $\alpha 6 \beta 4$ and disassembling hemidesmosomes. For details, see text. Cellular components of the hemidesmosome: Bullous Pemphigoid Antigens 1 and 2 (BPAG1, BPAG2); HD1/Plectin; $\alpha 6 \beta 4$ integrin; cytokeratins (CK).

Cells proximal to the wound margins migrate over a provisional, laminin-rich matrix until cell-cell contact is re-established. Hemidesmosome disassembly is apparent in wounded cells [17], a process that facilitates migration, and, as a consequence, $\alpha 6 \beta 4$ loses its basal localization. In fact, $\alpha 6 \beta 4$ has been localized at wound edges in actin protrusions [15*,18] and several studies have implicated the involvement of $\alpha 6 \beta 4$, as well as $\alpha 3 \beta 1$, in wound healing based on the use of function-blocking antibodies [14,15*]. Moreover, there is evidence that $\alpha 6 \beta 4$ may contribute to this process by stimulating $\alpha 3 \beta 1$ function [16*].

More definitive evidence to implicate $\alpha 6 \beta 4$ in migration comes from studies on invasive carcinoma cells. The process of malignant transformation can initiate an epithelial to mesenchymal transition that results in the acquisition of a motile, invasive phenotype. Furthermore, the expression of $\alpha 6 \beta 4$ is maintained, if not increased, in many invasive carcinomas even in the absence of hemidesmosome formation [19]. What is the function of $\alpha 6 \beta 4$ in such cells? In cells derived from aggressive, late-stage tumors, $\alpha 6 \beta 4$ has been localized in membrane protrusions associated with migration such as filopodia, lamellipodia and retraction fibers [20,21]. As discussed below, the adhesive properties of $\alpha 6 \beta 4$ contribute to the migration of such carcinoma cells. There is also evidence that the signaling properties of $\alpha 6 \beta 4$ stimulate carcinoma migration and invasion and that this function can be independent of its adhesive functions.

Mechanism(s) of hemidesmosome disruption and $\alpha 6 \beta 4$ mobilization

One implication of epithelial migration is that a mechanism must exist to mobilize $\alpha 6 \beta 4$ from hemidesmosomes and disrupt their structure in response to migratory stimuli. Insight into this mechanism has come from studies on cells such as A431 squamous carcinoma and 804G bladder carcinoma that form hemidesmosomes in culture [21,22]. Chemotactic factors

such as epidermal growth factor (EGF) stimulate a rapid disassembly of hemidesmosomes in these cells that is coincident with the formation of lamellipodia and membrane ruffles [21]. In fact, the mobilization of $\alpha 6 \beta 4$ from hemidesmosomes in response to EGF enables this integrin to be incorporated into these F-actin protrusions. These findings suggest that the association of $\alpha 6 \beta 4$ with cytokeratins and F-actin is dynamic and can be modulated by motility factors (Figure 2).

Stimuli such as EGF and PMA (phorbol myristate acetate) that disassemble hemidesmosomes increase the phosphorylation of the $\beta 4$ cytoplasmic domain. This phosphorylation occurs predominantly on serine residues as assessed by phosphoamino acid analysis ([21]; see also Update). Although the functional contribution of this serine phosphorylation remains to be determined, activation of protein kinase C (PKC)- α appears to be important for both $\beta 4$ phosphorylation and hemidesmosome disassembly [21]. It has been argued also that EGF stimulation increases tyrosine phosphorylation of the $\beta 4$ cytoplasmic domain and that this phosphorylation is also a mechanism for hemidesmosome disassembly [23*]. However, mutation of the major tyrosine phosphorylation sites provides only a partial protection from EGF-induced hemidesmosome disassembly suggesting the involvement of other mechanisms. Clearly, further work is needed to resolve this important issue. Also, the possibility that other components of hemidesmosomes are targets for stimuli that disrupt these structures needs to be considered.

Adhesive properties of $\alpha 6 \beta 4$ and cell migration

Do the adhesive properties of $\alpha 6 \beta 4$ provide the traction necessary for epithelial migration? The strongest evidence to support this function comes from studies on carcinoma cells that exhibit random and chemotactic migration on laminin-1 [20,21]. Antibodies specific for $\alpha 6 \beta 4$ inhibit the migration of these cells. Interestingly, these antibodies do not block cell attachment or spreading, processes that depend on $\beta 1$

integrins, but they do inhibit the formation of filopodia and lamellipodia. The observation that $\alpha 6 \beta 4$ interacts with F-actin in filopodia and lamellipodia [20] suggests that it can transmit forces to the substrate generated by the acto-myosin system. This novel aspect of $\alpha 6 \beta 4$ function is supported by the recent finding using a traction-force detection assay that forces are exerted through $\alpha 6 \beta 4$ in cells plated either on laminin-1 or on an anti- $\alpha 6 \beta 4$ antibody [24*]. These results demonstrate that $\alpha 6 \beta 4$ can transmit forces directly without the need to engage other integrins. Collectively, these data indicate that engagement of laminin-1 by $\alpha 6 \beta 4$ can stabilize actin-rich protrusions and mediate traction forces necessary for cell movement.

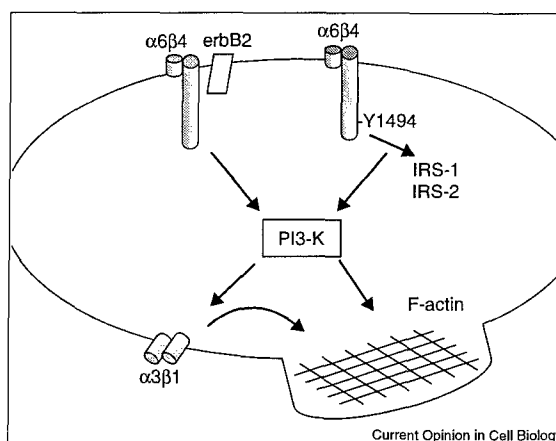
An important conundrum is the molecular nature of the association between $\alpha 6 \beta 4$ and F-actin. Most likely, this association is indirect because the $\beta 4$ cytoplasmic domain lacks a consensus actin-binding motif. One potential 'linker' molecule is HD-1/plectin, which also connects the $\beta 4$ subunit to intermediate filaments, because it contains an actin-binding site [25]. Although it has been reported that actin and $\beta 4$ compete for a similar site on HD-1 [26], this consideration would not necessarily exclude HD-1 as the connecting molecule. HD-1 is a rod-like tetramer with binding sites for actin and $\beta 4$ on each end; thus, it could bind $\beta 4$ on one end and F-actin on the other [27].

Signaling properties of $\alpha 6 \beta 4$ and cell migration and invasion

The $\alpha 6 \beta 4$ integrin has a significant impact on signaling pathways in epithelial and carcinoma cells, and this signaling influences their migration and invasion [16*,28,29,30–33*]. Signaling through $\alpha 6 \beta 4$ may not always depend on the adhesive functions of this integrin because its expression is sufficient to enhance migration and invasion, as well as lamellae formation, on non-laminin substrata such as collagen [29]. Moreover, these functions are not blocked by antibodies that inhibit $\alpha 6 \beta 4$ adhesive interactions, an observation that discounts the possibility of adhesion to laminins deposited on the substrate by the cells. Self-association of the $\beta 4$ cytoplasmic domains [34] could initiate intracellular signaling events independently of ligand binding. This possibility has profound implications for migration and invasion because it implies that the ability of $\alpha 6 \beta 4$ to stimulate those pathways is not limited to specific matrix environments.

Phosphatidylinositol 3-kinase (PI3-K) is probably the most important signaling molecule that is regulated by $\alpha 6 \beta 4$ with respect to migration (Figure 3; [28,31*,33*]). Pharmacological and genetic data indicate that this lipid kinase is essential for the chemotactic migration and invasion of epithelial-derived cells [28,33*,35], presumably because of its role in regulating actin dynamics. Several studies have observed that $\alpha 6 \beta 4$ is able to activate PI3-K more effectively than other integrins [16*,28,31*,32*], a finding that probably accounts for the marked stimulation of migration and invasion observed in response to $\alpha 6 \beta 4$ signaling. Importantly, activation of PI3-K by $\alpha 6 \beta 4$ may stimulate the function of other integrins, especially $\alpha 3 \beta 1$, that are also important for epithelial adhesion and

Figure 3



Central role of PI3-K in $\alpha 6 \beta 4$ -stimulated migration. The $\alpha 6 \beta 4$ integrin is able to activate PI3-K more effectively than other integrins. Proposed mechanisms for this activation include the association of $\alpha 6 \beta 4$ with erbB2 and possibly other growth factor receptors, and the activation of the IRS proteins by $\alpha 6 \beta 4$ signaling. A specific tyrosine residue (Y1494) located within a novel ITIM in the $\beta 4$ cytoplasmic domain is necessary for $\alpha 6 \beta 4$ -mediated phosphorylation of the IRS proteins, as well as for PI3-K activation. PI3-K plays a central role in regulating actin dynamics and promoting the formation of actin-protrusions involved in migration such as lamellipodia and filopodia. Activation of PI3-K by $\alpha 6 \beta 4$ may also stimulate the function of other integrins, especially $\alpha 3 \beta 1$, that are important for epithelial migration. For references, see text.

migration [16*]. Despite the evidence to support an important role for PI3-K in migration, it has been argued that $\alpha 6 \beta 4$ -stimulated PI3-K activity inhibits migration on laminin-5 based on the use of an antibody that activates $\beta 1$ integrins [32*]. Because the mechanism of this antibody-induced migration is not known, further studies are needed to explain how PI3-K reduces migration.

An important issue is the mechanism by which $\alpha 6 \beta 4$ activates PI3-K and the role of the $\beta 4$ cytoplasmic domain in this process (Figure 3). The ability of $\alpha 6 \beta 4$ to cooperate with specific growth factor receptors may be one mechanism for its robust activation of PI3-K. This integrin has been shown to associate with erbB2, a ligand orphan receptor of the EGFR family, on the surface of breast carcinoma cell lines [36]. And, expression of both $\alpha 6 \beta 4$ and erbB2 is required for PI3-K activation and the stimulation of chemo-invasion using a 3T3 cell model system [31*]. An alternative mechanism for PI3-K activation by $\alpha 6 \beta 4$ involves the insulin receptor substrates (IRS-1 and IRS-2), which contain multiple PI3-K-binding motifs [33*]. The IRS proteins are tyrosine phosphorylated and bind to PI3-K upon ligation of the $\alpha 6 \beta 4$ integrin by laminin or $\beta 4$ -specific antibodies. Interestingly, a critical tyrosine residue was identified in the third fibronectin Type III repeat of the $\beta 4$ A cytoplasmic domain (Y1494) (Figure 1) that appears to be necessary for the stimulation of IRS-2 phosphorylation and activation of PI3K by $\alpha 6 \beta 4$, as well as for the $\alpha 6 \beta 4$ -stimulation of chemo-invasion [33*].

Activation of mitogen-activated protein kinase (MAPK) signaling by $\alpha 6 \beta 4$ also has been implicated in epithelial motility [22]. A proposed mechanism of MAPK activation by $\alpha 6 \beta 4$ involves the direct binding of the adapter protein Shc to the $\beta 4$ cytoplasmic domain and the subsequent stimulation of Ras through the binding of Shc to Grb-2 and Sos [23*]. Tyrosine 1526, also located in the third fibronectin type III repeat of the $\beta 4A$ subunit, is reported to be a binding site for the PTB-domain of Shc. Mutation of this tyrosine residue inhibits Shc phosphorylation and activation of the MAPK Erk in response to $\alpha 6 \beta 4$ ligation [23*]. Additional mechanisms for the activation of MAPK through the $\alpha 6 \beta 4$ integrin are most likely to exist because $\alpha 6 \beta 4$ -dependent Shc phosphorylation has not been observed in all cells [33*]. One possible mechanism that warrants investigation is through Grb-2 recruitment to the IRS proteins because the Y1494 mutant of $\beta 4$ is unable to activate MAPK (see above).

The $\alpha 6 \beta 4$ integrin has been shown to stimulate activation of the Rac and RhoA GTPases, molecules that are central to migration [28,30*]. Activation of Rac is linked to $\alpha 6 \beta 4$ stimulation of PI3-K [28]. RhoA is also activated by $\alpha 6 \beta 4$ in carcinoma cells through a mechanism that involves cAMP and PKA [30*]. RhoA is important for migration because it can stimulate actomyosin contraction and generate contractile forces that are converted to traction by $\alpha 6 \beta 4$ and other integrins [24*,37].

In addition to the findings described above, recent studies are revealing new aspects of $\alpha 6 \beta 4$ signaling that could impact migration. The association of $\alpha 6 \beta 4$ with tetraspanins [38*,39*] is of particular interest because of the potential contribution of the tetraspanins to signaling and migration [40]. Also, the novel finding that $\alpha 6 \beta 4$ can stimulate the translation of cytokines such as VEGF through the PI3-K/mTOR pathway has significant implications for the generation of autocrine signaling loops that could influence migration (J Chung, AM Mercurio, unpublished data).

Conclusions

Important and distinct functions for the $\alpha 6 \beta 4$ integrin in the migration and invasion of epithelial cells are emerging that depend not only on the adhesive properties of this integrin but also on its potent signaling capacity. Much remains to be learned, however. The complex $\beta 4$ cytoplasmic domain, which is responsible for the many facets of $\alpha 6 \beta 4$, is largely unexplored. In this direction, the crystal structure of the first tandem pair of fibronectin Type III repeats in this domain has been reported providing a level of insight that should facilitate future structure-function studies [41]. The possibility that $\alpha 6 \beta 4$ exists on the cell surface as part of a large complex comprising growth factor receptor, tetraspanins and other molecules will influence approaches for studying this integrin and its role in migration. From a functional perspective, more work is needed on tissue specific knockouts of $\alpha 6 \beta 4$ not only to assess its contribution to tissue morphogenesis but, more importantly, to physiological and pathological stimuli that induce cell motility.

Update

The link between PKC-mediated serine phosphorylation of the $\beta 4$ subunit and hemidesmosome disassembly has been confirmed in another study [42].

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Protein Kinase A Regulates Rac and is Required for the Growth Factor-Stimulated Migration of Carcinoma Cells

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Abbreviations used in this paper are: EGF, epidermal growth factor; [cAMP]_i, intracellular concentration of cyclic AMP; IBMX, isobutylmethylxanthine; LPA, lysophosphatidic acid; MHC, major histocompatibility complex; PKA, cAMP-dependent protein kinase; PKI, protein kinase A inhibitor; PBD, Rac binding domain of Pak1; PDE, phosphodiesterase.

Abstract:

Members of the Rho family of small GTPases, such as Rho and Rac, are required for actin cytoskeletal reorganization during the migration of carcinoma cells. Phosphodiesterases (PDEs) are necessary for this migration because they alleviate cAMP/Protein kinase A (PKA)-mediated inhibition of RhoA (O'Connor et al, *J. Cell Biol.* 143:1749-1760; O'Connor et al, *J. Cell Biol.* 148:253-258). In this study, we report that migration of breast and squamous carcinoma cells toward either LPA or EGF involves not only PDE activity but also the cooperative signaling from PKA. Furthermore, we demonstrate that Rac1 activation in response to chemoattractant or $\beta 1$ integrin clustering is regulated by PKA and that Rac1 is required for this migration. Also, we find that $\beta 1$ integrin signaling stimulates the rapid and transient activation of PKA. A novel implication of these findings is that carcinoma cell migration is controlled by cAMP-dependent, as well as cAMP-inhibitory signaling mechanisms.

Cell migration in response to growth factors and chemoattractants is essential for embryonic development, tissue homeostasis and the immune response (1). It is also a major factor in the pathogenesis of many human diseases including cancer (2). This complex process involves dynamic and coordinated interactions among integrins, chemoattractant receptors and the actin cytoskeleton that result in actin polymerization at the leading edge and contraction of actin bundles within the cell body to promote translocation. These dynamic changes in the actin cytoskeleton are initiated by the engagement of chemoattractant and integrin receptors on the cell surface with their respective ligands. Such interactions trigger cascades of signaling events that result in the remodeling of the actin cytoskeleton and consequent directed movement (3). To understand cell migration at a mechanistic level, these signaling events need to be defined and linked to both cell surface receptors and actin dynamics. Members of the Rho family of GTPases including Rho, Rac and cdc42, in particular, are considered to be key signaling intermediates for cell migration (1,4-8). These GTPases are activated by signaling pathways initiated at the cell surface and, in their activated or GTP-bound state, stimulate downstream effectors that regulate actin polymerization and actin-myosin contraction (1,9). Despite considerable progress in understanding the function of the Rho GTPases, the mechanisms by which their activity is regulated by cell surface receptors and how this regulation relates to cell migration are understood poorly (10).

Recent studies have highlighted an important role for cAMP metabolism in the migration of carcinoma cells and in the regulation of RhoA function. Specifically, we found that cAMP-specific phosphodiesterases (PDEs) facilitate carcinoma cell migration, as well as lamellae formation, by lowering cAMP levels (11). More mechanistic studies have revealed that cAMP inhibits RhoA activity (8,12-14), which has been shown to be required for carcinoma migration and invasion (8,15,16). In fact, based on these observations, the current study was initiated to test the hypothesis that PKA activity inhibits carcinoma cell migration. Surprisingly, we observed that chemoattractant-stimulated migration requires PKA activity, as well as PDE

activity. In other terms, our results infer that this migration is controlled by cAMP-dependent as well as cAMP-inhibitory signaling mechanisms. We provide an explanation for this paradox by demonstrating that PKA is required for Rac activation by chemoattractants as well as $\beta 1$ integrins, a function that contrasts with its inhibition of RhoA. An important implication of these findings is that localized fluctuations in the $[cAMP]_i$ may provide a spatial and temporal regulation of PKA activity that influences Rac and RhoA function.

Experimental Procedures:

Cells: MDA-MB-435 human breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University. A431 squamous carcinoma cells were obtained from the American Type Culture collection. Cells were cultured in Dulbecco's-modified Eagle's medium (DMEM) with 10% fetal calf serum plus 1% L-glutamine, 1% penicillin, and 1% streptomycin (GIBCO-BRL, Gaithersburg, MD). For each experiment, subconfluent cell cultures were harvested with trypsin and rinsed with either DMEM or RPMI medium containing 250µg/ml heat-inactivated BSA (DMEM/BSA and RPMI/BSA, respectively).

Migration assays: Migration assays were performed as described previously (11). Briefly, Transwell chamber membranes (6.5mm diameter, 8µm pore size; Costar) were coated with 15µg/ml collagen I (trademark Vitrogen, Collagen Biomaterials). Lysophosphatidic acid (LPA; 100nM; Sigma) or Epidermal Growth Factor (EGF; 5ng/ml; Sigma) was added to the lower chambers. Cells (5×10^4) were added to the upper chamber and allowed to migrate for 4hrs at 37°C. Cells that had not migrated were removed from the upper chamber with a cotton swab. The remaining cells were fixed, stained with crystal violet, and counted as described previously (11). In some experiments, the following pharmacological inhibitors were added to the cell suspensions for 30 min. before cells were added to wells: H-89; IBMX and a myristoylated peptide derivative of PKI (Calbiochem). For checkerboard analysis of migration, LPA was placed in the bottom and/or top chambers of the Transwells, as noted, and then cells were added to the top chamber and assessed for migration as described above.

The involvement of Rac1 in migration was assessed using a dominant negative construct (N17Rac1). Briefly, cells were electroporated (8) with 1µg of pCS2-(n)β-gal and 4µg of either control vector or GST-tagged N17Rac1 construct (obtained from Chris Carpenter, BIDMC) at 250V and 500µFd. Cells were then cultured in growth medium containing 0.05% sodium butyrate for 16hrs and assayed for LPA- or EGF-stimulated migration 48hrs after the initial transfection as described above. N17Rac1 expression was confirmed by concentrating extracts of

transfected cells with glutathione-coupled Sepharose 4B beads (Pharmacia) and immunoblotting for Rac1 (mouse mAb; Transduction Laboratories).

Rac activity assays: Rac activity assays were based on established protocols (6,17). For these experiments, serum starved cells (3×10^6) were treated with 8 μ g of anti-integrin β 1 mouse mAb MC13 (obtained from Steve Akiyama, National Institutes of Health, Research Triangle Park, NC) for 30 min. at room temperature. Cells were then pelleted, resuspended in RPMI/BSA and either plated onto goat anti-mouse IgG (50 μ g; Jackson Immunochemicals) coated 60mm dishes or left in suspension. Cells were incubated at 37°C for the indicated time periods and then harvested with 50mM Tris, pH 7.4, 100mM NaCl, 1% NP-40, 10% glycerol, 2mM MgCl₂, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 μ g/ml pepstatin, 1mM PMSF. Cell extracts were cleared by centrifugation and 10% of the total volume was used for assessment of total Rac content. The remaining extract was combined with 2 volumes of binding buffer (25mM Tris, pH 7.5, 1mM DTT, 40mM NaCl, 30mM MgCl₂, 0.5% NP-40) and bacterially produced Rac/cdc42 binding domain of Pak (PBD)-GST fusion protein (obtained from Rick Cerione, Cornell University, ref. 17) coupled to glutathione beads and then incubated for 30 min. at 4°C. Beads were then rinsed three times with binding buffer and eluted in 2X Laemmli sample buffer. Aliquots of both total cell extracts and the eluents from the PBD beads were immunoblotted for Rac1.

To assess the impact of chemoattractants on Rac activation, cells were plated onto collagen-coated dishes in RPMI/BSA and allowed to attach and spread for 2-4 hours. Prior to chemoattractant stimulation, cells were treated with either carrier, 15 μ M H-89, 1 μ M PKI, or 1mM IBMX for 30 min. or with either the β 1 integrin-specific mAb MC13 or control IgG for 5 min. Cells were then treated with chemoattractant (5ng/ml EGF or 100nM LPA) for 10 min, unless indicated otherwise. Subsequently, cell extracts were assessed for Rac activity as described above.

PKA assays: For the PKA assays, cells were subjected to antibody mediated clustering of either β 1 integrins or MHC (using anti-HLA class I mAb, Sigma) or left in suspension as described above and harvested with a 50mM Tris buffer, pH 7.5 containing 5mM EDTA, 50mM NaF,

1mM sodium pyrophosphate and protease inhibitors. Cell extracts were sonicated and debris removed by centrifugation. Extracts were incubated for 5 min. at 30°C in reaction buffer (final concentration: 50mM Tris, pH 7.5, 10mM MgCl₂, 100μM ATP, 4nmol [γ -³²P] ATP, 0.25mg/ml BSA and 50μM Kemptide; Life Technologies) either alone (control) or in the presence of either 1μM PKI peptide (background), 10μM cAMP (total PKA activity) or PKI plus cAMP (total background activity). Samples were assayed in triplicate for each condition and quantified on a scintillation counter. PKI-inhibitable kinase activity was calculated and the data were reported as percent total PKA activity.

Results:

LPA-stimulated migration requires both PKA and PDE activities: PKA is activated in response to cAMP generated by adenylyl cyclases (18). We hypothesized that PKA is inhibitory for carcinoma cell migration based on our previous findings that chemotaxis requires PDE activity and that it is promoted by increasing PDE activity and reducing intracellular cAMP levels (11). To test this hypothesis, we assessed the migration of MDA-MB-435 breast carcinoma cells toward LPA in the presence or absence of the PKA-specific inhibitor, H-89. Checkerboard analysis indicated that this migration is predominantly chemokinetic (Fig. 1C). Surprisingly, treatment of these cells with concentrations of H-89 known to be specific for PKA *in vivo* (19) resulted in a concentration-dependent inhibition of migration (Fig. 1A). These concentrations of H-89 resulted in a dose-dependent decrease in total recoverable PKA activity (data not shown). The inhibitory effects of H-89 were confirmed using a myristoylated, cell permeable peptide derivative of the naturally occurring PKA Inhibitor (PKI; Fig. 1B).

The above results implicate PKA activity as a critical component of MDA-MB-435 chemokinesis. Together with our previous findings on PDEs (11), they suggest that chemoattractant-induced migration may involve both cAMP-dependent and cAMP-inhibitory signaling mechanisms. To determine if PDEs and PKA function either cooperatively or antagonistically in migration, cells were treated with suboptimal concentrations of both IBMX and H-89 prior to their use in the migration assay. As expected from our previous studies (8,11), the PDE inhibitor IBMX blocked the LPA-stimulated migration of MDA-MB-435 cells (Fig. 1A and ref. 11). Interestingly, the combined inhibitory effects of IBMX and H-89 treatment on migration were additive rather than compensatory. Collectively, these data emphasize the importance of PKA in migration and indicate that cAMP-dependent as well as cAMP-inhibitory signaling mechanisms work in a cooperative manner to regulate chemoattractant-induced migration of carcinoma cells.

Rac activation by $\beta 1$ integrins requires PKA: The small GTPase Rac is necessary for the migration and invasion of carcinoma cells (4-6) including MDA-MB-435 (Fig. 2A and ref. 4). Based on the findings that Rac and Rho often differ in their function and regulation and that cAMP impedes Rho function, we sought to determine whether that PKA activity is required for Rac activation in MDA-MB-435 cells.

First, we examined the involvement of LPA and integrins in Rac activation. For these experiments, we utilized the Rac/cdc42 binding domain (CRIB) of Pak fused to GST to affinity precipitate activated Rac (Rac assay) as described previously (6,17). In agreement with a previous report (20), we observed that Rac is activated in response to LPA. As shown in Fig. 2C, LPA stimulated a rapid and transient activation of Rac activation that peaked within 10 min. of stimulation. Interestingly, this activation was blocked by the addition of a $\beta 1$ integrin-specific mAb (Fig. 2C) indicating that the LPA stimulation of Rac is dependent on $\beta 1$ integrins. The addition of this mAb did not perturb the attachment of the cells to matrix (data not shown). Most likely, it prevented the formation of new $\beta 1$ integrins contacts in response to LPA.

Next, we assessed activation of Rac by integrin signaling. As shown in Fig. 3A, antibody-mediated clustering of $\beta 1$ integrins induced a time-dependent activation of Rac that was maximal at 30 min. Importantly, inhibition of PKA activity with either H-89 (Fig. 3B) or PKI (Fig. 3C) prevented Rac activation by $\beta 1$ clustering. These PKA inhibitors did not interfere with cell attachment to the Ab-coated wells (data not shown). Furthermore, the PDE inhibitor IBMX had no effect on the activation of Rac by $\beta 1$ integrin ligation (data not shown). These results indicate that Rac activation in response to clustering of $\beta 1$ integrins requires PKA activity.

$\beta 1$ integrin signaling stimulates PKA activity: In response to a chemoattractant such as LPA, new $\beta 1$ integrin-mediated contacts are formed at the leading edge to generate the traction necessary for migration (1,21). Given that both $\beta 1$ integrins (11) and PKA (Fig. 1) are required for cell migration on collagen, we tested the possibility that $\beta 1$ integrin signaling stimulates PKA activation. To address this hypothesis, $\beta 1$ integrins were clustered with a specific mAb for

varying periods of time and cell extracts were assayed for PKA activity (Fig. 4). As shown in Fig. 4A, mAb-mediated clustering of $\beta 1$ integrins resulted in a rapid, three-fold activation of PKA that decayed to baseline levels by 30 min. The specificity of this response was confirmed by the finding that clustering MHC class 1 molecules, which are expressed on the surface of MDA-MB-435 cells at levels comparable to $\beta 1$ integrins (data not shown), did not stimulate PKA activity (Fig. 4B). These data suggest that $\beta 1$ integrin signaling is a potential source of PKA activation required for chemotactic migration.

EGF-stimulated chemotactic migration and Rac activation also requires PKA. To extend the results obtained with MDA-MB-435 cells, we used another model of carcinoma cell migration that involves chemotaxis rather than chemokinesis. A431 cells are derived from a squamous carcinoma and chemotaxis efficiently toward low concentrations of EGF (Fig. 5 and refs. 22,23). Similar to the results obtained with MDA-MB-435 cells, chemotaxis of A431 cells toward EGF was sensitive to the inhibition of PDE and PKA activities by IBMX and H-89, respectively (Fig. 5A). Furthermore, A431 cell chemotaxis also required functional Rac (Fig. 5B) as determined by expression of a dominant negative mutant.

EGF stimulation of A431 cells results in the activation of Rac as assessed by the Pak binding assay (Fig. 6). In support of our hypothesis that PKA is required for Rac activation, we observed that pretreatment of cells with either H-89 (Fig. 6A) or PKI (data not shown) inhibited EGF-mediated Rac activation. In contrast, the PDE inhibitor IBMX had little effect on Rac activation (Fig. 6A). Together, these data substantiate the importance of PKA in chemoattractant-induced migration, which includes both chemotaxis and chemokinesis, and in the regulation of Rac.

Discussion

Recently, we established the importance of cAMP-dependent PDEs in the migration of carcinoma cells (8,11). This PDE activity is needed to lower cAMP levels within the cell and facilitate activation of RhoA. In our current study, we show that either LPA- or EGF-stimulated migration involves not only cAMP-inhibitory signaling that requires PDE activity but also a cAMP-dependent mechanism, mediated by PKA that involves Rac. Importantly, we find that activation of Rac in carcinoma cells, in response to either chemoattractant stimulation or β 1 integrin clustering, requires PKA activity.

Our results represent the first direct evidence that Rac activation can be controlled by cAMP/PKA. This finding is supported by other reports that have, indirectly, implicated Rac as a target of PKA. For example, PKA is necessary for cadherin-mediated cell-cell adhesion in epithelial cells (24), a process that is dependent on Rac (25). Also, Rac was shown to be an intermediate in the PKA-dependent activation of p38 mitogen-activated protein kinase (p38-MAPK) in response to thyroid-stimulating hormone (26). Here, we find that PKA is required for the Rac-dependent migration of carcinoma cells. In our study, we demonstrated that Rac activation in response to either β 1 integrin clustering or chemoattractant stimulation is blocked by pharmacological inhibitors of PKA and not by inhibition of phosphodiesterases. In support of this link between PKA and Rac activation, we reported that the Rac-independent migration of Clone A colon carcinoma cells does not require PKA (8).

An important implication of our findings is that PKA can contribute to the differential regulation of the Rac and Rho small GTPases. Recent studies have indicated a 'reciprocal' relationship between Rac and Rho activation and have provided evidence that Rac can inhibit Rho activity (7). Our data suggest that increased PKA activity would facilitate Rac activation and impede Rho activation, and *vice versa*, thus facilitating their reciprocal relationship. We and others have shown that cAMP/PKA mediated signaling has an inhibitory effect on RhoA activity (8,12-14). The basis for this inhibition involves the direct phosphorylation of RhoA by PKA (12,13). The mechanism by which PKA stimulates Rac activation, however, is unlikely to

involve its direct phosphorylation because Rac does not contain a consensus PKA phosphorylation site. PKA may regulate Rac indirectly by modifying the function of molecules that control Rac activation. For example, both Tiam-1 and Trio, which are guanine nucleotide exchange factors involved in Rac activation, have consensus PKA phosphorylation sites. Interestingly, Tiam-1 has been shown to be a target of LPA signaling (27) and, therefore, may provide a link between PKA and Rac activation.

The PKA dependence of Rac activation in the cells we examined contrasts with the reported PKA-mediated inhibition of Pak1 (28). Pak1, which is a down stream effector of both Rac and cdc42 (29), has been implicated in migration (30-32). One explanation for this discrepancy is that Rac activation may require only a transient burst of PKA activity, a possibility that is supported by our data on PKA activation in response to $\beta 1$ integrin clustering. The transient nature of PKA activation may permit Pak to be activated after complete Rac activation and diminution of PKA activity. Clearly, however, more studies are needed to understand the contribution of PKA to the regulation of Rac and Pak1 activation.

Recent studies by our group and others have revealed that integrins are involved in the regulation of the $[cAMP]_i$ and PKA activity (11,24,28,33,34). Moreover, our finding that $\beta 1$ integrins can activate PKA is in agreement with studies in other systems (24,33). Most likely, $\beta 1$ integrin signaling stimulates adenylyl cyclase activity and, consequently, a localized rise in $[cAMP]_i$ that increases PKA activity. Given the established importance of heterotrimeric (ht) G proteins in adenylyl cyclase activation and cAMP signaling, integrin-mediated PKA activation could be facilitated by ht G-proteins. In fact, several recent studies have provided evidence that supports this possibility. For example, mechanical stresses applied to the cell surface stimulate cAMP signaling by modulating local release of signals generated by activated integrins in a G-protein-dependent manner (33). Also, the $\alpha v\beta 3$ integrin has been reported to activate ht G_i by forming a complex with the integrin-associated protein CD47.

An important conclusion from our experiments involving pharmacological inhibition of PDE and PKA is that these enzymes, whose activities are counter-opposed, function

cooperatively to promote chemotactic migration. Their ability to signal cooperatively implies that these enzymes have distinct functions in migration. We suggest that they function together to create 'microgradients' of cAMP/PKA within a cell that are important for migration. This hypothesis is supported by the finding that localized gradients of the $[cAMP]_i$ regulate growth cone movement (35). The formation of cAMP/PKA gradient within migrating cells may differentially influence Rac and Rho resulting in spatial and temporal differences in the activation of Rac and Rho. Such differences could be manifested, for example, in the Rac-mediated lamellipodial protrusion and Rho-mediated contractility necessary for migration (1,29).

PKA regulation of Rac activation may have another important consequence for directed migration. It has been argued that the attractant gradient across a migrating cell is insufficient to signal directionality and that the chemotactic signals at the leading edge need to be amplified in order to orient and polarize a cell (36). Our results suggest that PKA signaling may provide one mechanism for amplifying chemotactic signals. During chemotaxis, new $\beta 1$ integrin contacts are formed at the leading edge of the cell in response to a chemoattractant gradient. It can be hypothesized that these new integrin contacts stimulate PKA and, as a consequence, amplify Rac activation. This scenario implies that activated Rac is localized at the leading edge of migrating cells. In fact, activated Rac has been localized at the leading edge of migrating cells using fluorescence resonance energy transfer (FRET) technology (37). Furthermore, Rac1 has been implicated in orienting Dictyostelium (38) and lymphocytes (39) during chemotaxis. In this context, it is worth noting that activation of $\beta 1$ integrins leads to the recruitment and colocalization of PKA regulatory subunit with integrin complexes and subsequent activation of PKA (24). In summary, our findings highlight the importance of cAMP and PKA in the regulation of small GTPase function and migration and identify important venues for future work.

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Figure Legends:

Figure 1. Chemotactic migration of MDA-MB-435 carcinoma cells requires both protein kinase A (PKA) and phosphodiesterase (PDE) activities. (A) MDA-MB-435 cells were treated for 30 min. with various concentrations of the PKA-specific inhibitor H-89 either alone (□) or in conjunction with 100μM (▣) or 500μM (■) of the PDE inhibitor IBMX. Cells (5×10^4) were then assayed for migration toward 100nM LPA in the continued presence of these inhibitors using a Transwell chamber assay as described in the Materials and Methods. (B) Cells were treated with either 1μM of a myristoylated, cell permeable peptide derivative of PKI or 15μM of H-89 and then assayed for migration as described above. (C) LPA (100nM) was placed in the either the bottom, top or both chambers of the Transwells as noted. Cells were then assessed for migration for 4hrs. Values reported represent the mean number of cells migrated per $\text{mm}^2 \pm$ standard deviation obtained from triplicate determinations.

Figure 2. (A) LPA-stimulated migration of MDA-435 cells requires Rac. Cells that had been co-transfected with a β-gal construct and either control vector or GST-tagged N17Rac were assayed for LPA stimulated migration as described in Fig. 1. Cells that had migrated were fixed and stained for β-gal. Transfected, β-gal positive cells were counted and reported as the relative number of cell migrated compared to the vector control. Bars represent mean $\pm$ standard error of triplicate determinations. P value for control versus N19 Rac = 0.02 (B) Transgene expression was confirmed by precipitating GST-Rac1 from extracts of cells in (A) with glutathione beads and immunoblotting for Rac1. (C) LPA stimulation of Rac activation is dependent on β1 integrins. Cells were plated onto collagen-coated dishes and allowed to attach and spread. Subsequently, either the β1 integrin-specific mAb MC13 or a control IgG was added to the cells for 5 min. and the cells were the stimulated with 100nM LPA for the indicated period of time. Cell extracts were assessed for Rac activity using the Pak binding domain (PBD) assay as described in Material and Methods. Representative experiments are shown.

Figure 3. Rac activation by $\beta 1$ integrins requires PKA. (A, B) Antibody-mediated clustering of $\beta 1$ integrins was performed as described in Fig. 2 either in the presence (A) or absence (B) of 15 μ M H-89 for times indicated. Cell extracts were assayed for Rac activation using the PBD assay as described in the Materials and Methods. (C) Antibody-mediated clustering of $\beta 1$ integrins was performed in either the presence or absence of the PKI peptide 30 min. and cell extracts were assayed for Rac activity as described above. Upper panels, Rac bound to the PBD; Lower panels, Total Rac expressed in cell extracts.

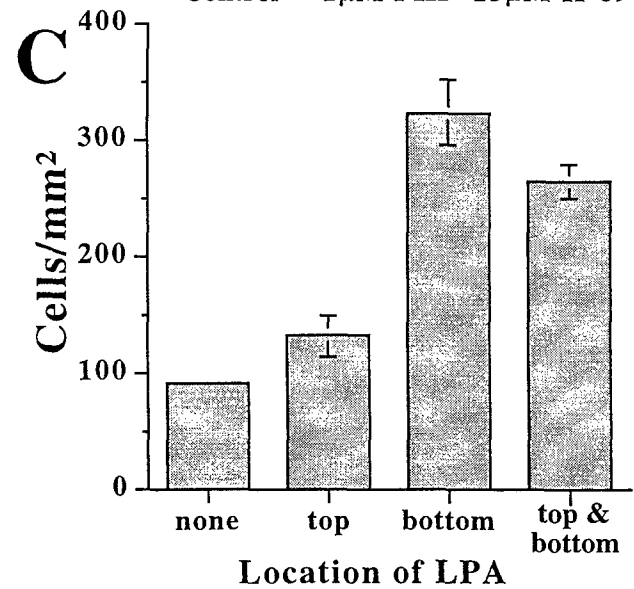
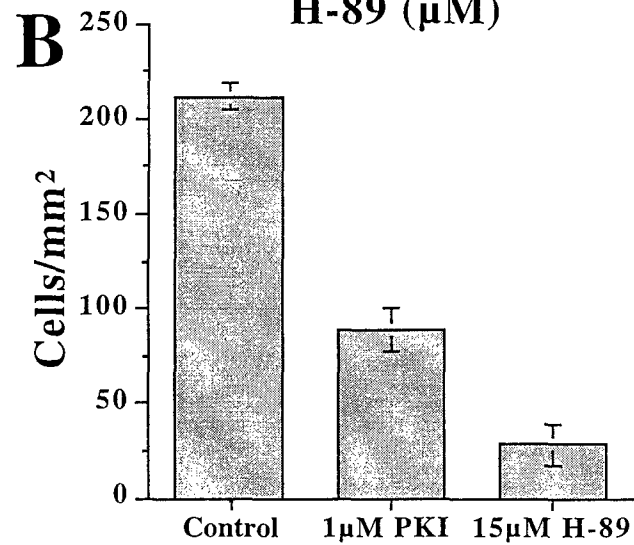
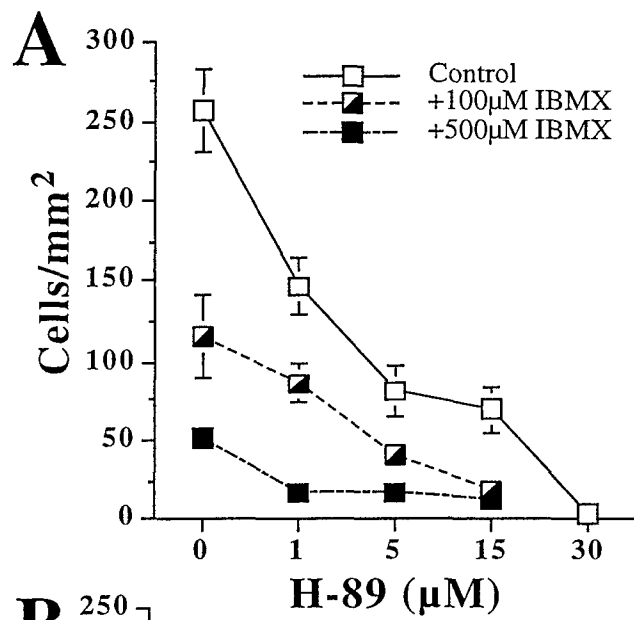
Figure 4. $\beta 1$ integrin signaling activates PKA. (A) MDA-MB-435 cells were incubated with MC13, a $\beta 1$ integrin-specific mouse mAb, for 30 min. Subsequently, the cells were added to tissue culture wells that had been coated with an anti-mouse IgG antibody. At the indicated times, cells were extracted and assayed for PKA activity as described in Materials and Methods. Values reported are the mean percent of total PKA activity $\pm$ standard deviation. (B) Cells were maintained in suspension or incubated with either the $\beta 1$ integrin mAb or an MHC-specific mAb and then added to tissue culture wells that had been coated with secondary antibody. After 5 min., cells were extracted and assayed for PKA activity. Values reported are the mean percent of total PKA activity $\pm$ standard error. Data are representative of four separate experiments. P value for suspension vs. $\beta 1$ clustered values = 0.001

Figure 5. Chemotactic migration of A431 cells toward EGF requires PKA, PDE and Rac1 activities. (A) A431 cells (5×10^4) were treated with either 15 μ M H-89 or 1mM IBMX for 30 min. and then assayed for chemotaxis toward 5ng/ml EGF for 4hr. as described in the Materials and Methods. (B) A431 cells were transfected with either vector alone or N17Rac as described in Fig. 3 and then assayed for EGF-stimulated chemotactic migration as described above. Transfected, β -gal positive cells were counted and reported as the relative number of cell

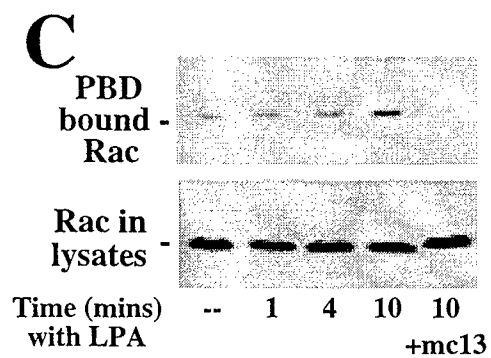
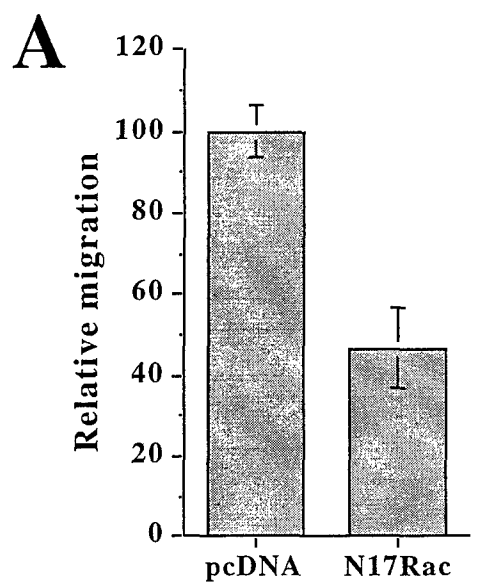
migrated compared to the vector control. Bars represent mean $\pm$ standard deviation of triplicate determinations (A, B). p value = 0.0002.

Figure 6. EGF treatment of A431 cells stimulates the PKA-dependent activation of Rac. A431 cells were plated on collagen I and then either left untreated or treated with 5ng/ml EGF alone or in the presence of 15 μ M H-89 or 1mM IBMX. Cells were then harvested and cell extracts assayed for either Rac as described in the Materials and Methods. (A) Representative immunoblots for active (top panel) and total (bottom panel) Rac1. (B) Quantitative analysis of the results obtained by densitometry. Bars represent mean of four separate experiments $\pm$ standard error.

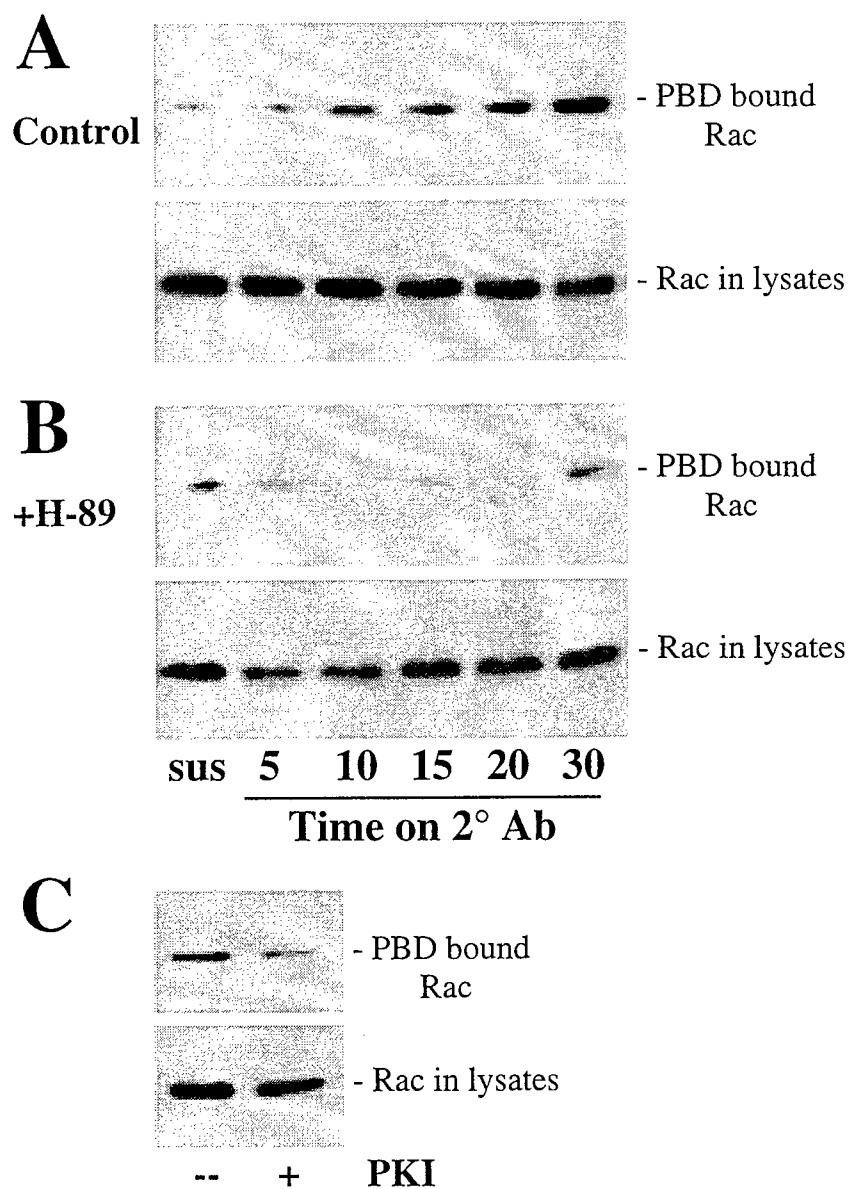
O'Connor and
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Figure 1



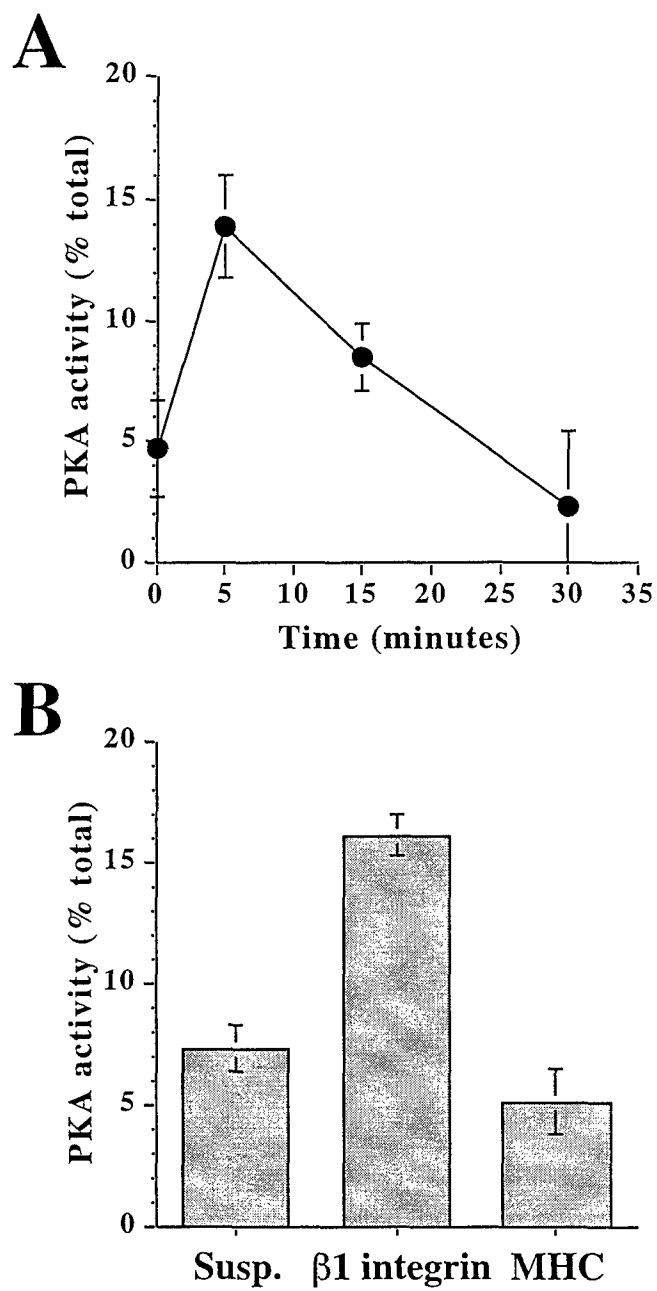
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Figure 2



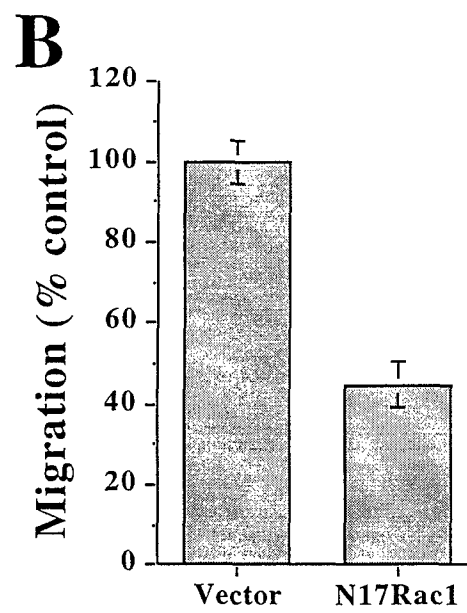
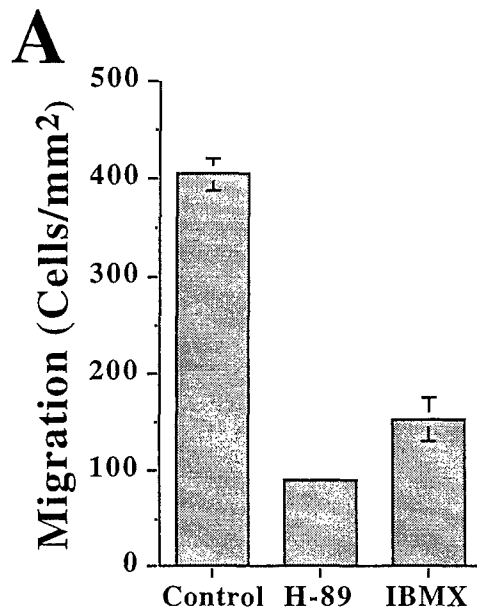
O'Connor and Mercurio
Figure 3



O'Connor and Mercurio
Figure 4



O'Connor and Mercurio
Figure 5



O'Connor and Mercurio
Figure 6

