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The β4 Integrin Subunit Rescues A431 Cells from Apoptosis through a PI3K/Akt Kinase Signaling Pathway

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To study whether α6β4 integrin regulates apoptosis, human A431 cells were plated on bacteria plates in the presence or absence of mAb β4. In the absence of mAb β4, A431 cells demonstrated morphological characteristics of apoptosis by 24 h and most cells died by 48 h. In contrast, in the presence of mAb β4, cells remained viable, and at the end of 48 h, 70–80% of cells survived. Treatment of A431 cells with mAb β4 resulted in tyrosine phosphorylation of the p85 subunit of PI3 kinase; PI3 kinase activity increased within 15 min and peaked at 60 min. Stimulation of β4 in A431 cells resulted in a time-dependent phosphorylation of Akt with a concomitant and parallel phosphorylation of Bad. Inactivation of PI3 kinase with inhibitors blocked the anti-apoptotic effect induced by mAb β4. These are the first results to suggest that ligation of α6β4 integrin protects cells from apoptosis through a PI3K/Akt kinase signaling pathway.

Key Words: **A431 cell; apoptosis; integrin β4; PI3K; PKB/Akt.**

The integrin subunit β_4 associates with α_6 to form a multivalent laminin receptor (1, 2). High expression of β_4 is found in most epithelia, in Schwann cells, and in several tumors of epithelial origin (21). The sequence of the β_4 cytoplasmic domain is 1045 amino acids long and it is not homologous to any other β subunits (3). This large and structurally unique cytoplasmic domain of the β_4 integrin subunit associates with cytoskeletal elements and signaling molecules and such associations provide the basis for the distinct functions associated with $\alpha_6\beta_4$ (3). In squamous and transitional epithelia, β_4 is highly enriched in hemidesmosomes, providing firm mechanical links between the basal lamina and the intermediate filament cytoskeleton (22). Loss of function of β_4 both in human genetic diseases and in gene knockout mice results in hemidesmosome disruption, blistering, and is usually lethal perinatally (4). In addition to its structural role in hemidesmosomes, $\alpha_6\beta_4$ is involved in the adhesion and migration of carcinoma cells (2), mediating cell interaction with laminin matrices. Expression and redistribution of integrin $\alpha_6\beta_4$ have been correlated with the progression and metastatic potential of several different tumors (5, 24). It has been implicated that $\alpha_6\beta_4$ integrin plays a key role in carcinoma migration and invasion through its ability to interact with the actin cytoskeleton and mediate the formation and stabilization of lamellae (15). This dynamic function of $\alpha_6\beta_4$ in cell migration and invasion involves its preferential activation of a phosphoinositide 3-OH kinase (PI3-K)/Rac signaling pathway (17) and concomitant suppression of the intracellular cAMP concentration by activation of a cAMP-specific phosphodiesterase (16). Although the involvement of $\alpha_6\beta_4$ integrin in the migration and invasion of carcinoma cells has been well documented, its role in other cellular responses related to tumor progression are relatively unknown and controversial as in the case of apoptosis (19, 23). In this study, we report that antibody ligation of β_4 prevented human epidermoid carcinoma A431 cells from undergoing apoptosis, activated PI3 kinase and protein kinase B/Akt, and subsequently phosphorylated the proapoptotic protein Bad. Our data illustrate a novel function of β_4 integrin in promoting cell survival through the PI3-K/Akt signaling pathway.

Abbreviations used: PI3K, phosphatidylinositide 3-OH kinase; LY 294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PKB/Akt, protein kinase B; A431 cell, human epidermoid carcinoma; PtdIns, phosphatidylinositol; FAK, focal adhesion kinase; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Antibodies and reagents. Antibodies to p85α of PI3-kinase and the anti-phosphotyrosine Ab PY20 were from Transduction Lab (Lexington, KY). Antibodies against phospho-Akt and phospho-Bad were from New England Biolabs, Inc. (Beverly, MA). A human β4 integrin-specific mAb 3E1 and a anti-human β1 P4C10 mAb were from GIBCO BRL (Gaithersburg, MD). Antibody to human integrin α6 CD49f mAb was from PHARMINGIN (San Diego, CA). TLC plates

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were from EM SCIENCE (Gibbstown, NJ). Phosphatidylinositol was from Sigma (St. Louis, MO).

Cell culture. Human epidermoid carcinoma A431 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured (at 37°C, 5% CO_2) in DMEM containing 4.5 g of glucose, 10% FCS, streptomycin (100 μg/ml), and penicillin (100 units/ml) (Gibco, Grand Island, NY). A431 cells were passaged with 0.68 mM EDTA and 0.25% trypsin in RPMI.

Characterization of apoptosis by light microscopy. Cells cultured in 10 cm bacterial dishes or tissue culture dishes were treated with the β4 mAb. Cell morphology was observed using a Nikon Diaphot inverted microscope. Photomicrographs were taken using TMAX $\times$400 black and white films. Apoptotic cells were determined on the basis of their typical morphology, i.e., shrunken cell body, membrane blebbing, and appearance of apoptotic bodies.

DNA fragmentation assay. 2.5×10^6 cells were grown in 10 cm untreated bacterial dishes in DMEM medium without FCS in the absence or presence of a mAb to integrin β4 for 48 h. Subsequently, cells were harvested by rubber policeman and fragmented DNA extracted with 200 μl of the lysis buffer for 5 min. Samples were centrifuged at 500*g* for 5 min and the resultant supernatants transferred to a clean set of eppendorf tubes where the pellets were redissolved in 200 μl lysis buffer and extracted for 2 min. Samples were recentrifuged and the resultant supernatants combined with the previous supernatants. Subsequently, SDS and DNase-free RNase (Ambion, Austin, TX) were added to the pooled supernatants to final concentrations of 1% and 5 mg/ml, respectively, and samples incubated at 56°C for 2 h. At the end of RNase treatment, proteinase K (2.5 mg/ml, Ambion, Austin, TX) was added and samples further incubated for 2 h at 37°C. Samples were then extracted once with alkaline phenol/chloroform/isoamyl alcohol (25:24:1) and DNA precipitated with 0.3 M NaAc (pH 5.2) and ethanol. DNA from equal number of cells (typically, 20 μg) was run on a 1.2% agarose gel and the DNA ladder formation visualized by ethidium bromide staining (6).

Trypan blue dye exclusion assay. 2.5×10^6 tumor cells were plated into 10 cm bacterial dishes and cultured in DMEM without 10% FCS in the absence or presence of mAb β4. At 48 h, dead cells were gently removed and surviving cells counted using the trypan blue dye exclusion assay. Trypan blue-stained cells and those cells with typical apoptotic morphology (i.e., membrane blebbing) were excluded from counting. Cell survival was expressed as the percentage of the control (i.e., cells grown in tissue culture dishes). Each condition was run in triplicate or quadruplicate and the experiments repeated twice.

Immunoprecipitation. Cells were lysed in a cold lysis buffer consisting of 1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8.0, 0.2 mM sodium ortho-vanadate, 0.2 mM PMSF, 0.5% NP-40. The lysate was clarified by centrifugation at 10,000 g for 10 min. The supernatants were immunoprecipitated with 1–2 μg antibody against PI3-Kinase (p85α subunit) or the anti-phosphotyrosine Ab PY20 for 2 h, followed by 40 μl Sepharose 4B-conjugated protein G at 4°C overnight. Immune complexes were washed three times in the lysis buffer, and the pellets were either suspended in SDS sample buffer for SDS-PAGE electrophoresis or washed further for kinase activity assay.

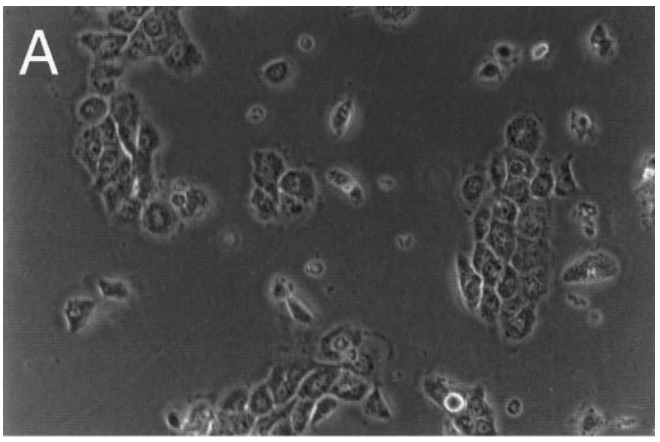
Western blotting. Aliquots of total cell lysate were mixed with 1 vol. of SDS sample buffer (85 mM Tris-HCl, pH 6.8, containing 1.4 (w/v) SDS, 14% (v/v) glycerol, 5% (v/v) mercaptoethanol and a trace of bromophenol blue, boiled for 5 min, and subjected to SDS-PAGE on 8% acrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes. After transfer, nonspecific sites were blocked with 5% (w/v) nonfat-dry milk in TTBS (0.1% Tween-20, 20 mM Tris base, 137 mM NaCl, 3.8 mM HCl, pH 7.6) for 2 h at 25°C followed by probing with primary antibody. After washing the blot three times in TTBS, the membranes were incubated for 1 h at 25°C with horseradish peroxidase-conjugated secondary antibodies (dilution: 1:4500. Amersham, Arlington Heights, IL). After the blot was washed again in TTBS, then developed using ECL according to the manufacturer's instruction (Amersham, Arlington Heights, IL).

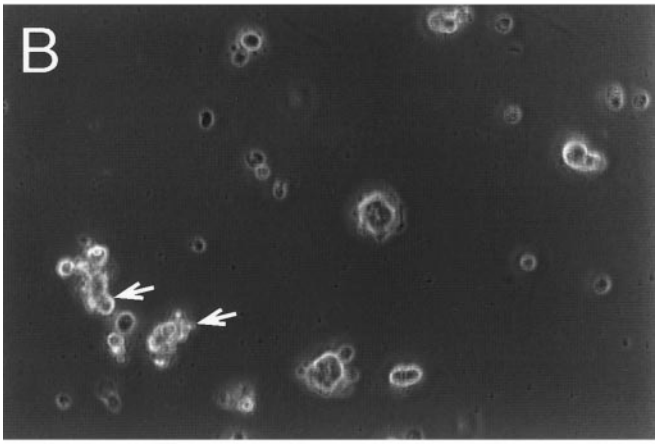
In vitro PI3 kinase assay. Immunoprecipitated PI3K (as described the above) was washed three times in lysis buffer and the activity of PI3K measured as described (7). PI3-kinase assay was performed using phosphatidylinositol (PtdIns) as substrate in a final volume of 50 μl containing: 20 mM Tris/HCl pH 7.5, 100 mM NaCl, 0.5 mM EGTA, 0.5 mg/ml sonicated PtdIns, 20 mM $MgCl_2$, 10 μCi [γ-^{32}P]ATP and 50 μM ATP. Reactions were carried out for 10 min at room temperature and terminated by the addition of 150 μl chloroform/methanol/concentrated HCl (100:200:2) and the lipids extracted after addition of 100 μl chloroform. The organic phase was washed in methanol/1 M HCl (1:1), and analyzed by ascending chromatography on silica gel thin-layer plates in chloroform/methanol/25% ammonium hydroxide/water (90:90:9:19) followed by autoradiography. Radioactivity was quantified by liquid scintillation counting.

RESULTS

β4 ligation rescues A431 cells from apoptosis. A431 cells are normally cultured in tissue culture flasks or dishes. When cultured on non-adhesive bacterial plates, most cells undergo apoptosis (23). When A431 cells were cultured on bacterial plates with and without serum we observed apoptosis under either condition, however cells cultured without serum were more sensitive. For example, in the absence of serum 100% of cells were dead, predominantly by apoptosis, in 48 h whereas in the presence of serum 90% of cells were apoptotic with the remaining 10% attached and exhibiting morphological evidence of mitosis. This system was used as a model to study the role of integrin β4 on A431 cell survival. When A431 cells were plated onto untreated bacterial plates in DMEM without serum, they demonstrated morphological changes characteristic of apoptosis i.e. cell rounding, shrinkage, membrane blebbing and formation of apoptotic bodies (Fig. 1A–1B) by 24 h. By 48 h more than 90% of cells were dead. In contrast, under the same conditions but in the presence of a mAb directed against the β4 integrin subunit, cells attached to substrate and at the end of 48 h, 76% of cells survived (Fig. 4C). In contrast, normal mouse IgG used as an antibody control, had no effect on A431 cell survival, nor did α6 antibody and unrelated β integrin subunit β1 antibody (data not shown). In addition, cells treated with mAb β4 showed less evidence of oligonucleosomal DNA fragmentation (Fig. 1C). These results suggest that β4 ligation rescues A431 cells from apoptosis.

β4 ligation activates PI3 kinase in A431 cells. Since integrin α6β4 was reported to activate PI3K during the promotion of carcinoma invasion, we questioned whether β4 prohibits A431 cells from apoptosis in our model through its ability to activate PI3K/Akt. To understand the mechanism of β4 promotion of A431 cell survival, we cultured cells in tissue culture dishes to test whether mAb β4 can stimulate PI3K activity.





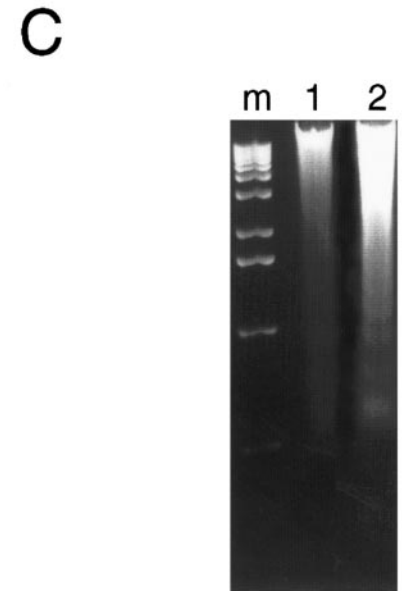


FIG. 1. β4 ligation rescues A431 cells from apoptosis. (A, B) Phase-contrast photomicrographs of A431 cells cultured on untreated bacterial plates in DMEM medium with (A) or without (B) integrin β4 mAb for 24 h. Original magnification 300×. (A) Viable, spread, and dividing A431 cells. (B) A431 cells showing evidence of cell rounding, shrinkage, membrane ruffling, and formation of apoptotic bodies (arrows). (C) DNA fragmentation in A431 cells after being cultured on bacterial plates in DMEM without serum for 48 h. m, molecular weight marker; 1, cells treated with mAb β4 (5 μg/ml); 2, without mAb β4.

A431 cell were serum starved overnight, followed by stimulation with mAb β4 for various time intervals. Activity of PI3K in these cells was assessed by immunoprecipitation of endogenous enzyme and in vitro kinase assay using PtdIns as a substrate. As can be seen in Fig. 2, mAb β4 (3E1 clone) stimulates tyrosine phosphorylation of the p85 subunit of PI3K in time-dependent manner with a peak response at 60 min. By laser densitometer scanning, the reading for each band was 0.70, 0.72, 0.88, 1.38, 1.57, 0.85 at control, 5, 15, 30, 60, 90 min respectively. The results of PI3K phosphorylation were repeated with either immunoprecipitation using anti-p85 antibody followed by probing with a general anti-tyrosine phosphorylation antibody PY20 (Fig. 2A) or vice versa (data not shown). To further confirm if β4 can stimulate PI3K activity, in vitro kinase assays were performed. After ligation of the β4 subunit with mAb β4, extracts were immunoprecipitated with a phosphotyrosine-specific antibody to capture the activated population of PI3K, and those immunoprecipitates were assayed for their ability to phosphorylate phosphatidylinositol. As shown in Fig. 2B, PI3K activity increased in time-dependent manner, as indicated by the appearance of PtdIns-3-P upon mAb stimulation. The amount of radiolabeled PtdIns-3-P were quantitated for each treatment by liquid scintillation counting (Fig. 2C). The results were consistent with the immunoprecipitation assay. These data suggest that β4 ligation increases PI3K activity in A431 cells. Several studies demonstrated that integrin activation of PI3K is mediated by the focal adhesion kinase (FAK) (20). Interestingly, in our study we observed no change in the phosphorylation of FAK following mAb β4 ligation in A431 cells (data not shown).

Akt and Bad phosphorylation by β4 ligation. One target of PI3K is the serine-threonine kinase Akt, also known as PKB (protein kinase B) and RAC-PK (related to A and C protein kinase) (8). To test whether Akt is activated by PI3K in A431 cells after β4 ligation, we tested for the level of phosphorylated Akt by Western Blotting (Fig. 3A). After mAb β4 stimulation, the level of Akt phosphorylation was elevated in a time-dependent manner (maximum at 60 min), which coincided with the increased PI3K activity. The density of each band was scanned by the laser densitometer. The reading for each band was 0.64, 0.65, 1.58, 1.74, 2.0, 0.86 at control, 5, 15, 30, 60, 90 min respectively. As shown, the intensity of Akt and PI3K phosphorylation were not exactly comparable at all time intervals, but both were initiated at 15 min and reached a maximum at 60 min treatment. There may exist several possibil-

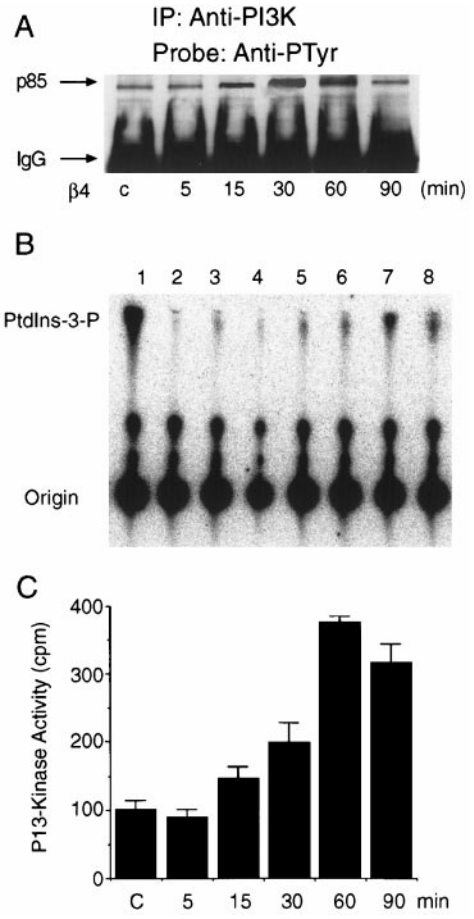


FIG. 2. β4 ligation activates PI3 kinase in A431 cells. Immunoprecipitation assay analysis of the phosphorylation of PI3 kinase p85 subunit. Cells were serum-starved overnight and then treated with β4 mAb at the indicated times. (A) PI3 kinase was immunoprecipitated from cell lysates using an anti-p85 antibody and detected with an anti-phosphotyrosine Ab PY20. (B, C) PI3 kinase activity assay. PI3 kinase was immunoprecipitated from cells lysates using an anti-phosphotyrosine antibody. PI3 kinase activity was assayed (as described under Materials and Methods) using phosphatidylinositol as substrate. 3-Phosphorylated lipids were resolved using thin-layer chromatography (TLC), identified by autoradiography (B), and quantified by liquid scintillation counting (C). The data shown are the mean values ($\pm$SEM) from three experiments. Lanes: 1, total cell lysate positive control; 2, protein-G beads control; 3, control, without treatment of mAb β4; 4, treatment with mAb β4 for 5 min; 5, for 15 min; 6, for 30 min; 7, 60 min; 8, 90 min.

ities for this difference in phosphorylation intensity, such as the difference of sensitivity between the methods used to detect each (i.e. IP and WB) or the fact that Akt could be phosphorylated by a combination of PI3 kinase and other kinases activated by the products of PI3 kinase (8). One mechanism by which Akt may promote survival is through the inhibition of a component (i.e. Bad) of the cell death machinery (9). Therefore we next determined whether Bad phosphorylation was regulated by mAb β4 stimulation. Under serum-starved conditions, cells were treated with mAb β4 over various time intervals and Bad detected with anti-phospho-Bad by Western blotting (Fig. 3B). The ratio of the upper and lower bands, representing phospho- and non-phospho-Bad, was altered at the indicated times (Fig. 3B). Within 60 min treatment, the ratios were increased, but at 90 min they decreased. These results demonstrated that the level of phosphorylation of Bad after mAb β4 ligation were comparable with Akt activity at the different time points. Taken together, these results suggest that Akt and Bad are phosphorylated by β4 ligation.

Inhibition of β4-promoted survival by PI3 kinase inhibitors. To test whether β4-ligation rescues A431 cells from apoptosis by phosphorylation of Bad through the PI3K/Akt signaling pathway, we pretreated A431 cells with a specific inhibitor of PI3K, LY294002 (Fig. 4A–B) or wortmannin (data not shown) for 30 min. These inhibitors completely eliminated the mAb β4-dependent increase in Akt/Bad phosphorylation. We next examined the inhibitor effects on cell survival. A431 cells were

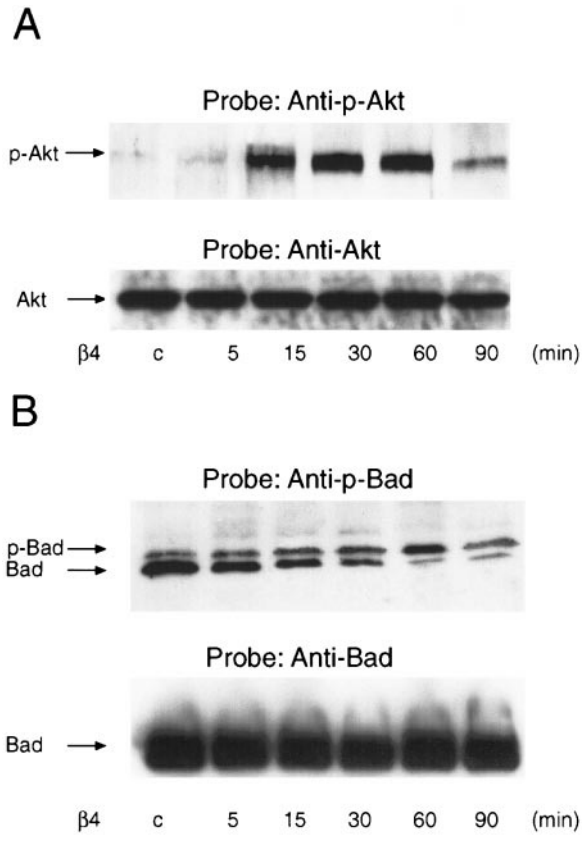


FIG. 3. Akt and Bad phosphorylation by β4 ligation. Western blotting analysis of phosphorylated Akt and Bad when cells were stimulated by mAb β4 at the indicated times. Cells were serum-starved overnight and then treated with mAb β4. After treatment, cells lysates were probed with an anti-phospho-Akt antibody (A, upper panel) and anti-Akt antibody (A, low panel), and probed with an anti-phospho-Bad antibody (B, upper panel) and anti-Bad antibody (B, low panel).

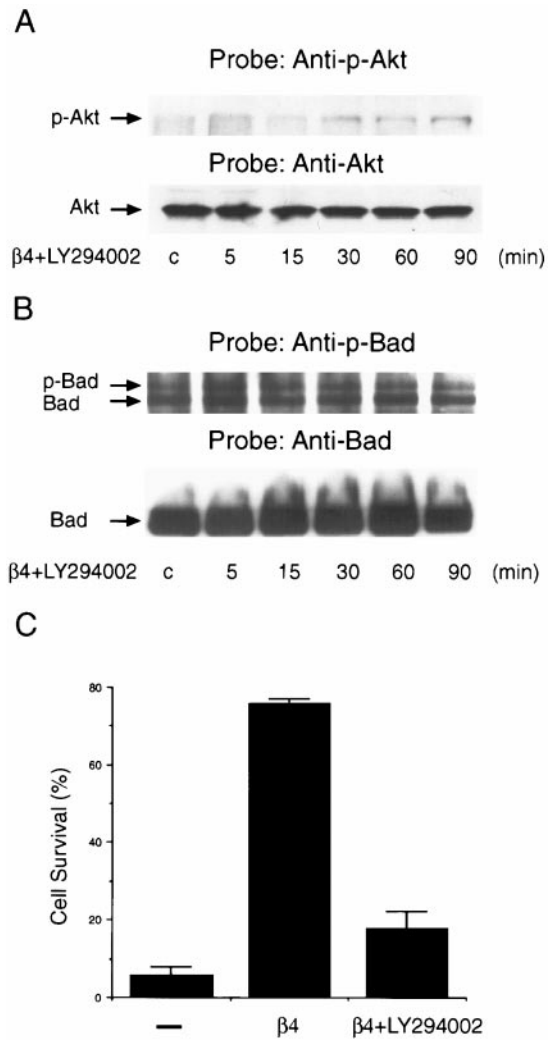


FIG. 4. Inhibition of β4-promoted survival by the PI3 kinase inhibitor LY294002. (A) Cells were treated with mAb β4 in the presence of the PI3 kinase inhibitor LY294002 (20 μM), and then cell lysates were probed with antibodies to phosphorylated Akt (A, upper panel) and Bad (B, upper panel). After the blots were stripped, they were reprobed with anti-Akt or anti-Bad antibodies as a loading control (A/B, lower panel). (C) Cells were placed onto untreated bacterial plates and tissue culture plates in DMEM without FCS in the presence or absence of LY294002 (20 μM) and mAb β4. After 48 h, cell survival as determined by trypan blue dye-exclusion assay. Survival on tissue culture plates without treatment is defined as 100% for comparison. LY294002 inhibited the promotion of survival by β4 ligation. Data are from three experiments; error bars indicate SEM.

placed on untreated bacterial plates as described above, treated with mAb β4 plus LY294002, mAb β4 or vehicle (Fig. 4C). Cell survival was increased by 76% by the addition of mAb β4 into the seeding media, however cell survival was dramatically reduced to 17% after addition of LY294002 plus mAb β4. These data suggest that the inhibitor of PI3K prohibited β4-promoted cell survival. In the absence of treatment or in the presence of normal mouse IgG control (data not shown), cells underwent rapid apoptosis.

DISCUSSION

In this study we found that ligation of integrin β4 can promote survival of A431 cells by inhibiting apoptosis. Ligation of β4 stimulates PI3 kinase activity and via this pathway activates the downstream signaling molecule PKB/Akt, which phosphorylates Bad, an apoptosis-promoting member of the family of Bcl-2 proteins. Our data suggest that β4 is linked to a PI3K/Akt signaling pathway to deliver an anti-apoptotic signal.

Accumulated evidence indicates that the β4 integrin has an important role in tumorigenesis and tumor progression. β4 integrin expression is altered during pulmonary, pancreatic, and uterine cervical carcinogenesis (12, 13). An example of altered expression is in thyroid carcinomas. Normal thyroid follicular cells do not express β4-integrin at all, but thyroid follicular carcinoma cells express a high level of this integrin (10). Enhanced β4 integrin expression also is associated with the early recurrence of squamous cell carcinoma (11). The level of β4 integrin expression has been shown to correlate with the degree of invasiveness of colon carcinoma and with high metastatic potential of lung cancer and melanoma. Colon carcinoma cells transfected with β4 integrin have an increased ability to invade laminin matrices (12, 13). The signaling mechanism(s) involved are not completely understood although it has been shown that the β4 integrin regulates keratinocyte proliferation through the Ras-mitogen-activated protein kinase signaling pathway (14). The enhanced cell migration resulting from formation and stabilization of actin-containing structures may account for the aggressive phenotype of β4-integrin expressing cancers. Release of cAMP gating by the β4 integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells (16). The β4 integrin promotes carcinoma invasion by its activation of a PI3-K/Rac signaling pathway (17). Here we present evidence that β4 integrin rescues A431 tumor cells from apoptosis through PI3K/Akt anti-apoptotic signaling pathway. Our results indicate that engagement of β4 integrin may contribute to tumorigenesis and progression via the suppression of tumor cell apoptosis. The study underscores the concept that tumor progression is not only a function of cell proliferation but may also result from changes in cell survival due to aberrant suppression of apoptosis.

The consequences of activation of PI3 kinase by stimulation of β4 integrin are dependent upon the downstream effectors of PI3 kinase. Previous studies have shown that PI3K provides an anti-apoptotic signal in hematopoetic cells as well as in other types of cells. Further studies demonstrated that PKB/Akt, a downstream target of PI3K, is a key serine/threonine kinase required for inhibition of apoptosis (18). The PI3K/Akt signaling pathway is activated by a variety of cytokines and growth factors, but this activation does not solely deliver an anti-apoptotic signal as demonstrated by the

reported increase in carcinoma cell invasion (17). Akt is a general mediator of growth factor-induced survival and has been shown to suppress the apoptotic death of a number of cell types induced by a variety of stimuli, including growth factor withdrawal, cell-cycle discordance, loss of cell adhesion, and DNA damage (9). Phosphorylation of Akt on Thr-308 and Ser-473 is required for its full activation. Among molecules central to the regulation of cell death in eukaryotes are members of the BCL-2 family of proteins. It has been shown that Akt phosphorylation of Bad couples survival signals to the intrinsic cell death machinery. Phosphorylation of Bad has been correlated with the binding of Bad to the 14-3-3 protein, which may sequester Bad from BCL-XL/BCL-2, thus promoting cell survival. In addition to PI3K/Akt pathway, focal adhesion kinase (FAK) is an important protein tyrosine kinase which mediates several integrin signaling pathways. FAK interacts with a number of signaling and cytoskeletal proteins, including Src, phosphatidylinositol 3-kinase, Grb2, p130cas and paxilin. Focal adhesion kinase activated by integrins can suppress apoptosis (20). This apoptosis-suppressing effect of FAK may be mediated through PI3 kinase and the Akt oncoprotein (20). However in this study, we did not observe FAK activation after β4 ligation, suggesting that PI3 kinase activation is not mediated through the FAK signaling pathway. If FAK is not involved then presently it is not known how the β4 integrin molecule is linked to an upstream modulator of PI3 kinase. Studies are ongoing to identify the upstream activators of PI3 kinase which are triggered by β4 ligation.

It has been shown that overexpression of the cytoplasmic domain of β4 integrin can induce apoptosis in RKO cells by the activation of the p21 (WAF/Cip1) pathway (19). We could not demonstrate significant alteration of expression of p21 in A431 cells following β4 ligation (unpublished observation). Therefore, in this study, we report that β4 rescues A431 cells from apoptosis through a PI3 kinase/Akt anti-apoptotic pathway. In addition, integrins may regulate the expression of Bcl-2 proteins, however in this study the Bcl-2 protein level was unchanged. Finally, treatment of A431 cells with an antibody directed to the α6 subunit did not rescue cells from apoptosis and did not activate the PI3 kinase/Akt pathway (unpublished observation) suggesting that only the β4 subunit participates in the antiapoptotic effect. This result is in agreement with other studies of the β4 integrin preventing vascular endothelial cells from apoptosis and stimulating cell proliferation (23, 25, 11, 14).

In summary, we present evidence that β4 ligation prevents A431 cells from entering the apoptotic pathway through the PI3K/Akt anti-apoptotic signaling pathway. Our findings may help to explain the complex role of the β4 integrin in tumorigenesis.

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