

Parathyroid Hormone-Related Protein Regulates Cell Survival Pathways via Integrin $\alpha_6\beta_4$ -Mediated Activation of Phosphatidylinositol 3-Kinase/Akt Signaling

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Abstract

Parathyroid hormone-related protein (PTHrP) is expressed by human prostatic tissues and cancer cell lines. PTHrP enhances tumor cell growth and metastasis *in vivo* and up-regulates proinvasive integrin $\alpha_6\beta_4$ expression *in vitro*. Hallmarks of malignant tumor cells include resistance to apoptosis and anchorage-independent cell growth. In this study, we used the human prostate cancer cell lines C4-2 and PC-3 as model systems to study the effects of PTHrP on these processes. We report that PTHrP protects these cells from doxorubicin-induced apoptosis and promotes anchorage-independent cell growth via an intracrine pathway. Conversely, autocrine/paracrine PTHrP action increases apoptosis in C4-2 cells and has no effect on apoptosis in PC-3 cells. The intracrine effects of PTHrP on apoptosis are mediated via activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. PTHrP also affects the phosphorylation state of Akt substrates implicated in apoptosis suppression, including glycogen synthase kinase-3 and Bad. The prosurvival effects of PTHrP are accompanied by increases in the ratio of antiapoptotic to proapoptotic members of the Bcl-2 family and in levels of c-myc. PTHrP also increases nuclear factor- κ B activity via a PI3K-dependent pathway. Integrin $\alpha_6\beta_4$ is known to activate PI3K. Here, we also show that knockdown of integrin $\alpha_6\beta_4$ negates the PTHrP-mediated activation of the PI3K/Akt pathway. Taken together, these observations provide evidence of a link between PTHrP and the PI3K/Akt signaling pathway through integrin $\alpha_6\beta_4$, resulting in the activation of survival pathways. Targeting PTHrP production in prostate cancer may thus prove therapeutically beneficial. (Mol Cancer Res 2009;7(7):1119–31)

Introduction

Prostate cancer is the most common noncutaneous cancer and the second leading cause of cancer-related deaths in males in the United States (1). The prostate is strongly dependent on androgens for normal development and physiologic functions. However, additional factors, including growth factors, neuroendocrine peptides, and cytokines, also play important roles in the prostate (2); one of these factors is parathyroid hormone-related protein (PTHrP). PTHrP is expressed by most fetal and adult tissues, including the prostate. The protein has been localized to normal neuroendocrine cells and the glandular epithelium of normal and benign prostatic hyperplasia tissues (3, 4). Cultured epithelial cells derived from normal and benign prostatic hyperplasia tissues, and immortalized prostate cancer cell lines, secrete PTHrP (5). The progression of normal prostate epithelium to benign prostatic hyperplasia as well as to carcinoma is accompanied by an increase in PTHrP expression (6). PTHrP plays a major role in both the initial osteoblastic phase and the later osteolytic phase, which is characteristic of prostate cancer (7).

Oncogenic cell transformation is a multistage process in which multiple genetic lesions result in alterations in cellular physiology (8). This results in cells acquiring new capabilities, including an increased ability to migrate and invade surrounding tissues and escape apoptotic death (8). Cell migration and invasion involve cell detachment from the extracellular matrix and would result in apoptosis if not accompanied by increased expression of cell survival factors (8). Resistance to apoptosis increases in prostate cancer cells with increasing metastatic potential (9, 10). Specifically, the term “anoikis” describes apoptosis induced by loss of anchorage during dissemination in lymph or blood (11). This term was originally defined by Frisch to describe apoptotic cell death as a consequence of insufficient cell-matrix interactions (11) and has since been recognized as a significant player in tumor metastasis and angiogenesis (11–13).

Because prostate cancer progression is accompanied by increased PTHrP expression, and given the role of PTHrP in prostate cancer metastasis, in this study, we asked whether PTHrP plays a role in prostate cancer cell survival and anchorage-independent cell growth and investigated the mechanisms involved. We used the C4-2 and PC-3 cell lines as model systems. The C4-2 cell line is a second-generation LNCaP subline that is androgen-independent and metastasizes to the lymph node and bone when injected orthotopically into nude mice (14, 15). C4-2 cells produce mixed lytic/blastic lesions (7).

Received 12/17/08; revised 3/18/09; accepted 3/20/09; published OnlineFirst 7/7/09.
Grant support: NIH grant CA83940.

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doi:10.1158/1541-7786.MCR-08-0568

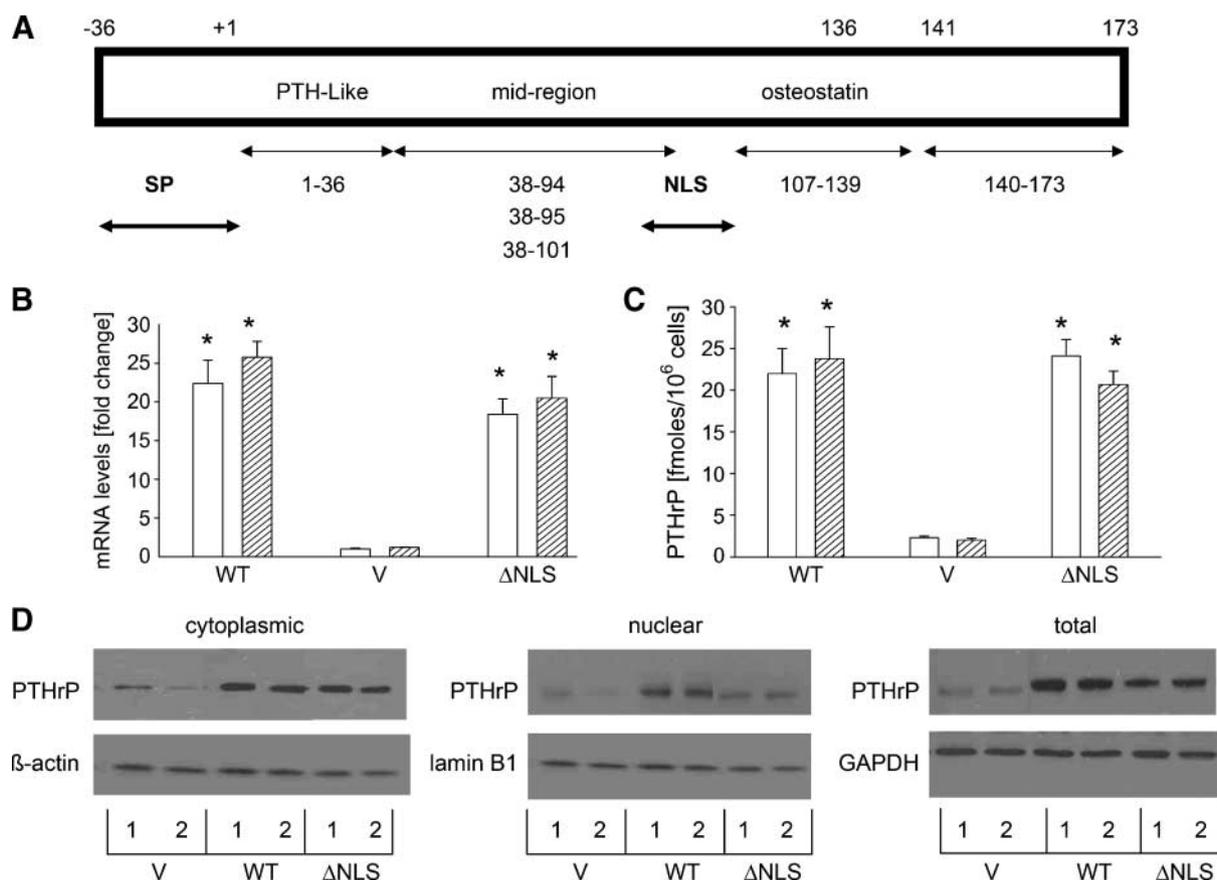


FIGURE 1. **A.** Schematic representation of PTHrP structure and processing. *SP*, signal peptide (amino acids -36 to -1); *NLS*, nuclear localization signal (amino acids 88-91 and 102-106). 136, 141 and 173 represent the three human PTHrP isoforms. The 1-36 region interacts with PTH1R. Mid-region PTHrP is represented by the 38-94, 38-95 and 38-101 isoforms, and C-terminal PTHrP is represented by the 107-139 (osteostatin) and 140-173 isoforms. **B** to **D.** Characterization of C4-2 cells overexpressing wild-type PTHrP or PTHrP mutated over the NLS. **B.** mRNA levels were measured by reverse transcription/real-time PCR. Values are expressed relative to the corresponding V value, set arbitrarily at 1.0. **C.** PTHrP secretion was measured using an immunoradiometric assay. Values are expressed as PTHrP secretion in fmol/10⁶ cells. **B** and **C.** Each bar is the mean ± SEM of three independent experiments. *, significantly different from control (V) value ($P < 0.001$). **D.** Western blot analysis of cytoplasmic, nuclear, and total cellular fractions. PTHrP was detected as an ~18 kDa protein. Equal loading was confirmed by re-probing with cytoplasmic (β -actin), nuclear (lamin B1), or total cell (GAPDH) protein markers. WT, wild-type PTHrP-overexpressing cells; V, empty vector controls; Δ NLS, NLS-mutated PTHrP-overexpressing cells. **B** to **D.** Data for each of two independent clones.

The androgen-independent PC-3 cell line was initiated from a bone metastasis and produces predominantly lytic lesions.

PTHrP is only detected in the circulation in normal subjects during pregnancy and lactation and in cancer patients with the accompanying syndrome of humoral hypercalcemia of malignancy. In normal subjects and in cancer patients in the absence of humoral hypercalcemia of malignancy, PTHrP exerts its effects via autocrine/paracrine and intracrine pathways. The mature PTHrP species is post-translationally processed to NH₂-terminal, mid-region, and COOH-terminal secretory forms (Fig. 1A; 16). NH₂-terminal PTHrP exerts its autocrine/paracrine effects via interaction with the parathyroid hormone/PTHrP 1 receptor (PTH1R; ref. 17). Autocrine/paracrine PTHrP action via the PTH1R has been reported to inhibit or enhance cell proliferation in a cell type-specific manner (18-21). PTHrP functions in an intracrine manner after translocation to the nucleus or nucleolus. Intracrine PTHrP action is mediated via a bipartite nuclear localization signal (NLS) located at amino acids 88 to 91 and 102 to 106 (18, 22). One proposed mechanism for nuclear entry in-

volves PTHrP secretion then endocytosis to the nucleus after complexing with its receptors. A second mechanism involves alternative translation start sites at CUG codons that occur in the context of a Kozak sequence. Translational initiation at these CUGs disrupts the signal peptide function, allowing the nascently translated peptide to remain in the cytosol and enter the nucleus in a phosphorylation-dependent manner (22). Intracrine PTHrP action increases C4-2 and PC-3 cell proliferation (21).⁴ PTHrP also increases cell migration and invasion and up-regulates proinvasive integrin $\alpha_6\beta_4$ expression via an intracrine pathway (23, 24).

The molecular mechanisms that control the susceptibility of cells to anoikis involve the overexpression of antiapoptotic factors and/or the activation of several kinases, including phosphatidylinositol 3-kinase (PI3K; ref. 25). This pathway plays

⁴ Bhatia and Falzon, unpublished observations.

a central role in apoptosis and in the anchorage-independent survival of cancer cells (25). In this study, we also asked whether the prosurvival effects of PTHrP are mediated via the PI3K pathway. Because integrin $\alpha_6\beta_4$ is known to signal synergistically with growth factor receptors to activate the PI3K pathway (26), we also asked whether up-regulation of integrin $\alpha_6\beta_4$ expression by PTHrP (24, 27, 28), in turn, activates PI3K. PTHrP is involved in both osteolytic and osteoblastic phases of prostate cancer, so elucidating the mechanisms via which it exerts its effects in prostate cancer may identify therapeutically useful targets for drug development.

Results

Characterization of C4-2 and PC-3 Cell Lines Overexpressing Wild-type or NLS-Mutated PTHrP

Cell survival plays a critical role in cancer cell progression. PTHrP exerts its effects via autocrine/paracrine and intracrine pathways. In this study, we asked whether PTHrP alters prostate cancer cell survival and the pathways involved. We therefore established C4-2 and PC-3 cells overexpressing wild-type or NLS-mutated PTHrP. Empty vector transfectants served as controls. These cells were characterized in terms of PTHrP mRNA and cellular protein levels as well as secreted protein levels.

Transfecting C4-2 cells with the constructs expressing wild-type or NLS-mutated PTHrP resulted in significant (~20- to 25-fold) increases in PTHrP mRNA levels and secreted PTHrP levels when compared with control cells (Fig. 1B and C). Total and cytoplasmic PTHrP levels were elevated in cells overexpressing wild-type or NLS-mutated PTHrP when compared with control cells (Fig. 1D). Nuclear PTHrP levels were significantly higher in wild-type PTHrP-overexpressing cells compared with NLS-mutated PTHrP-overexpressing cells or control cells (Fig. 1D). Reprobing the blots with antibodies specific for lamin B1 and β -actin, specific for nuclear and cytoplasmic markers, respectively, confirmed that there was no cross-contamination of the nuclear or cytoplasmic fractions (data not shown). Total cellular PTHrP levels were higher in cells overexpressing wild-type PTHrP compared with NLS-mutated PTHrP-overexpressing and control cells (Fig. 1D). An ~18 kDa PTHrP isoform was present in total, cytoplasmic, and nuclear fractions; this moiety represents a processed form of PTHrP (29). There was no difference in PTHrP mRNA and secreted protein levels in control versus parental cells (data not shown). The PC-3 clones overexpressing wild-type or NLS-mutated PTHrP have been described (24). PTHrP mRNA and secreted protein levels were 8- to 10-fold higher in PC-3 than in C4-2 cells. However, Western blot analysis showed that total cellular PTHrP levels were only ~2- to 3-fold higher in PC-3 versus C4-2 cells (data not shown), indicating that a large proportion of PTHrP synthesized by the cell is secreted.

We also ascertained that overexpression of wild-type or NLS-mutated PTHrP does not alter PTH1R expression and activity and therefore that differences in the response of PTHrP-overexpressing cells versus control cells are not due to differences in PTH1R expression and/or activity. The PTH1R was expressed at low levels in control C4-2 cells; PC-3 cells expressed ~15-fold higher PTH1R levels (data not

shown). In Fig. 2, data are presented for C4-2 cells; PC-3 cells showed the same profile. Overexpressing wild-type or NLS-mutated PTHrP did not alter PTH1R mRNA levels (Fig. 2A). To compare PTH1R activity, we measured cyclic AMP (cAMP) accumulation in response to human PTHrP(1-34). C4-2 cells showed an increase in cAMP production in response to this peptide (Fig. 2B). The ability of PTHrP(1-34) to induce a cAMP response was dose related between 0.1 and 100 nmol/L; cAMP production reached a plateau at 10 nmol/L (Fig. 2B). There was no difference in the response to PTHrP(1-34) in control cells and in cells overexpressing wild-type or NLS-mutated PTHrP (Fig. 2B). As a control, cells were treated with the structurally unrelated peptides salmon calcitonin and secretin, which do not interact with the PTH1R. These peptides did not affect PTH1R activity in control or PTHrP-overexpressing cells (data not shown). Similarly, two PTHrP antagonists, [Leu¹¹, D-Trp¹²] hPTHrP-(7-34) amide and [Asn¹⁰, Leu¹¹] hPTHrP-(7-34) amide, and the parathyroid hormone antagonist PTH(3-34) did not

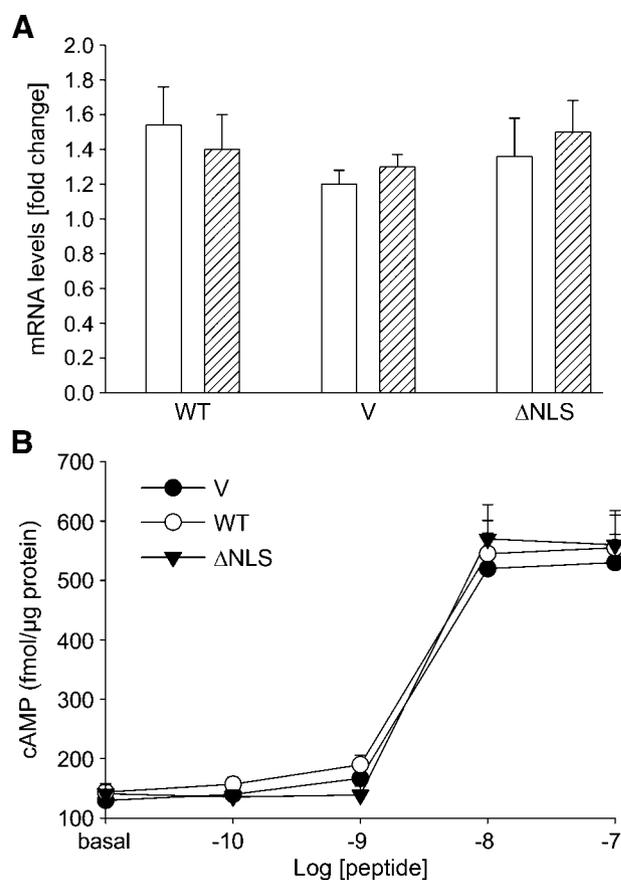


FIGURE 2. Parathyroid hormone 1 receptor expression (**A**) and activity (**B**) in C4-2 cells overexpressing wild-type or NLS-mutated PTHrP. **A.** mRNA levels were measured by reverse transcription/real-time PCR. Values are expressed relative to the corresponding V value, set arbitrarily at 1.0. Each bar is the mean \pm SEM of three independent experiments. Data for two independent clones are presented. **B.** Dose-dependent cAMP response of C4-2 cells. Cells were treated with human PTHrP (1-34). cAMP activity was measured using the Parameter cAMP assay kit. Each point is the mean \pm SEM of three independent experiments for each of two independent clones. **A** and **B.** WT, wild-type PTHrP-overexpressing cells; V, empty vector controls; Δ NLS, NLS-mutated PTHrP-overexpressing cells.

increase cAMP production in any of the cells tested (data not shown).

Wild-type PTHrP Supports Anchorage-Independent Cell Growth

To investigate the effect of PTHrP on anchorage-independent cell growth, C4-2 and PC-3 cells overexpressing wild-type or NLS-mutated PTHrP were grown in soft agar for 15 days. Data are shown for C4-2 cells; a similar profile was obtained with PC-3 cells, except that the size and number of colonies obtained with PC-3 cells were ~3-fold greater than those obtained with C4-2 cells. Wild-type PTHrP facilitated soft-agar clone formation. Thus, overexpressing PTHrP increased both the number and the size of the colonies in soft agar (Fig. 3). NLS-mutated PTHrP had no effect on the number and size of colonies in soft agar; there was no significant difference in these parameters between cells transfected with NLS-mutated PTHrP and cells transfected with the empty vector (Fig. 3). Increasing the incubation time from 15 to 21 days did not increase the number of colonies formed by any of the clones (data not shown). There was no significant difference in colony formation between parental and empty vector transfectants (data not shown).

Wild-type PTHrP Protects C4-2 and PC-3 Cells from Doxorubicin-Induced Apoptosis

To ask whether PTHrP modulates apoptosis, we compared this parameter in cells overexpressing wild-type PTHrP versus control cells. In C4-2 cells, the basal level of apoptosis was not significantly different in PTHrP-overexpressing and control cells (Fig. 4A). In PC-3 cells, PTHrP caused a small, but signif-

icant, decrease in basal apoptosis (Fig. 4B). Wild-type PTHrP protected both C4-2 and PC-3 cells against apoptosis induced by the DNA-intercalating agent doxorubicin. Thus, treating C4-2 cells with 0.3 $\mu\text{g}/\text{mL}$ doxorubicin for 16 h induced apoptosis in both PTHrP-overexpressing and control cells (Fig. 4A). However, the degree of apoptosis induced in control cells was significantly higher than that in wild-type PTHrP-overexpressing cells (Fig. 4A). Similar effects were observed when cells were treated with 0.1 $\mu\text{g}/\text{mL}$ doxorubicin, although the overall degree of apoptosis induced was significantly lower (Fig. 4A). Similar results were obtained in PC-3 cells, although a 72 h treatment was required to induce apoptosis (Fig. 4B). To determine whether the antiapoptotic effects of PTHrP are mediated via an intracrine pathway, doxorubicin-induced apoptosis was compared in cells overexpressing wild-type and NLS-mutated PTHrP. NLS-mutated PTHrP did not protect cells from apoptosis (Fig. 4A and B).

The autocrine/paracrine effects of PTHrP are mediated via interaction with the PTH1R. To assess the contribution of autocrine/paracrine signaling to the effects of PTHrP on apoptosis, parental C4-2 and PC-3 cells were treated with an anti-PTHrP (1-34) antibody or an anti-PTH1R antibody (at a concentration of 1 or 2 $\mu\text{g}/\text{mL}$) to neutralize the autocrine/paracrine effects of PTHrP. Treatment of C4-2 cells with either of these antibodies did not enhance doxorubicin-induced apoptosis; rather, it caused an ~40% decrease in apoptosis compared with cells treated with control IgG (Fig. 4C). These antibodies had no effect on apoptosis of PC-3 cells (data not shown). To confirm these results, cells were transfected with a small interfering RNA (siRNA) targeting the PTH1R and then treated with doxorubicin (0.3 $\mu\text{g}/\text{mL}$) to induce apoptosis. To eliminate

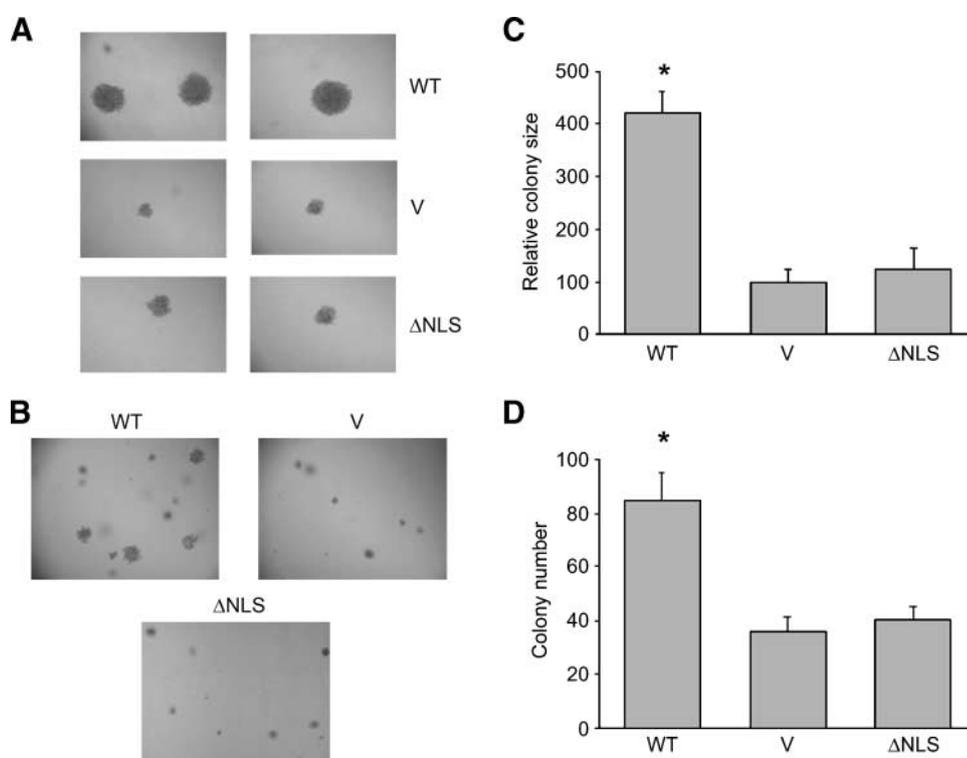


FIGURE 3. Effects of PTHrP on anchorage-independent cell growth. Assays to determine colony formation in soft agar were performed in 60 mm dishes containing a bottom layer consisting of 1.5 ml culture medium plus 0.4% (w/v) agar. Cells (1×10^4) were plated in a top layer of 0.3% agar. **A.** After 2 wk in culture, the plates were photographed at $40 \times$ magnification, and clone size was measured using the ImageJ software (NIH). **B.** Photographs were also taken at $10 \times$ magnification to measure clone frequency. **C.** Colony size. Each bar is the mean \pm SEM of 20 colonies for each of two independent clones overexpressing WT PTHrP (WT) or NLS-mutated PTHrP (ΔNLS), or transfected with empty vector (V). **D.** Average number of colonies. All clones in focus $>50 \mu\text{m}$ in size were measured. Each bar is the mean \pm SEM of three fields per plate for each of the cell lines described in **C.** **C** and **D.** *, significantly different from the control (V) value ($P < 0.001$).

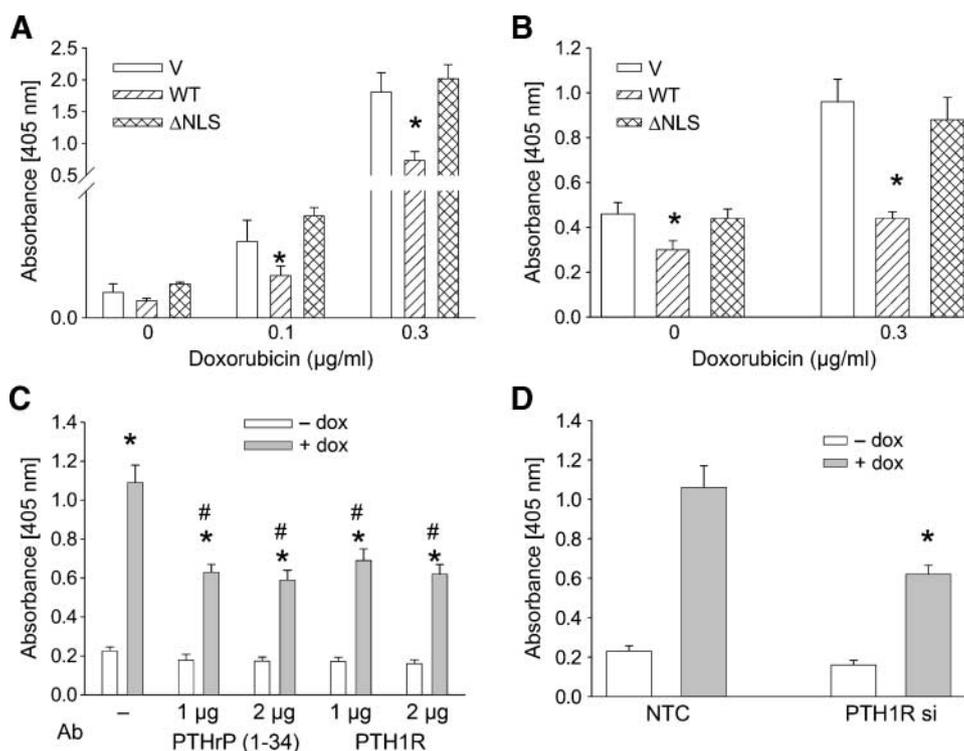


FIGURE 4. Cell apoptosis of C4-2 (**A**) and PC-3 (**B**) cells overexpressing WT or NLS-mutated PTHrP and treated with doxorubicin. Cells (1×10^4) were plated in 96-well dishes in medium containing 10% FBS. After 48 h, the cells were treated with the indicated concentration of doxorubicin. Apoptosis was measured after 16 h (**A**) or 72 h (**B**) as described in Materials and Methods. WT, wild-type PTHrP-overexpressing cells; Δ NLS, NLS-mutated PTHrP-overexpressing cells; V, empty vector-transfected cells. Each bar is the mean \pm SEM of three independent experiments for each of three independent clones. *, significantly different from control (V) value ($P < 0.001$). Effect on apoptosis after treating parental C4-2 cells with anti-PTHrP (1-34) or anti-PTH1R antibody (**C**) or transfection with PTH1R siRNA (**D**). **C.** At 48 h after plating, the cells were treated with the indicated antibodies. -, IgG control. **D.** At 48 h after plating, the cells were transfected with an siRNA targeting the PTH1R. NTC, non-target control. **C** and **D.** After 48 h, doxorubicin (0.3 μ g/ml) was added, and apoptosis was measured after 16 h. Each bar is the mean \pm SEM of three independent experiments (**C**) or three independent experiments for each of two independent siRNAs (**D**). **C.** *, Significantly different from the - dox value ($P < 0.001$); #, significantly different from the IgG control value (-) treated with dox ($P < 0.001$). **D.** *, Significantly different from the NTC +dox value ($P < 0.001$).

the potential for off-target effects, two independent PTH1R-specific siRNAs were used. Transfection with either of the two PTH1R-specific siRNAs caused an $\sim 75\%$ decrease in PTH1R mRNA levels compared with the nontarget control (NTC; as measured by reverse transcription/real-time PCR; data not shown). Suppressing PTH1R expression in C4-2 cells caused an $\sim 35\%$ decrease in doxorubicin-induced apoptosis compared with the NTC controls similarly treated with doxorubicin (Fig. 4D). Suppression of PTH1R expression had no effect on apoptosis in PC-3 cells (data not shown).

Wild-type PTHrP Alters the Expression of Apoptosis-Regulating Proteins of the Bcl-2 Family

The Bcl-2 family of proteins plays an important role in the regulation of apoptosis; different members of this family either inhibit or promote apoptosis (30). Figure 5A shows that the pro-survival effects of wild-type PTHrP in C4-2 cells were accompanied by an increase in the levels of the antiapoptotic proteins Bcl-2 and Bcl_{XL} as well as p-Bad and a decrease in the levels of Bax, which exert proapoptotic effects. Conversely, NLS-mutated PTHrP had minimal effects on the levels of Bcl-2, Bcl_{XL}, Bax, and p-Bad (Fig. 5A). There was no difference

in the levels of any of the proteins measured in control versus parental cells (data not shown). Similar effects of PTHrP on Bcl-2, Bcl_{XL}, Bax, and p-Bad levels were observed in PC-3 cells (data not shown).

Heterodimerization between proapoptotic and antiapoptotic members of the Bcl-2 family and the relative levels of the two types of proteins play a role in the susceptibility to apoptosis (30). Figure 5B and C show that wild-type, but not NLS-mutated, PTHrP expression is associated with an increased ratio of antiapoptotic (Bcl-2 and Bcl_{XL}) to proapoptotic (Bax) proteins.

PTHrP Modulates the Levels of p-Akt, p-glycogen Synthase Kinase-3, and c-myc

The PI3K/Akt pathway plays a major role in cancer cell survival (25). We therefore compared the levels of p-Akt in C4-2 and PC-3 cells overexpressing wild-type or NLS-mutated PTHrP. We observed a correlation between the expression of wild-type PTHrP and p-Akt levels (Fig. 5D; data not shown). Conversely, p-Akt levels in cells overexpressing NLS-mutated PTHrP were comparable with those in control cells (Fig. 5D; data not shown). PTHrP had no effect on total Akt levels (Fig. 5D; data not shown).

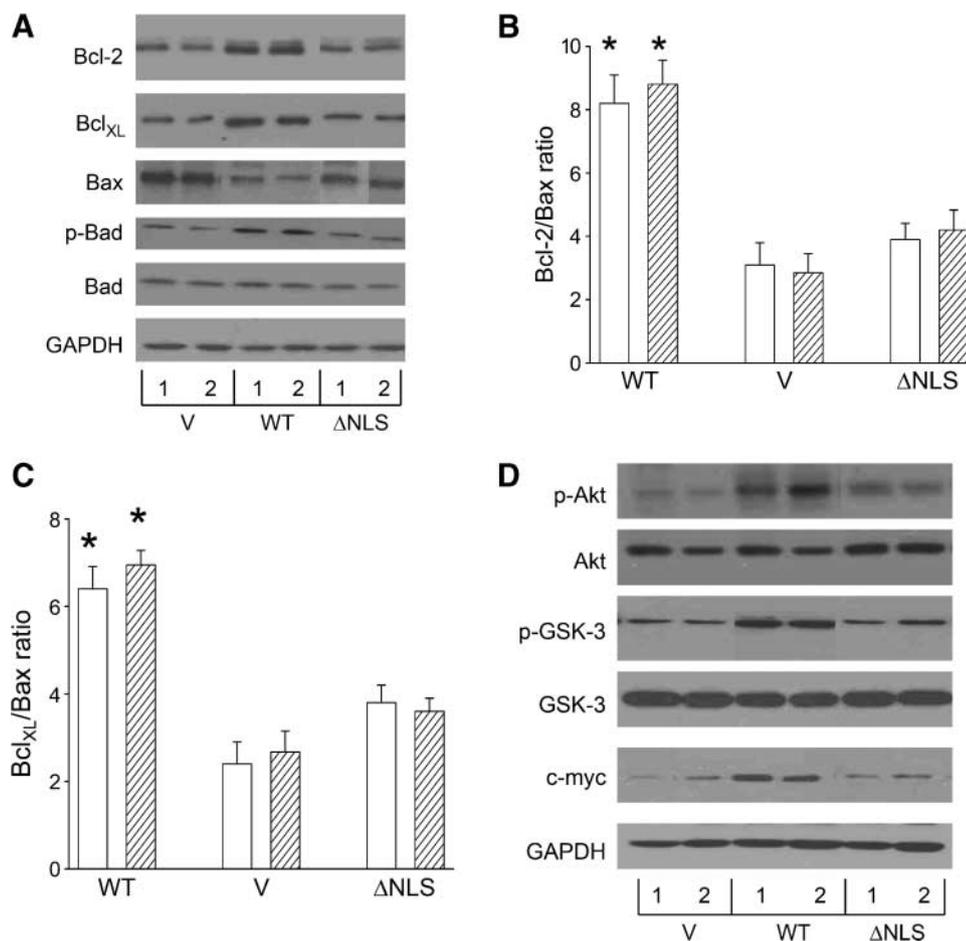


FIGURE 5. Levels of apoptosis-regulating proteins (**A–C**), and levels of phosphorylated and total Akt and GSK-3, and of c-myc (**D**) in C4-2 cells overexpressing wild-type or NLS-mutated PTHrP. **A.** Levels of Bcl-2, Bcl_{XL}, Bax, p-Bad, and total Bad. **B** and **C.** Ratio of anti-apoptotic to proapoptotic proteins, obtained after densitometric scanning of the Western blots and normalization to GAPDH. Each bar is the mean \pm SEM of three independent experiments. Data for two independent clones are presented. *, significantly different from V ($P < 0.001$). **D.** Cells were plated in medium containing 10% FBS. At ~80% confluence, they were transferred to medium in the absence of FBS. After 16 h, medium containing 10% FBS was added. The cells were harvested after 30 min and lysates were prepared for Western blotting. **A** and **D.** Within each group of transfectants, 1 and 2 refer to two independent clones. Each figure is representative of three independent experiments.

Analysis of the effect of PTHrP overexpression on downstream signaling molecules of the Akt pathway involved in cell survival revealed that wild-type PTHrP also induced phosphorylation of glycogen synthase kinase (GSK)-3 β in C4-2 cells, resulting in inhibition of its activity. NLS-mutated PTHrP had no effect on GSK-3 β phosphorylation (Fig. 5D). Wild-type, but not NLS-mutated, PTHrP expression was also linked to elevated levels of c-myc (Fig. 5D). Similar effects were observed in PC-3 cells (data not shown).

Increased Cell Survival in Wild-type PTHrP-Overexpressing Cells Is Mediated via the PI3K Pathway

We next asked whether regulation of the PI3K pathway by PTHrP plays a role in the observed antiapoptotic effects of PTHrP. For this purpose, PI3K levels and activity were suppressed by transfection with siRNAs targeting the p110 α subunit or by treating with the PI3K inhibitor LY49002 (25 or 50 μ mol/L). Transfecting C4-2 cells with the p110 α siRNA caused a significant decrease in p110 α mRNA (Fig. 6A) and protein levels (Fig. 6B), which were accompanied by significant decreases in the levels of p-Akt (Fig. 6B). Treating C4-2 and PC-3 cells with LY49002 also caused a significant decrease in p-Akt levels (data not shown).

Doxorubicin (0.3 μ g/mL) was more effective in inducing apoptosis in control cells and in cells overexpressing NLS-mutated PTHrP than in cells overexpressing wild-type PTHrP (Figs. 4 and 6C and D). Inhibiting PI3K activity in C4-2 cells by transfection with p110 α siRNA (in the absence of doxorubicin) caused a significant increase in apoptosis in the control cells and the cells overexpressing both wild-type and NLS-mutated PTHrP (Fig. 6C). Down-regulation of PI3K activity by transfection with the siRNA targeting p110 α abolished the differences in the extent of apoptosis induced by doxorubicin in wild-type PTHrP-overexpressing versus control cells (Fig. 6C). Thus, the degree of apoptosis, expressed as a ratio for untreated versus doxorubicin-treated cells, was not significantly different in control and PTHrP-overexpressing cells transfected with the p110 α siRNA (2.3 for p110 α transfectants in wild-type PTHrP-overexpressing cells versus 2.4 for p110 α transfectants in control cells; Fig. 6C). In cells transfected with the NTC siRNA, this ratio was significantly higher in control cells (4.5) than in cells overexpressing wild-type PTHrP (1.9; Fig. 6C). Cells overexpressing NLS-mutated PTHrP presented the same profile as the control cells (Fig. 6C). A similar profile was observed in both C4-2 and PC-3 after treating with LY49002, in that suppression of PI3K activity negated the differences in sensitivity

to doxorubicin of PTHrP-overexpressing versus control cells (Fig. 6D; data not shown).

PTHrP Up-Regulates Nuclear Factor- κ B Transcriptional Activity in a PI3K-Dependent Manner

PI3K signaling activates the nuclear factor- κ B (NF- κ B) pathway, leading to enhanced cell survival (31). Here, we show that transcriptional activity from a NF- κ B reporter construct is significantly higher in PTHrP-overexpressing cells than in control cells (Fig. 6E). Treating C4-2 cells with LY49002 (25 or 50 μ mol/L) caused a significant decrease in NF- κ B transcriptional activity in PTHrP-overexpressing cells, such that there was no significant difference in promoter activity in PTHrP-overexpression and control cells after LY204002 treatment (Fig. 6E; data not shown). The decrease in promoter activity in control cells was significantly less than that in PTHrP-overexpressing cells after LY49002 treatment (Fig. 6E; data not shown).

Integrin $\alpha_6\beta_4$ Expression Is Required for the PTHrP-Mediated Activation of PI3K

The data presented above indicate that PTHrP activates the PI3K pathway via an intracrine mechanism of action. We have shown previously that nuclear PTHrP is required for induction of integrin α_6 and β_4 expression at both mRNA and protein levels (23, 24). Because it has been shown that integrin $\alpha_6\beta_4$ signals synergistically with growth factor receptors to activate the PI3K pathway (26), here we asked whether activation of PI3K in wild-type PTHrP-overexpressing cells occurs as a result of up-regulation of integrin $\alpha_6\beta_4$ expression. The expression of these integrin subunits was suppressed using siRNAs. In cells transfected with the NTC siRNA, integrin α_6 and β_4 mRNA levels were higher in wild-type PTHrP-overexpressing cells than in control cells (Fig. 7A and B). Deletion of the NLS negated the effects of PTHrP on integrin expression (Fig. 7A and B). After transfection with the integrin α_6 - or β_4 -specific

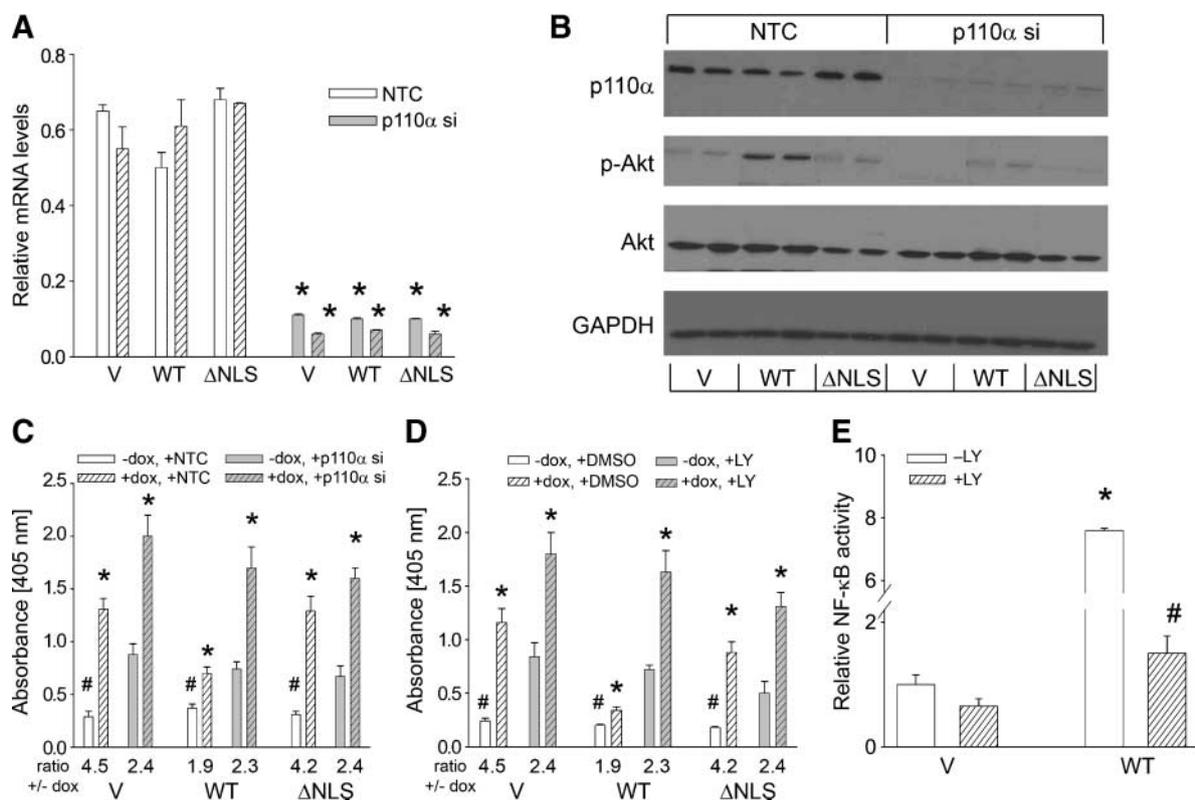


FIGURE 6. **A** to **D**. Effect of suppression of PI3-K activity on apoptosis after doxorubicin treatment of control C4-2 cells and C4-2 cells overexpressing wild-type or NLS-mutated PTHrP. **A**. Reverse transcription/real-time PCR analysis for p110 α mRNA levels in cells transfected with siRNA targeting the p110 α subunit of PI3-K. Each bar is the mean \pm SEM of two experiments for each of two independent clones, each transfected with one of two independent siRNAs. **B**. Western blot analysis for p110 α , p-Akt and total Akt in cells transfected with siRNA targeting p110 α on apoptosis induced by doxorubicin (0.3 μ g/ml). NTC, transfected with non-target control. Each bar is the mean \pm SEM of two experiments for each of two independent clones, each transfected with one of two independent siRNAs. **C**. Effect of LY49002 (25 μ M) treatment on doxorubicin-induced apoptosis. Each bar is the mean \pm SEM of three experiments for each of two independent clones. **C** and **D**. WT, wild-type PTHrP-overexpressing cells; Δ NLS, NLS-mutated PTHrP-overexpressing cells; V, empty vector-transfected cells. + dox, treated with doxorubicin; - dox, untreated control. The ratio of the absorbance [405 nm] in the presence and absence of doxorubicin is indicated under each set of bars. *, significantly different from the respective - dox control; #, significantly different from the respective p110 α siRNA-transfected cells (**C**) or LY49002-treated cells (**D**; $P < 0.001$). **E**. Effect of LY49002 on NF- κ B activity in control and wild-type PTHrP-overexpressing C4-2 cells. Luciferase activity was measured in empty vector-transfected (V) and PTHrP-overexpressing (WT) C4-2 cells treated with LY49002 as described in **D**. Values were normalized to Renilla luciferase activity. Each bar is the mean \pm SEM of three experiments for each of two independent clones. *, significantly different from the untreated V value; #, significantly different from the respective untreated value ($P < 0.001$).

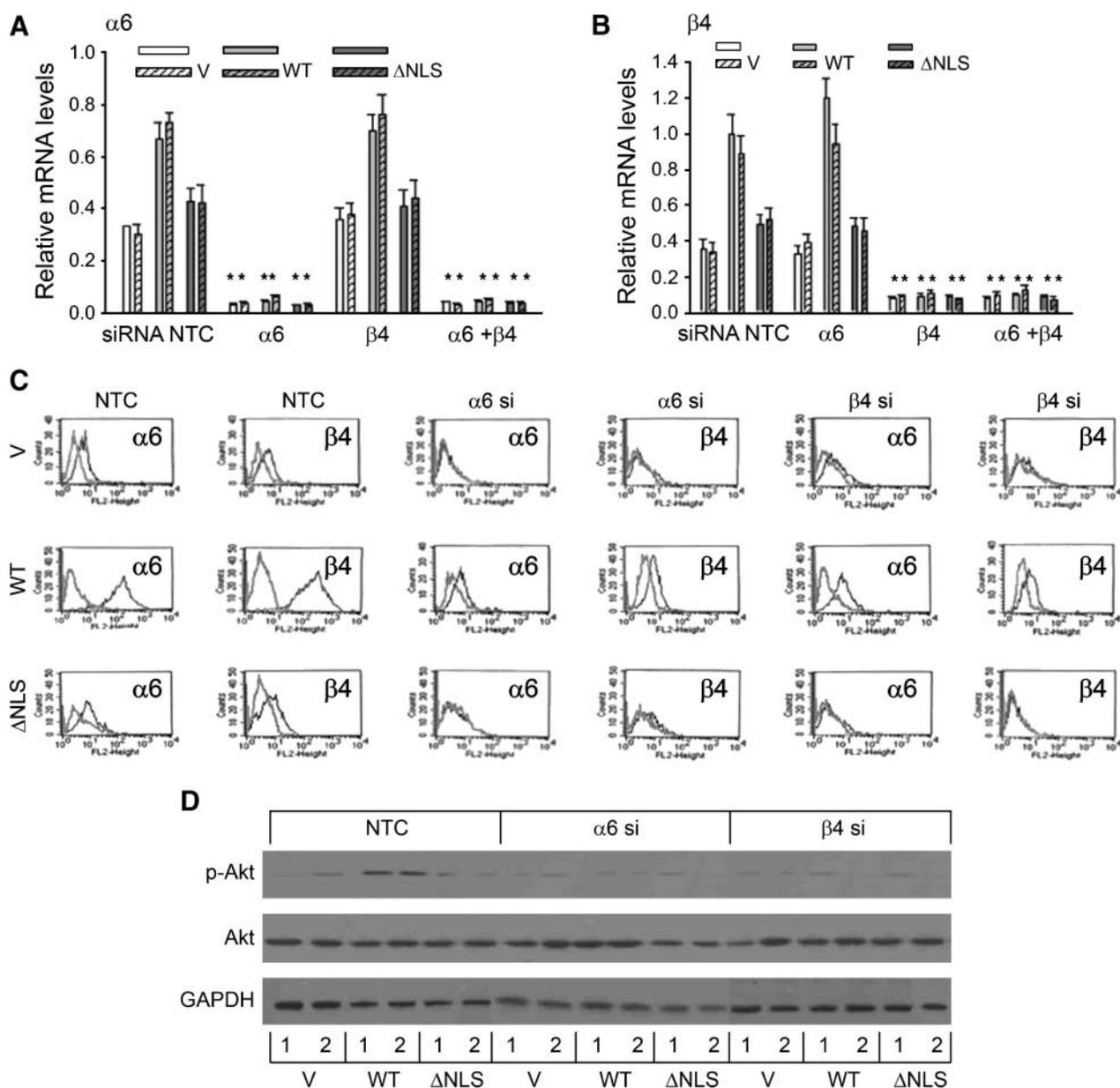


FIGURE 7. Effect of suppressing integrin $\alpha 6$ and $\beta 4$ expression on p-Akt levels in C4-2 cells overexpressing wild-type or NLS-mutated PTHrP. **A** and **B.** Reverse transcription/real-time PCR analysis of integrin $\alpha 6$ or $\beta 4$ mRNA levels in cells transfected with siRNA targeting the integrin $\alpha 6$ or $\beta 4$ subunit. Each bar is the mean \pm SEM of three experiments for each of two independent siRNAs. Data for two independent clones are presented. *, significantly different from the respective NTC control ($P < 0.001$). **C.** FACS analysis for cell surface integrin $\alpha 6$ and $\beta 4$ expression. The cells were stained with phycoerythrin-labeled antibodies against the integrin $\alpha 6$ and $\beta 4$ subunits and analyzed by FACS. The dashed line represents the scan obtained with isotype control antibody and the thick line represents the scan obtained with the integrin-specific antibody. **D.** Western blot analysis for p-Akt and total Akt in cells transfected with siRNAs targeting the integrin $\alpha 6$ or $\beta 4$ subunit. Within each group of transfectants, 1 and 2 refer to two independent clones. NTC, transfected with non-target control. WT, wild-type PTHrP-overexpressing cells; Δ NLS, NLS-mutated PTHrP-overexpressing cells; V, empty vector-transfected cells.

siRNAs, there was a significant decrease in the mRNA levels for the corresponding subunit (Fig. 7A and B). The mRNA levels of both integrin $\alpha 6$ and $\beta 4$ subunits were suppressed in cells cotransfected with siRNAs targeting both subunits (Fig. 7A and B).

Cell-surface integrin $\alpha 6$ and $\beta 4$ protein levels were also significantly higher in wild-type PTHrP-overexpressing cells but not in NLS-mutated PTHrP-overexpressing cells (Fig. 7C). Transfection with the integrin $\alpha 6$ siRNA caused a significant

decrease in the cell-surface levels of both $\alpha 6$ and $\beta 4$ subunits (Fig. 7C). Transfection with the integrin $\beta 4$ subunit also caused a significant decrease in $\alpha 6$ and $\beta 4$ levels (Fig. 7C). Because the $\alpha 6$ subunit can dimerize with both $\beta 1$ and $\beta 4$ subunits (32), these data indicate that, in C4-2 cells, the preferential partner for integrin $\alpha 6$ is the $\beta 4$ subunit.

Transfection of wild-type PTHrP-overexpressing cells with the siRNA targeting the integrin $\alpha 6$ and/or $\beta 4$ subunit negated the PTHrP-mediated increase in p-Akt levels (Fig. 7D; data

not shown). p-Akt levels were higher in wild-type PTHrP-overexpressing cells transfected with NTC (Fig. 7D). Transfection with the integrin α_6 and/or β_4 siRNAs had no effect on total Akt levels (Fig. 7D; data not shown).

Discussion

Multiple studies have shown that PTHrP expression is increased in prostate cancer cells compared with normal prostate epithelium and benign prostatic hyperplasia (5, 6). PTHrP also plays a role in the development of the osteoblastic/osteolytic lesions that accompany prostate cancer (7). However, the consequences of PTHrP dysregulation in prostate cancer have not yet been fully elucidated. Our main findings are that intracrine PTHrP increases anchorage-independent cell growth, a hallmark of malignant tumor cells (33). Given the correlation between anchorage-independent growth *in vitro* and cellular tumorigenicity in nude mice, these findings may explain the consequences of PTHrP expression in prostate cancer. Wild-type, but not NLS-mutated, PTHrP also activates Akt, a downstream effector of the PI3K pathway. Notably, the prosurvival effects of intracrine PTHrP are mediated via the PI3K pathway, and PI3K signaling in turn is dependent on expression of integrin $\alpha_6\beta_4$.

The ability of cells to migrate and invade through the basement membrane into surrounding tissues is one of the essential hallmarks of cancer and is a necessary step for local tumor progression and metastatic spread. Migration and invasion require that a cell acquires anchorage independence. This property of cancer cells has been recognized as a necessity for cell transformation for many years (33). Here, we report that PTHrP increases C4-2 and PC-3 cell growth as colonies in soft agar in the absence of adhesion to the extracellular matrix. Anchorage independence can lead to apoptosis if not counterbalanced by increased survival (8). In particular, anoikis, a term that describes apoptosis induced by loss of anchorage, is known to limit the spread of cells outside the tissue environment (11). Cancer cells are more anoikis-resistant than their normal counterparts, and the level of anoikis correlates with *in vivo* tumorigenicity and metastatic capability (13, 34). One mechanism via which cancer cells overcome anoikis is through the overexpression of antiapoptotic factors, such as members of the Bcl-2 family (30, 35). Multiple studies have shown that modulation of the expression of these proteins plays a role in resisting anoikis (30, 35). Here, we show that PTHrP increases the ratio of antiapoptotic (Bcl-2 and Bcl_{XL}) to proapoptotic (Bax) proteins in the cell. Levels of p-Bad are also increased in PTHrP-overexpressing cells. p-Bad is thought to bind to the 14-3-3t protein, resulting in the sequestration of Bad from Bcl_{XL} and enhanced cell survival (36). Thus, one of the pathways via which PTHrP may inhibit anoikis is by regulating members of the Bcl-2 family.

Androgen deprivation therapy is widely used to treat patients with advanced prostate cancer (37). However, this treatment is not curative, because when tumors eventually recur, they are typically less differentiated and more metastatic than the parent androgen-sensitive tumors (38, 39). The emergence of hormone-refractory disease does not signify a lack of dependence on androgen receptor signaling (40), and it has been

shown that the PI3K signaling pathway plays a central role in promoting androgen independence in an androgen receptor-dependent manner (40). This pathway has also been linked to protection from anoikis (25, 41, 42). In this study, we show that PTHrP increases p-Akt levels and protects against doxorubicin-induced apoptosis through the PI3K/Akt signaling pathway (Fig. 8). We also report that Akt activation in wild-type PTHrP-overexpressing cells is accompanied by increased phosphorylation and therefore inactivation of GSK-3 (Fig. 8). GSK-3 phosphorylates c-myc and targets it to the ubiquitin-proteasome pathway (43). Thus, the observed increase in c-myc levels in wild-type PTHrP-overexpressing cells may also be mediated via the PI3K/Akt pathway. These results therefore establish PI3K signaling as one of the primary mediators of PTHrP-enhanced cell survival under conditions of cell stress.

The effects of PTHrP on anoikis and on the expression of members of the Bcl-2 family of proteins may also be mediated via activation of the PI3K pathway. PI3K signaling is known to activate the NF- κ B pathway, leading to the survival of cells in suspension (31). Here, we show that PTHrP activates the NF- κ B pathway in a PI3K-dependent manner. Several genes with antiapoptotic properties that are regulated by NF- κ B have been identified; these include Bcl-2 and Bcl_{XL} (31). The increase in p-Bad in PTHrP-overexpressing cells is also likely mediated via the PI3K/Akt pathway, because Akt has been reported to phosphorylate Bad (36, 44).

PTHrP functions via both autocrine/paracrine and intracrine pathways (16, 18, 22). Here, we report that the intracrine pathway appears to mediate the antiapoptotic effects of PTHrP, because deleting the NLS negates the protective effect of PTHrP

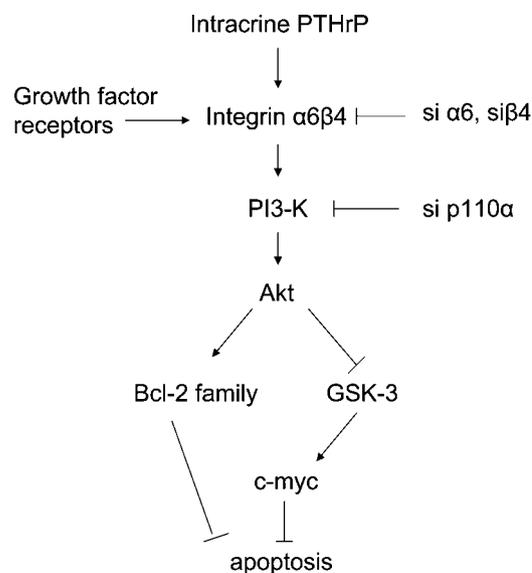


FIGURE 8. Proposed mechanism of intracrine PTHrP regulation of apoptosis, involving integrin $\alpha_6\beta_4$ and the PI3-K/Akt pathway. PTHrP increases cell surface levels of integrin $\alpha_6\beta_4$, which signals synergistically with growth factor receptors to activate the PI3-K/Akt pathway. This results in phosphorylation, and therefore inactivation of GSK-3. Since GSK-3 is known to phosphorylate c-myc, thereby targeting it to the ubiquitin-proteasome pathway, inactivation of GSK-3 results in increased c-myc levels and therefore decreased apoptosis. The PI3-K/Akt pathway also regulates the Bcl-2 family of proteins, resulting in decreased apoptosis. Thus, the net effect of intracrine PTHrP signaling in C4-2 cells is increased cell survival.

against doxorubicin-mediated apoptosis, and there is no significant difference in the levels of members of the Bcl-2 family and in the ratio of antiapoptotic to proapoptotic proteins in control versus NLS-mutated PTHrP-overexpressing cells. Moreover, we show that neutralizing autocrine/paracrine PTHrP action in C4-2 cells using PTHrP- or PTH1R-specific antibodies or siRNAs directed to PTH1R results in decreased, rather than increased, apoptosis. These data suggest that autocrine/paracrine PTHrP action exerts a proapoptotic effect in these cells. PTHrP(1-87) has also been detected in the nucleus of the prostate cancer cell lines PC-3 and PPC-1, resulting in elevated interleukin-8 secretion and increased cell proliferation (45). However, in this study, we did not observe any effects of PTHrP deleted over the NLS (amino acids 88-91 and 102-106) on C4-2 or PC-3 cell apoptosis. PTHrP(1-87) may thus affect cell proliferation but not apoptosis.

The magnitude of the increase in PTHrP secretion in PTHrP-overexpressing versus control cells (~25-fold) is greater than that for PTHrP retained in the cell (5- to 10-fold; Fig. 1), indicating that a greater proportion of the PTHrP generated by the cell is secreted. It therefore appears that relatively small changes in nuclear PTHrP levels may be enough to exert significant effects in the cell. Translocation of PTHrP into the nucleus is cell cycle dependent and involves phosphorylation by p34^{cdc2} at Thr⁸⁵ immediately upstream of the NLS. Phosphorylation results in cytoplasmic retention/nuclear exclusion (46). Although no studies have directly addressed whether exertion of environmental pressure on the cells influences the ratio of secreted to internalized PTHrP, factors that alter cytoplasmic PTHrP levels may indirectly affect its secretion.

Nuclear PTHrP staining has been detected in several tumors, including those of the prostate (reviewed in ref. 47). However, secreted PTHrP levels *in vivo* can only be measured in cases of humoral hypercalcemia of malignancy, which is not prevalent in prostate cancer patients. Therefore, the relative contribution of the autocrine/paracrine versus the intracrine pathway of PTHrP action in patients is not clearly delineated. However, animal studies show a role for intracrine PTHrP action *in vivo*, in that wild-type, but not NLS-mutated, PTHrP increases C4-2 xenograft growth in nude mice; this effect is accompanied by increased nuclear PTHrP immunostaining.⁵ Recently, it has also been shown that knock-in mice expressing PTHrP deleted over the NLS/COOH-terminal domain exhibit retarded growth, early senescence, and malnutrition, leading to a rapid demise postnatally (48). Taken together, these studies underline the importance of intracrine PTHrP action *in vitro* and *in vivo*.

In this and previous studies, we show that PTHrP up-regulates integrin α_6 and β_4 expression at the mRNA and protein levels, with intracrine PTHrP action playing a major role in this effect (23, 24). Integrin $\alpha_6\beta_4$ signals synergistically with growth factor receptors, such as ErbB2, ErbB3, and c-Met (49-51), resulting in activation of the PI3K pathway (26). Here, we show that integrin $\alpha_6\beta_4$ expression is required for activation of PI3K by PTHrP, because PTHrP fails to increase p-Akt levels in wild-type PTHrP-overexpressing cells with suppressed integrin α_6 or β_4 expression (Fig. 7D). We have shown previ-

ously that the prosurvival and promigration effects of PTHrP in MDA-MB-231 cells are mediated via up-regulation of integrin $\alpha_6\beta_4$ expression (52). We therefore propose the following model for intracrine PTHrP action (Fig. 8). PTHrP increases the expression of integrin $\alpha_6\beta_4$ at the mRNA and protein levels, which results in increased integrin $\alpha_6\beta_4$ cell-surface levels. Integrin $\alpha_6\beta_4$ signals synergistically with growth factor receptors, resulting in activation of the PI3K/Akt pathway and increased cell survival.

In conclusion, the results presented here show that PTHrP increases cell survival and anchorage-independent cell growth via an intracrine pathway. The protective effects of PTHrP against doxorubicin-induced cell apoptosis are mediated through the PI3K/Akt pathway and downstream effectors of Akt including GSK-3 β and c-myc as well as by modulating the expression of members of the Bcl-2 family of proteins. These results establish Akt signaling as one of the primary mediators of PTHrP-enhanced cell survival under conditions of cellular stress, with integrin $\alpha_6\beta_4$ providing the link between PTHrP and PI3K/Akt. Strategies aimed at decreasing PTHrP production in prostate cancer may thus provide therapeutic benefits.

Materials and Methods

Materials

Fetal bovine serum (FBS) was obtained from Atlanta Biologicals. Tissue culture supplies were purchased from Life Technologies. Antibodies for Western blot analysis were obtained from Santa Cruz Biotechnology and Cell Signaling Technology. The R-phycoerythrin-conjugated anti- α_6 (clone GoH3) and anti- β_4 antibody (clone 439-9B), as well as the respective isotype control antibodies, were obtained from Pharmingen. The siRNAs targeting the p110 α subunit of PI3K, PTH1R, and the integrin α_6 and β_4 subunits, as well as the NTC siRNA, were purchased from Dharmacon. Doxorubicin and LY49002 were purchased from Calbiochem. The Parameter cAMP assay kit was purchased from R&D Systems.

Plasmid Constructs

A cDNA encoding human PTHrP (obtained from Genentech) was cloned into the *EcoRI* and *HindIII* sites of the expression vector pcDNA3.1(+) (Invitrogen). This construct was used to prepare the PTHrP construct deleted over the NLS (amino acids 88-91 and 102-106). These constructs have been described (21). These constructs, as well as the empty vector control pcDNA3.1(+), were transfected into C4-2 and PC-3 cells by electroporation. The construct containing the NF- κ B site in the context of the -162/+44 human IL-8 promoter, cloned upstream of the luciferase reporter (hIL-8/LUC), has been described (53).

Cell Culture and Transfection

C4-2 cells were purchased from UroCor and grown at 37°C in humidified 95% air/5% CO₂ in RPMI 1640 containing 10% FBS and L-glutamine. PC-3 cells were purchased from the American Type Culture Collection and grown at 37°C in humidified 95% air/5% CO₂ in Ham's F-12 medium supplemented with 10% FBS and L-glutamine. The cells were stably transfected with the PTHrP constructs or with the empty vector by electroporation. Two days after transfection, 600 μ g/mL G418 (geneticin; Life Technologies) was added, and resistant

⁵ Bhatia et al., unpublished observations.

clones were selected. Single clones of stably transfected cells, isolated by limiting dilution in 96-well plates, were transferred to individual flasks and cultured in medium containing 150 $\mu\text{g}/\text{mL}$ G418. Individual clones overexpressing wild-type or NLS-mutated PTHrP were tested for PTHrP mRNA levels by reverse transcription/real-time PCR (27) and for PTHrP secretion using an immunoradiometric assay (Diagnostics Systems Laboratories; ref. 20). The PC-3 clones have been described (21, 24). For assays to measure NF- κB promoter activity, cells (1×10^5) were plated into 6-well dishes. After 48 h, the cells were transfected with 100 ng DNA using Lipofectamine (Invitrogen). After 4 h, the medium was replaced with complete medium containing LY49002 (25 or 50 $\mu\text{mol}/\text{L}$). Luciferase activity was measured after 24 h using the Luciferase Dual-Assay kit (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Measurement of Intracellular cAMP Levels

Cells were plated in 24-well dishes at 5×10^4 per well. When the cells were $\sim 90\%$ confluent, they were treated for 20 min with human PTHrP(1-34) (concentration, 0.1-100 nmol/L) or the PTHrP antagonists [Leu¹¹, D-Trp¹²] hPTHrP-(7-34) amide and [Asn¹⁰, Leu¹¹] hPTHrP-(7-34) amide (25 and 75 nmol/L). Salmon calcitonin and secretin (25 and 75 nmol/L) were used as negative controls. The supernatant was then collected, and the protein concentration of the harvested cell monolayer was determined using the Bio-Rad protein assay. The cAMP concentration in the supernatant was measured using the Parameter cAMP assay kit (R&D Systems), per the manufacturer's protocol.

Western Blot Analysis

Nuclear and cytoplasmic fractions were prepared using the NE-PER extraction kit (Pierce). In all experiments, fractionation was confirmed by probing the blots with anti-lamin B1 and anti- β -actin antibodies, specific for nuclear and cytoplasmic markers, respectively. Western blot analyses using whole-cell, cytoplasmic, and nuclear extracts were done as described previously (27).

Specific Gene Silencing Using siRNA

Cells were plated in 6-well plates at 2×10^5 per well in medium containing 10% FBS (Atlanta Biologicals). After 48 h, the cells were transfected with ON-Target plus siRNAs directed against the human p110 α subunit of PI3K, the PTH1R, or the integrin α_6 or β_4 subunits (100 nmol/L; Dharmacon). In some experiments, the cells were transfected with the integrin α_6 plus integrin β_4 siRNAs. To eliminate the potential for off-target effects, two independent siRNAs were used. As a control, cells were transfected with ON-Target plus NTC siRNAs. Transfections were done using the DharmaFECT 3 transfection reagent (Dharmacon) following the manufacturer's protocol. To measure the mRNA levels, the cells were harvested 48 h after transfection. To measure protein levels, the cells were harvested 72 h after transfection and processed for Western blot or fluorescence-activated cell sorting analysis. In some experiments, the cells were transferred to serum-free medium 56 h after transfection. After 16 h, medium containing 10% FBS was added. Cells were harvested after 30 min and processed for Western blot analysis.

Induction of Cell Apoptosis

Cells were plated in 96-well dishes (1×10^4 per well) in medium containing 10% FBS. After 48 h, the cells were treated with doxorubicin (Calbiochem) at 0.1 or 0.3 $\mu\text{g}/\text{mL}$ for 16 h (C4-2 cells) or 0.3 $\mu\text{g}/\text{mL}$ for 72 h (PC-3 cells) to induce apoptosis. In some experiments, the cells were transfected with a siRNA targeting the p110 α subunit of PI3K, or the PTH1R, or with a NTC siRNA (100 nmol/L; Dharmacon) 48 h after plating. Doxorubicin (0.3 $\mu\text{g}/\text{mL}$) was then added 48 h after transfection. In another set of experiments, the cells were treated with monoclonal antibodies raised against PTHrP(1-34) (Santa Cruz Biotechnology) or the PTH1R(143-169) (Calbiochem). IgG was used as a control. After 48 h, the cells were treated with 0.3 $\mu\text{g}/\text{mL}$ doxorubicin. For LY49002 treatment, cells were grown to 80% confluence and then treated with LY49002 (25 or 50 $\mu\text{mol}/\text{L}$) for 4 h followed by doxorubicin (0.3 $\mu\text{g}/\text{mL}$). Apoptosis was measured after 16 h (C4-2 cells) or 72 h (PC-3 cells) using the Cell Death Detection ELISA PLUS kit (Roche Applied Science). The cells were lysed for 30 min at room temperature by incubation with 200 μL lysis buffer. After centrifugation (10 min at $200 \times g$), 20 μL of the supernatant were transferred onto a streptavidin-coated microplate for quantitation at 405 nm per the manufacturer's protocol.

Quantitative Real-time PCR

Total RNA was extracted using the RNAqueous isolation kit (Ambion), per the manufacturer's protocol. RNA concentrations were determined by spectrophotometry. Reverse transcription/real-time PCRs were done as described (27). The following TaqMan inventoried products were used: PTHrP, Hs00174969_m1; PTHR1, Hs00174895_m1; PIK3CA (PI3K p110 α), Hs00180679_m1; integrin α_6 , Hs00173952_m1; integrin β_4 , Hs00173995_m1; and the predeveloped 18S rRNA primers (VIC-dye labeled probe, TaqMan assay reagent, P/N 4319413E), and were obtained from Applied Biosystems, as was the universal PCR master mix reagent kit (P/N 4304437).

Measurement of Levels of p-Akt and Total Akt, GSK-3, and c-myc

Cells were plated in 100 mm dishes. When the cells had reached 70% to 80% confluence, they were serum starved for 16 h. FBS (10%) was then added for 30 min, and the cells were processed for Western blot analysis. The following antibodies were used: anti-p-Akt (Ser⁴⁷³) and anti-total Akt (Santa Cruz Biotechnology), anti-GSK-3 α/β (Ser²¹/Ser⁹) and anti-total GSK-3 β (Cell Signaling Technology), and anti-c-myc antibody (Santa Cruz Biotechnology).

Anchorage-Independent Cell Growth

Colony formation in soft agar was assayed as described previously (28). Photographs were taken after 2 weeks at high magnification to measure the clone size, using the imaging software ImageJ (NIH). Photographs were also taken at low magnification to measure clone frequency. Five fields per plate were photographed and all clones in focus $>50 \mu\text{m}$ in size were measured. At least two independent experiments were done in triplicate.

Flow Cytometry

Integrin α_6 and β_4 cell-surface levels were measured after staining with R-phycoerythrin-labeled antibodies to the respective subunits, as described (24). Scans are presented as a comparison with the shift obtained using an isotype control antibody as negative control (24).

Statistics

Numerical data are presented as mean \pm SE. Data were analyzed by ANOVA followed by a Bonferroni post-test to determine the statistical significance of differences. All statistical analyses were done using InStat Software (GraphPad Software). $P < 0.05$ was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. David Konkel for critical reading of the article.

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Mol Cancer Res 2009;7:1119-1131. Published OnlineFirst July 7, 2009.

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