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13. ABSTRACT *(Maximum 200 words)*

During the first year of this career development award, we have made progress in determining the contribution of the α6 integrin receptors to breast carcinoma progression. In previous work we had established that the α6β1 receptor contributes to the growth and survival of breast carcinoma metastases. In addition, we had demonstrated that de novo expression of the integrin β4 subunit in breast carcinoma cell lines that lack this integrin subunit increases their invasive potential. Since submitting the initial proposal, we have demonstrated that the ability of the α6β4 integrin to promote carcinoma invasion is related to its activation of phosphoinositide 3-OH kinase (PI3K) and the small GTP-binding protein Rac. In the past year we have identified PKC-epsilon as a critical effector for invasion and we will continue to investigate how this kinase contributes to this complex process. In addition we have identified a cooperative action of α6β1 with an important survival growth factor, IGF-1. Interestingly, our data indicate that the activation of PI3K appears to be an essential component of both α6-dependent pathways and this underscores the importance of understanding in more detail how this lipid kinase is activated and which downstream effectors are involved.

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Leslie M Shaw 9/29/98

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Introduction

A major advance 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).

Although several integrin heterodimers can function as receptors for members of the laminin family, the α6 integrins, α6β1 and α6β4, are the predominant laminin receptors in many cell types (7). The possibility that the a6 integrins play an important role in breast carcinoma progression is supported by a study by Imhof and colleagues (8) which examined the expression of α6 in invasive breast carcinomas of 119 women and correlated levels of expression with patient survival. The results demonstrated that high expression levels of the α6 subunit correlated with reduced patient survival time. In fact, α6 integrin expression was superior in predicting reduced survival than other known factors alone including estrogen receptor status. These data implicate a key role for the α6 integrins in deadly breast carcinomas. Data to support the involvement of the α6 integrins in the progression of other carcimomas has also been reported. Expression of the α6 integrins in prostate carcinoma cell lines correlates directly with the invasive potential of these cells in nude mice (9). In addition, expression of the α6β4 integrin has been reported to correlate with the progression of squamous, gastric, and colon carcinomas (10-12). Taken together, these data, in conjunction with the Imhof data, provide a compelling case for the involvement of α6 integrins in carcinoma progression. However, these studies only emphasize the potential importance of the α6 integrins in breast cancer, but they do not provide mechanistic information. Until this is established, the full potential of α6 for diagnosis, or as a target for therapeutic development, will not be known.

In previous work we had established that the α6β1 receptor contributes to the growth and survival of breast carcinoma metastases (13). In addition, we had demonstrated that de novo expression of the integrin β4 subunit in colon and breast carcinoma cell lines that lack this integrin subunit increases their invasive potential (14,15). The aim of this grant is to investigate these α6-dependent functions in more molecular detail. In this regard, since submitting the initial proposal we have demonstrated that the ability of the α6β4 integrin to promote carcinoma invasion is related to its activation of phosphoinositide 3-OH kinase (PI3K) and the small GTP-binding protein Rac (15).

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The involvement of a PI3K-dependent signaling pathway in invasion is supported by other studies (16) and adds to previous data that have implicated PI3K in tumor promoting functions including transformation (17), cell survival (18, 19), anchorage-independent growth (20), and motility (21). Taken together, these findings support a central role for PI3K in carcinoma progression and highlight the need to investigate in more detail how this pathway promotes the invasive phenotype. The functions of PI3K are mediated through its lipid products which bind and recruit signaling molecules to the plasma membrane where they can interact with other regulatory and effector molecules (22). To understand how PI3K enhances carcinoma invasion, it is necessary to identify the downstream effectors that are activated by the D3 phosphoinositides and that control

cellular functions that are required for the invasive phenotype. Using a genetic approach, we initially identified the small GTP-binding protein Rac as one of the essential downstream effectors of PI3K for invasion. Transient transfection of dominant negative Rac inhibited the invasion of a breast carcinoma cell line, MDA-MB-435, that expressed the α6β4 integrin (15). Rac most likely contributes to carcinoma invasion through its ability to promote lamellae formation, a function we have demonstrated is dependent upon PI3K activity (15) and that is required for cell motility (23). Interestingly, transient transfection of a constitutively active Rac did not increase the invasion of the MDA-MB-435 cells, suggesting that Rac is essential, but not sufficient to promote invasion (15). These findings support the probability that other downstream effectors of PI3K must cooperate with Rac to increase the invasive potential of carcinoma cells.

PKC Epsilon is required for carcinoma invasion. Several members of the Protein Kinase C (PKC) family, a group of homologous serine/threonine kinases, are activated by the lipid products of PI3K (22). The PKC isoforms that can be activated by the D3 phosphoinositides *in vitro* are the novel PKC-ε and -η and the atypical PKC-ζ (24). In addition, PKC-ε and PKC-ζ have both been demonstrated to be activated *in vivo* in a PI3K dependent manner (25,26). The regulation by PI3K makes these PKC isoforms strong candidates to participate in the PI3K-dependent promotion of invasion. In support of this, we had demonstrated in the initial grant application that a PKC family member was in fact required for invasion because Calphostin C, a specific PKC inhibitor, inhibited the invasion of both the mock and the b4-transfectants (Fig. 1A). To investigate the role of a specific PKC isoform in carcinoma invasion, we used the genetic approach that we used to address the involvement of Rac in invasion (15). Wild type and kinase inactive PKC isoform cDNAs were transfected into the MDA-MB-435/b4 transfectants and they were analyzed for their ability to invade. The kinase inactive mutants have been demonstrated to function as dominant negative mutants presumably by competing with endogenous protein for essential interactions. As shown in Figure 1B, transfection of wild type (WT) PKC-epsilon and -zeta, or kinase inactive (KI) PKC-zeta did not alter the ability of the MDA-MB-435/β4 cells to invade. However, transfection of the KI-epsilon inhibited invasion by approximately 50%. These data indicate that PKC-epsilon, but not PKC-zeta, is required for carcinoma invasion.

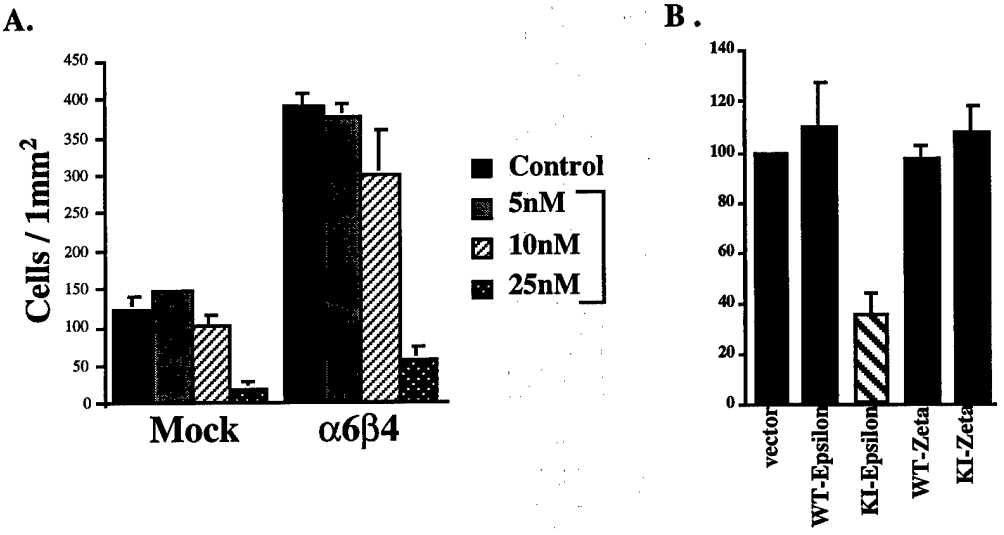

Figure 1: Involvement of PKC-epsilon in invasion. A) Calphostin C inhibited the invasion of both the mock and β4 transfectants. B) Expression of kinase inactive (KI) PKC-epsilon inhibited the invasion of the MDA-MB-435/β4 transfectants.

PKC Epsilon is activated by α6β4. To examine the possibility that PKC-epsilon is a downstream mediator of an α6β4 signaling pathway, in vitro kinase activity assays were performed using the mock and MDA-MB-435/β4 transfectants. After ligation of the α6 integrins with antibodies, extracts were immunoprecipitated with a PKC-epsilon-specific antibody and these immunoprecipitates were assayed for their ability to phosphorylate myelin basic protein. To date we have been unsuccessful in demonstrating the activation of PKC-epsilon in an *in vitro* kinase assay in this manner. However, this may be explained by the fact that PKC-epsilon can be regulated by diacylglycerol which makes it difficult to maintain the kinase in an active state in an *in vitro* kinase assay. There have been several reports that PKC-epsilon can be translocated to the cytoskeleton upon activation. Therefore, we examined the localization of PKC-epsilon after ligation of the α6β4 receptor with α6-specific antibodies. As shown in Fig. 2, significantly more PKC-epsilon was extracted with the cytoskeletal fraction after ligation of α6β4 that after ligation of α6β1. We are presently investigating if the activation of PKC-epsilon by α6β4 is dependent upon PI3K.

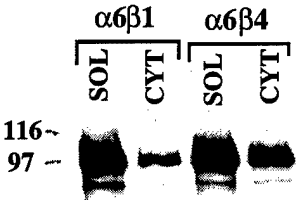

Figure 2: PKC-epsilon is activated by α6β4. PKC-epsilon is translocated to the cytoskeleton upon ligation of the α6β1 and α6β4 receptors. There is significantly more translocation of PKC-epsilon after clustering the α6β4 receptor than in response to α6β1 clustering.

PDK1 is required for α6β4-dependent invasion. The ability of PI3K to activate PKC-epsilon involves, in part, the phosphorylation of PKC-epsilon by the phosphatidylinositol-dependent kinase 1 (PDK1). PDK1 requires the lipid products of PI3K to be translocated to the plasma membrane where it can phosphorylate its substrates. We examined if PDK1 is required for PI3K-dependent invasion by transiently expressing either wild type PDK1 or kinase inactive PDK1 (PDK1-KI) cDNAs in the MDA-MB-435/β4 transfectants and assaying their ability to invade. As shown in Figure 3, expression of PDK1-KI, but not the wild type PDK1, inhibited invasion significantly. These results suggest that downstream substrates of PDK1, which include PKC-epsilon, are required for invasion.

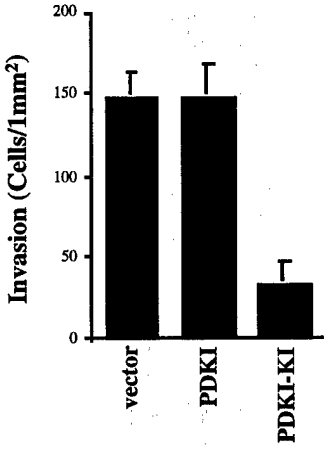

Figure 3: PDK1 is required for breast carcinoma invasion. Expression of kinase inactive (KI) PDK1 inhibited the invasion of the MDA-MB-435/β4 transfectants.

PI3K is required for the survival of MDA-MB-435 cells. The α6β1 integrin is required for the growth and survival of MDA-MB-435 cell in vivo. To understand the mechanisms involved in the contribution of α6β1 to survival, we established conditions *in vitro* that mimicked the survival differences of the mock and α6β4-ΔCYT transfected cells *in vivo*. Using this assay, we examined the signaling pathways that are involved in α6β1-dependent survival. As shown in Fig. 4, there is a 2.5 fold difference in the amount of apoptosis observed for the α6β4-ΔCYT transfectants when compared with the mock transfectants. Treatment of the mock transfectants with an inhibitor that prevents activation of the mitogen activated protein kinases (MAPK) Erk1 and Erk2 did not alter the survival of these cells. In contrast, treatment of the mock transfectants with wortmannin, an inhibitor of PI3K, resulted in a level of apoptosis that was comparable to that observed for the α6β4-ΔCYT transfectants. These data suggest that α6β1 promotes the survival of MDA-MB-435 cells through activation of a PI3K signaling pathway.

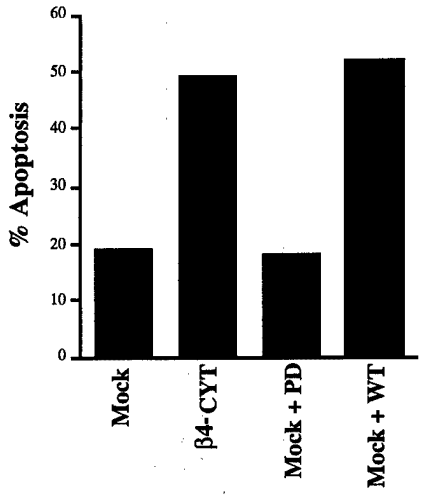

Figure 4: PI3K is required for a6b1-dependent breast carcinoma survival. Inhibition of PI3K by wortmannin (WT) increased the amount of apoptosis observed in the mock transfectant cells to the level observed for the α6β4-ΔCYT transfectants. Inhibition of MEK by PD98059 did not alter the survival of the mock transfectants.

The α6β1 receptor cooperates with the IGF-1 receptor to activate PI3K. In a previous report, we had demonstrated that the α6β1 receptor can cooperate preferentially with IGF-1 to activate Akt, a PI3K substrate that is involved in cell survival. To further examine the contribution of the α6β1 receptor to the growth and survival of breast carcinoma cells, we compared the ability of the α6β1 and α6β4-ΔCYT receptors to cooperate with IGF-1 to activate PI3K. As shown in Fig. 5 the α6β4-ΔCYT receptor is impaired in its ability to cooperate with the IGF-1 receptor to activate downstream signals, including PI3K. Significantly more PI3K p85 regulatory subunit was associated with a phosphotyrosine immunoprecipitation (A) after clustering the α6β1 receptor in the presence of IGF-1 than when α6β4-ΔCYT was clustered (B). Taken together, these data suggest that the decreased survival of the α6β4-ΔCYT transfectants could be the result of a decreased ability to respond to survival growth factors such as IGF-1.

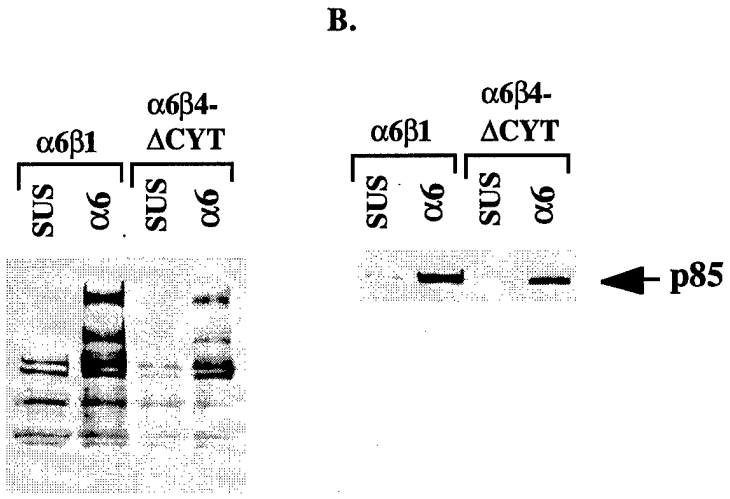

Figure 5: The α6β1 receptor cooperates with IGF-1 to activate PI3K. The increase in total cellular tyrosine phosphorylation that was observed after clustering the α6β1 integrin in the presence of IGF-1 was significantly greater than after clustering the α6β4-ΔCYT receptor (A). The amount of p85 PI3K regulatory subunit that was associated with these phosphotyrosine immunoprecipitates was also higher after clustering the α6β1 receptor (B).

Conclusions

The focus of this grant proposal is to understand the contribution of the α6 integrins to breast carcinoma progression. In this regard, we have begun to identify specific signaling pathways that are activated by the α6β1 and α6β4 receptors to promote carcinoma survival and invasion respectively. Since the submission of the initial grant application, we have made significant progress toward these aims (See reprint attached). The activation of PI3K appears to be an essential component of both α6-dependent pathways which underscores the importance of understanding in more detail how this lipid kinase is activated and which downstream effectors are involved. In the past year we have identified PKC-epsilon as a critical effector for invasion and we will continue to investigate how this kinase contributes to this complex process. In addition we have identified a cooperative action of α6β1 with an important survival growth factor, IGF-1. Future experiments will investigate this relationship in more detail.

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Activation of Phosphoinositide 3-OH Kinase by the α6β4 Integrin Promotes Carcinoma Invasion

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Summary

We demonstrate that the α6β4 integrin promotes carcinoma invasion through a preferential and localized targeting of phosphoinositide-3 OH kinase (PI3K) activity. Stable expression of α6β4 increased carcinoma invasion in a PI3K-dependent manner, and transient expression of a constitutively active PI3K increased invasion in the absence of α6β4. Ligation of α6β4 stimulated significantly more PI3K activity than ligation of β1 integrins, establishing specificity among integrins for PI3K activation. α6β4-regulated PI3K activity was required for the formation of lamellae, dynamic sites of motility, in carcinoma cells. The small G protein Rac is required downstream of PI3K for invasion. These studies define a mechanism by which the α6β4 integrin promotes carcinoma invasion and invoke a novel function for PI3K signaling.

Introduction

Understanding the progression from carcinoma in situ to invasive carcinoma is one of the most complex and challenging problems in the pathobiology of cancer. Because the biology of invasive 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 (Rodriguez-Boulan and Nelson, 1989). The progression to invasive carcinoma has been shown to involve perturbations in these features resulting in the acquisition of a motile, mesenchymal phenotype. This progression involves alterations in the expression and function of surface receptors that maintain the epithelial phenotype. Most notably, invasive carcinoma is characterized by a loss of function of cadherins, cell–cell adhesion receptors (Takeichi, 1993; Birchmeier et al., 1995). Integrins, in contrast, are essential for both normal epithelial function and for mediating dynamic processes associated with invasive carcinoma such as migration (Hynes, 1992). For this reason, it is assumed that the altered expression and function of specific integrins contribute significantly to invasive carcinoma (Juliano and Varner, 1993). Although considerable progress has been made in understanding how integrins regulate cell function, relatively little is known about integrin specificity and integrin-mediated signaling events in the pathogenesis of invasive carcinoma.

The integrin α6β4, a receptor for the laminins, is likely to play a pivotal role in the biology of invasive carcinoma (Rabinovitz and Mercurio, 1996). This integrin is essential for the organization and maintenance of epithelial structure (Dowling et al., 1996; Vanderneut et al., 1996). In many epithelia, this integrin mediates the formation of stable adhesive structures termed hemidesmosomes that link the intermediate filament cytoskeleton with the extracellular matrix (Borradori and Sonnenberg, 1996; Green and Jones, 1996). Expression of the α6β4 integrin persists, however, in many tumor cells that do not form stable adhesive contacts but rather exhibit the motile phenotype characteristic of invasive carcinoma. Indeed, numerous pathological studies have correlated α6β4 expression and localization with invasive carcinoma (Falcioni et al., 1986; Van Waes et al., 1991; Serini et al., 1996). Notable examples of the association between α6β4 expression and carcinoma include the finding that this integrin is not expressed in normal thyroid, but induction of its expression correlates with the progression to invasive thyroid carcinoma (Serini et al., 1996). Also, expression of the α6β4 integrin is enhanced at the invading fronts of gastric carcinomas (Tani et al., 1996). Such correlative studies have been substantiated by our finding that expression of α6β4 in a β4-deficient colon carcinoma cell line dramatically increases the invasive potential of these cells (Chao et al., 1996). Also, we demonstrated recently that α6β4 mediates the migration of invasive colon carcinoma cells on laminin-1 through its ability to associate with the actin cytoskeleton and promote the formation and stabilization of filopodia and lamellae (Rabinovitz and Mercurio, 1997). This finding implies that the function and cytoskeletal association of α6β4 in invasive carcinoma cells are distinct from its established role of anchoring epithelial cells to the basement membrane through its association with cytokeratins. Together, the current data indicate a key role for the α6β4 integrin in promoting carcinoma invasion, and they suggest that understanding the signaling pathways regulated by this integrin should provide insight into the mechanism of invasion.

Much of the work on signaling through the α6β4 integrin to date has focused on the structurally distinct, 1000 amino acid cytoplasmic domain of the β4 subunit (Hemler et al., 1989; Kajiji et al., 1989; Kennel et al., 1989). In keratinocytes, the β4 cytoplasmic domain has been shown to bind the adaptor protein Shc and activate the Ras-Mitogen activated protein kinase (MAPK) pathway, a pathway implicated in α6β4-mediated regulation of keratinocyte proliferation (Mainiero et al., 1997). The argument could be made that activation of MAPK by α6β4 is relevant for invasion because of the report linking MAPK to cell migration through its ability to phosphorylate myosin light chain kinase (Klemke et al., 1997).

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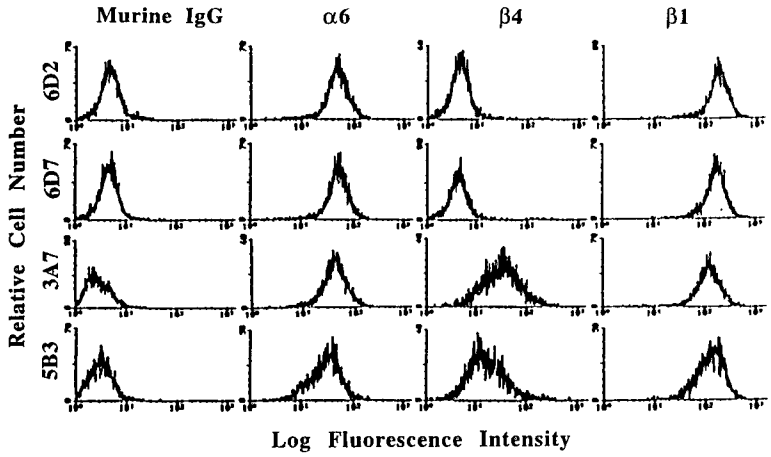

Figure 1. Surface Expression of Integrin Subunits in MDA-MB-435 Transfectants

Subclones of transfected MDA-MB-435 cells expressing β4 on the cell surface were isolated by FACS using UM-A9, a MAb specific for the β4 integrin subunit. MDA-MB-435 cells transfected with vector alone (6D2 and 6D7) or the human β4 integrin subunit (3A7 and 5B3) were analyzed by flow cytometry using monoclonal antibodies specific for the indicated integrin subunits.

No study, however, has addressed the possibility that MAPK or other signaling molecules downstream of α6β4 contribute to carcinoma invasion and metastasis. Indeed, the possibility exists that the signaling pathways required for invasion differ from those involved in cell proliferation. In addition, functions of α6β4 that contribute to invasion may also differ either qualitatively or quantitatively from signaling pathways regulated by other integrins, especially given the size and structural diversity of the β4 cytoplasmic domain.

In this study, we sought to identify signaling pathways through which the α6β4 integrin promotes invasion. For this purpose, we used a model system in which expression of this integrin in α6β4-deficient breast carcinoma cells markedly increases their rate of invasion. We also used a colon carcinoma model in which the α6β4 integrin is known to mediate motile events required for invasion. The data obtained reveal that PI3K (phosphoinositide-3 OH kinase) and the downstream effector Rac are required for carcinoma invasion. We also demonstrate that α6β4 activates PI3K preferentially over α6β1 and other β1 integrins and that this α6β4-regulated PI3K activity is required for the formation of lamellae, dynamic sites of motility in invasive carcinoma cells.

Results

Expression of the α6β4 Integrin Increases the Invasiveness of MDA-MB-435 Cells

The MDA-MB-435 cells used in this study do not express the α6β4 integrin, although they express the α6β1 integrin (Shaw et al., 1996). Stable subclones of these cells were generated that express either the α6β4 integrin or a mutated α6β4 that lacks the β4 cytoplasmic domain with the exception of the four amino acids proximal to the transmembrane domain (β4-ΔCYT). The relative surface expression of the α6, β4, and β1 subunits on the subclones used in this study is shown in Figure 1. Expression of the β4 subunit did not alter surface expression of the α6 subunit (Figure 1) or other integrin α subunits (data not shown) on these cells. However, a slight decrease in β1 surface expression was observed in the β4 transfectants that probably reflects a decrease in α6β1 expression at the expense of α6β4 expression (Figure 1).

The possibility that expression of the α6β4 integrin stimulates the invasion of carcinoma cells was examined by comparing the ability of mock transfectants (6D2 and 6D7) and β4 transfectants (3A7 and 5B3) to invade Matrigel in a standard chemoinvasion assay (Albini et al., 1987). As shown in Figure 2A, the rate of invasion of the β4 transfectants was approximately 3- to 4-fold greater than that of the mock transfectants in a 4 hr assay. The β4-ΔCYT transfectants invaded at a slightly slower rate than that of the mock transfectants (Figure 2A), indicating that the β4 cytoplasmic domain is essential for stimulating invasion. Interestingly, the rate of adhesion to laminin was not greater in the β4 transfectants than in the mock transfectants (data not shown).

Antibodies Specific for the α6β4 Integrin Stimulate Invasion of MDA-MB-435 Cells

To examine the contribution of integrin receptors to the invasion of MDA-MB-435 cells, Matrigel chemoinvasion assays were performed in the presence of integrin subunit-specific antibodies. A β1-specific antibody (MAb 13) inhibited invasion of the mock and β4 transfectants (Figure 2B). An α6-specific MAb (2B7) inhibited invasion of the mock transfectants by approximately 60% (Figure 2B), in agreement with our previous result that these cells use α6β1 as a major laminin receptor (Shaw et al., 1996). However, the same antibody increased the rate of invasion of the β4 transfectants by approximately 30% (Figure 2B). The stimulation of invasion observed for the α6 antibody in the MDA-MB-435/β4 transfectants suggests that α6β4 is not required for the adhesive functions involved in invasion but rather acts as a signaling receptor whose function can be enhanced by antibody binding. Such a phenomenon has been observed for stimulation of melanoma invasion by αv-specific antibodies (Seftor et al., 1992). This possibility is also supported by the finding mentioned above that α6β4 expression did not increase the rate of adhesion of MDA-MB-435 cells to laminin-1. These data indicate that the adhesive functions of the MDA-MB-435/β4 transfectants required for invasion are mediated largely by β1 integrins.

Invasion of MDA-MB-435 Cells Is Dependent on PI3K

As a prelude to identifying the signaling mechanism by which the α6β4 integrin stimulates invasion, we assessed first the effects of the MAPK kinase inhibitor

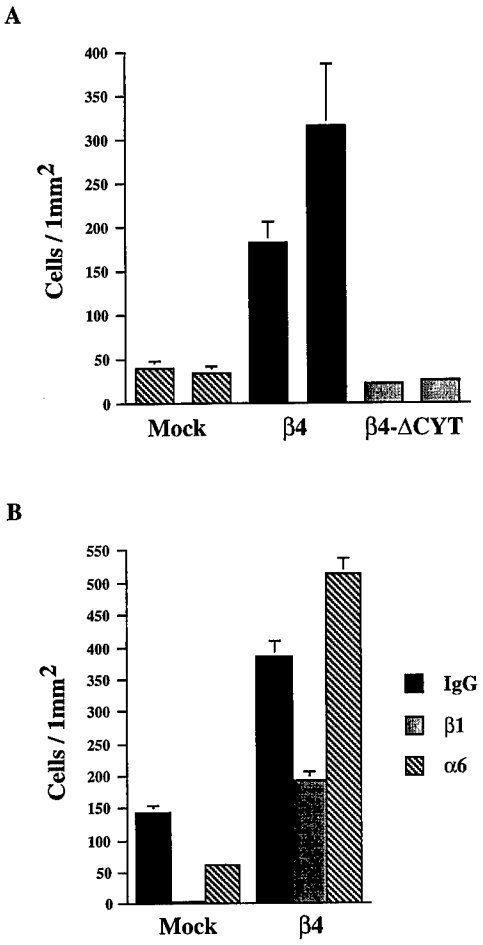

Figure 2. Invasion of the MDA-MB-435 Transfectants

(A) MDA-MB-435 transfectants were assayed for their ability to invade Matrigel. Matrigel was diluted in cold distilled water, added to the upper well of Transwell chambers, and dried in a sterile hood. The Matrigel was reconstituted with medium and the transfectants (5×10^4) were added to each well. Conditioned NIH-3T3 medium was added to the bottom wells of the chambers.

(B) MDA-MB-435 transfectants were assayed for their ability to invade Matrigel in the presence of monoclonal antibodies specific for the indicated integrin subunits. Cells were preincubated for 30 min in the presence of antibodies before addition to the Matrigel-coated wells. After 4 hr at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in Experimental Procedures.

Mock, MDA-MB-435 cells transfected with vector alone; β4, MDA-MB-435 cells transfected with the full-length β4 subunit; β4-ΔCYT, MDA-MB-435 cells transfected with the β4 subunit lacking the cytoplasmic domain; IgG, nonspecific antibody; β1, MAb 13; α6, 2B7. The data shown are from (A) two individual subclones of each transfectant and are the mean values (±SD) of a representative experiment done in duplicate, (B) the mean values (±SEM) of a representative experiment done in triplicate.

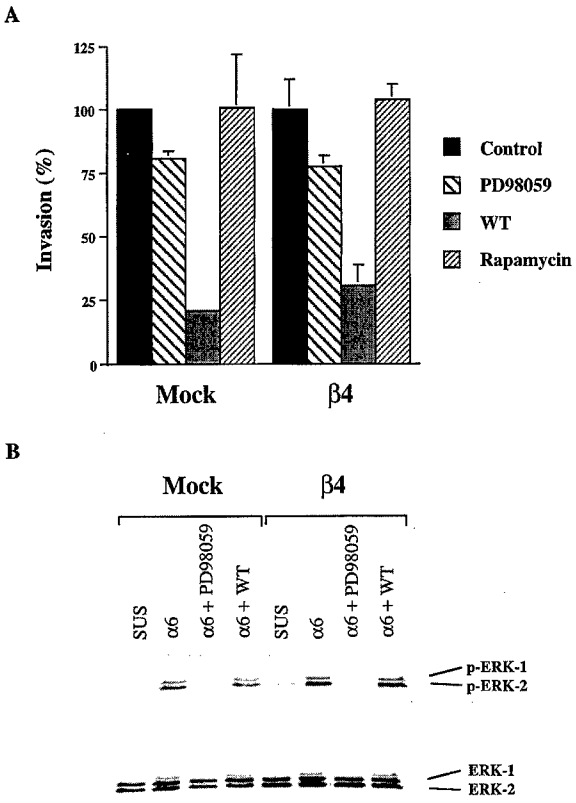

Figure 3. Analysis of MAPK, PI3K, and p70 S6K Involvement in MDA-MB-435 Invasion

(A) MDA-MB-435 transfectants were assayed for their ability to invade Matrigel in the presence of either the MEK inhibitor PD98059 (25 μM), the PI3K inhibitor wortmannin (100 nM), or the p70 S6K inhibitor rapamycin (20 ng/ml). Cells were preincubated for 10 min in the presence of the inhibitors before addition to the Matrigel-coated wells. After 4 hr at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in Experimental Procedures. The data shown are the mean values (±SD) of a representative experiment done in duplicate.

(B) MDA-MB-435 transfectants were maintained in suspension or incubated with α6-specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 min in the absence or presence of the indicated inhibitors. Aliquots of total cell extracts were normalized for protein content and resolved by 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and immunoblotted using an ERK polyclonal antibody that recognizes the phosphorylated isoforms of ERK-1 and ERK-2 (upper panel). The blots were then stripped and reprobed with an ERK-1 antibody that recognizes both ERK-1 and ERK-2 (lower panel).

Mock, MDA-MB-435 cells transfected with vector alone; β4, MDA-MB-435 cells transfected with the full length β4 subunit; WT, wortmannin; SUS, cells maintained in suspension.

PD98059 on MDA-MB-435 invasion (Dudley et al., 1995; Pang et al., 1995). As shown in Figure 3A, pretreatment of these cells with PD98059 (25 μM) resulted in only a modest inhibition (20%) of invasion. To confirm that PD98059 inhibits MAPK activity in these cells, an antibody that recognizes the phosphorylated, active isoforms of ERK1 and ERK2 was used. Antibody-induced clustering of the α6 integrins in both the mock and β4 transfectants using 2B7, an α6-specific Ab, stimulated activation of MAPK as assessed by reactivity with the phospho-specific MAPK Ab (Figure 3B). This activation was inhibited by PD98059 (25 μM). Of note, α6β4 expression did not have a significant impact on α6-induced MAPK activation in these cells.

We next targeted PI3K as a mediator of invasion because of its central involvement in multiple signaling pathways (Toker and Cantley, 1997). The PI3K inhibitor wortmannin (WT) (Ui et al., 1995) inhibited invasion of

both the mock and β4 transfectants by 70%–80% (Figure 3A). In contrast to PD98059, WT did not inhibit activation of MAPK by antibody-induced clustering of the α6 integrins in either the mock or β4 transfectants (Figure 3B). Taken together, these results suggest that PI3K, but not MAPK, is necessary for the invasion of MDA-MB-435 cells. The total amount of ERK-1 and ERK-2 protein was not altered by either PD98059 or WT (Figure 3B).

Activation of PI3K by the α6β4 Integrin

To determine if the α6β4 integrin can stimulate PI3K activity, in vitro kinase assays were performed using the mock, β4, and β4-ΔCYT transfectants of MDA-MB-435 cells. After ligation of the α6 integrins with 2B7, extracts were immunoprecipitated with a phosphotyrosine-specific antibody to capture the activated population of PI3K, and these immunoprecipitates were assayed for their ability to phosphorylate crude brain phosphoinositides. As shown in Figure 4A, an increase in PI3K activity, indicated by the appearance of PtdIns-3,4,5-P_3, was observed upon clustering the α6β1 integrin in the mock transfectants and the α6β4 integrin in the β4 transfectants. More importantly, PI3K activity stimulated by clustering the α6β4 integrin was markedly greater than that observed after clustering the α6β1 receptor. This enhanced stimulation of PI3K was also seen using a β4-specific MAb to ligate the α6β4 integrin in the β4 transfectants (Figure 4B).

PI3K activity was higher in the β4 transfectants than in the mock transfectants after adhesion to laminin-1 (Figure 4A). This observation suggests that interactions with laminin through this receptor can stimulate PI3K activity even though α6β4 is not used as an adhesion receptor in these cells. PI3K activity was not increased upon ligation of the α6β4-ΔCYT receptor, and little activity was evident when the transfectants were maintained in suspension (Figure 4).

Our data suggested that the ability of the α6β4 integrin to activate PI3K may be quantitatively greater than that of β1 integrins in MDA-MB-435 cells. This possibility was examined by comparing PI3K activation in the β4 transfectants in response to antibody ligation of either β1 integrins or the α6β4-integrin. As shown in Figure 4B, ligation of the α6β4 integrin with β4-specific antibodies stimulated PI3K activity approximately 2-fold greater than β1 integrin ligation, demonstrating that PI3K is activated preferentially by the α6β4 integrin. The differences between the abilities of the α6β4 and β1 integrins to activate PI3K are most likely even greater than what was observed given the 2- to 3-fold higher level of expression of β1 than β4 integrins on the cell surface (Figure 1).

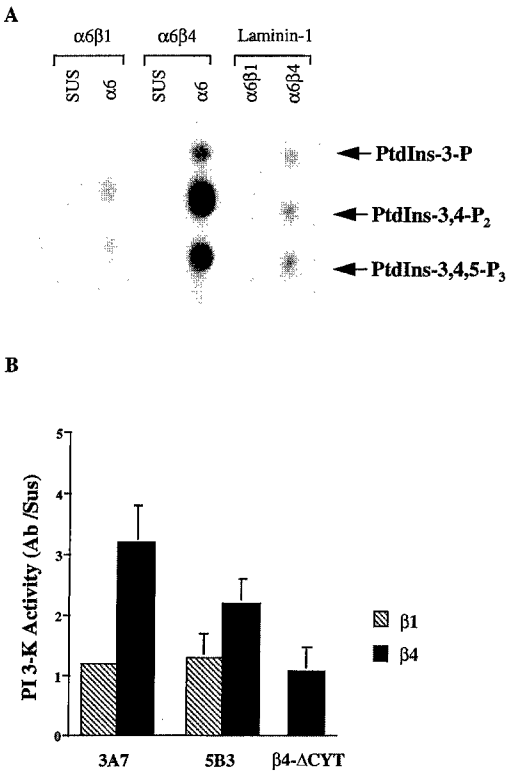

Figure 4. Analysis of PI3K Activity in the MDA-MB-435 Transfectants

(A) MDA-MB-435 transfectants were maintained in suspension or incubated with integrin-specific antibodies and allowed to adhere to anti-mouse IgG coated plates or laminin-1 coated plates for 30 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with the anti-phosphotyrosine MAb 4G10 and Protein A sepharose for 3 hr. After washing, the beads were resuspended in kinase buffer and incubated for 10 min at room temperature. The phosphorylated lipids were resolved by thin layer chromatography. The D3-phosphoinositides are indicated by arrows. SUS, cells maintained in suspension; α6, cells clustered with the α6-specific antibody.

(B) The amount of radiolabeled PtdIns-3,4,5-P_3 was determined for each condition by densitometry. The integrin-activated levels of PtdIns-3,4,5-P_3 were compared to the level observed for the cells that were maintained in suspension. The value from this ratio was determined to be the relative PI3K activity stimulated by each integrin subunit. The data shown are the mean values (±SD) from two representative experiments. 3A7, subclone of MDA-MB-435 cells transfected with the full-length β4 subunit; 5B3, subclone of MDA-MB-435 cells transfected with the full-length β4 subunit; β4-ΔCYT, MDA-MB-435 cells transfected with the β4 subunit lacking the cytoplasmic domain; β1, cells clustered with the β1-specific antibody (MAb 13); β4, cells clustered with the β4-specific antibody (A9).

Constitutively Active PI3K Stimulates Invasion of MDA-MB-435 Cells

The hypothesis that the α6β4 integrin promotes invasion of MDA-MB-435 cells by enhancing the activity of PI3K implies that expression of a constitutively active form of PI3K in the parental cells should increase their invasion in the absence of α6β4 expression. To validate this prediction, a constitutively active, membrane-targeted PI3K (Myr-p110-Myc) was expressed transiently in the parental MDA-MB-435 cells, and the ability of these cells to invade Matrigel was compared to cells transfected with an empty vector. As shown in Figure 5A, constitutively active PI3K increased invasion 2-fold, and this invasion was inhibited by wortmannin. Expression of the transiently expressed p110 subunit was confirmed by immunoblotting using a myc-specific antibody (Figure 5A).

α6β4-Dependent Invasion Requires PI3K Activity

If the α6β4 integrin promotes invasion of MDA-MB-435 cells by enhancing the activity of PI3K, expression of a

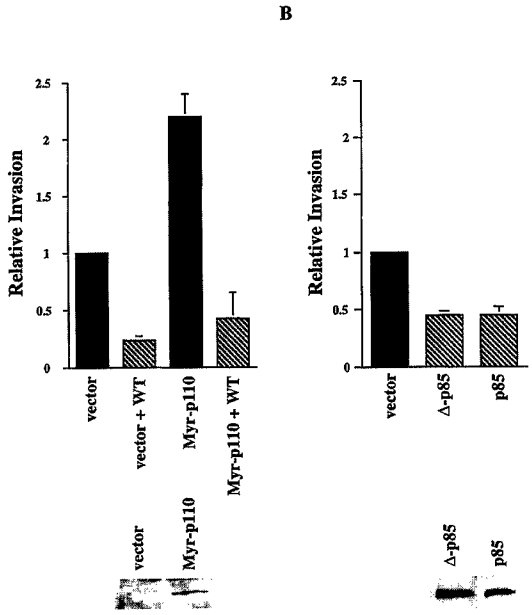

Figure 5. Analysis of PI3K Involvement in Invasion of MDA-MB-435 Cells by Transient Transfections

(A) MDA-MB-435 cells were transiently transfected with 1 μg pCS2-(n)β-gal and 4 μg of either the vector alone or a Myc-tagged, constitutively active form of the PI3K p110 catalytic subunit (Myr-p110) and assayed for their ability to invade Matrigel in the absence or presence of wortmannin (100 nM). The data shown are the mean values (±SD) of two (wortmannin) or three (without wortmannin) experiments done in triplicate.

(B) MDA-MB-435/β4 transfectants were transiently transfected with 1 μg pCS2-(n)β-gal and either the vector alone, 6 μg of a PI3K p85 subunit deleted in the p110 binding site (Δp85), or 6 μg of a wild-type PI3K p85 regulatory subunit (p85) and assayed for their ability to invade Matrigel. After 5 hr at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed and stained as described in Experimental Procedures. Invasion was quantitated by counting the cells that stained positively for β-galactosidase expression. Relative invasion was determined by comparing the amount of invasion obtained for the experimental transfections to that observed for the cells transfected with the vector alone, which was given the value of 1. The expression of the transfected cDNAs was confirmed by immunoblotting and is shown below each graph. The data shown are the mean values (±SD) of two experiments done in triplicate.

dominant negative PI3K subunit in the β4 transfectants should decrease their invasion. Transient expression of a GST-tagged, PI3K p85 subunit deleted in the p110 binding site (Δp85) inhibited invasion of the MDA-MB-435/β4 transfectants significantly (Figure 5B). A similar inhibition of invasion was observed after transient expression of a wild-type p85 subunit. It has been shown that overexpression of the wild-type p85 subunit blocks PI3K activation by binding to phosphotyrosine-containing proteins and inhibiting the binding of endogenous p85/p110 to these proteins (Rameh et al., 1995). The data obtained with the Δp85 and wild-type p85 subunits substantiate the wortmannin data shown in Figure 3A and confirm the involvement of PI3K signaling in α6β4-dependent invasion. Expression of the transiently expressed Δp85 and p85 subunits was confirmed by immunoblotting using GST- (Δp85) or myc-specific (wt p85) antibodies (Figure 5B).

The Akt/PKB Kinase and p70 S6 Kinase, Downstream Effectors of PI3K, Are Not Required for Invasion

The Akt/PKB serine/threonine kinase (Akt) and the p70 S6 kinase (S6K) are activated downstream of PI3K and, for this reason, could play important roles in invasion (Chou and Blenis, 1995; Franke et al., 1997). This possibility was supported by our finding that ligation of the α6β4 integrin in the MDA-MB-435/β4 transfectants activated both Akt and S6K (Figure 6A). Based on these observations, we examined the ability of parental MDA-MB-435 cells that expressed a constitutively active form of Akt (Myr-Akt) to invade Matrigel. Surprisingly, this constitutively active form of Akt actually decreased the rate of invasion in comparison to the control cells even though it was expressed at relatively high levels (Figure 6B). Most likely, the exogenously expressed active Akt sequestered a significant fraction of D3 phosphoinositides and precluded the use of these lipids in those signaling pathways downstream of PI3K that are required for invasion. We examined the involvement of S6K in invasion using rapamycin, a specific inhibitor of S6K activation (Chung et al., 1992; Kuo et al., 1992; Price et al., 1992). As shown in Figure 3, rapamycin did not decrease the invasion of either the mock or β4 transfectants. Based on these results, we conclude that Akt and S6K are not required for MDA-MB-435 invasion.

The Small G Protein Rac Is Required for MDA-MB-435 Invasion

The Rho family of small G proteins are involved in the actin rearrangements that result in the formation of stress fibers, membrane ruffles and lamellae, and filipodia (Nobes and Hall, 1995). The ability of cells to form these actin-containing structures is linked to their motility and therefore could influence their invasive potential (Sheetz, 1994; Rabinovitz and Mercurio, 1997). To examine this possibility, constitutively active mutants of either Rho (V14Rho), Rac (V12Rac), or Cdc42 (V12Cdc42) were transiently expressed in the parental MDA-MB-435 cells. As shown in Figure 6B, independent expression of these constitutively active small G proteins did not significantly alter the invasion of MDA-MB-435 cells, indicating that they are not sufficient by themselves to increase invasion.

To determine if either Rac or Cdc42 contributed to α6β4-dependent invasion, dominant negative mutants of Rac (N17Rac) and Cdc42 (N17Cdc42) were expressed in the MDA-MB-435/β4 transfectants. A significant reduction (50%) in invasion was observed when N17Rac was transiently expressed in the MDA-MB-435/β4 transfectants. In contrast, expression of N17Cdc42 did not inhibit invasion significantly (Figure 6C). To examine the role of Rac in PI3K-stimulated invasion of parental MDA-MB-435 cells, N17Rac was transiently expressed along with the Myr-p110-Myc construct. As shown in Figure 6D, coexpression of N17Rac inhibited the increased invasion that was observed when the constitutively active p110 subunit of PI3K was expressed alone. Taken together, these results demonstrate that Rac is an essential downstream mediator of the α6β4/PI3K signaling pathway involved in invasion. The inability of the constitutively active mutant of Rac to significantly increase

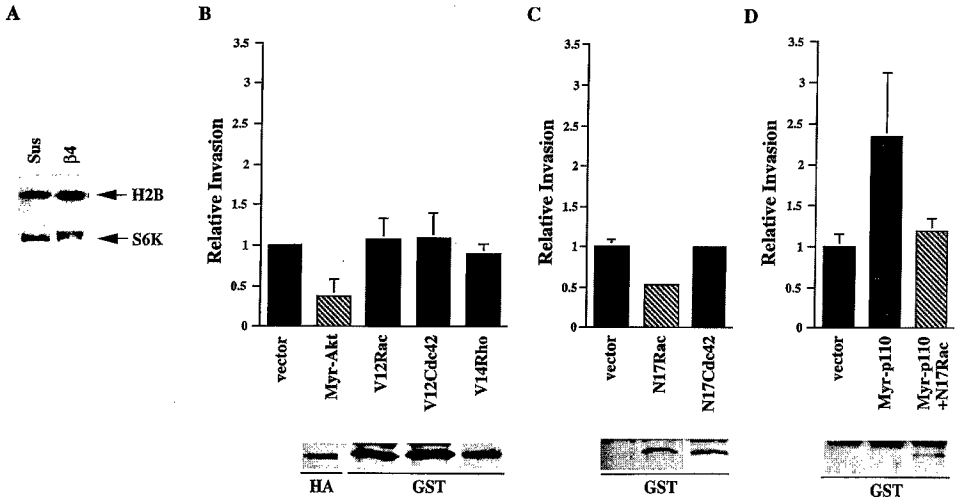

Figure 6. Analysis of Downstream Effectors in PI3K-Dependent Invasion of MDA-MB-435 Cells

(A) MDA-MB-435/β4 transfectants were maintained in suspension or incubated with α6-specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 min. (Upper panel) Aliquots of cell extracts that contained equivalent amounts of protein were incubated with a polyclonal anti-Akt antibody and a 1:1 mixture of ProteinA/Protein G. After washing, the beads were resuspended in kinase buffer and incubated for 20 min at room temperature. The phosphorylated substrate, histone H2B, is indicated. (Lower panel) Aliquots of total cell extracts were normalized for protein content and resolved by 8% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and immunoblotted using a polyclonal antibody that recognizes the C-terminal end of p70 S6K.

(B) MDA-MB-435 cells were transiently transfected with 1 μg of pCS2-(n)β-gal and either 4 μg of the vector alone or 4 μg of constitutively active mutants of Akt (Myr-Akt), Rac (V12Rac), Cdc42 (V12Cdc42), or Rho (V14Rho) and assayed for their ability to invade Matrigel. After 5 hr at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed and stained as described in Experimental Procedures. Invasion was quantitated by counting the cells that stained positive for β-galactosidase expression. Relative invasion was detemined by comparing the amount of invasion obtained for the experimental transfections to that observed for the cells transfected with the vector alone, which was given the value of 1. The data shown are the mean values (±SD) of 3 experiments done in triplicate.

(C) MDA-MB-435/β4 transfectants were transiently transfected with 1 μg of pCS2-(n)β-gal and either 4 μg of vector alone or 4 μg of dominant negative mutants of Rac (GST-N17Rac) or Cdc42 (N17Cdc42) and assayed for their ability to invade Matrigel as described above. The data shown are the mean values (±SD) of a representative experiment done in triplicate.

(D) MDA-MB-435 cells were transiently transfected with 1 μg of pCS2-(n)β-gal and either 5 μg of the vector alone, 3 μg of the vector alone, and 2 μg of a Myc-tagged constitutively active form of the PI3K p110 catalytic subunit (Myr-p110) or 2 μg of Myr-p110 and 3 μg N17Rac and assayed for their ability to invade Matrigel as described above. The data shown are the mean values (±SD) of a representative experiment done in triplicate. The expression of the transfected cDNAs was confirmed by Western blotting and is shown below each graph.

the invasion of the MDA-MB-435 cells suggests that other PI3K downstream effectors, in addition to Rac, are important for invasion in these cells.

Involvement of PI3K in the α6β4-Dependent Migration of Invasive Colon Carcinoma Cells

We wished to confirm that the activation of PI3K by α6β4 in the MDA-MB-435 transfectants also occurred in a carcinoma cell line that endogenously expresses the α6β4 integrin and that this activation was related to the invasive properties of the cell. For this purpose we chose clone A cells, an invasive colon carcinoma cell line that we have characterized extensively. Clone A cells express relatively high levels of the α6β4 integrin and no α6β1 integrin (Lee et al., 1992). Importantly, these cells use the α6β4 integrin as an adhesion receptor for laminin-1 (Lotz et al., 1990; Lee et al., 1992) in contrast to the MDA-MB-435/β4 transfectants. As shown in Figure 7A, the Matrigel invasion of clone A cells is inhibited by α6-specific antibodies.

Recently, we demonstrated that the α6β4 integrin performs an essential role in the migration of clone A cells on laminin-1 by promoting the formation and stabilization of filopodia and lamellae (Rabinovitz and Mercurio, 1997). Based on the data obtained with MDA-MB-435 cells, the prediction can be made that the α6β4-dependent formation of actin-containing motility structures is dependent on PI3K and that ligation of α6β4 stimulates PI3K activity in these cells. To examine this possibility, clone A cells were treated with wortmannin (100 nM) prior to their plating on laminin-1, and their behavior was then monitored by video microscopy. Wortmannin had no effect on the attachment of clone A cells to laminin or on their initial spreading. However, wortmannin inhibited the formation of lamellae by 80% (Figure 7B). Interestingly, the effect of wortmannin on the formation of lamellae was very similar to the effect of a function-blocking α6 antibody that recognizes only the α6β4 integrin in these cells (Figure 7C).

To examine whether α6β4 activates PI3K in clone A cells, we performed in vitro kinase assays on cell extracts prepared from cells that had attached to laminin-1 in the presence of either nonspecific IgG or an α6-specific antibody. As shown in Figure 8, PI3K activity was increased after attachment to laminin-1, but this increase was inhibited when the α6β4 receptor was blocked

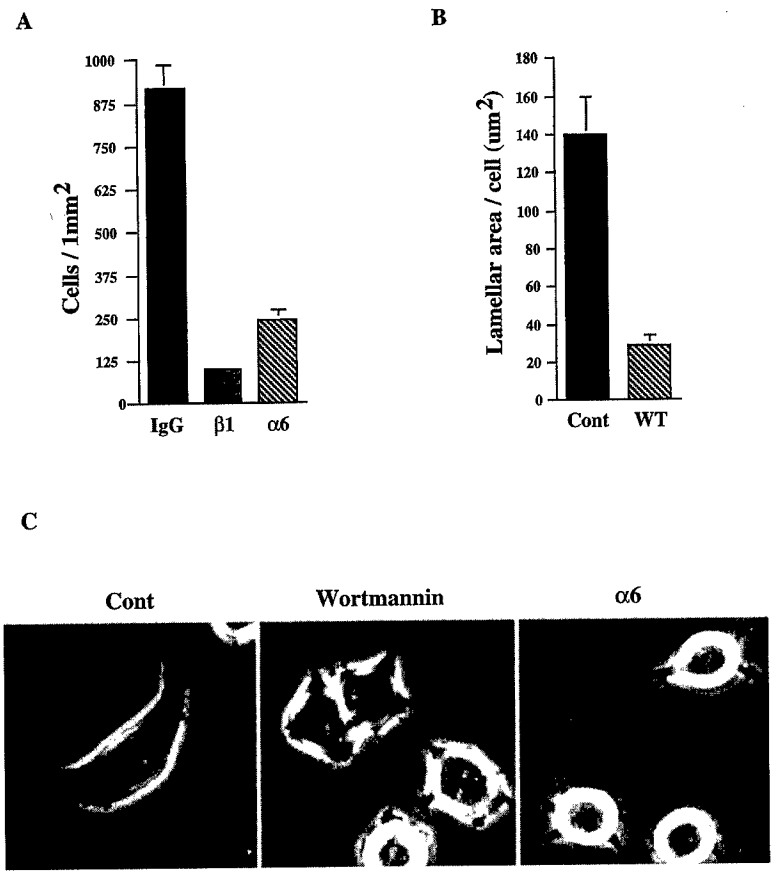

Figure 7. Analysis of α6β4 Function in an Invasive Colon Carcinoma Cell Line

(A) Clone A cells were assayed for their ability to invade Matrigel in the presence of monoclonal antibodies specific for the indicated integrin subunits. Cells were preincubated for 30 min in the presence of antibodies before addition to the Matrigel-coated wells. After 24 hr at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in Experimental Procedures. The data shown are the mean values (±SD) of a representative experiment done in duplicate.

(B) Clone A cells were allowed to adhere to laminin-1 in the absence or presence of wortmannin (100 nM) for 45 min. Images were obtained at this time using a Nikon Diaphot 300 inverted microscope with phase contrast optics. This microscope was connected to a CCD camera (Dage-MTI), a frame-grabber (Scion), and a 7600 Power Macintosh computer to capture the images. The lamellar area for the cells in each condition was determined using IPlab Spectrum image analysis software. The data shown are the mean values (±SEM) for ≥50 cells. WT, wortmannin.

(C) Clone A cells were allowed to adhere to laminin-1 in the absence or presence of wortmannin (100nM) or the α6-specific antibody 2B7, as indicated, for 45 min. Representative images from cells in each condition are shown.

IgG, non-specific antibody; β1, MAb 13; α6, 2B7; Cont, control.

by the α6 antibody. Although clone A cells still adhere to laminin-1 in the presence of the α6 antibody using the α2β1 integrin (Lotz et al., 1990; Lee et al., 1992; Figure 7C), there was no increase in PI3K activity in these cells compared to cells maintained in suspension. Therefore, a differential ability of the α6β4 and β1 integrins to activate PI3K is evident in clone A cells, as was observed in the MDA-MB-435/β4 transfectants. In summary, α6β4 is required for PI3K activation and formation of lamellae in response to laminin-1 attachment in clone A cells, functions that are required for the invasion of these cells.

Discussion

Our results establish that the coupling of a specific integrin, α6β4, to the PI3K signaling pathway promotes the invasion of carcinoma cells. In addition, we demonstrate specificity in integrin activation of this lipid kinase pathway because α6β4 activated PI3K better than α6β1 and other β1 integrins in the cells examined. An essential role for PI3K in invasion constitutes a novel function for this kinase and implies that downstream effectors of PI3K are critical for the invasive process. We provide evidence, in fact, that the small GTP-binding protein Rac is downstream of PI3K in the cells examined and that it is involved in invasion. In contrast, the serine/threonine kinases Akt and S6K do not contribute to the invasive process even though they are regulated by PI3K and are activated by the α6β4 integrin. Collectively, our findings provide a mechanism for the involvement of α6β4 in promoting carcinoma invasion and reveal a specific PI3K signaling pathway that is essential for this process.

Invasion is a defining event in carcinoma progression. In general, this process represents the ability of epithelial cells to acquire a mesenchymal phenotype characteristic of the breast and colon carcinoma cells used in this study. Numerous studies have demonstrated that the progression to invasive carcinoma involves loss of function of adhesion molecules involved in maintenance of the epithelial phenotype, namely, cadherins and catenins. These studies support this epithelial–mesenchymal transition model (Takeichi, 1993; Birchmeier et al., 1995). An issue that has been less clear, however, is an understanding of the mechanisms used by mesenchymal-like carcinoma cells to invade through basement membranes and stroma. Specifically, the role of epithelial integrins in invasion has been difficult to study because the progression to invasive carcinoma does not always involve gross alterations in their expression. More likely, the transition from a polarized epithelium to invasive cancer involves changes in integrin function that contribute to the invasive process as exemplified by our work on the α6β4 integrin. Although this integrin contributes to the polarized phenotype of epithelial cells through its ability to form stable adhesive contacts at the basal cell surface (Dowling et al., 1996; Vanderneut

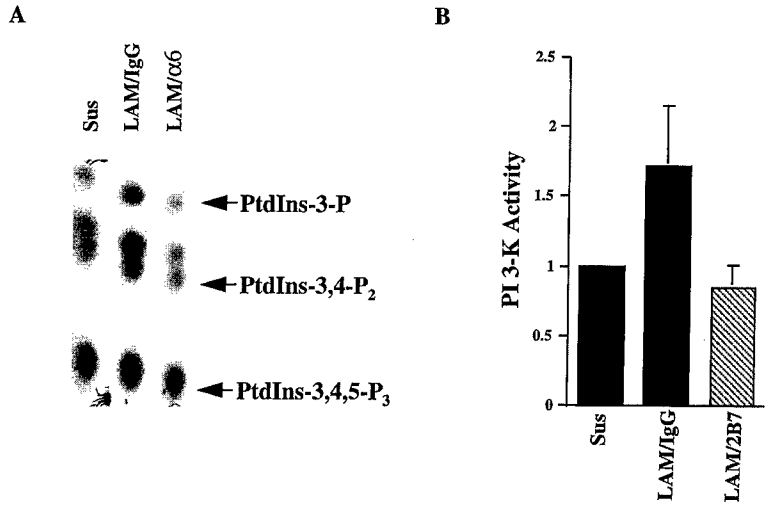

Figure 8. Analysis of PI3K Activity in Clone A Cells

(A) Clone A cells were maintained in suspension or allowed to adhere to laminin-1 in the presence of nonspecific IgG or α6-specific antibodies for 45 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with the anti-phosphotyrosine MAb 4G10 and Protein A sepharose for 3 hr. After washing, the beads were resuspended in kinase buffer and incubated for 10 min at room temperature. The labeled lipids were resolved by thin layer chromatography. The D3-phosphoinositides are indicated by arrows.

(B) The amount of radiolabeled PtdIns-3,4,5-P_3 was determined for each condition by densitometry. The adhesion-dependent levels of PtdIns-3,4,5-P_3 were compared to the level observed for the cells that were maintained in suspension, which was given the value of 1. The value from this ratio was determined to be the relative PI3K activity stimulated by adhesion. The data shown are the mean values (±SEM) from two experiments.

et al., 1996), the many pathological studies that have associated α6β4 with invasion suggested that its functions are also important for the invasive phenotype (Rabinovitz and Mercurio, 1996). We substantiated this possibility initially by showing that expression of α6β4 increased the invasion of both colon (Chao et al., 1996) and breast carcinoma cells (Figure 2A). Importantly, we now establish a mechanism for this increase by demonstrating that the α6β4 integrin activates a PI3K signaling pathway in carcinoma cells that is necessary for the invasive ability of these cells and that this α6β4-mediated pathway is linked to cell motility. Although protease activity is essential for invasion, no differences in protease expression or localization were observed between the mock and β4 transfectants of MDA-MB-435 cells (K. O'Connor, unpublished observation). Based on these findings, we postulate that the α6β4 integrin is critical for invasion because it promotes carcinoma motility through a PI3K-dependent pathway.

A central role for PI3K and its lipid products in carcinoma progression is indicated by our findings that wortmannin and dominant interfering p85 subunits of PI3K inhibited invasion of MDA-MB-435 cells and that a constitutively active p110 subunit of PI3K increased their invasion. The involvement of a PI3K-dependent signaling pathway in invasion adds to previous data that have implicated PI3K in tumor-promoting functions such as transformation, cell survival, anchorage-independent growth, and motility (Chang et al., 1997; Derman et al., 1997; Dudek et al., 1997; Kennedy et al., 1997; Khwaja et al., 1997). Interestingly, integrins have been implicated in many of these processes as well (Meredith et al., 1993; Frisch and Francis, 1994; Huttenlocher et al., 1995). However, how such PI3K-dependent pathways are influenced by integrin signaling is an area that has not been well explored. Recent reports that MDCK cell adhesion to collagen (Khwaja et al., 1997) and COS cell adhesion to fibronectin (King et al., 1997) can increase the levels of PtdIns-3,4,5-P_3 and PtdIns-3,4-P_3 suggested that integrins regulate PI3K. We now provide evidence that integrins can differ in their ability to activate PI3K based on the preferential activation of PI3K by α6β4 compared to other β1 integrins in both the MDA-MB-435 and clone A cells and that this difference is linked to a specific cellular response.

Our data on the PI3K-dependent formation of lamellae in clone A cells indicate that the localized regulation of PI3K activity by α6β4 may provide an efficient mechanism for targeting downstream functional effects of this kinase. The migration of invasive carcinoma cells involves the dynamic formation of actin-containing motility structures such as lamellae and filopodia. In fact, we have shown recently that α6β4 is localized in lamellae and filipodia of clone A cells and that the migration of these cells on laminin-1 involves the α6β4 integrin–dependent formation of these structures (Rabinovitz and Mercurio, 1997). An important finding in the present study is that the formation of these lamellae is dependent on PI3K. Quite surprisingly, wortmannin inhibited the formation of lamellae but had little effect on the adhesion of these cells. These observations implicate a rather specific role for α6β4-regulated PI3K activity in inducing the formation of actin-containing motility structures in carcinoma cells. The lipid products of PI3K, the D3 phosphoinositides, could play a direct role in their formation because they can bind to a number of proteins that regulate actin assembly (Hartwig et al., 1996; Lu et al., 1996), and they have been shown to contribute to filopodial actin assembly in platelets (Hartwig et al., 1996).

An issue that arises from the data presented is the mechanism by which the α6β4 integrin activates PI3K. The preferential activation of PI3K by α6β4 compared to α6β1, as well as other β1 integrins, in the MDA-MB-435 cells suggests that the mechanism by which α6β4 activates PI3K differs either quantitatively or qualitatively from these integrins. We know that the β4 cytoplasmic domain is required for PI3K activation because a cytoplasmic domain mutant of the β4 subunit failed to increase PI3K activity upon ligation. Although the

sequence of the β4 cytoplasmic domain is different from other integrin β subunits, it does not contain the consensus sequence for p85 binding via SH2 domains, YMXM (Cantley and Songyang, 1994), thus diminishing the possibility of a direct association with PI3K. In fact, we have not been able to detect such an association in our experiments. More likely, signaling intermediates are involved. In keratinocytes, for example, the β4 cytoplasmic domain can bind to the adaptor protein Shc and link the α6β4 integrin to the activation of Ras and downstream MAPK pathways (Mainiero et al., 1997). Although MAPK does not appear to be essential for the invasion of MDA-MB-435 cells even though its activity is stimulated by α6β4 ligation, the issue of whether α6β4 activation of Ras itself is involved in PI3K activation is relevant because Ras has been shown to activate PI3K (Rodriguez-Viciana et al., 1994, 1996).

One downstream effector of PI3K that we demonstrate is involved in carcinoma invasion is the small GTP-binding protein Rac (Nobes and Hall, 1995). This observation supports the previous reports that both constitutively active Rac and Tiam-1, a GDP-dissociation stimulator for Rac, can induce the invasion of lymphoid cells (Habets et al., 1994; Michiels et al., 1995). Interestingly, neither constitutively active Rac nor constitutively active Rho and Cdc42 were able to increase invasion of MDA-MB-435 cells, suggesting that activation of other PI3K dependent pathways is also required in these cells. Given the potential importance of Rac to invasion, subsequent studies on the α6β4-PI3K pathway will focus on downstream effectors of Rac. One downstream effector of Rac that we have determined is not involved in invasion is p70 S6K (Chou and Blenis, 1995, 1996). Although p70 S6K is activated by α6β4 ligation in the MDA-MB-435 cells, rapamycin, which blocks p70 S6K activation (Chung et al., 1992; Kuo et al., 1992; Price et al., 1992), did not inhibit invasion. Other possible effectors of Rac are the family of related p21-activated serine/threonine kinases (PAK1, -2, -3) that bind to and are activated by both Rac and Cdc42 (Manser et al., 1994; Martin et al., 1995). The current data on the role of the PAKs in mediating the effects of Rac are conflicting (Harden et al., 1996; Joneson et al., 1996; Lamarche et al., 1996; Westwick et al., 1997). However, these kinases and their downstream effector JNK can mediate actin rearrangements in some cell types and, for this reason, could contribute to the motile events required for invasion. This possibility is interesting because results from primary keratinocytes suggest that the α6β4 integrin can activate JNK in a Rac-dependent manner and that this activity is inhibited by wortmannin (Mainiero et al., 1997).

The PI3K signaling pathways involved in invasion appear distinct from those involved in PI3K-dependent cell survival. Even though the Akt kinase is activated by α6β4 ligation, it is not required for invasion of the breast carcinoma cells we examined. In fact, expression of a constitutively active form of Akt actually inhibited the invasion of these cells, most likely because activated Akt uses the D3-phosphoinositide products of PI3K at the expense of those PI3K-dependent pathways that are involved in invasion. A fascinating problem is raised by these observations because carcinoma progression involves both tumor cell invasion and survival. The recent demonstration that Akt is required for the survival of several cell types (Dudek et al., 1997; Kennedy et al., 1997), coupled with our finding that PI3K is required for invasion, suggests that two essential functions of progression may require the products of PI3K and that the balance between the use of these pathways may impact tumor cell invasion or survival.

In summary, we have identified a specific integrin-mediated pathway involving PI3K that promotes carcinoma invasion. This pathway involves the small GTP-binding protein Rac. These findings are particularly important because they suggest that this PI3K signaling pathway is a potential target for inhibiting tumor spread.

Experimental Procedures

Cells

The MDA-MB-435 breast carcinoma cell line was obtained from the Lombardi Breast Cancer Depository at Georgetown University. The MDA-MB-435 cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (GIBCO) and 1% penicillin-streptomycin (GIBCO). Clone A cells were grown in RPMI supplemented with 25 mM HEPES (RPMI-H), 10% fetal calf serum, and 1% penicillin-streptomycin.

The cloning of the human β4 cDNA, the construction of the β4 cytoplasmic domain deletion mutant (β4-ΔCYT), and their insertions into the pRc/CMV (β4) and pcDNA3 (β4-ΔCYT) eukaryotic expression vectors, respectively, have been described previously (Clarke et al., 1995). The vectors containing the full-length and mutant β4 cDNAs, as well as the pcDNA3 vector alone, were transfected into the MDA-MB-435 cell line using Lipofectin (GIBCO) according to the manufacturer's instructions. Neomycin-resistant cells were isolated by selective growth in medium containing G418 (0.6 mg/ml; GIBCO). The stable transfectants were pooled and populations of cells that expressed the human β4 subunit on the cell surface were isolated by FACS. A human β4 integrin–specific MAb, UM-A9 (obtained from Tom Carey), was used for this sorting and for subsequent analysis of the transfectants. The sorting was repeated sequentially to enrich for homogeneous populations of cells expressing high levels of the transfected β4 and β4-ΔCYT subunits on the cell surface. Subclones were isolated from these populations by FACS. Surface labeling and immunoprecipitation with A9 were done to confirm that the α6β4 heterodimer was expressed on these subclones.

Analysis of Integrin Surface Expression

The relative surface expression of integrin subunits on the mock and β4 transfectants of the MDA-MB-435 cells was assessed by flow cytometry. For this purpose, aliquots of cells (5×10^5) were incubated for 45 min at room temperature with RPMI-H and 0.2% BSA (RH/BSA) and the following integrin-specific Abs: 2B7 (α6; prepared in our laboratory); MAb 13 (β1; provided by Stephen Akiyama); A9 (β4; provided by Thomas Carey); as well as mouse IgG (Sigma). The cells were washed two times with RH/BSA and then incubated with goat $F(ab')_2$ anti-mouse IgG coupled to fluorescein (Tago) for 45 min at room temperature. After washing two times with RH/BSA, the cells were resuspended in the same buffer and analyzed by flow cytometry.

Invasion Assays

Matrigel invasion assays were performed as described (Shaw et al., 1996) using 6.5 mm Transwell chambers (8 μm pore size; Costar). Matrigel, purifed from the EHS tumor, was diluted in cold distilled water, added to the Transwells (2–10 μg/well), and dried in a sterile hood. The Matrigel was then reconstituted with medium for an hour at 37°C before the addition of cells. Cells were resuspended in serum-free medium containing 0.1% BSA, and cells were added to each well. Conditioned NIH-3T3 medium was added to the bottom wells of the chambers. After 4–6 hr, the cells that had not invaded were removed from the upper face of the filters using cotton swabs, and the cells that had invaded to the lower surface of the filters were fixed in methanol and then stained with a 0.2% solution of

crystal violet in 2% ethanol. Invasion was quantitated by visual counting. The mean of five individual fields in the center of the filter where invasion was the highest was obtained for each well. In some assays, the cells were preincubated for 30 min before addition to the Matrigel-coated wells with either wortmannin (Ui et al., 1995), PD98059 (Dudley et al., 1995; Pang et al., 1995), or rapamycin (Chung et al., 1992; Kuo et al., 1992; Price et al., 1992). For assays that used the transient transfectants described below, the cells were fixed for 30 min in 4% paraformaldehyde and then stained with PBS containing 1 mg/ml bluo-gal (Boehringer Mannheim), 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide.

Kinase Assays

Cells were removed from their dishes with trypsin and washed twice with RH containing 0.2% heat-inactivated BSA. After washing, the cells were resuspended in the same buffer at a concentration of 2×10^6 cells/ml and incubated for 30 min with integrin-specific antibodies or in buffer alone. The cells were washed once, resuspended in the same buffer, and added to plates that had been coated overnight with either anti-mouse IgG or laminin-1. After a 30 min incubation at 37°C, the cells were washed twice with cold PBS and solubilized at 4°C for 10 min in a 20 mM Tris buffer (pH 7.4), containing 0.14 M NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 2 mM PMSF, 5 μg/ml aprotinin, pepstatin, and leupeptin. Nuclei were removed by centrifugation at 12,000 × g for 10 min.

To assay PI3K activity, aliquots of cell extracts that contained equivalent amounts of protein were incubated for 3 hr at 4°C with either the anti-phosphotyrosine MAb 4G10 (UBI) or a p85 subunit–specific PI3K antibody and Protein A sepharose (Pharmacia). The sepharose beads were washed twice with solubilization buffer and twice with a 10 mM HEPES buffer (pH 7) containing 0.1 mM EGTA (kinase buffer). After removal of the last wash, the beads were resuspended in kinase buffer containing 10 μg of sonicated crude brain lipids (Sigma), 100 μM ATP, 25 mM $MgCl_2$, and 10 μCi [γ-^{32}P]ATP and incubated for 10 min at room temperature. The reaction was stopped by the addition of 60 μl 2N HCl and 160 μl of a 1:1 mixture of chloroform and methanol. Lipids were resolved by thin layer chromatography plates coated with potassium oxalate.

To assay MAP kinase, total cell extracts, prepared as described above, were resolved by electrophoresis on SDS-polyacrylamide gels (10%), transferred to nitrocellulose, and blotted with a phospho-specific ERK polyclonal antibody that recognizes the phosphorylated isoforms of ERK-1 and ERK-2 (New England BioLabs, Inc.). Immune complexes were detected using a secondary antibody conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence (Amersham, Inc.). The blots were then stripped and reprobed with an ERK-1 antibody (provided by John Blenis) that recognizes both ERK-1 and ERK-2.

To assay Akt kinase activity, total cell extracts containing equivalent amounts of protein were precleared with a 1:1 mixture of Protein A/Protein G and then incubated with a polyclonal antibody that recognizes the C-terminal end of Akt (provided by Thomas Franke) for 3 hr at 4°C. After a 1 hr incubation with the Protein A/Protein G mixture, the beads were washed 3 times with solubilization buffer, once with H_20, and once with a 20mM HEPES buffer (pH 7.4) containing 10 mM $MgCl_2$ and 10 mM $MnCl_2$ (kinase buffer). After removal of the last wash, the beads were resuspended in 30 μl of kinase buffer containing 5 μM ATP, 1 mM DTT, 10 μCi [γ-^{32}P]ATP, and 1.5 μg of Histone H2B (Boehringer Mannheim) and incubated for 20 min at room temperature. The reaction was stopped by the addition of 5× Laemmli sample buffer and resolved by electrophoresis on SDS-polyacrylamide gels (12%).

To assay p70 S6K activation, total cell extracts, prepared as described above, were resolved by electrophoresis on SDS-polyacrylamide gels (8%), transferred to nitrocellulose, and blotted with a polyclonal antibody that recognizes the C-terminal end of p70 S6K (provided by John Blenis). Immune complexes were detected using a secondary antibody conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence (Amersham, Inc.).

Transient Transfections

The constitutively active PI3K catalytic p110 subunit was a generous gift of Julian Downward, ICRF, London. The small GTP-binding proteins V14Rho, V12Rac, V12Cdc42, N17Rac, and N17Cdc42, in the pEBG vector, were a kind gift of Margaret Chou, University of Pennsylvania. The constitutively active Akt was kindly provided by Philip Tsichlis, Fox Chase Cancer Center, PA. The dominant negative PI3K p85 subunit was kindly provided by Brian Schaffhausen, Tufts University. The pCS2-(n)β-Gal was a gift from Sergei Sokol, Beth Israel Deaconess Medical Center.

Cells were cotransfected with 1 μg pCS2-(n)β-Gal and the cDNAs specified in the figure legends using Lipofectamine (GIBCO) according to manufacturer's instructions. Cells were harvested 24 hr after transfection and added to Matrigel invasion assays as described. Transfected cells were also plated in 48 wells to stain for β-galactosidase expression to determine transfection efficiency. The remaining cells were collected and extracted in RIPA buffer (phosphate buffered saline [pH 7.4] containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF, and 5 μg/ml aprotinin, leupeptin, and pepstatin). These cell extracts were immunoprecipitated with HA- (Myr-Akt; Boehringer Mannheim), Myc- (Myr-p110 and p85; Oncogene Science), or GST- (V14Rho, V12Rac, V12Cdc42, N17Rac, N17Cdc42, and Dp85; Santa Cruz) specific antibodies, resolved by electrophoresis on SDS-polyacrylamide gels (10%), and transferred to nitrocellulose. The expression of the tagged proteins was detected by blotting with the same antibodies.

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