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**13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)**
The work performed during this grant has verified and extended our initial hypothesis. Specifically, we have established that the a6b1 integrin facilitates the survival of breast carcinoma cells in hypoxia by a mechanism that involves its ability to activate HIF-1 and stimulate the transcription of VEGF. The second major discovery made from the work funded by the proposal is that another a6 integrin, a6b4, contributes to the survival of breast carcinoma cells by an entirely different mechanism. This mechanism involves the ability of this integrin to regulate the translation of VEGF through the mTOR pathway. This is a very exciting finding for several reasons. VEGF is probably the 'tip of the iceberg' and there are likely to be several other proteins important for breast carcinoma survival that are regulated by a6b4-mediated control of mTOR. Another important aspect of this finding is that mTOR was defined as a target of the drug rapamycin and derivatives of rapamycin are being used with success for adjuvant therapy of breast cancer. Collectively, the work funded by this proposal has revealed the importance of the a6 integrins for the survival of breast carcinoma cells and the mechanisms involved. This work provides a solid foundation for additional mechanistic studies, as well as the potential to exploit the pathways we have highlighted as therapeutic targets.

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# Table of Contents

**Introduction**: Understanding the mechanisms involved in the progression from carcinoma *in situ* to metastatic disease is one of the most important problems in breast cancer research. In simple terms, progression to metastatic disease requires that breast carcinoma cells acquire the abilities to invade, stimulate angiogenesis and survive in non-breast tissues. Recently, progress has been made in identifying specific molecules that contribute to each of these critical events. These molecules include growth and angiogenic factors, as well as their corresponding receptors, and cell adhesion molecules. The challenge ahead is to understand how these molecules cooperate and interact to promote progression. Studies by our group and others have implicated a critical role for the $\alpha 6$ integrins in breast cancer progression (1) (2-4) (5). The goal of this IDEA proposal was to define the mechanism by which an integrin, such as the $\alpha 6$ integrins contributes to the survival of metastatic breast carcinoma cells. We postulated that these integrins regulate the expression of HIF-1$\alpha$, a key regulator of VEGF expression and angiogenesis in breast and other cancers. The studies completed during the tenure of this grant enabled us to verify and extend our original hypothesis.

**Body**:
*Integrin ($\alpha 6\beta 4$) regulation of eIF-4E activity and VEGF translation: a survival mechanism for breast carcinoma cells*: Consistent with the goals of this proposal, we defined a novel mechanism by which the $\alpha 6$ integrins regulate growth factor expression and the survival of carcinoma cells. Specifically, we demonstrated that the $\alpha 6\beta 4$ integrin enhances VEGF translation in breast carcinoma cells. The mechanism involves the ability of this integrin to stimulate the phosphorylation and inactivation of 4E-Binding Protein (4E-BP1), a translational repressor that inhibits the function of translation initiation factor 4E (eIF-4E). The regulation of 4E-BP1 phosphorylation by $\alpha 6\beta 4$ derives from the ability of this integrin to activate the PI-3K/Akt pathway and, consequently, the rapamycin-sensitive kinase mTOR that can phosphorylate 4E-BP1. Importantly, we show that this $\alpha 6\beta 4$-dependent regulation of VEGF translation plays an important role in the survival of metastatic breast carcinoma cells by sustaining a VEGF autocrine signaling pathway that involves activation of PI3-K and Akt. These findings revealed that integrin-mediated activation of PI-3K/Akt is amplified by integrin-stimulated VEGF expression and they provided a mechanism that substantiates the reported role of $\alpha 6\beta 4$ in carcinoma progression. The details of this study can be found in Chung et al., Journal of Cell Biology (2002). A copy of this publication is included in the Appendix.

*Hypoxia-induced VEGF transcription and protection from apoptosis are dependent upon a6b1 integrin in breast carcinoma cells:* An important role for the a6b1 integrin in breast cancer progression has been indicated by several studies (1-3). The involvement of this integrin in progression was suggested first by the finding that high expression of the $\alpha 6$ subunit in women with breast cancer correlated significantly with reduced survival times (1). 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. This study is consistent with the report that the metastatic potential of MDA-MB-435 cells correlates with their level of $\alpha 6\beta 1$ expression (3). Moreover, when a6b1-deficient MDA-MB-435 cells were inoculated into the mammary fat pads of nude mice, primary tumor size was significantly diminished compared to the parental cells because of increased apoptosis (2). 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 (2).

Interestingly, these α6β1-deficient cells did not differ in their ability to survive *in vitro* under standard culture conditions suggesting that α6β1 is needed for survival within the tumor microenvironment. Given that the microenvironment of solid tumors is often hypoxic and lacks the rich growth factor milieu present in culture medium, we examined the hypothesis that this integrin contributes to the survival of MDA-MB-435 cells in such conditions. Interestingly, the data obtained indicate that hypoxia protects these cells from apoptosis induced by serum deprivation and that this protection depends on a6b1 expression. Protection from apoptosis under these conditions requires autocrine VEGF and further analysis revealed that α6β1 is necessary for VEGF expression because it functions in concert with hypoxia to activate HIF-1 and stimulate VEGF transcription by a mechanism that involves PKC-α. This study validates the major hypothesis of the proposal. The details of this study can be found in Chung et al., Cancer Research (2004). A copy of this publication is included in the Appendix.

**Key Research Accomplishments:**
- Established the ability of the α6β4 integrin to regulate protein translation in breast carcinoma cells.
- Demonstrated that VEGF is regulated by α6β4 and that it is necessary for breast carcinoma survival.
- Established assays for measuring VEGF and HIF-1α in metastatic breast cells.
- Developed effective RNAi strategy for inhibiting expression of α6 integrins in breast carcinoma cells.
- Demonstrated the importance of α6β1 integrin for the survival of breast carcinoma cells in hypoxia using this RNAi strategy.
- Established that α6β1 regulates the activation of HIF-1α in hypoxia but not its expression.
- Provided evidence that VEGF transcription in breast carcinoma cells is HIF-1α dependent.
- Established the importance of autocrine VEGF for the survival of breast carcinoma cells.

**Reportable Outcomes:**
Chung, J., Bachelder, R.E., Lipscomb, E., Shaw, L.M. and <u>A.M. Mercurio</u>. Integrin (α6β4) regulation of VEGF translation: A survival mechanism for carcinoma cells. *J. Cell Biology* 2002, 158:165-174.

Mercurio AM, Bachelder RE, Bates RC, Chung JC. Autocrine signaling in carcinoma: VEGF and the α6β4 integrin. **Seminars in Cancer Biology**, 2004; 14:115-122.

Chung J, Mercurio AM. Contributions of the α6 integrins to breast carcinoma survival and progression. **Molecules and Cells** 2004, 17:203-209.

Chung J, Yoon S, Datta K, Bachelder RE, Mercurio AM. Hypoxia-induced VEGF transcription and protection from apoptosis are dependent upon α6β1 integrin in breast carcinoma cells. **Cancer Research** 2004, In Press.

**Conclusions:**
The work performed during this grant has verified and extended our initial hypothesis. Specifically, we have established that the α6β1 integrin facilitates the survival of breast

carcinoma cells in hypoxia by a mechanism that involves its ability to activate HIF-1 and stimulate the transcription of VEGF. In related work, we have reported that VEGF is essential for the survival of metastatic breast carcinoma cells. The second major discovery made from the work funded by the proposal is that another α6 integrin, α6β4, contributes to the survival of breast carcinoma cells by an entirely different mechanism. This mechanism involves the ability of this integrin to regulate the translation of VEGF through the mTOR pathway. This is a very exciting finding for several reasons. VEGF is probably the 'tip of the iceberg' and there are likely to be several other proteins important for breast carcinoma survival that are regulated by α6β4-mediated control of mTOR. Another important aspect of this finding is that mTOR was defined as a target of the drug rapamycin and derivatives of rapamycin are being used with success for adjuvant therapy of breast cancer.

Collectively, the work funded by this proposal has revealed the importance of the α6 integrins for the survival of breast carcinoma cells and the mechanisms involved. This work provides a solid foundation for additional mechanistic studies, as well as the potential to exploit the pathways we have highlighted as therapeutic targets. We are most grateful to the DOD for their support of our work.

**References:**
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5. Mukhopadhyay, R., Theriault, R. L., and Price, J. E. (1999) Increased levels of alpha6 integrins are associated with the metastatic phenotype of human breast cancer cells. *Clin Exp Metastasis* 17, 325-332

**Appendix:**

Chung, J., Bachelder, R.E., Lipscomb, E., Shaw, L.M. and <u>A.M. Mercurio</u>. Integrin (α6β4) regulation of VEGF translation: A survival mechanism for carcinoma cells. *J. Cell Biology* 2002, 158:165-174.

Chung J, Yoon S, Datta K, Bachelder RE, Mercurio AM. Hypoxia-induced VEGF transcription and protection from apoptosis are dependent upon α6β1 integrin in breast carcinoma cells. **Cancer Research** 2004, In Press.

# Article

# Integrin (α6β4) regulation of eIF-4E activity and VEGF translation: a survival mechanism for carcinoma cells

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We define a novel mechanism by which integrins regulate growth factor expression and the survival of carcinoma cells. Specifically, we demonstrate that the α6β4 integrin enhances vascular endothelial growth factor (VEGF) translation in breast carcinoma cells. The mechanism involves the ability of this integrin to stimulate the phosphorylation and inactivation of 4E-binding protein (4E-BP1), a translational repressor that inhibits the function of eukaryotic translation initiation factor 4E (eIF-4E). The regulation of 4E-BP1 phosphorylation by α6β4 derives from the ability of this integrin to activate the PI-3K–Akt pathway and, consequently, the rapamycin-sensitive kinase mTOR that can phosphorylate 4E-BP1. Importantly, we show that this α6β4-dependent regulation of VEGF translation plays an important role in the survival of metastatic breast carcinoma cells by sustaining a VEGF autocrine signaling pathway that involves activation of PI-3K and Akt. These findings reveal that integrin-mediated activation of PI-3K–Akt is amplified by integrin-stimulated VEGF expression and they provide a mechanism that substantiates the reported role of α6β4 in carcinoma progression.

## Introduction

An understanding of the mechanisms that sustain the survival of tumor cells in adverse physiological conditions is one of the most important problems in cancer biology. As argued recently, cancer progression is an evolutionary process that selects for cells that exhibit the capacity for survival in environmental conditions not present in normal tissue (Fearon, 1999; Hanahan and Weinberg, 2000). One survival strategy used by tumor cells is the secretion of proteins that elicit an angiogenic response, such as vascular permeability factor or vascular endothelial growth factor (VEGF).* VEGF appears to be an essential factor for the progression of many solid tumors (Shweiki et al., 1992; Brown et al., 1999; Dvorak et al., 1999). It is widely assumed that the function of VEGF produced by tumor and tumor stromal cells is to stimulate angiogenesis by acting in a paracrine fashion on vicinal endothelium (Hanahan and Folkman, 1996; Brown et al., 1999). Another mechanism for tumor cell survival is the establishment of autocrine signaling loops that act on tumor cells directly (Scotlandi et al., 1996; Tokunou et al., 2001; Wong et al., 2001). Although the significance of this mechanism has been overshadowed by angiogenesis, recent studies have substantiated the importance and necessity of such signaling loops for tumor survival (Scotlandi et al., 1996; Bachelder et al., 2001; Tokunou et al., 2001; Wong et al., 2001). Indeed, this mechanism probably contributes to the ability of cells to survive in hypoxic, poorly vascularized regions of tumors. In this direction, we described recently the existence of a VEGF autocrine signaling pathway in metastatic breast carcinoma cells that is essential for their survival (Bachelder et al., 2001).

An important issue that arises from the contribution of VEGF autocrine signaling to tumor survival is an understanding of the mechanisms that regulate VEGF expression. Such mechanisms are important not only for VEGF signaling in tumor cells, but also for angiogenesis as well. Clearly, hypoxia is a strong inducer of VEGF transcription and mRNA stability (von Marschall et al., 2001), but other factors are likely to be involved. Of note, our finding that the α6β4 integrin can promote the survival of breast carcinoma cells in stress conditions is intriguing (Bachelder et al., 1999b) and raised the novel possibility that a specific integrin, which has been implicated in cancer progression, could regulate VEGF expression. This possibility is substantiated by the finding reported here that the ability of the α6β4 integrin to promote survival is VEGF dependent.

The results described above prompted us to investigate the relationship between the $\alpha6\beta4$ integrin and VEGF expression. We observed that the expression and signaling properties of this integrin have no impact on steady-state VEGF mRNA levels. Surprisingly, however, we detected a significant influence of $\alpha6\beta4$ on VEGF translation and protein expression in these cells, an observation that reveals the ability of this integrin to regulate translation. The mechanism by which $\alpha6\beta4$ regulates VEGF expression involves its ability to stimulate the phosphorylation of 4E-binding protein (4E-BP1). 4E-BP1 is phosphorylated by mammalian target of rapamycin (mTOR), a protein kinase whose catalytic domain is structurally related to that of phosphatidylinositol 3-kinase (PI-3K) (Dennis et al., 1999; Schmelzle and Hall, 2000). Phosphorylation of 4E-BP1 by mTOR disrupts its binding to eukaryotic translation initiation factor eIF-4E, which is present in rate-limiting amounts in most cells (De Benedetti and Harris, 1999; McKendrick et al., 1999). eIF-4E plays a critical role in the recruitment of the translational machinery to the 5' end of mRNA, which is demarcated by an m7GpppN cap (where m is a methyl group and N is any nucleotide) (Raught and Gingras, 1999). The m7 cap is essential for the translation of most mRNAs including VEGF (De Benedetti and Harris, 1999; Raught and Gingras, 1999). Dissociation of 4E-BP1 from eIF-4E enables eIF-4E to initiate translation (Gingras et al., 1999, 2001b). The regulation of 4E-BP1 phosphorylation by $\alpha6\beta4$ derives from the ability of this integrin to activate the PI-3K–Akt pathway and, consequently, mTOR. Our findings reveal a novel mechanism of tumor cell survival and they highlight the ability of a specific integrin to regulate protein translation by influencing eIF-4E activity.

## Results

### The ability of the $\alpha6\beta4$ integrin to promote the survival of carcinoma cells is VEGF dependent

To examine the hypothesis that the ability of the $\alpha6\beta4$ integrin to promote survival is VEGF dependent, we used MDA-MB-435 cells, which lack expression of this integrin. Stable expression of $\alpha6\beta4$ in these cells enhances their ability to survive in stressful conditions (Bachelder et al., 1999b). Importantly, however, expression of $\alpha6\beta4$ does not alter the expression of other integrin subunits in these cells and does not influence their adhesion to matrix (Shaw et al., 1997). As shown in Fig.1 A, a significant level of apoptosis was observed after 24 h of serum deprivation in the parental MDA-MB-435 cells and mock transfectants, as well as in transfectants that express $\alpha6\beta4$ containing a cytoplasmic domain deletion of the $\beta4$ subunit that lacks the ability to signal (Shaw et al., 1997). Stable subclones that express the intact $\alpha6\beta4$ integrin, however, were protected from apoptosis under these conditions. Based on these results and our previous finding that the survival of metastatic breast carcinoma cells is dependent on VEGF, we used a VEGF antisense oligonucleotide to reduce VEGF expression in the MDA-MB-435/$\beta4$ transfectants and assessed the impact of reducing VEGF expression on their survival (Fig. 1, B and C). The VEGF antisense oligonucleotide reduced VEGF protein expression significantly in the $\beta4$ transfectants (Fig.
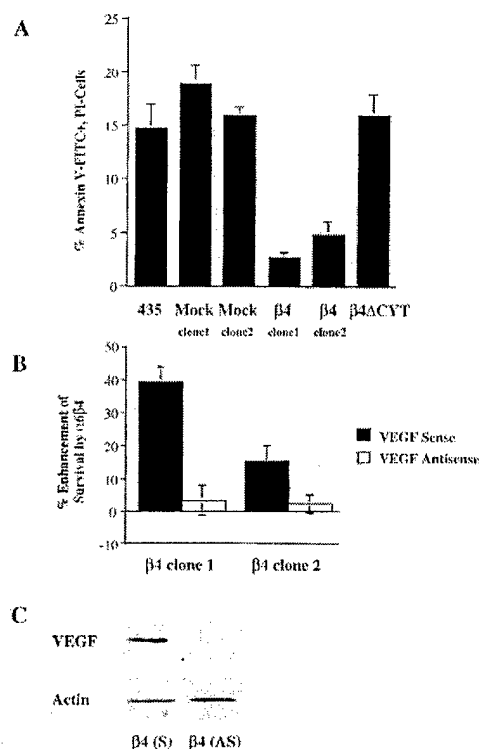


Figure 1. **The $\alpha6\beta4$-mediated survival of breast carcinoma cells is VEGF dependent.** (A) Parental, mock (clone 1, 6D2; clone 2, 6D7), $\beta4$-$\Delta$CYT–expressing (cytoplasmic tail deletion mutant), and $\beta4$ integrin–expressing (clone 1, 3A7; clone 2, 5B3) MDA-MB-435 subclones were maintained in low serum (0.5% FBS) medium for 24 h. To assess the level of apoptosis, these cells were stained with annexin V–FITC and propidium iodide (PI), and analyzed on a Becton Dickinson flow cytometer using CellQuest software. The percentage of annexin-positive, PI-negative cells ($\pm$ SD) is indicated. Results were obtained from three independent experiments. Apoptosis was minimal in the presence of 10% FBS (unpublished data). (B) Mock-transfected clone 6D7 and $\beta4$ integrin– (clone 1, 3A7; clone 2, 5B3) expressing MDA-MB-435 subclones were transiently transfected with VEGF sense or antisense oligonucleotides and maintained in low serum (0.5% FBS) medium. After 24 h, the level of apoptosis in these cells was assessed as described above. The data are presented as the mean difference ($\pm$ SD) in annexin positivity between mock-transfected and $\alpha6\beta4$-expressing MDA-MB-435 cells. Similar results were observed in two separate experiments. (C) The relative amount of VEGF protein in extracts obtained from the MDA-MB-435/$\beta4$ cells transfected with either the VEGF sense (S) or antisense (AS) oligonucleotide was determined by immunoblotting using a polyclonal anti-VEGF immune serum.

1 C). As shown in Fig. 1 B, this reduction in VEGF expression abrogated the survival-enhancing effect of $\alpha6\beta4$ under conditions of serum deprivation.

### The $\alpha6\beta4$ integrin increases VEGF protein but not mRNA expression

Given that the survival effect of $\alpha6\beta4$ expression is VEGF dependent, the novel possibility arose that VEGF expression could be regulated by this integrin. VEGF expression can be regulated at the level of both transcription and mRNA stability (Nabors et al., 2001; von Marschall et al., 2001), mechanisms that would alter the steady-state levels of VEGF

A

B



C
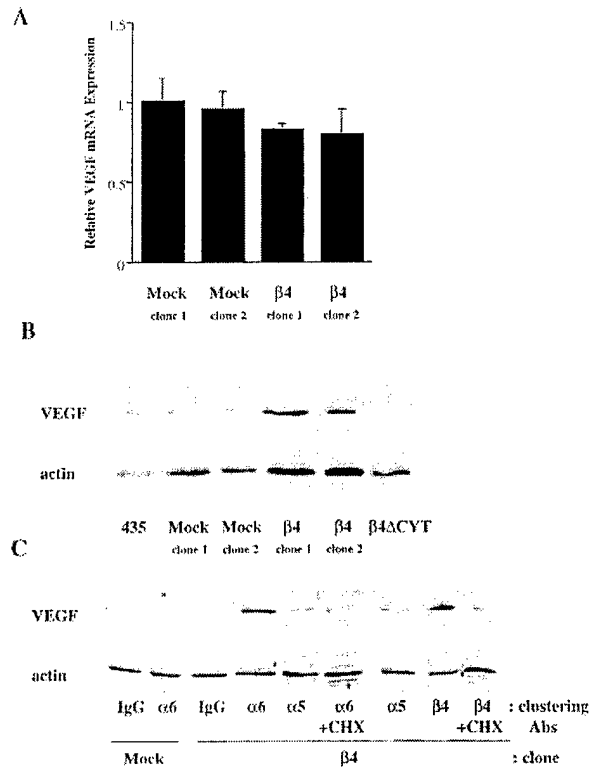
Figure 2.   **Expression of the α6β4 integrin increases VEGF protein but not steady-state mRNA.** (A) The amount of VEGF mRNA in extracts obtained from mock- (clone 1, 6D2; clone 2, 6D7) and β4 integrin– (clone 1, 3A7; clone 2, 5B3) transfected MDA-MB-435 subclones was quantified by real-time PCR. The data are presented as the mean ratio of VEGF to β-actin mRNA (± SD) obtained from triplicate samples. (B) Parental (435), mock (clone 1, 6D2; clone 2, 6D7), β4-ΔCYT–expressing (clone 1E10), and β4 integrin–expressing (clone 1: 3A7, clone 2: 5B3) MDA-MB-435 subclones were cultured in low serum (0.5% FBS) medium for 24 h. Extracts of these cells containing equivalent amounts of protein were analyzed for their relative expression of VEGF and actin by immunoblotting. Similar results were observed in four independent experiments. (C) Mock (clone 6D7) and β4 integrin–expressing (clone 3A7) MDA-MB-435 subclones were maintained in low serum (0.5% FBS) medium for 24 h. These cells were detached with trypsin and incubated with integrin-specific antibodies (α6 integrin, 2B7; β4 integrin, A9; α5 integrin, Sam1) or IgG for 30 min in suspension and allowed to adhere on anti-IgG–coated plates for 60 min before lysis. In addition, cells were preincubated in cycloheximide (CHX) at a concentration of 10 μg/ml for 30 min and then incubated with either the α6 or β4 integrin antibodies in the presence of cycloheximide. Extracts of these cells containing equivalent amounts of protein were analyzed for their relative expression of VEGF and actin by immunoblotting. Similar results were observed in two independent experiments.

mRNA. In addition, regulation can also occur at the level of VEGF translation (Kevil et al., 1996; Akiri et al., 1998; Stein et al., 1998). As shown in Fig. 2 A, quantitative analysis of VEGF mRNA levels in two clones of MDA-MB-435/mock and β4 transfectants using real-time PCR revealed no significant difference in the steady-state mRNA levels in these two populations. However, we detected a substantial increase in VEGF protein expression in the MDA-MB-435/β4 transfectants relative to either the parental cells, mock

transfectants, or cells that express a cytoplasmic domain deletion of the β4 subunit (β4-ΔCYT) (Fig. 2 B). These results indicate that the α6β4 integrin regulates VEGF protein expression. It is also worth noting that the level of apoptosis observed in these populations in response to serum deprivation correlates inversely with their expression of VEGF (Fig. 1 A and Fig. 2 B).

To substantiate the regulation of VEGF expression by α6β4, integrin-specific antibodies were used to cluster either α6β4 or α5β1 and the effects of integrin-mediated clustering on VEGF expression were assessed by immunoblotting. Of note, the MDA-MB-435/β4 transfectants express equivalent levels of α6β4 and α5β1 (unpublished data). An α6-specific antibody (mAb 2B7) was used to cluster the α6β1 integrin in the mock transfectants and the α6β4 integrin in the β4 transfectants, a β4-specific antibody (mAb A9) was used to cluster the α6β4 integrin in the β4 transfectants, and an α5-specific antibody (mAb Sam1) was used to cluster α5β1 in both the mock and β4 transfectants. A substantial induction of VEGF expression was observed upon α6β4 integrin clustering in the β4 transfectants but not in the mock transfectants, and no induction was seen in response to α5β1 clustering (Fig. 2 C). Importantly, the induction of VEGF expression that occurs in response to α6β4 clustering was inhibited by cycloheximide (Fig. 2 C). This result, together with the real-time PCR data (Fig. 2 A), indicates that α6β4 is influencing VEGF translation.

To obtain more definitive evidence that α6β4 is regulating VEGF translation, we performed polysome analysis of the VEGF message. mRNA isolated from the MDA-MB-435/mock and β4 transfectants was fractionated on a sucrose gradient (Fig. 3 A) and the relative amount of VEGF mRNA in each fraction was determined by real-time PCR (Fig. 3 B). As shown in Fig. 3 B, a striking difference in the distribution of VEGF mRNA was evident in the two populations of cells. In the MDA-MB-435/β4 transfectants, VEGF mRNA fractionated in the heavy polysomal region, whereas in the mock transfectants, the majority of VEGF mRNA was associated with light polysomal to ribosomal subunit fractions. This result indicates that the translation of VEGF in the MDA-MB-435/β4 transfectants is cap dependent.

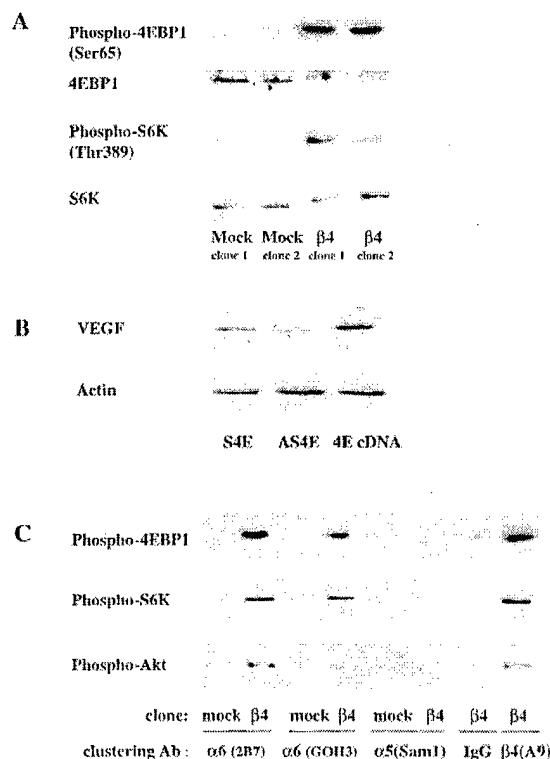## Identification of an α6β4 integrin–mediated signaling pathway that regulates VEGF expression

Our finding that α6β4 regulates the cap-dependent translation of VEGF prompted us to assess the ability of this integrin to stimulate the activity of the eIF-4E translation initiation factor. The α6β4 integrin is a potent activator of the PI-3K–Akt signaling pathway in MDA-MB-435 and other carcinoma cells (Shaw et al., 1997; Bachelder et al., 1999a; Gambaletta et al., 2000; Nguyen et al., 2000; Hintermann et al., 2001), and this pathway has been linked to the regulation of protein translation. Specifically, the serine/threonine kinase mTOR is activated by Akt-mediated phosphorylation events (Sekulic et al., 2000). Phosphorylation of 4E-BP1 by mTOR disrupts its binding to eIF-4E, enabling eIF-4E to initiate translation of VEGF and other molecules (De Benedetti and Harris, 1999). We hypothesized, based on this information, that α6β4 regulates 4E-BP1 phosphorylation and, as a consequence, VEGF expression. Initially, we as-
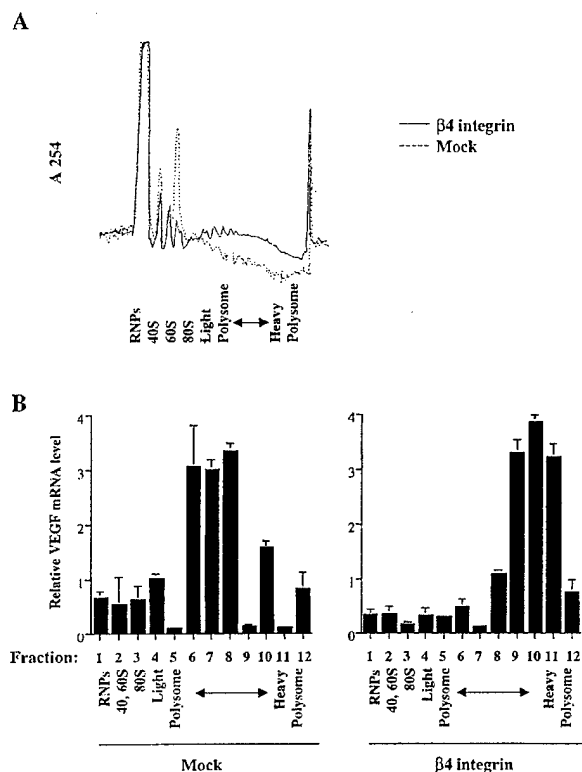
Figure 3. **Polysome analysis of VEGF mRNA.** (A) The distribution of RNA from MDA-MB-435/β4 and mock transfectants that had been fractionated on sucrose gradients as described in the Materials and methods was determined by measuring the $A_{254}$ of each fraction. (B) The relative VEGF mRNA content of each sucrose gradient fraction was measured by real-time PCR as described in the Materials and methods. Fraction 1 contains unbound RNA present in the ribonucleoprotein fraction, fraction 2 contains 40S and 60S monosomes, fraction 3 contains 80S monosomes, fractions 4–7 contain light polysomes, and fractions 8–12 contain heavy polysomes. The data are presented as the mean ratio of VEGF to β-actin mRNA (± SD) obtained from triplicate samples. Similar results were obtained from three independent experiments.



Figure 4. **The α6β4 integrin stimulates the phosphorylation of Akt, 4E-BP1, and p70$^{S6K}$.** (A) MDA-MB-435 parental cells, mock transfectants, and β4 transfectants were maintained in medium containing low serum (0.5% FBS) for 24 h. The phosphorylation status of 4E-BP1 on Ser 65 and S6K on Thr 389 was assessed in extracts from these cells using phosphospecific antibodies as described in the Materials and methods. In addition, the total amount of 4E-BP1 and p70$^{S6K}$ in these extracts was assessed by immunoblotting. (B) The MDA-MB-435/β4 cells were transiently transfected with either an eIF-4E sense (S) or antisense (AS) oligonucleotide, or a full-length eIF-4E cDNA (4E). Extracts of these cells containing equivalent amounts of protein were analyzed for their relative expression of VEGF and actin by immunoblotting. (C) MDA-MB-435 mock (clone 6D7) and β4 (clone 3A7) transfectants were maintained in low serum (0.5% FBS) medium for 24 h. These cells were detached with trypsin and incubated with integrin-specific antibodies (α6 integrin, 2B7; α6 integrin, GOH3; α5 integrin, Sam1; β4 integrin, A9) or IgG for 30 min as described in the legend to Fig. 2. The phosphorylation status of 4E-BP1 (Ser 65), S6K (Thr 389), and Akt (Ser 473) was assessed in extracts from these cells using phosphospecific antibodies. Similar results were observed in four independent experiments.

sessed the steady-state phosphorylation levels of 4E-BP1 and S6 kinase (p70$^{S6K}$), which are both downstream targets of mTOR, in cells that had been serum deprived for 24 h. Indeed, a marked increase in the level of phosphorylation of 4E-BP1 (on Ser65) and p70$^{S6K}$ (on Thr389) was evident in the MDA-MB-435/β4 transfectants relative to either the mock transfectants or the parental cells (Fig. 4 A). Phosphorylation of Ser65 of 4E-BP1 has been shown to be critical for dissociation of 4E-BP from eIF-4E (Gingras et al., 2001a). The reduced expression of 4E-BP1 in the β4 transfectants compared with the mock transfectants that is apparent in Fig. 4 A may reflect the possibility that the 4E-BP Ab does not recognize the hyperphosphorylated form of the protein as well as it recognizes the hypophosphorylated form.
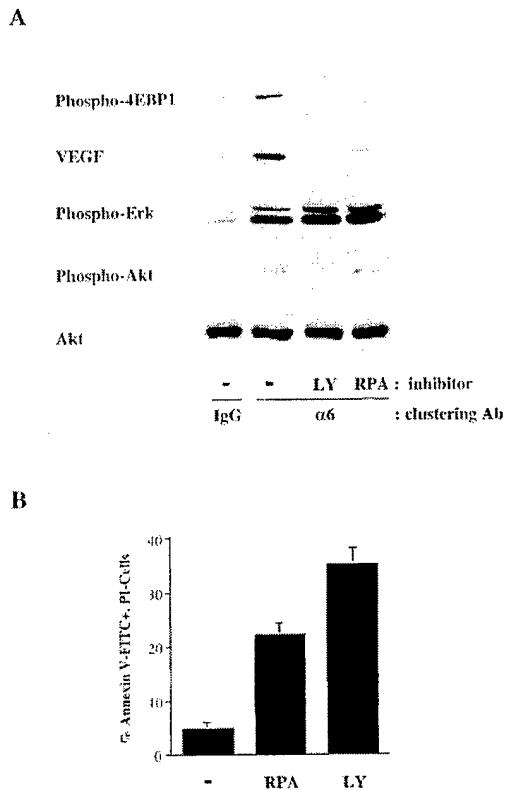
The involvement of eIF-4E in VEGF translation was confirmed by the expression of an antisense eIF-4E oligonucleotide in the MDA-MB-435/β4 transfectants. As shown in Fig. 4 B, expression of this oligonucleotide reduced the level of VEGF protein significantly. In contrast, expression of the full-length eIF-4E cDNA increased the VEGF protein level by ap-

proximately twofold. These results, together with the polysome analysis data (Fig. 3), indicate that α6β4 regulates VEGF expression by eIF-4E–mediated, cap-dependent translation.

To confirm the specificity of the α6β4 integrin in mTOR signaling, the effects of integrin-mediated clustering on 4E-BP1 phosphorylation were assessed. A substantial induction of Akt, 4E-BP1, and p70$^{S6K}$ phosphorylation was observed upon α6β4 integrin clustering in the β4 transfectants but not in the mock transfectants (Fig. 4 C). In contrast, clustering of the α5β1 integrin did not stimulate phosphorylation of these molecules in either the mock or β4 transfectants. Collectively, these data demonstrate the preferential ability of the α6β4 integrin to regulate the mTOR signaling pathway and, more importantly, the phosphorylation of 4E-BP1.

A



Phospho-4EBP1

VEGF

Phospho-Erk

Phospho-Akt

Akt

- - LY RPA : inhibitor

IgG α6 : clustering Ab

B



- RPA LY

Figure 5. Stimulation of 4E-BP1 phosphorylation, VEGF expression, and survival by the α6β4 integrin requires PI-3K and mTOR.
(A) MDA-MB-435 β4 transfectants (clone 3A7) were incubated with either DMSO (−), the PI-3K inhibitor LY 294002 (10 μM) (LY), or the mTOR-specific inhibitor rapamycin (50nM) (RPA) for 30 min and then incubated with either IgG or the α6 integrin antibody 2B7 as described in the legend to Fig. 2. Extracts of these cells were immunoblotted for phospho–4E-BP1 (Ser65), VEGF, phospho-Erk (recognizing phosphorylated isoforms of ERK1 and ERK2), phospho-Akt (Ser 473), and total Akt. Similar data were obtained in three experiments. (B) MDA-MB-435 β4 transfectants (clone 3A7) were maintained at low serum (0.5%) medium for 24 h in the presence of either rapamycin (50nM) (RPA), LY 294002 (10 μM) (LY), or DMSO (−). Apoptosis was assayed as described in the Materials and methods and is reported as the percentage of annexin V–FITC-positive, PI-negative cells. The data shown are mean values (± SD) of a representative experiment performed in triplicate.

A



VEGF

Phospho-4EBP1

Actin

435 mock β4 Y1494

B



435 Mock β4 Y1494 Y1494
+ VEGF

Figure 6. Y1494 in the β4 cytoplasmic domain is required for α6β4 stimulation of 4E-BP1 phosphorylation, VEGF expression, and survival. (A) MDA-MB-435 parental cells (435), mock transfectants (clone 6D7), wild-type β4 transfectants (clone 3A7), and Y1494F mutant transfectants (clone E1h) were maintained in low serum (0.5% FBS) for 24 h. Extracts from these cells were analyzed by immunoblotting to assess the relative expression of VEGF and 4E-BP1 phosphorylation. The relative amount of actin was also determined as a control for protein loading. Similar results were obtained in three experiments. (B) Aliquots of the same cell populations described in A were assayed for the level of apoptosis after a 24-h incubation in low serum (0.5% FBS) medium. Apoptosis was assayed as described in the Materials and methods and is reported as the percentage of annexin V–FITC-positive, PI-negative cells. The data shown are mean values (± SD) of three experiments performed in triplicate.

To establish that PI-3K and mTOR are required for 4E-BP1 phosphorylation and VEGF expression, we performed the antibody clustering experiments in the presence of the PI-3K–specific inhibitor LY294002 and the mTOR-specific inhibitor rapamycin (Fig. 5). As shown in Fig. 5 A, both of these inhibitors blocked the α6β4-mediated induction of 4E-BP1 phosphorylation and VEGF expression. Although rapamycin did not block Akt phosphorylation, LY294002 did inhibit its phosphorylation, confirming that Akt acts upstream of mTOR and downstream of PI-3K (Fig. 5 A). These inhibitors did not block the phosphorylation of ERK1 and ERK2 (Fig. 5 A).

Finally, we investigated the importance of the mTOR pathway in survival, using rapamycin and LY294002. As shown in Fig. 5 B, rapamycin treatment increased the apop-

tosis of the MDA-MB-435/β4 transfectants fivefold and LY294002 treatment increased their apoptosis eightfold. These results indicate that the PI-3K–mTOR pathway is critical for the survival of these cells.

## Identification of a specific tyrosine residue in the β4 cytoplasmic domain required for α6β4 stimulation of 4E-BP1 phosphorylation and VEGF expression

Recently, a critical tyrosine residue (Y1494) was identified in the third fibronectin type III repeat of the β4 cytoplasmic domain, and this tyrosine was shown to be essential for activation of PI-3K by α6β4 (Shaw, 2001). To assess the importance of Y1494 in 4E-BP1 phosphorylation and VEGF expression, stable subclones of MDA-MB-435 cells were generated that expressed α6β4 containing a Y1494F mutation. As shown in Fig. 6 A, VEGF protein expression was barely detectable in these transfectants compared with the wild-type transfectants. Also, the steady-state level of 4E-BP1 phosphorylation was substantially lower in the Y1494F mutant transfectants than in the wild-type β4 transfectants. Interestingly, these mutant transfectants also exhibited an
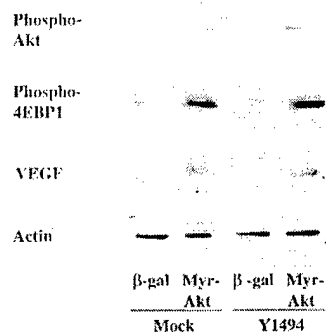
Figure 7. **Expression of a constitutively active Akt construct mimics the effects of α6β4 integrin expression and signaling.** MDA-MB-435 mock transfectants (clone 6D7) and Y1494F mutant transfectants (clone E1h) were infected with adenoviruses that expressed either β-galactosidase or Myr-Akt as described in the Materials and methods. Subsequently, the cells were incubated in low serum (0.5% FBS) medium for 24 h. Extracts of these cells were immunoblotted to assess the relative phosphorylation of Akt and 4E-BP1, as well as total expression of VEGF and actin.

eightfold higher level of apoptosis than the wild-type β4 transfectants in response to serum deprivation (Fig. 6 B). The apoptosis of the mutant cells was reduced substantially by the addition of recombinant VEGF (Fig. 6 B), a result that substantiates the importance of VEGF in the survival of these cells. Together, these findings highlight the importance of the β4 cytoplasmic domain and PI-3K signaling in the regulation of VEGF expression and tumor cell survival.

## Expression of constitutively active Akt stimulates 4E-BP1 phosphorylation and VEGF expression in the absence of α6β4 signaling

The hypothesis that activation of Akt is a major determinant for the stimulation of 4E-BP1 phosphorylation and VEGF expression was assessed by expressing a constitutively active Akt construct in MDA-MB-435 cells that are deficient in α6β4 signaling. For this purpose, we used MDA-MB-435/ mock transfectants that lack α6β4 expression and the MDA-MB-435/β4 Y1494F transfectants, described above, which are deficient in α6β4-mediated activation of PI-3K. These cells were infected with adenoviruses that encoded either a myristoylated Akt (Myr-Akt) construct or β-galactosidase as a control. As shown in Fig. 7, expression of Myr-Akt stimulated 4E-BP1 phosphorylation and VEGF expression substantially in both populations of transfectants in comparison to cells that expressed β-galactosidase. This result indicates the critical importance of Akt activation by α6β4 for stimulating VEGF expression.

## α6β4 regulates 4E-BP1 phosphorylation, VEGF expression, and survival in carcinoma cells that express this integrin endogenously

Given that the data reported above are based on the exogenous expression of α6β4 in α6β4-deficient carcinoma cells, we sought to extend our findings to cells that express this integrin endogenously, a pattern that is typical of most carcinoma cells. For this purpose, we used MDA-MB-231 breast carcinoma cells because they express the α6β4 and α5β1 in-
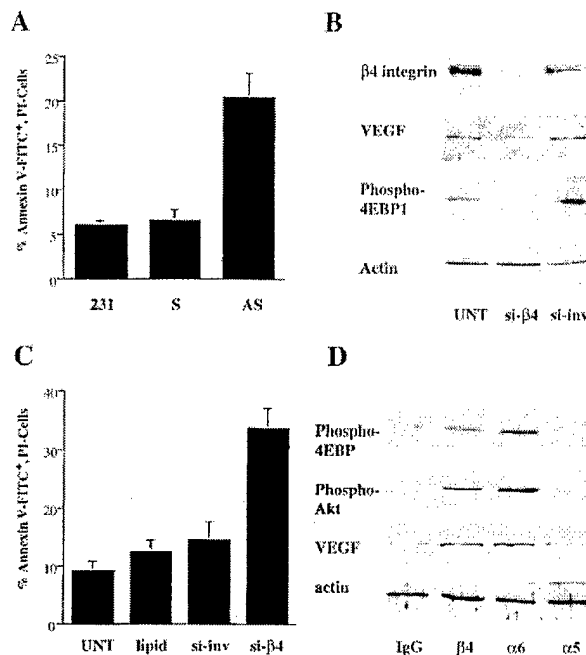


Figure 8. **α6β4 regulates 4E-BP1 phosphorylation, VEGF expression, and survival in carcinoma cells that express this integrin endogenously.** (A) Parental MDA-MB-231 cells and cells transfected with antisense or sense VEGF oligonucleotides were maintained in low serum (0.5% FBS) medium for 24 h. Apoptosis was assayed as described in the Materials and methods and is reported as the percentage of annexin V–FITC-positive, PI-negative cells. The data shown are mean values (± SD) of two separate experiments performed in triplicate. (B) MDA-MB-231 cells were left untreated (UNT) or were transfected with either an RNAi specific for the β4 integrin (si-β4) or the corresponding inverted sequence (si-inv). After 72 h, the cells were placed in medium containing low serum (0.5% FBS) for an additional 24 h and then extracted. Extracts of these cells were immunoblotted as described in the legend to Fig. 4 to assess expression of β4 integrin, VEGF, and actin, as well as the phosphorylation of 4E-BP1. Similar results were observed in three independent trials. (C) Apoptosis was assessed in the same populations of cells and is reported as the percentage of annexin V–FITC-positive, PI-negative cells. The data shown are mean values (± SD) of three independent experiments performed in triplicate. (D) MDA-MB-231 cells were maintained in low serum (0.5% FBS) medium for 24 h and harvested by trypsin treatment. The suspended cells were incubated with integrin-specific antibodies (β4 integrin, A9; α6 integrin, 2B7; α5 integrin, Sam1) or IgG for 30 min in suspension and allowed to adhere on anti-IgG–coated plates for 30 min. Extracts of these cells were immunoblotted with phosphospecific antibodies to assess the relative phosphorylation of Akt and 4E-BP1, as well as with antibodies specific for VEGF and actin. Similar results were obtained in five experiments.

tegrins (Plopper et al., 1998; Mukhopadhyay et al., 1999; Saad et al., 2000). Initially, we confirmed that the survival of these cells is dependent on their expression of VEGF. As shown in Fig. 8 A, expression of a VEGF antisense oligonucleotide in these cells (Bachelder et al., 2001) resulted in an approximate fourfold increase in annexin V staining upon serum starvation compared with either untreated cells or cells that expressed the sense oligonucleotide.

To establish a role for α6β4 in regulating VEGF expression and survival rigorously, we used a small interfering

RNA (RNAi) approach to inhibit β4 expression in MDA-231 cells. RNAis specific for the β4 subunit and the corresponding inverted sequence were designed and expressed in these cells by transfection. The cells were maintained in low serum (0.5%) for 24 h after transfection and then analyzed. As shown in Fig. 8 B, cells transfected with the RNAi specific for β4 exhibited a significant reduction in β4 expression in comparison with either untransfected cells or cells transfected with the inverted sequence. Importantly, the reduction in β4 expression by RNAi coincided with a marked reduction in 4E-BP1 phosphorylation and in the steady-state level of VEGF (Fig. 8 B), as well as an approximate threefold increase in annexin V staining (Fig. 8 C). These results link α6β4 expression directly to 4E-BP1 phosphorylation, VEGF expression, and survival in a carcinoma cell line that expresses endogenous α6β4.

Subsequently, we performed antibody clustering experiments to substantiate the regulation of VEGF expression by α6β4 (Fig. 8 D). Clustering of the α6β4 integrin with either an α6 integrin–specific antibody (mAb 2B7) or a β4 integrin–specific antibody (mAb A9) stimulated the phosphorylation of 4E-BP1 and Akt, and increased VEGF expression. In contrast, no induction of VEGF expression or stimulation of either 4E-BP1 or Akt phosphorylation was observed upon clustering with an α5 integrin–specific antibody (mAb Sam1) or IgG.

## Discussion

This study establishes a novel mechanism by which integrins regulate growth factor expression. Specifically, our findings demonstrate the ability of a specific integrin (α6β4), which has been implicated in carcinoma progression (Mercurio and Rabinovitz, 2001), to stimulate the translation of VEGF and sustain a VEGF autocrine loop that is essential for survival. More specifically, we define a signaling pathway regulated by α6β4 that involves the preferential ability of this integrin to stimulate the phosphorylation of 4E-BP1 by activating the PI-3K–Akt pathway. As shown previously, this phosphorylation event dissociates 4E-BP1 from eIF-4E, enabling this key elongation factor to mediate the translation of VEGF and other functionally important molecules (De Benedetti and Harris, 1999; Gingras et al., 1999, 2001b; McKendrick et al., 1999). Moreover, the polysome analysis and antisense eIF-4E results we provide indicate that α6β4 stimulation of VEGF translation is cap dependent and probably doesn't involve the internal ribosome entry sites that are present in the VEGF mRNA (Huez et al., 1998; van der Velden and Thomas, 1999). Our data extend earlier reports on the involvement of eIF-4E, VEGF, and α6β4 in carcinoma progression by linking these molecules in a common signaling pathway that promotes tumor survival. Furthermore, they reveal a role for integrins in regulating growth factor expression by stimulating protein translation.

An important and novel aspect of our findings is that they add a new dimension to the understanding of how integrins promote cell survival. The widely accepted notion is that integrins, often in concert with growth factor receptors, activate specific signaling pathways that sustain survival (Taylor et al., 1999; Liu et al., 2000). We demonstrate here that the

survival function of integrins may not only be mediated by the activation of a key survival kinase such as Akt and the consequent effects of Akt on apoptotic signaling (Datta et al., 1999) but also by the Akt-dependent translation and expression of growth factors, such as VEGF, that promote survival in an autocrine, and possibly paracrine, fashion. In other terms, our results reveal that VEGF is a novel target of Akt signaling by integrins that is important for survival and distinct from known survival factors that are downstream of Akt, such as Bad (Datta et al., 1999). Importantly, our recent observation that VEGF stimulates the PI-3K–Akt pathway in carcinoma cells (Bachelder et al., 2001), in conjunction with our finding that α6β4 signaling enhances VEGF expression, leads to the conclusion that integrin-mediated activation of PI-3K–Akt is amplified by integrin-stimulated VEGF expression. Moreover, we show that this amplification of PI-3K–Akt activity is important for carcinoma survival.

Although α6β4 activates PI-3K in carcinoma cells (Gambaletta et al., 2000; Nguyen et al., 2000; Hintermann et al., 2001; Trusolino et al., 2001), no attempt had been made to link this signaling event with downstream effectors that regulate protein translation, namely mTOR and 4E-BP1. One reason that this possibility had not been explored is because a role for α6β4 in regulating either protein translation or growth factor expression was not obvious. In fact, almost all of the functional studies on α6β4 in carcinoma cells have focused on its role in promoting migration and invasion, and on the mechanism by which α6β4-mediated signaling influences these processes (Mercurio, 1990; Shaw et al., 1997; Gambaletta et al., 2000; Trusolino et al., 2001). Our motivation to examine a possible connection between α6β4 and VEGF translation was based on our interest in understanding the mechanisms by which these molecules promote the survival of carcinoma cells. Indeed, our results establish a role for α6β4 in survival signaling by regulating VEGF translation, but the implications of these findings are more widespread. For example, recent studies that have argued that α6β4 is necessary for growth factor receptor (erbB2, c-met) activation of PI-3K (Gambaletta et al., 2000; Trusolino et al., 2001) raise the interesting possibility of an intimate functional association among specific growth factor receptors, α6β4, VEGF, and PI-3K, all of which have been implicated in tumor progression.

Surprisingly, few studies have examined the role of integrin signaling in regulating protein translation (e.g., Pabla et al., 1999). Indeed, there has been much more interest in defining the contribution of integrins to transcription. The ability of integrins to regulate translation, however, provides a mechanism for altering cell function rapidly, by increasing the expression of specific proteins. This possibility is exemplified by our finding that ligation of the α6β4 integrin resulted in a significant increase in VEGF protein within 60 min (Fig. 2 C). Given the fact that eIF-4E is rate limiting for the translation of proteins involved in growth control and other critical cell functions (De Benedetti and Harris, 1999), the hypothesis can be formulated that integrin-mediated regulation of translation contributes to the ability of cells to alter their behavior rapidly in response to changes in their microenvironment. This hypothesis is particularly relevant to our interest in the regulation of VEGF expression. Al-

though much of the work in this area has focused on the ability of hypoxia to stimulate VEGF transcription and increase the stability of VEGF mRNA (von Marschall et al., 2001), it has become apparent that translational control is also important (Kevil et al., 1996; De Benedetti and Harris, 1999). Moreover, our recent finding that VEGF is essential for the survival of breast carcinoma cells in normoxia substantiates the functional importance of integrin-mediated regulation of VEGF expression (Bachelder et al., 2001).

The fact that our data implicate eIF-4E in tumor cell survival is of considerable interest because recent studies have revealed an important role for this elongation factor in cancer (DeFatta et al., 1999, 2000; Ernst-Stecken, 2000; Berkel et al., 2001). Overexpression of this factor in NIH3T3 cells, as well as other "normal" cells, stimulates division and can induce their transformation (Fukuchi-Shimogori et al., 1997). These findings are consistent with the reports that the expression of eIF-4E is elevated in solid tumors compared with normal tissue (De Benedetti and Harris, 1999). Moreover, hypoxia, a pathophysiological stress that provides a selective pressure for the survival of aggressive tumor cells, enhances eIF-4E expression (DeFatta et al., 1999). Together, these observations highlight an important role for translational control in human cancer. This role is substantiated by the fact that eIF-4E controls the translation of not only VEGF but also other molecules that influence tumor growth and survival such as c-Myc, cyclin D1, and FGF-2 (De Benedetti and Harris, 1999). From our perspective, we are intrigued by the reports that the α6β4 integrin is associated with the progression of many solid tumors, and its expression has been correlated with a poorer prognosis in patients with some of these tumors (Mercurio and Rabinovitz, 2001). Our finding that α6β4 can induce the translational function of eIF-4E by regulating the phosphorylation of 4E-BP1 provides one mechanism to account for the role of this integrin in cancer.

# Materials and methods

## Cells

MDA-MB-231 and MDA-MB-435 breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University. They were grown in low glucose DME containing 10% FBS, 1% penicillin–streptomycin, and 25 mM Hepes. For inhibitor experiments, cells were harvested by trypsinization and suspended cells were incubated with rapamycin (Calbiochem) at 100 nM or LY 294002 (Calbiochem) at 10 μM on ice for 30 min before they were plated at 37°C for the experiment.

The generation of MDA-MB-435 subclones expressing the α6β4 integrin has been described previously (Shaw et al., 1997). Tyrosine residue 1494 in the β4 subunit was mutated to a phenylalanine residue using the Quickchange site-directed mutagenesis kit (Stratagene), and stable subclones of MDA-MB-435 cells that expressed α6β4 containing this mutant β4 subunit were generated (Shaw, 2001).

For adenoviral infection, cells were grown in DME containing 10% FBS until they reached 50% confluency. At this point, the culture medium was changed to DME containing 0.5% FBS. Viral dilutions were prepared from purified viral stocks in DME containing 0.5% FBS and the cells were infected for 4 h. At the end of the infection period, the virus-containing medium was removed and the cells were washed once with PBS, and incubated for an additional 12 h in DME containing 10% FBS.

## Apoptosis assays

To induce apoptosis, cells were incubated in DME containing 0.5% FBS for 24 h at 37°C. Subsequently, both adherent and nonadherent cells were harvested and their level of apoptosis was assessed using annexin V–FITC. In brief, cells were washed once with serum-containing medium, once

with PBS, once with annexin V–FITC buffer (10 mM Hepes-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂), and then incubated for 15 min at room temperature with 5 μg/ml annexin V–FITC (Biosource International). After washing once with annexin V buffer, the samples were resuspended in the same buffer and analyzed by flow cytometry. Immediately before the analysis, 5 μg/ml propidium iodide (PI) (Biosource International) was added to distinguish apoptotic cells from necrotic cells.

## Quantitative real-time PCR

Quantitative analysis of VEGF mRNA expression was performed by real-time PCR using an ABI Prism 7700 sequence detection system (Perkin-Elmer) and SYBR green master mix kit as described previously (Bachelder et al., 2001). Sequences of primers and probes were as follows: VEGF forward primer, 5'-GAAGTGGTGAAGTTCATGGATGTCTA-3'; VEGF reverse primer, 5'-TGGAAGATGTCCACCAGGGT-3'; VEGF probe, 5'-/TET/AGCGCAGCTACTGCCATCCAATCG/TAM/-3'; β-actin forward primer, 5'-TCACCATGGATGATGATATCGC-3'; β-actin reverse primer, 5'-AAGCCGGCCTTGCACAT-3'; and β-actin probe, 5'-/FAM/CGCTCGTCGTCGA-CAACGGCT/TAM/-3'. The data obtained are presented as the mean ratio of VEGF to β-actin mRNA (± SD) obtained from triplicate samples.

## VEGF antisense oligonucleotide experiments

A VEGF antisense 2'-O-methyl phosphorothioate oligodeoxynucleotide (5'-CACCCAAGACAGCAGAA-3') and a sense 2'-O-methyl phosphorothioate oligodeoxynucleotide (5'-CTTTCTGCTGTCTTGGGTG) (provided by Greg Robinson, Children's Hospital, Boston, MA) were used to transfect MDA-MB-435 β4 transfectants at a concentration of 0.3 μM in the presence of lipofectin reagent (2 μg/ml; GIBCO BRL). The experimental details for this approach have been described previously (Bachelder et al., 2001). In addition, the same protocol was used to express antisense and sense eIF-4E oligonucleotides, which were gifts from Arigo De Benedetti (Louisiana State University, Shreveport, LA) (DeFatta et al., 2000).

## RNAi experiments

An RNAi specific for the β4 integrin subunit (GAGCUGCACGGAGUGU-GUC) as well as the inverted sequence (CUGUGUGAGGCACGUCGAG) were designed and synthesized by Dharmacon, Inc. MDA-231 cells at 30% confluency were transfected with 300 pmoles of RNAi using TKO lipids (Mirus). Subsequently, the cells were maintained in complete medium for 72 h and in medium containing 0.5% FBS for an additional 24 h before analysis.

## Polysome analysis

Cells (3 × 10⁷) were maintained in medium containing low serum (0.5% FBS) for 24 h and then pretreated with 100 μg/ml cycloheximide (Calbiochem) for 15 min at 37°C before being harvested. After washing once with PBS containing 100 μg/ml cycloheximide, the cells were resuspended in 0.5 ml of a modified U+S buffer (Davies and Abe, 1995). This buffer was composed of 200 mM Tris-HCl (pH 8.8), 25 mM MgCl₂, 5 mM EGTA (pH 8.0), 150 mM KCl, 10 μg/ml heparin, 5 mM DTT, 1% sodium deoxycholate, 2% polyoxyethylene 10-tridecy ester, 100 μg/ml cycloheximide, and 200 mM sucrose. Ribonuclease inhibitor (Amersham Biosciences) was added to a final concentration of 0.5 U/μl. Cells were homogenized with 20–25 strokes in a Kontes tissue homogenizer, followed by centrifugation for 5 min at 14,000 g. The supernatant was collected and frozen at −80°C until further use. Sucrose gradients (15–50%, wt/wt) were layered with 300 μl of cleared cell extract, which was then centrifuged at 160,000 g for 2 h. Fractions (0.75–0.375 ml) were withdrawn from the top of the gradient and monitored for absorbency at 254 nm using an ISCO syringe pump with UV-6 detector. Total RNA from the sucrose gradient fractions was extracted using Trizol LS (Life Technologies) according to the manufacturer's instructions. Quantitative real-time PCR was used to measure the VEGF mRNA level in each fraction as described above.

## Integrin signaling experiments

Cells were harvested by trypsin treatment and washed twice with DME containing 25 mM Hepes and 0.1% BSA. After washing, the cells were resuspended in the same buffer at a concentration of 2 × 10⁶ cells/ml and incubated for 30 min with integrin-specific antibodies (4 μg/ml) or with either mouse or rat IgG (4 μg/ml). The cells were washed once, resuspended in the same buffer, and added to plates that had been coated overnight with either the anti–mouse or rat IgG. After a 60-min incubation at 37°C, the cells that had attached to integrin-specific antibodies were washed twice with cold PBS and solubilized at 4°C for 10 min using RIPA buffer (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). The IgG-treated cells were harvested by centrifugation and solubilized with RIPA buffer.

## Protein analysis

Aliquots of cell extracts containing equivalent amounts of protein were solubilized using 5× sample buffer containing 100 mM DTT and then incubated at 100°C for 15 min. These extracts were resolved by SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked for 1 h using a 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl, 0.05% Tween-20 (TBST), and 5% (wt/vol) Carnation dry milk. The filters were incubated overnight in the same buffer containing antibodies specific for p70S6K, 4EBP antibodies (Santa Cruz Biotechnology, Inc.), actin (ICN Biomedicals), and VEGF (clone 618, provided by Donald Senger, Beth Israel Deaconess Medical Center). After three, 10-min washes in TBST, the filters were incubated for 1 h in blocking buffer containing HRP-conjugated secondary antibodies. After three 10-min washes in TBST, proteins were detected by ECL (Pierce Chemical Co.).

For immunoblots involving phosphospecific antibodies, the filters were blocked for 1 h using a 10 mM Tris buffer, pH 7.5, containing 0.5 M NaCl, 0.1% Tween-20, and 2% (wt/vol) BSA. The filters were washed briefly and then incubated overnight at 4°C in the same blocking buffer containing antibodies specific for phospho-p70S6K (Thr-389; Cell Signaling Technology), phospho-4E-BP1 (Ser-65; Cell Signaling Technology), phospho-Erk (E10; Cell Signaling Technology), and phospho-Akt (Ser-473 clone 4E2; Cell Signaling Technology). After washing, the filters were incubated for 1 h in blocking buffer containing HRP-conjugated secondary antibody and the proteins were detected by ECL.

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*Advances in Brief*

# Hypoxia-Induced Vascular Endothelial Growth Factor Transcription and Protection from Apoptosis Are Dependent on α6β1 Integrin in Breast Carcinoma Cells

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## Abstract

AQ: A    The α6β1 integrin has been implicated in breast carcinoma progression, but the mechanisms involved remain elusive. MDA-MB-435 cells engineered to be deficient in α6β1 expression form primary tumors that are highly apoptotic and unable to metastasize, although they exhibit no increased apoptosis *in vitro* under standard culture conditions. Based on the hypothesis that α6β1 is necessary for the survival of these cells in the tumor microenvironment, we report here that hypoxia protects these cells from apoptosis induced by serum deprivation and that hypoxia-mediated protection requires α6β1 expression. We investigated the influence of α6β1 on vascular endothelial growth factor (VEGF) expression because autocrine VEGF is necessary for the survival of serum-deprived cells in hypoxia. The results obtained indicate that α6β1 is necessary for VEGF expression because the ability of hypoxia to activate HIF-1 and to stimulate VEGF transcription in MDA-MB-435 cells is dependent on α6β1 expression by a mechanism that involves protein kinase C-α.

## Introduction

An important role for the α6β1 integrin in breast cancer progression has been indicated by several studies (1–3). The involvement of this integrin in progression was suggested first by the finding that high expression of the α6 subunit in women with breast cancer correlated significantly with reduced survival times (1). In an analysis of 119 patients with invasive breast carcinoma, all of the patients with low or absent α6 expression survived, whereas the mortality rate of the patients with a high level of α6 expression was 19%. Of note, 30 of 34 of the patients that presented with distant metastases were highly positive for α6 expression. This study is consistent with the report that the metastatic potential of MDA-MB-435 cells correlates with their level of α6β1 expression (3). Moreover, when α6β1-deficient MDA-MB-435 cells were inoculated into the mammary fat pads of nude mice, primary tumor size was significantly diminished compared with the parental cells because of increased apoptosis (2). The α6β1-deficient cells did not form metastases in the lung, as did the parental cells, because of their inability to survive in this organ (2). Interestingly, these α6β1-deficient cells did not differ in their ability to survive *in vitro* under standard culture conditions, suggesting that α6β1 is needed for survival within the tumor microenvironment. Given that the microenvironment of solid tumors is often hypoxic and lacks the rich growth factor milieu present in culture medium, we examined the hypothesis that this integrin contributes to the survival of MDA-MB-435 cells in such conditions. Interestingly, the data obtained indicate that hypoxia protects these cells from apoptosis induced by serum deprivation and that this protection depends on

α6β1 expression. Protection from apoptosis under these conditions requires autocrine vascular endothelial growth factor (VEGF), and additional analysis revealed that α6β1 is necessary for VEGF expression because it functions in concert with hypoxia to activate HIF-1 and to stimulate VEGF transcription by a mechanism that involves protein kinase C (PKC)-α.

## Materials and Methods

**Cells and Reagents.** MDA-MB-435 breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University (Washington, DC). MDA-MB-435 cells that had been sorted by fluorescence-activated cell sorting to obtain populations that express relatively high or low α6β1 expression were provided by Beth Israel Deaconess Medical Center (Boston, MA). Myrsitylated PKC-α and PKC-ε constructs were obtained from Dr. Alex Toker (Beth Israel Deaconess Medical Center). The HIF-1 dominant-negative mutant constructs A26E and K29E (4, 5) were provided by Dr. Dev Mukhopadhyay (Beth Israel Deaconess Medical Center).

The following antibodies were used: an HIF-1α monoclonal antibody (Novus Biological, Newington, NH); a rabbit p300 polyclonal antibody (Upstate Biotechnology, Lake Placid, NY); an α6 integrin monoclonal antibody, clone 2B7 (prepared in our laboratory); and a rabbit actin antibody (Sigma, St. Louis, MO). Rabbit polyclonal anti-VEGF serum (clone 618) was obtained from Dr. Don Senger (Beth Israel Deaconess Medical Center).

**Small Interfering RNA (siRNA) Experiments.** A siRNA specific for the α6 integrin subunit (GGUCGUGACAUGUGCUCAC) and a scrambled-sequence control (AUGCAGAGUGGCGCUCUCU) were synthesized by Dharmacon, Inc. (Lafayette, CO). A siRNA specific for α5 integrin subunit (UGGCUCAGACAUUCGAUCC) and control siRNA were synthesized by Qiagen, Inc. (Valencia, CA). Cells ($1 \times 10^5$) were plated onto 35-mm tissue culture dishes a day before the transfection of 200 nM siRNA duplex with 25 mg of TransIT-TKO transfection reagent (Mirus, Madison, WI) in the presence of serum. A day after transfection, the transfection medium was aspirated from cells, and fresh complete medium was added and incubated for an additional 48–72 h. For each transfection, flow cytometry was used to assess α6 integrin expression.

**VEGF Antisense Strategy.** Either a VEGF antisense 2′-O-methyl phosphorothioate oligodeoxynucleotide (5′-CACCCAAGACAGCAGAAG-3′) or a VEGF sense 2′-O-methyl phosphorothioate oligodeoxynucleotide (5′-CTT-TCTGCTGTCTTGGGTG) at a concentration of 0.3 μM was transfected into MDA-MB-435 cells as described previously (6).

**Protein Analysis.** For the analysis of protein expression, cells were extracted in radioimmunoprecipitation assay buffer [20 mM Tris buffer (pH 7.4) containing 0.14 M NaCl; 1% NP40; 10% glycerol; 1 mM sodium orthovanadate; 2 mM phenylmethylsulfonyl fluoride; and 5 μg/ml aprotinin, pepstatin, and leupeptin], and immunoblotting was performed as described previously (7). For experiments that assessed HIF-1 expression and HIF-1 association with p300, nuclei were isolated and extracted as described previously (8, 9) and used for immunoblotting and immunoprecipitation experiments.

**Apoptosis Assay.** Adherent and nonadherent cells were harvested and assayed for apoptosis using annexin V-FITC (Biosource, Sunnyvale, CA) and propidium iodide (Biosource) as described previously (7). In some experiments, cells were incubated in low serum [0.5% fetal bovine serum (FBS)] in hypoxia with or without recombinant $VEGF_{165}$ (final concentration, 100 ng/ml; R&D Systems) along with 1 μg/ml heparin (Sigma) before the apoptosis assay.

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**Quantitative Real-Time PCR.** Quantitative analysis of VEGF mRNA expression was performed by real-time PCR as described previously (7).

**Transcription Assays.** Human VEGF promoters (either the full-length 2.6-kb promoter or 0.35-kb deletion mutant lacking the HRE) were cloned into pGL3basic vector (Promega, Madison, WI) and used to assess VEGF promoter activity using firefly luciferase as the reporter gene (10). Cells at a confluency of 85–95% were used for all experiments and incubated with 20 μM ZVAD-FMK (Promega) to prevent apoptosis during the experiment. Plasmids were transiently transfected with the Effectene transfection kit (Qiagen, Inc.) according to the manufacturer's protocol. Thirty h after transfection, cells were washed with PBS and lysed with reporter buffer (Promega) at room temperature for 15 min followed by the luciferase assay. Luciferase activity was measured with a luminometer (MicroLumat LB96P; Berthold Technologies, Germany) using the Dual-Glo luciferase assay kit (Promega). Renilla luciferase construct was used as an internal control to normalize the result. Triplicate readings were taken for each experiment, and SDs were calculated.

## Results and Discussion

**Hypoxia Protects Serum-Deprived MDA-MB-435 Cells from Apoptosis: Role of α6β1 Integrin.** To assess the importance of the α6β1 integrin for the survival of tumor cells in stress conditions, we used MDA-MB-435 cells, which express α6β1 but not α6β4 (2, 11). Two approaches were used to modulate α6β1 expression in these cells. In the first approach, cells were sorted by fluorescence-activated cell sorting to obtain two distinct clonal populations that exhibited either relatively high or low α6 integrin surface expression. As shown F1 in Fig. 1A, these populations differed by almost 4-fold in their mean fluorescence of α6 integrin expression. The second approach involved expression of a siRNA oligonucleotide specific for the α6 integrin subunit. Expression of this siRNA in MDA-MB-435 cells that expressed high levels of α6β1 resulted in an approximate 70% reduction in the surface expression of this integrin (Fig. 1B). This siRNA did not alter expression of the α5 integrin subunit (Fig. 1B).

The cell populations described above were maintained in either a normoxic or hypoxic environment in the presence of either 10 or 0.5% FBS for 24 h, and apoptosis was assessed by annexin-FITC staining (Fig. 1C). In normoxia, serum deprivation (0.5% FBS) increased the level of apoptosis by approximately 3–4-fold compared with cells maintained in 10% serum (Fig. 1C). Hypoxia, however, protected cells from apoptosis induced by serum deprivation as a function of α6β1 expression. More specifically, both the low-expressing α6 cells and the α6 siRNA-treated cells were significantly ·more apoptotic under these conditions than were the corresponding high-α6β1-expressing control cells (Fig. 1C). To assess the integrin specificity of these results, we used a siRNA to reduce expression of the α5β1 integrin, which is expressed in MDA-MB-435 cells at levels comparable with α6β1. As shown in Fig. 2A, expression of a siRNA specific F2 for the α5 integrin subunit reduced surface expression of α5β1 by approximately 30%. This reduction in α5β1 expression, however, had no impact on apoptosis induced by serum deprivation under either normoxic or hypoxic conditions as assessed by annexin-V-FITC staining (Fig. 2B).

**Hypoxic Stimulation of VEGF Transcription Is Dependent on α6β1.** Given the recent reports that autocrine VEGF is necessary for the survival of breast and other carcinoma cells (6, 7, 12–15), we assessed whether the ability of hypoxia to prevent apoptosis is VEGF F3 dependent. As shown in Fig. 3A, expression of an antisense VEGF oligonucleotide in high-α6-expressing cells reduced hypoxia-induced VEGF expression by approximately 60% in comparison with the sense oligonucleotide, and it increased the apoptosis of serum-deprived cells in hypoxia 2-fold. Furthermore, incubation of low-α6-expressing cells with exogenous recombinant VEGF$_{165}$ inhibited the apoptosis induced by serum deprivation significantly (Fig. 3A). These findings led us to examine the hypothesis that α6β1 influences hy-

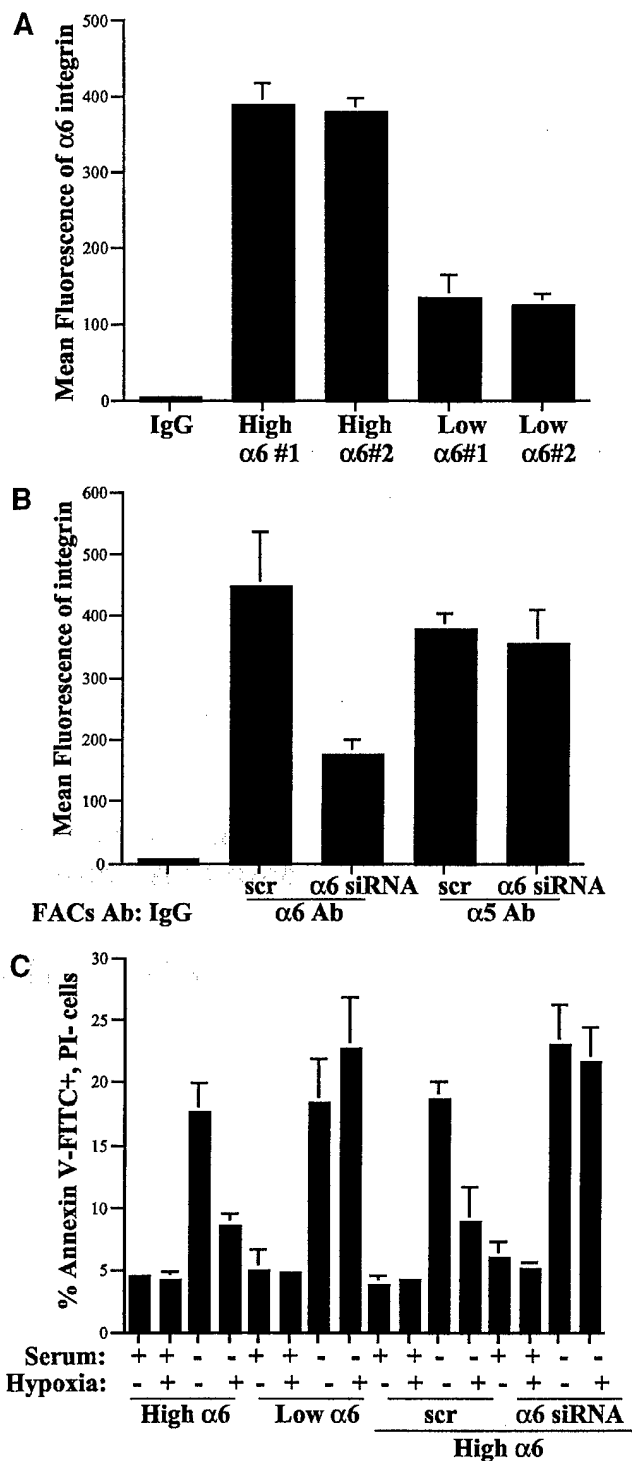Fig. 1. Expression of α6β1 integrin is needed for protection from apoptosis by hypoxia. A, MDA-MB-435 cells that exhibited either *high* or *low* α6 expression were analyzed by flow cytometry, and data are reported as mean channel fluorescence (±SD) of α6 expression relative to a nonspecific IgG. B, siRNAs specific for α6 and a scrambled (*scr*) siRNA were transfected into cells that expressed high levels of α6, and cells were analyzed by flow cytometry 72 h later for α6 and α5 surface expression. Data are reported as in A. C, the cells characterized in A and B were incubated with either 10% FBS (*+serum*) or 0.5% FBS (*−serum*) in either normoxia (−) or hypoxia (+) for 24 h and stained with annexin V-FITC and propidium iodide (*PI*). *Ab*, antibody.

poxia-induced VEGF expression. In the presence of 10% serum, VEGF expression is relatively high and independent of α6β1 expression in MDA-MB-435 cells (data not shown). Serum deprivation for 24 h, however, reduced VEGF expression to nearly undetectable

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**A**



FACs Abs :  IgG    $\alpha6$ integrin    $\alpha5$ integrin

**B**
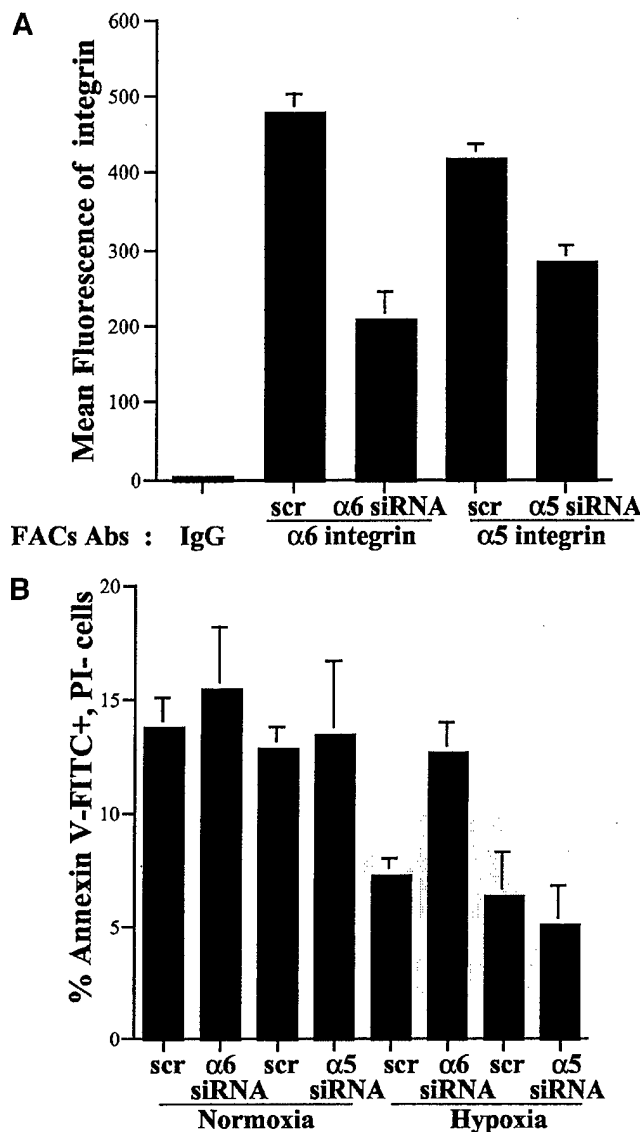


Fig. 2. Expression of $\alpha6\beta1$ but not $\alpha5\beta1$ integrin is needed for protection from apoptosis by hypoxia. *A*, cells that express high levels of $\alpha6\beta1$ were transfected with siRNAs specific for $\alpha6$ or $\alpha5$ or with scrambled sequences (*scr*). After 72 h, integrin expression was assessed by flow cytometry as in Fig. 1*A*. *B*, the populations of cells described above were assessed for apoptosis under the conditions described in Fig. 1*C*. Apoptosis is reported as the percentage of annexin V-FITC$^{+}$, propidium iodide (*PI$^{-}$*) cells. The data shown are mean values ($\pm$SD) of a representative experiment performed in triplicate. *Ab*, antibody.

levels in normoxia (Fig. 3*B*). In contrast, hypoxia sustained both total VEGF and secreted VEGF as a function of $\alpha6\beta1$ expression (Fig. 3*B*). These data indicate that serum induces VEGF expression independently of $\alpha6\beta1$, but in the absence of serum, VEGF expression decreases and apoptosis increases. Hypoxia prevents this increase in apoptosis in serum-deprived cells by inducing VEGF expression in an $\alpha6\beta1$-dependent manner.

To address the mechanism by which $\alpha6\beta1$ influences hypoxia-induced VEGF expression, we quantified VEGF mRNA expression by real-time PCR. Hypoxia increased VEGF mRNA expression approximately 2-fold in serum-deprived cells with high $\alpha6\beta1$ expression but not in cells with reduced expression of this integrin (Fig. 3*C*). This finding prompted us to evaluate the mechanism by which this integrin contributes to VEGF transcription. For this purpose, cells were transfected with a reporter construct that consisted of the intact VEGF promoter conjugated to luciferase. The relative luminescence of the

reporter construct increased approximately 3-fold in hypoxia but only in those populations of cells that expressed high levels of $\alpha6\beta1$ (Fig. 3*D*). The ability of hypoxia to increase VEGF promoter activity in cells selected for low $\alpha6\beta1$ expression or treated with the $\alpha6$ siRNA was negligible (Fig. 3*D*). To exclude the possibility that the apoptosis induced by these conditions affects VEGF promoter activity, cells were incubated with the general caspase inhibitor ZVAD-FMK. The use of this inhibitor prevented the apoptosis triggered by serum deprivation and low $\alpha6$ expression (data not shown). The effect of $\alpha6\beta1$ on VEGF transcription appears to be independent of its ligation because we observed comparable VEGF expression when cells were plated on either laminin, an $\alpha6\beta1$ ligand, or other matrix proteins that are not ligands for this integrin such as collagen (data not shown).

MDA-MB-435 cells lack expression of VEGFR-1 (*flt*-1) and VEGFR-2 (KDR, *flk*-1; Refs. 16 and 17). However, they express neuropilin-1 (17), and the expression of this VEGF receptor did not differ between high- and low-a6-expressing cells, as evidenced by immunoblotting (data not shown) suggesting that the $\alpha6\beta1$ integrin does not influence the expression of VEGF receptors.

**The $\alpha6\beta1$ Integrin Regulates the Transcriptional Activity of HIF-1.** We examined the hypoxic induction of HIF-1$\alpha$ expression as a function of $\alpha6\beta1$ expression because HIF-1 plays a pivotal role in stimulating the transcription of VEGF and many other genes in hypoxia (18). Interestingly, as shown in Fig. 4*A*, the level of HIF-1$\alpha$ F4 induction in hypoxia was comparable between the high and low $\alpha6$ clones, suggesting that $\alpha6\beta1$ integrin is not involved in either the expression or stabilization of HIF-1$\alpha$. To assess HIF-1 activation directly, we monitored the association of HIF-1 with its coactivator, p300 (Fig. 4*A*). HIF-1 binding to HRE itself is not sufficient to activate target gene transcription, and HIF-1 association with p300 is required to recruit the RNA polymerase II complex to initiate transcription (19). Therefore, association of HIF-1 with p300 is an indicator of HIF-1 activation. Association of HIF-1 with p300 was maximal at 6 h in cells that expressed high levels of $\alpha6\beta1$ and diminished by 24 h (Fig. 4*A*). In cells that expressed low levels of $\alpha6\beta1$, however, association of HIF-1 with p300 was barely detectable (Fig. 4*A*). The total level of p300 expression is similar in both populations of cells (Fig. 4*A*). These data indicate that the $\alpha6\beta1$ integrin influences HIF-1 activation but not its expression.

To confirm the involvement of HIF-1 in VEGF transcription under hypoxia, we used a luciferase construct conjugated with a VEGF promoter (0.35 kb) that lacks the HRE (Fig. 4*B*). We also expressed HIF-1 dominant-negative mutants that block HIF-1 binding to HRE. Expression of this mutant promoter, as well as the two different dominant-negative HIF-1 mutants, in high-$\alpha6$-expressing cells level did not result in hypoxia-induced increase in VEGF promoter activity (Fig. 4*B*), indicating that HIF-1 is playing a major role in $\alpha6\beta1$-mediated VEGF transcription in hypoxia.

Our consideration of possible mechanisms involved in $\alpha6\beta1$ regulation of HIF-1 activation led us to PKC because of the report that this integrin associates with specific PKC isoforms (20) and other studies that have linked PKCs to HIF-1 activation, VEGF expression, apoptosis, and survival signaling (21–24). Pharmacological inhibition of PKC-$\alpha$ activity using Go6983 prevented $\alpha6\beta1$-mediated HIF-1 activation as measured by p300 association (Fig. 4*C*). The finding that PKC-$\alpha$ activity is necessary for HIF-1 activation raised the possibility that HIF-1 activation could be induced in cells that expressed low levels of $\alpha6\beta1$ by expression of a constitutively active form of PKC-$\alpha$. Indeed, as shown in Fig. 4*C*, expression of a constitutively active form of PKC-$\alpha$ increased HIF-1 activity dramatically in cells that normally could not activate HIF-1 under hypoxia (Fig. 4*A*). Expression of a constitutively active form of PKC-$\epsilon$, in contrast, did not rescue the ability of these cells to activate HIF-1 under hypoxia
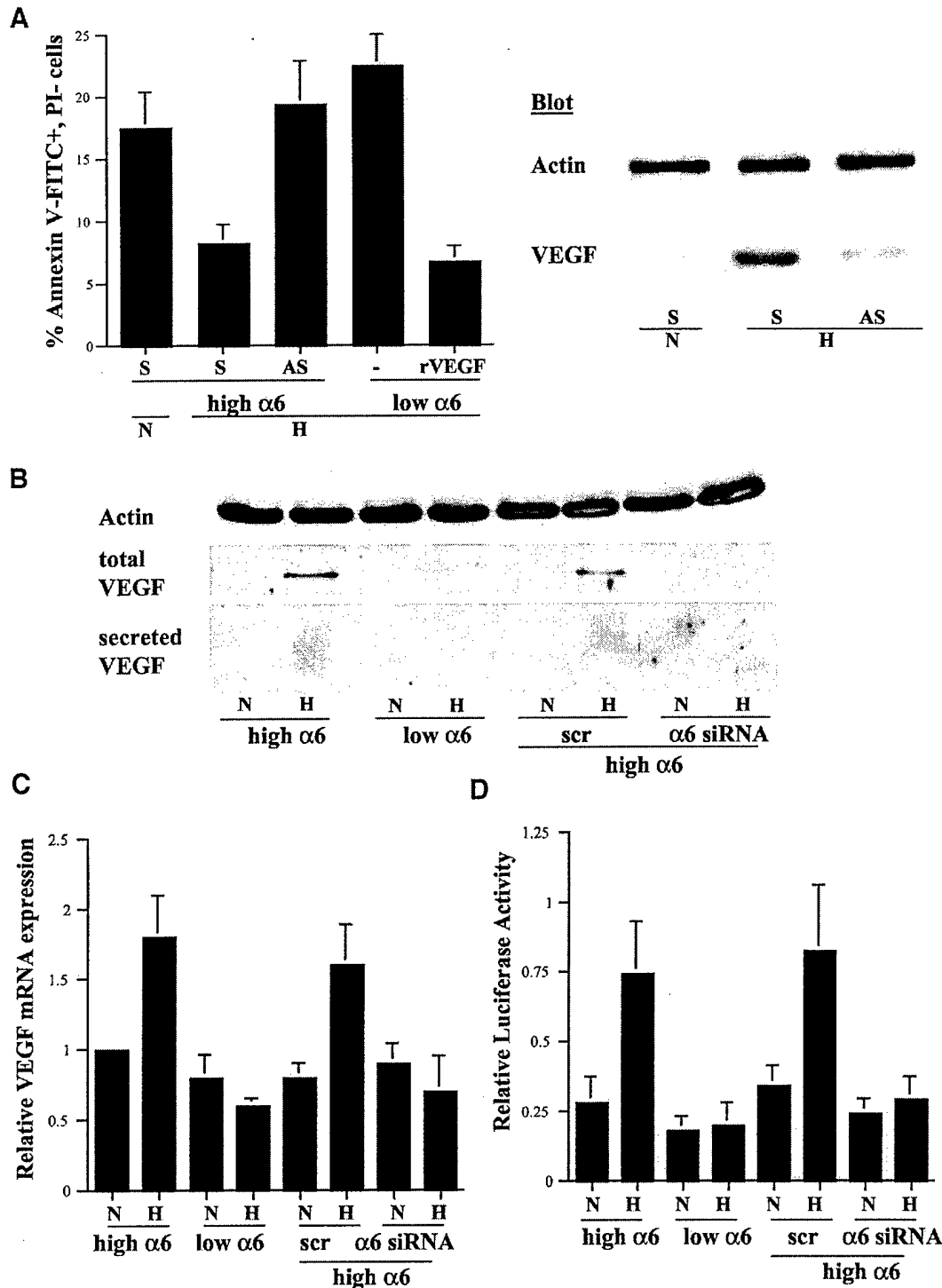
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Fig. 3. α6β1 integrin is necessary for hypoxia-induced VEGF expression. *A*, MDA-MB-435 cells expressing high α6 integrin were transfected with either the VEGF sense (*S*) or antisense (*AS*) oligonucleotide and recovered in complete medium overnight, followed by incubation in low serum (0.5% FBS) either in normoxia (*N*) or hypoxia (*H*) for 24 h. In addition, cells expressing low α6 integrin were incubated in low serum (0.5% FBS) in hypoxia with or without recombinant VEGF$_{165}$ (*rVEGF*; final concentration, 100 ng/ml; R&D Systems) along with 1 μg/ml heparin. The level of apoptosis is indicated as the percentage of annexin V-FITC$^+$, propidium iodide (*PI$^-$*) cells. Extracts of these cells were analyzed for their relative expression of VEGF and actin by immunoblotting. *B*, extracts of the cells described in Fig. 1*C* were analyzed for their relative expression of VEGF and actin by immunoblotting (*total VEGF*). Culture media from the cells described in Fig. 1*C* were concentrated and analyzed for VEGF expression by immunoblotting (*secreted VEGF*). *C*, VEGF mRNA was quantified by real-time PCR in the cells described in Fig. 1*C* that had been maintained in either normoxia or hypoxia for 24 h in the presence of low serum. Data are presented as the mean ratio of VEGF to β-actin mRNA (±SD). *D*, a luciferase construct conjugated to the VEGF promoter was transfected into the cells described in Fig. 1*C*. Luciferase activity (±SD) was assayed after the cells had been maintained in either normoxia or hypoxia for 24 h in the presence of low serum. *scr*, scrambled.

(Fig. 4*D*). Collectively, these data highlight a key role for PKC-α in HIF-1 activation and VEGF transcription.

To date, the mechanism of HIF-1 activation has focused on two different posttranslational modifications at the COOH-terminal activation domain of HIF-1α that are known to regulate the association of p300 with HIF-1α. One modification is the hydroxylation of a specific asparagine residue (amino acid 803 of HIF-1α) at the COOH-terminal activation domain that prevents the association
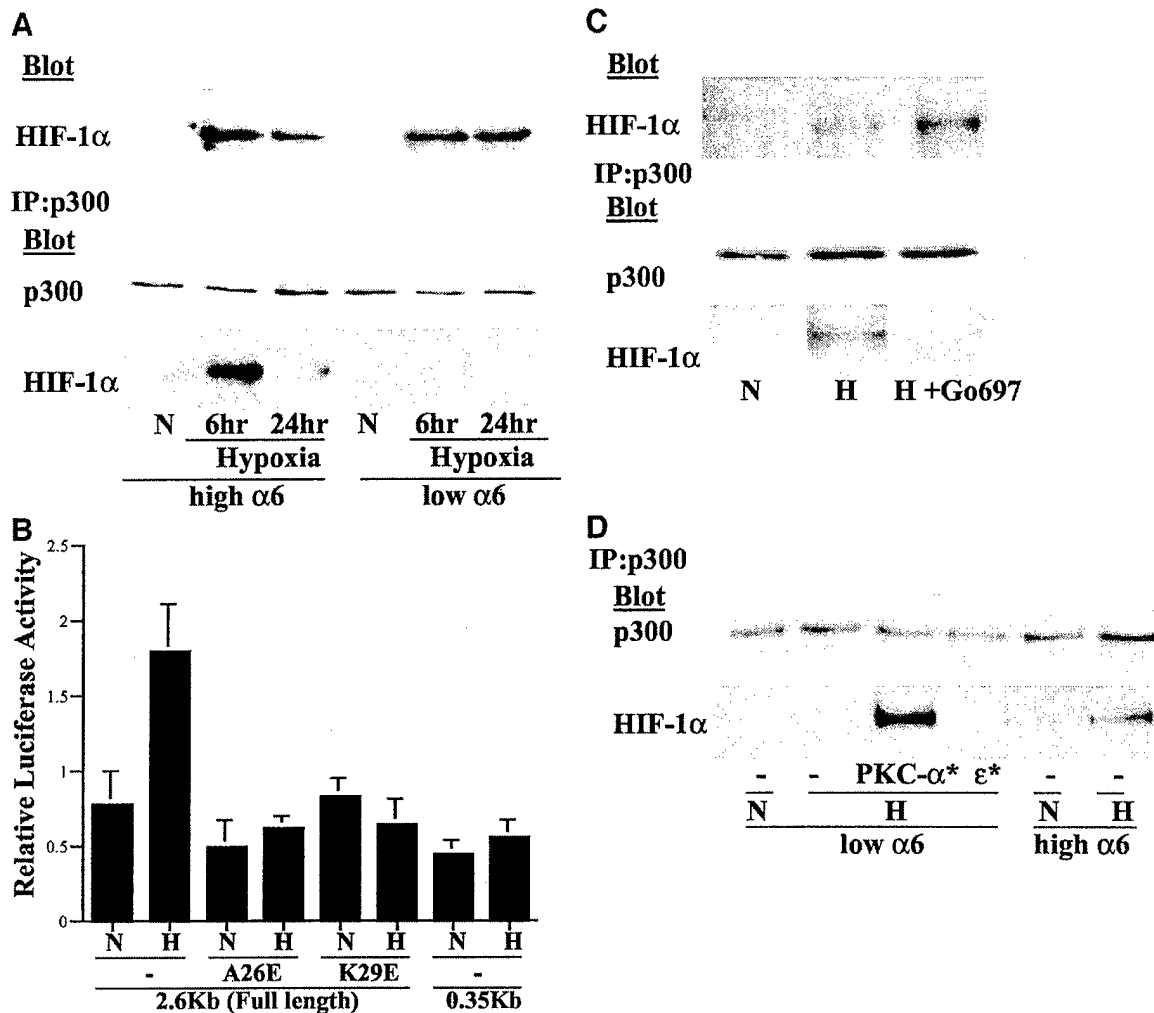
Fig. 4. α6β1 is involved in HIF-1 activation via PKC-α. *A*, HIF-1α expression was assessed by immunoblotting nuclear extracts obtained from cells that expressed either *high* or *low* levels of α6 and that had been maintained in either normoxia for 24 h or hypoxia for 6 or 24 h in the presence of low serum. p300 was immunoprecipitated from the nuclear extracts described above. The immunoprecipitates were analyzed by immunoblotting for p300 and HIF-1α expression. *B*, luciferase constructs conjugated to either the full-length promoter (2.6 kb) or a deletion mutant lacking the HRE (0.35 kb) were transfected into cells that expressed high levels of α6 integrin. Dominant-negative HIF-1 constructs (*A26E* and *K29E*) were cotransfected with a luciferase construct conjugated with the full-length promoter (2.6 kb) into MDA-MB-435 cells that expressed high levels of α6 integrin. VEGF promoter activity was assessed by the luciferase assay as described in Fig. 3*D*. *C*, cells that express high levels of α6β1 were incubated with 0.5% FBS for 6 h in normoxia or hypoxia with or without 1 μM Go6976. HIF-1α expression and association with p300 were assessed as described above. *D*, cells that express low levels of α6β1 were transfected with a constitutively active PKC-α construct (*Lane 3*), a constitutively active PKC-ε construct (*Lane 4*), or an empty vector (*Lanes 1* and *2*) and analyzed for HIF-1α and p300 association in hypoxia as described above. Cells that express high levels of α6β1 were used as positive control (*Lanes 5* and *6*).

of p300 with HIF-1 in normoxia (25, 26). The other modification is the phosphorylation of a threonine residue (amino acid 844 of HIF-1α) that is required for their association (27). An important issue that arises, therefore, is how PKC-α facilitates HIF-1 activation and how this signaling pathway is linked to these other signaling pathways that have been implicated in HIF-1 activation. Our data indicate that PKC-α regulates the association of HIF-1 and p300 in hypoxia, which excludes a role for PKC-α in regulating asparagine hydroxylase because it is inactive in hypoxia. More likely, PKC-α is directly or indirectly involved in COOH-terminal activation domain phosphorylation to activate HIF-1.

Overall, our studies highlight a potential function for the α6β1 integrin in stimulating VEGF transcription and providing a selective survival advantage for carcinoma cells in the tumor microenvironment in which both nutrients and oxygen supply are limited. Moreover, these findings reveal a mechanism that could account for the involvement of the α6β1 integrin in tumor survival that has been observed *in vivo,* and they are consistent with the finding reported here and elsewhere (2, 3) that breast carcinoma cells depend upon autocrine

VEGF for survival. These findings may also bear on other aspects of VEGF function in cancer, as well as other targets of VEGF transcription that could contribute to tumor survival. The best-characterized functions of VEGF, produced by both tumor and stromal cells, are to increase vascular permeability (28) and to stimulate angiogenesis (18, 29). Thus, it can be inferred that the α6 integrin influence on VEGF transcription will also impact these core VEGF functions. In a different direction, HIF-1 is known to regulate the transcription of more than 40 target genes, many of which can contribute to the survival of tumor cells in hypoxia (30). For this reason, it is conceivable that such genes function in concert with VEGF and α6β1 to affect tumor survival. In addition, our data highlight a role for PKC-α in HIF-1 activation and VEGF transcription.

## Acknowledgments

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