p53 Inhibits α6β4 Integrin Survival Signaling by Promoting the Caspase 3-dependent Cleavage of AKT/PKB

Robin E. Bachelder,‡† Mark J. Ribick,‡† Alessandra Marchetti,§ Rita Falcioni,§ Silvia Soddu,§ Kathryn R. Davis,‡* and Arthur M. Mercurio‡*

*Division of Cancer Biology and Angiogenesis, Department of Pathology, Beth Israel Deaconess Medical Center and †Harvard Medical School, Boston, Massachusetts 02215; and ‡Regina Elena Cancer Institute, Rome, 00158 Italy

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

Key words: p53 • integrin • AKT/PKB • survival • caspase

Primary epithelial (Frisch and Francis, 1994) and endothelial (Meredith et al., 1993) cells are prone to anoikis, a form of programmed cell death, when grown in the absence of growth factors and extracellular matrix proteins. This default apoptotic pathway is thought to be important in preventing cell growth at inappropriate anatomical sites. Survival signaling pathways associated with both growth factor receptors and cell adhesion molecules are important in protecting cells from anoikis. For example, growth factors such as EGF, PDGF, and insulin can promote the survival of serum-starved epithelial cells (Meral et al., 1995; Rampalli and Zelenka, 1995; Rodeck et al., 1997). Similarly, the binding of integrins such as αvβ3 (Stromblad et al., 1996), α5β1 (Zhang et al., 1995), and α6β1 (Howlett et al., 1995; Wewer et al., 1997; Farrelly et al., 1999) to the appropriate extracellular matrix protein can inhibit anoikis. These survival signals have been attributed to the ability of integrins to activate numerous molecules including focal adhesion kinase (Frisch et al., 1996), integrin-linked kinase (Radeva et al., 1997), AKT/PKB (Khwaja et al., 1997), and bcl-2 (Zhang et al., 1995; Stromblad et al., 1996). In addition, integrin survival functions have been associated with their ability to inhibit the activity of p53 (Stromblad et al., 1996; Ilic et al., 1998) and Rb (Day et al., 1997) tumor suppressors. Tumor cells acquire a partial resistance to anoikis as a result of their transformation, which is thought to activate survival signaling pathways in these cells constitutively (Frisch and Francis, 1994). For this reason, the identification of molecules that can inhibit survival signaling is crucial for developing strategies aimed at blocking tumor cell growth.

The α6β4 integrin, a receptor for the laminin family of extracellular matrix proteins, plays an important role in diverse cellular activities. In addition to serving an important structural role in the assembly of hemidesmosomes in epithelial cells (Borradori and Sonnenberg, 1996; Green and Jones, 1996), α6β4 promotes carcinoma cell migration and invasion (Tozeren et al., 1994; Chao et al., 1996; Shaw et al., 1997; O’Connor et al., 1998) in a phosphoinositide

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3-O H kinase–dependent manner (Shaw et al., 1997). The β4 subunit of this integrin, which contains a cytoplasmic tail of ~1,000 amino acids (Hemler et al., 1989; K ajii et al., 1989; K ennel et al., 1989), has been shown to be crucial in the ability of this integrin to activate numerous signaling molecules, including phosphoinositide 3-O H kinase (Shaw et al., 1997), Shc (M ainiero et al., 1997), R as (M ainiero et al., 1997), J nk (M ainiero et al., 1997), p21W A F 1/CIP1 (C lárke et al., 1995), and p53 (B achelder et al., 1999). The diverse activities of this integrin are exemplified by its ability to promote both the survival of keratinocytes (D owling et al., 1996) as well as the apoptosis of a number of carcinoma cell lines (C lárke et al., 1995; K im et al., 1997; S un et al., 1998; B achelder et al., 1999). These apparently contradictory functions likely reflect the activation of distinct signaling pathways by this integrin in different cell types as well as the influence of other signaling pathways on α β4 function.

In the present study, we define opposing signaling pathways that are activated by the α β4 integrin that promote either carcinoma cell survival or apoptosis, depending on whether these cells express wild-type or functionally inactive mutants of p53. Specifically, we show that α β4 can promote the A KT/P KB–dependent survival of p53-deficient carcinoma cells. However, this activity contrasts with the ability of α β4 to stimulate the caspase-dependent cleavage and inactivation of A KT/P KB in p53 wild-type carcinoma cells. The ability of wild-type p53 to inhibit α β4-associated survival signals suggests that the p53 status of an α β4-expressing carcinoma cell influences its growth potential.

**Materials and Methods**

**Cells**

The RKO colon carcinoma cell line was obtained from M. B rattain (U niversity of Texas, San A ntoinio, T X), and M DA–M B–435 breast carcinoma cells were obtained from the Lombardi Breast Cancer D epository (G eorgetown U niversity).

The cloning of the human β4 C DNA, the construction of the β4 cytoplasmic domain deletion mutant (β4Δcyt), and their insertions into the pR C/CM V (β4) and pCDNA 3 (β4Δcyt) eukaryotic expression vectors, respectively, have been described (C lárke et al., 1995). RKO β4/4Δcyt clone 3E 1, RKO β4/β4 clone D 4 (RKO β4/4 clone 1), RKO β4/β4 clone A 7 (RKO β4 clone 2), M DA–M B–435/β4Δcyt clone 3C 12, M DA–M B–435/β4 clone B 3 (M DA–M B–435/β4 clone 1), and M DA–M B–435/β4 clone 3A 7 (M DA–M B–435/β4 clone 2) were selected for analysis based on their expression of similar surface levels of α β4 and α β4Δcyt, as we have previously demonstrated (C lárke et al., 1995; S haw et al., 1998; B achelder et al., 1999).

Dominant negative p53-expressing RKO β4/4Δcyt and RKO β4/β4 subclones were obtained by cotransfecting RKO β4/4Δcyt clone 3E 1 and RKO β4/β4 clone D 4 with plasmids expressing the p53α-resistant resistance gene (M orgenstern and L and, 1990) and a dominant negative p53 (dpn53) construct (provided by M. O ren, W eizmann I nstitute for S cience, I srael) that encodes for a carboxy-terminal domain of p53 that can heterodimerize with endogenous p53 and inhibit its transcriptional activity. D np53-expressing subclones were obtained and those subclones expressing high levels of dnp53 were selected by F ACS using the P ab122 mAb (B oehringer M annheim), which recognizes a conserved, denaturation stable epitope in dnp53. In addition, RKO β4 and RKO β4Δcyt cells were transfected with the p53α-resistant resistance gene plasmid alone to obtain p53α-resistant mock transfectants. All assays were performed using cell maintained below passage 10.

**Antibodies**

The following antibodies were used: 439-98, a rat mAb specific for the β4 integrin subunit (F alcon et al., 1998), control rat IgG (S igma C hemical C o.), control rabbit polyclonal serum specific for p53 (B oehringer M annheim); goat anti-human p53; rabbit polyclonal anti-A KT/P KB raised against a peptide corresponding to mouse A KT/P KB residues 466-479 (N ew E ngland B iolabs); rabbit polyclonal anti-A KT/P KB phosphoryserine 473 (N ew E ngland B iolabs); rabbit anti-actin (S igma C hemical C o.); and mouse anti-hemagglutinin (B oehringer M annheim). Goat anti-mouse IgG and goat anti-rat IgG secondary antibodies, as well as HRP conjugates of these antibodies, were obtained from J ackson I mmunoR esearch L aboratories, I nc. HRP-conjugated donkey anti-goat IgG was obtained from B ioS oource I nternational.

**Apoptosis Assays**

To induce apoptosis in the RKO and M DA–M B–435 transfectants, the cells were plated in complete medium for 8 h in tissue culture wells (12-well plate; 2.5 × 105 cells/well) that had been coated overnight at 4°C with poly-L-lysine (S igma C hemical C o.; 2 ml of 25 µg/ml stock) and blocked with 1% B SA. A fter 8 h, this medium was replaced with serum-free culture medium containing 1% B SA. A fter 15 h at 37°C, adherent and suspension cells were harvested, combined, and the level of apoptosis in these cells was assessed as described below.

For annexin V stains, cells were washed once with serum-containing medium, once with PBS, once with annexin V-FITC buffer (10 mM Hepes/N aOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2), and incubated for 15 min at room temperature with annexin V-FITC (B ender M edSystems) at a final concentration of 2.5 µg/ml in annexin V buffer. A fter washing once with annexin V buffer, the samples were resuspended in the same buffer and analyzed by flow cytometry. I mmediately before analysis, propidium iodide was added to a final concentration of 5 µg/ml to distinguish apoptotic from necrotic cells, and 5,000 cells were analyzed for each sample.

F or A popTag reactions, cells were harvested as described above, fixed in 1% paraformaldehyde for 15 min on ice, and washed twice with PBS. The samples were resuspended in 1 ml ice-cold 70% ethanol and stored at −20°C overnight. A fter centrifugation at 2,500 rpm for 15 min, cells were washed two times in PBS before performing A popTag reactions (O ncor) according to the manufacturer’s recommendations. These samples were analyzed by flow cytometry.

F or in situ analysis of apoptosis in cells transfected transiently with the green fluorescent protein (G FP)–expressing vector pEGFP-1 (C LONE T ECH Laboratories) and d nA KT/P KB, the transfected cells were stained with annexin V-PE (P harMingen) according to the manufacturer’s directions, and plated on coverslips. The percentage of G FP-positive cells that was annexin V-PE–positive was determined by fluorescence microscopy. A total of at least 80 G FP-positive cells from at least 10 microscopic fields were analyzed for each data point.

**Analysis of AKT/PKB Expression and Activity**

To assess the expression of endogenous A KT/P KB protein, cells were in-
cubated with either rat Ig or 439-9B as described above in the presence of either D M S O (1:500), a caspase 3 inhibitor (Z-D-E-V-D-F-M-K; Calbiochem-Novabiochem; 4 μg/ml), or a caspase 8 inhibitor (Z-I-E-T-D-F-M-K; Calbiochem-Novabiochem; 4 μg/ml). A fter washing with PBS, the cells were plated in serum-free medium containing 1% BSA in wells of a 12-well plate that had been coated with anti-rat Ig (13.5 μg/ml) and blocked for 1 h at 37°C with 1% BSA-containing medium. A fter a 1-h stimulation, adherent and suspension cells were harvested and extracted with AK T/PKB lysis buffer (20 mM Tris, pH 7.4, 0.14 M NaCl, 1% NP-40, 10% glycerol, 2 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 50 μg/ml leupeptin, 1 mM sodium orthovanadate). A fter removing cellular debris by centrifugation at 12,000 g for 10 min, equivalent amounts of total cell protein from these extracts were resolved by SDS-PAGE (8%) and transferred to nitrocellulose. The blots were probed with a rabbit anti-AKT/PKB antiserum specific for active AKT/PKB, followed by HRP-conjugated goat anti–rabbit IgG. Phospho-AKT/PKB was visualized by enhanced chemiluminescence. These blots were also probed with a rabbit antiserum specific for actin to confirm the loading of equivalent amounts of protein. Relative AK T/PKB and actin expression levels were assessed by densitometry using IP Lab Spectrum software (Scanalytics).

To determine the level of serine 473-phosphorylated AK T/PKB, cells were transfected transiently using the Lipofectamine reagent (G BCO BR L) with an HA-tagged AK T/PKB cDNA (provided by A. Toker, Boston Biomedical Research Institute, Boston, MA). 2 h after transfection, these cells were harvested by trypsinization and subjected to antibody-mediated integrin clustering. Specifically, cells were incubated on ice for 30 min with either control rat IgG or 439-9B at a concentration of 10 μg/ml. A fter washing with PBS, the cells were plated in serum-free medium containing 1% BSA onto wells of a 60-mm tissue culture dish that had been coated at 4°C with anti-rat Ig (13.5 μg/ml) and blocked for 1 h at 37°C in 1% BSA-containing medium. A fter 1 h, adherent and suspension cells were harvested and washed twice with PBS. Proteins from these cells were extracted with AK T/PKB lysis buffer (see above). A fter removing cellular debris by centrifugation at 12,000 g for 10 min at 4°C, equivalent amounts of total cellular protein were precleared with a 1:1 mixture of protein A and protein G-Sepharose for 1 h at 4°C. Immunoprecipitations were performed for 1 h on these precleared lysates using an HA-specific mAb b (1 μg; Boehringer Mannheim) and protein A/protein G-Sepharose beads. Proteins from these immunoprecipitates were subjected to reducing SDS-PAGE (8%), transferred to nitrocellulose, and probed with an AK T/PKB phosphoserine 473-specific rabbit antiserum (New England Biolabs) followed by HRP-conjugated goat anti–rabbit IgG. Phospho-AKT/PKB was detected on these blots by chemiluminescence (Pierce Chemical Co.). These samples were also probed with rabbit anti-AKT/PKB. The relative intensity of phosphoserine AK T/PKB and AK T/PKB bands was assessed by densitometry, as described above.

Analysis of AKT/PKB Proteolysis

Baculovirus-expressed AK T/PKB (0.5 μg; provided by A. Toker) was incubated with either recombiant caspase 8 (2 mg; Calbiochem-Novabiochem) or active recombinant caspase 3 (2 μg; Calbiochem-Novabiochem) for 30 min at 37°C, followed by HRP-conjugated goat anti–rabbit IgG. Phospho-AKT/PKB was detected on these blots by chemiluminescence (Pierce Chemical Co.). These samples were also probed with rabbit anti-AKT/PKB. The relative intensity of phosphoserine AK T/PKB and AK T/PKB bands was assessed by densitometry, as described above.

Results

The α6β4 Integrin Promotes the Survival of p53-deficient, but Not p53 Wild-type Carcinoma Cells

For our initial experiments, we used stable β4 transfectants of two α6β4-deficient carcinoma cell lines that differ in their p53 status: R K O colon carcinoma cells, which express wild-type p53 (Nagawa et al., 1995); and M D A-M B-435 breast carcinoma cells, which express a mutant, inactive form of p53 (Lesoon-Wood et al., 1995). We also used R K O and M D A-M B-435 cells that express a cytoplasmic domain deletion mutant of α6β4 (R K O/β4-Δcyt; M D A/β4-Δcyt) that is signaling deficient. The character-

Figure 1. p53 inhibits α6β4-mediated survival. M D A-M B-435, R K O, and R K O + dpn53 cells that expressed either α6β4 (β4) or α6β4-Δcyt (β4-Δcyt) were plated on poly-L-lysine-coated tissue culture wells and cultured in the absence of serum. A fter 15 h, the cells were harvested, subjected to either A popT ag reactions (A) or annexin V-FITC staining (B), and analyzed by flow cytometry. A survival effect of α6β4 was quantified by subtracting the percentage of α6β4-expressing cells that were positive for either A popTag (A) or annexin V-FITC (B) staining from the percentage of α6β4-Δcyt-expressing cells that were positive for these markers. This value was plotted on the bar graphs shown in A and B, with positive values indicating that the specified β4 clone exhibits increased survival relative to the relevant β4-Δcyt subclone, and negative values indicating an increased apoptosis of the indicated clone relative to the appropriate β4-Δcyt clone. The data in A represent the means (± SE M) from three independent experiments. Similar results to those shown in B were observed in three separate trials.

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

<table>
<thead>
<tr>
<th>Clone</th>
<th>Percent propidium iodide-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA/Mock</td>
<td>21</td>
</tr>
<tr>
<td>β4 Clone 1</td>
<td>13</td>
</tr>
<tr>
<td>β4 Clone 2</td>
<td>9</td>
</tr>
<tr>
<td>RKO/Mock</td>
<td>32</td>
</tr>
<tr>
<td>β4 Clone 1</td>
<td>49</td>
</tr>
<tr>
<td>β4 Clone 2</td>
<td>47</td>
</tr>
</tbody>
</table>

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

ferase end labeling reactions (A poptag) to detect DNA fragmentation (Fig. 1). In addition, we assessed the viability of these serum-deprived cells by measuring the cellular uptake of propidium iodide (Table I). The ability of α6β4 to promote the survival of these cells was determined by subtracting the percent apoptotic α6β4-expressing cells from the percent apoptotic α6β4Δcyt–expressing cells. The expression of α6β4 in MDA-MB-435 cells significantly increased the survival of these cells relative to MDA-MB-435 cells expressing α6β4Δcyt, as assessed by annexin V-FITC staining (Fig. 1), a popTag staining (Fig. 1), and propidium iodide uptake (Table I). In contrast, the expression of α6β4 in RKO cells did not increase the survival of these cells relative to either the mock (Table I) or RKO/β4Δcyt transfecants (Fig. 1). In fact, we observed a higher level of apoptosis and cell death in serum-starved RKO/β4 as compared with RKO/β4Δcyt cells, in agreement with our previous demonstration that α6β4 can promote apoptosis in wild-type p53 carcinoma cells (Bachelder et al., 1999).

Based on the fact that RKO and MDA-MB-435 cells differ in their p53 status, we reasoned that the ability of α6β4 to promote cell survival may be inhibited by p53. This hypothesis was examined by investigating the effect of α6β4 expression on the survival of RKO cells in which p53 activity had been inhibited by the expression of a dnP53 construct. Indeed, α6β4 expression promoted the survival of serum-starved, dpn53-expressing RKO cells as determined by a popTag and annexin V-FITC staining (Fig. 1). These results demonstrate that p53 can suppress the survival signaling mediated by α6β4 in serum-starved carcinoma cells.

α6β4-Mediated Survival in p53-deficient Carcinoma Cells Is Inhibited by Dominant Negative AKT/PKB

Given the importance of the AKT/PKB kinase in numerous survival signaling pathways (Ahmed et al., 1997; Atta et al., 1997; Dudev et al., 1997; Songyang et al., 1997; Blume-Jensen et al., 1998; Crowder and Freeman, 1998; Gerber et al., 1998), we investigated whether the survival function of α6β4 in serum-starved, p53-deficient carcinoma cells was AKT/PKB-dependent. The MDA-MB-435/β4-transfected clones, as well as the parental cells, were cotransfected with plasmids encoding for GFP and an HA-tagged, kinase-deficient AKT/PKB mutant that acts as a dominant negative construct (dnA K T/PKB) (Dudev et al., 1997; Skorski et al., 1997; Eves et al., 1998). Expression of this dnA K T/PKB construct was confirmed by immunoblotting extracts from these transfected cells with an HA-specific mAb (data not shown). After 15 h of serum starvation, the level of apoptosis in GFP-positive cells was assessed by annexin V-PE staining. As shown in Fig. 2, MDA-MB-435/β4 clones demonstrated significantly less apoptosis than parental MDA-MB-435 cells in agreement with the data shown in Table I. Importantly, dnA K T/PKB expression inhibited this α6β4 survival function in each of the two MDA-MB-435/β4 clones examined, but it did not alter the level of apoptosis in parental MDA-MB-435 cells.

p53 Inhibits the Activation of AKT/PKB by α6β4

To understand the mechanism by which p53 inhibits α6β4-mediated survival, we investigated the possibility that p53 alters the ability of this integrin to activate AKT/PKB. Initially, we examined whether the antibody-mediated clustering of α6β4 in MDA-MB-435 cells resulted in the phosphorylation of AKT/PKB on serine 473, an event that has been shown to correlate with AKT/PKB activation (Alessi et al., 1996). MDA-MB-435/β4 subclones were transfected with an HA-tagged AKT/PKB construct. These cells were incubated with either a control rat IgG or the β4-specific antibody 459-9B and plated in the absence of serum on secondary antibody–coated tissue culture wells for 1 h. HA immunoprecipitates were performed on extracts from these cells, and the levels of serine-phosphorylated AKT/PKB were assessed by blotting these immunoprecipitates with an antiserum specific for AKT/PKB molecules phosphorylated on serine residue 473. As shown in Fig. 3A, the antibody-mediated clustering of α6β4 stimulated an increase in the level of serine-phosphorylated AKT/PKB in each of the two MDA-MB-435/β4 subclones relative to control cells (2.1-fold increase, β4 clone 1; 5.5-fold increase, β4 clone 2). This α6β4-induced increase in A K T/
PKB signaling was dependent on α6β4 signaling based on the inability of α6β4-Δcyt clustering to increase the level of the serine 473-phosphorylated A K T/PKB in M D A -β4, A C T/PKB in M D A -β4, A C T/PKB in M D A -β4 subclones (data not shown).

To investigate the influence of p53 on the activation of AKT/PKB by α6β4, we explored whether α6β4 clustering induced the phosphorylation of AKT/PKB on serine residue 473 in M D A -β4 subclones that had been reconstituted with functional p53. Specifically, M D A -β4 subclones were transfected with a temperature-sensitive mutant of AKT/PKB at 37°C (Zhang et al., 1994). This construct has been used extensively to study the influence of p53 on signaling pathways involved in cell growth and apoptosis (Kobayashi et al., 1995; Owen-Schaub et al., 1995). Stable transfectants of these cells were selected, and tsp53 expression was confirmed by immunoblotting (data not shown). Tsp53 and mock-transfected cells were transfected transiently with H A - A C T/PKB. After incubating these cells with either rat IgG or 439-9B, they were plated on secondary antibody-coated wells and subjected to a 32°C incubation to stimulate p53 activity, followed by a 37°C incubation to activate A K T/PKB. HA immunoprecipitations were performed on extracts from these cells, and these immunoprecipitates were subjected to immunoblotting with phosphoserine 473 A K T/PKB-specific rabbit antiserum. A s shown in Fig. 3 B, the clustering of α6β4 significantly increased the level of phosphoserine 473 A K T/PKB in mock-transfected M D A -β4 cells (7.9-fold increase), but not in tsp53-expressing M D A -β4 cells (1.2-fold increase). The importance of p53 in the inhibition of the α6β4-associated activation of A K T/PKB was indicated by the finding that α6β4 clustering increased the level of phosphoserine 473 A K T/PKB in M D A -β4 + tsp53 transfectants that had been incubated at 37°C, the nonpermissive temperature for this tsp53 construct (data not shown).

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

α6β4 Stimulation Induces the Caspase 3-dependent Cleavage of AKT/PKB in a p53-dependent Manner
To define the mechanism by which p53 inhibits the ability of α6β4 to activate A K T/PKB, we investigated whether p53 alters A K T/PKB expression levels in response to α6β4 clustering. RKO-β4 and RKO-β4 + dnp53-expressing cells were incubated with either rat Ig or 439-9B and stimulated from these lysates was assessed by immunoblotting. Immunoprecipitations were performed on extracts from these cells, and these immunoprecipitates were subjected to immunoblotting with phosphoserine 473 A K T/PKB in mock-transfected M D A -β4 cells (7.9-fold increase), but not in tsp53-expressing M D A -β4 cells (1.2-fold increase). The importance of p53 in the inhibition of the α6β4-associated activation of A K T/PKB was indicated by the finding that α6β4 clustering increased the level of phosphoserine 473 A K T/PKB in M D A -β4 + tsp53 transfectants that had been incubated at 37°C, the nonpermissive temperature for this tsp53 construct (data not shown).

Based on the reported ability of caspases to cleave signaling molecules that promote cell survival (Cheng et al.,
Figure 4. Clustering of the α6β4 integrin reduces A K T/P K B protein levels in p53-wild type but not in p53-deficient carcinoma cells. R KO /β4 (A and B) and R KO /β4 + d n p 53 (B) -expressing cells were incubated with either rat Ig or 439-9B and plated on secondary antibody-coated wells for 1 h in the absence of serum. Equivalent amounts of total protein from lysates from these cells were resolved by S D S-P A G E (8%), transferred to nitrocellulose, and probed with an A K T/P K B-specific rabbit antisera (New England Biolabs) followed by H R P-conjugated goat anti-rabbit IgG. These blots were also probed with an actin-specific rabbit antisera (Sigma Chemical Co.) to confirm the loading of equivalent amounts of protein. The A K T/P K B and actin bands were detected by enhanced chemiluminescence, and are indicated by arrows. These bands were quantified by densitometry. α6β4 clustering decreased A K T/P K B levels in R KO /β4 subclones (1.7-fold decrease, β4 clone 1; 1.9-fold decrease, β4 clone 2), but not in R KO /β4 + d n p 53 cells. Similar results were observed in four additional trials.

Figure 5. A caspase 3 inhibitor blocks α6β4-associated reductions in A K T/P K B protein levels. R KO /β4 cells were incubated with either rat Ig or 439-9B in the presence of D M S O (1:500), a caspase 3 inhibitor (Z-D E V D-F MK ; 4 μg/ml), or a caspase 8 inhibitor (Z-I E T D-F MK ; 4 μg/ml). These cells were washed with PBS and plated onto secondary antibody-coated wells in the presence of the same drugs for 1 h in serum-free medium. Equivalent amounts of total protein were resolved by S D S-P A G E (8%), transferred to nitrocellulose, and probed with an A K T/P K B-specific rabbit antisera (New England Biolabs) followed by H R P-conjugated goat anti-rabbit IgG. A K T/P K B was detected by enhanced chemiluminescence and quantified by densitometry. The antibody-mediated clustering of α6β4 decreased the level of A K T/P K B in D M S O-treated cells (2.0-fold decrease, β4 clone 1; 1.9-fold decrease, β4 clone 2), as well as in cells pretreated with a caspase 8 inhibitor (1.9-fold decrease). In contrast, the pretreatment of these cells with a caspase 3 inhibitor partially restored A K T/P K B levels in R KO /β4 cells subjected to α6β4 clustering (1.1-fold decrease, β4 clone 1; 1.1-fold decrease, β4 clone 2). By probing these blots with an actin-specific rabbit antisera (Sigma Chemical Co.), we confirmed that equivalent amounts of actin were present in each lane (data not shown). Similar results were observed in three experiments.
Although previous studies have demonstrated that cas-
signaling, similar to the phosphatase activities of PTEN.

Our results suggest that the α6β4 integrin suppresses anoikis exclusively in carcinoma cells that lack functional p53. Furthermore, we demonstrate that this α6β4-associated survival function depends on the ability of this integrin to activate the serine/threonine kinase AKT/PKB in p53-deficient cells. Finally, we provide evidence that p53 inhibits the α6β4-mediated activation of AKT/PKB by promoting the caspase 3-dependent cleavage of this kinase. Collectively, our findings establish that p53 can inhibit an integrin-associated survival function, a phenomenon that has important implications for tumor cell growth.

Our results suggest that the α6β4 integrin can enhance the survival of carcinoma cells in an AKT/PKB-dependent manner. Although previous studies have shown that cell attachment to matrix proteins promotes the survival of primary epithelial cells (Khwaja et al., 1997; Farrelly et al., 1999), α6β4 is the first specific integrin to be implicated in the delivery of a DN AkT/PKB construct containing inactivating mutations in the catalytic domain to inhibit the survival effect of α6β4 in serum-starved MDAMB-435 cells. Although this DNA AKT/PKB has been used extensively to implicate AKT/PKB in survival pathways, it is possible that it associates with phosphoinositide-dependent kinases and inhibits their activity. However, our observation that the expression of a constitutively active AKT/PKB in MDAMB-435 enhances their survival (data not shown) strongly suggests that α6β4 expression promotes the survival of these cells by activating AKT/PKB.

Our demonstration that p53 can inhibit AKT/PKB kinase activity is of interest in light of the recent finding that the PTEN tumor suppressor can also inhibit cell growth by inhibiting AKT/PKB in a manner that is dependent on its lipid phosphatase activity (Miers et al., 1998; Stambolic et al., 1998; Davies et al., 1999; Ramaswamy et al., 1999; Sun et al., 1999). Together, our current findings on p53 and the previously described activities of PTEN highlight the impact of tumor suppressors on integrin-mediated functions. Moreover, our demonstration that p53 inhibits α6β4 survival signaling by promoting the caspase-dependent cleavage of AKT/PKB provides a mechanistic link between tumor suppressor function and the regulation of integrin signaling, similar to the phosphatase activities of PTEN. Although previous studies have demonstrated that caspases can be activated by p53 in both cell-free systems (Ding et al., 1998) as well as in response to DNA damage (Fuchs et al., 1997; Yu and Little, 1998), our findings suggest that caspases can also be activated by an integrin in a p53-dependent manner. Indeed, it will be informative to determine if other activators of p53 such as DNA damage (Siegel et al., 1995; Komarova et al., 1997) can promote the caspase-dependent cleavage of AKT/PKB.

The finding that AKT/PKB activity can be regulated by caspase 3 substantiates the hypothesis that caspases play an important role in many forms of apoptosis based on their ability to cleave signaling molecules that influence cell survival. For example, caspases have been shown to cleave and inactivate an inhibitor of caspase-activated deoxyribonuclease (CAD). Importantly, the cleavage of this inhibitor results in the activation of CAD, which is the enzyme responsible for the DNA fragmentation that is characteristic of apoptosis (Enari et al., 1998; Sakahira et al., 1998). Caspase 3 has also been shown to cleave bcl-2, resulting in an inhibition of its anti-apoptotic function (Cheng et al., 1997). While AKT/PKB has been suggested to be a target of caspase activity based on the reduced levels of this kinase observed in T cells in response to fas stimulation (Widmann et al., 1998), our results extend this finding by establishing definitively that AKT/PKB is cleaved by caspase 3. More importantly, we provide evidence that this cleavage event results in the inhibition of AKT/PKB kinase activity, and implicate this event in the inhibition of α6β4 integrin survival function.

It is important to consider the mechanism by which the α6β4-induced, caspase-dependent cleavage of AKT/PKB inhibits its kinase activity. We detected an AKT/PKB fragment (M, 49 kD) after the in vitro incubation of AKT/PKB.
PKB with recombinant caspase 3. This fragment was recognized by a rabbit antiserum raised against a peptide corresponding to the extreme carboxy-terminal amino acids of the molecule, suggesting that caspase 3 cleaves AKT/PKB at its amino terminus. Interestingly, the pleckstrin homology domain, which resides in the amino terminus of AKT/PKB, is important in both the translocation of this kinase to the membrane and its subsequent activation (Franke et al., 1995; A njelkovic et al., 1997). It is possible that the caspase 3–dependent cleavage of AKT/PKB prevents the membrane translocation of this kinase, thus, preventing its activation. However, we were unable to identify an AKT/PKB fragment in vivo after the clustering of α6β4, despite our detection of reduced AKT/PKB levels under these conditions. This result suggests that after the initial cleavage of AKT/PKB by caspase 3, this kinase is subjected to further cleavage by other caspases, as has been shown for ICAD (Tang and Kidd, 1998). Moreover, our inability to detect AKT/PKB fragments in vivo after the clustering of α6β4 suggests that AKT/PKB cannot be detected by immunoblotting after its cleavage by multiple caspases. The ability of a caspase 3 inhibitor to restore PKB activity in these studies may relate to the kinetics of AKT/PKB activation. Specifically, the rapid stimulation of AKT/PKB may impede p53 or caspase activation. In contrast, the ability of α6β4 clustering to promote the caspase 3–dependent inactivation of AKT/PKB in p53 wild-type carcinoma cells may relate to the fact that α6β4 signaling stimulates caspase activity before AKT/PKB activity in these cells. Alternatively, it is possible that the ability of caspase 3 to cleave AKT/PKB was not observed in previous studies because insufficient amounts of endogenous caspase activity were present to inhibit the activity of exogenously introduced, active AKT/PKB. Nonetheless, these results suggest that an intimate crosstalk exists between AKT/PKB and caspases that contributes to the regulation of cell survival.

We have previously demonstrated that the α6β4 integrin activates p53 function (Bacheler et al., 1999). The current studies describe an important consequence of this α6β4 activity, namely the inhibition of AKT/PKB activity and its associated cell survival function. Similar to previous results from our laboratory (Clarke et al., 1995; Shaw et al., 1997; O’Connor et al., 1998) and others (Kim et al., 1997; Sun et al., 1998), the current studies demonstrate that the survival function of α6β4 is ligand-independent in β4-transfected, p53-deficient carcinoma cells. This ligand-independent survival function may be attributable to the ability of the β4 cytoplasmic domain to self-associate (Rezniczek et al., 1998).

In addition to demonstrating that p53 inhibits α6β4-mediated survival, we observed that α6β4 increases the level of apoptosis observed in serum-starved p53 wild-type carcinoma cells. This result suggests that the apoptotic signaling pathway activated by α6β4 can augment the apoptotic signaling initiated by serum deprivation. Although p53 has been implicated in the apoptosis induced in endothelial cells upon their detachment from matrix (Ilic et al., 1998), others have reported that epithelial cell anoikis is p53-independent (Boudreau et al., 1995). In agreement with the results of the latter study, we observed apoptosis in p53-deficient cells, including MDA-MB-435 cells and dnp53-expressing RKO cells, upon their detachment from matrix. These results indicate that carcinoma cells are subject to a p53-independent form of anoikis. In combination with our previous observation that α6β4 apoptotic signaling requires p53 activity (Bacheler et al., 1999), our findings suggest that the p53-independent apoptosis of carcinoma cells that occurs in response to matrix detachment can be enhanced by p53-dependent, α6β4 apoptotic signaling.

The current studies may explain why the α6β4 integrin has been implicated in the apoptosis of some cells and the survival of others. Specifically, α6β4 has been shown to induce growth arrest and apoptosis in several carcinoma cell lines (Clarke et al., 1995; Kim et al., 1997, Sun et al., 1998) as well as in endothelial cells (Miao et al., 1997). However, this integrin has also been shown to promote the proliferation (Mainiero et al., 1997; Murgia et al., 1998) and survival (Dowling et al., 1996) of keratinocytes. These apparently contradictory functions of α6β4 may relate to the fact that the functions of α6β4 are cell type–specific. The current studies establish that the p53 tumor suppressor is one critical signaling molecule that may influence α6β4 function in different cell types because this integrin promotes apoptosis only in wild-type p53–expressing cells and survival only in p53-deficient cells. Interestingly, the reported ability of α6β4 to promote keratinocyte survival (Dowling et al., 1996) may relate to the reported deficiency of p53 activity in these cells (Nigro et al., 1997).

One implication of our findings is that the α6β4 integrin is similar to a number of oncogenes that promote cell proliferation in some settings and cell death in others. The recent observation that oncogenes can deliver such death signals has led to their seemingly contradictory categorization as tumor suppressors in select environments. For example, although the stimulation of c-myc and E2F normally promotes cell proliferation, the activation of these oncogenes induces apoptosis in the presence of secondary stress signals such as p53 expression, serum starvation or hypoxia (E van et al., 1992; Shi et al., 1992, Hermeking and Eick, 1994; Qin et al., 1994, Wu and Levine, 1994). The ability of these stress signals to stimulate oncogene–dependent apoptosis is thought to be important in eliminating tumor cells that escape normal proliferation checkpoints as a result of oncogene expression. Similarly, the α6β4 integrin, which promotes the survival of p53-deficient cells, could also be classified loosely as a tumor suppressor based on its apoptotic function in carcinoma cells that express wild-type p53. The current studies demonstrate that, similar to the activity of oncogenes, integrin function and signaling can be profoundly influenced by physiological stimuli that activate other signaling pathways in a cell.

In summary, we have described the ability of the α6β4...
integrin to promote the survival of the p53 mutant, but not p53 wild-type carcinoma cells. This ability of p53 to influence integrin-mediated functions so markedly derives from its ability to activate the caspase 3-dependent cleavage of AKT/PKB. The fact that AKT/PKB overexpression has been suggested to contribute to the transformed phenotype of tumor cells (Bellacosa et al., 1995) suggests that the introduction of the α6β4 integrin into p53 wild-type tumors may inhibit their growth by inducing the cleavage of this transforming protein. The ability of α6β4 to induce the p53-dependent cleavage of AKT/PKB also suggests that the acquisition of inactivating mutations in either p53 or caspase 3 will provide a selective growth advantage for carcinoma cells by stimulating α6β4-mediated AKT/PKB-dependent survival signaling. Moreover, given our previous demonstration that α6β4 promotes carcinoma cell migration and invasion (Chao et al., 1996, Shaw et al., 1997; O'Connor et al., 1998), we suggest that carcinoma cells that express α6β4 and mutant forms of p53 or caspase 3 will have a distinct advantage in their ability to disseminate and survive as metastatic lesions.

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