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Parathyroid hormone-related protein regulates integrin $\alpha 6$ and $\beta 4$ levels via transcriptional and post-translational pathways

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Abstract

Parathyroid hormone-related protein (PTHrP) enhances prostate cancer (CaP) growth and metastasis *in vivo*. PTHrP also increases cell survival and migration, and upregulates pro-invasive integrin $\alpha 6 \beta 4$ expression. We used the human CaP cell lines C4-2 and PC-3 as model systems to study the mechanisms via which PTHrP regulates $\alpha 6 \beta 4$ levels. We report that PTHrP regulates $\alpha 6$ and $\beta 4$ levels via a transcriptional pathway; $\beta 4$ regulation involves the NF- κ B pathway. PTHrP also regulates $\beta 4$ levels at the post-translational level. PTHrP inhibits caspase-3 and -7 activities. Post-translational regulation of $\beta 4$ by PTHrP is mediated via attenuation of its proteolytic cleavage by these caspases. Since $\alpha 6$ dimerizes with $\beta 4$, increased $\beta 4$ levels result in elevated $\alpha 6 \beta 4$ levels. Suppressing $\beta 4$ using siRNA attenuates the effect of caspase inhibition on apoptosis and cell migration. These results provide evidence of a link between PTHrP, integrin $\alpha 6 \beta 4$ levels as a function of caspase activity, and cell survival and migration. Targeting PTHrP in CaP cancer, thereby reversing the effect on caspase activity and $\alpha 6 \beta 4$ levels, may thus prove therapeutically beneficial.

Keywords

PTHrP; Integrin $\alpha 6 \beta 4$; Caspase; Apoptosis; Migration

Introduction

Cancer of the prostate (CaP) is the most commonly diagnosed malignancy in western men, and is the second most common cause of male cancer deaths worldwide [1]. Due to effective CaP screening programs, an increasing number of men are diagnosed and treated for clinically localized CaP. However, the majority of these will not develop life-threatening disease. Therefore, identification of men who will suffer recurrence of CaP constitutes a great challenge for therapy [2]. Identification of changes associated with lethal disease is a prerequisite for achieving effective patient-based target therapy [3].

Multiple studies have established a role for PTHrP in the prostate. PTHrP is expressed in both normal neuroendocrine cells and the glandular epithelium of normal and benign

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.yexcr.2013.03.003>.

prostatic hyperplasia (BPH) tissues [4,5]. PTHrP expression is increased with progression of normal prostate epithelium to BPH and then to carcinoma [6]. Moreover, there is a positive correlation between CaP stage, as assessed using the Gleason score, and PTHrP levels [7,8]. CaP frequently metastasizes to the bone, and PTHrP is also present in bone metastases sections from human CaP patients [9,10]. The protein is involved in both the initial osteolytic phase and the ensuing osteoblastic phase [11].

Aberrant kinase signaling is a frequent occurrence in CaP and other cancers [12–14]. One of the key pathways that is activated in CaP and that leads to CaP progression is the phosphatidylinositol 3-kinase (PI3-K) pathway. PI3-K activation is associated with increased cell survival and migration [12,14,15]. A key activator of the PI3-K pathway in CaP cells is PTHrP [16]. PTHrP also increases cell-surface levels of the pro-invasive integrin $\alpha_6\beta_4$, this integrin functions as an intermediate in the PTHrP-mediated activation of the PI3-K pathway [16]. Therefore, regulation of integrin $\alpha_6\beta_4$ levels by PTHrP plays a crucial role in the anti-apoptotic and pro-migratory effects of PTHrP. Here we investigated the pathways via which PTHrP regulates levels of the integrin α_6 and β_4 subunits, leading to increased cell survival and migration, parameters which are crucial in CaP progression. 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 [17,18]. C4-2 cells produce mixed lytic/blastic lesions [11]. The androgen-independent PC-3 cell line was initiated from a bone metastasis and produces predominantly lytic lesions.

Deregulated kinase signaling plays a critical role in cancer progression, and specific targeting of pathways that lead to kinase activation holds great therapeutic potential. Integrin $\alpha_6\beta_4$ functions as an intermediate in the PTHrP-mediated activation of the PI3-K pathway. Thus, understanding the pathways via which PTHrP regulates integrin $\alpha_6\beta_4$ levels may lead to the development of strategies that disrupt the PTHrP-mediated activation of the PI3-K pathway in CaP, with potential therapeutic benefits.

Materials and methods

Materials

Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA). Tissue culture supplies were purchased from Life Technologies, Inc. (Gaithersburg, MD). Antibodies for Western blot analysis were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and from Cell Signaling Technology (Danvers, MA). Doxorubicin was purchased from Calbiochem (San Diego, CA). zVAD-fmk and Z-FA-fmk, and the Caspase-Glo 3/7 kit were purchased from Promega (Madison, WI). The Cell Death Detection ELISA PLUS kit was purchased from Roche Applied Science (Indianapolis, IN). The FluoroBlok inserts for analysis of migration were purchased from BD PharMingen (San Jose, CA). Calcein-AM was obtained from Molecular Probes (Eugene, OR).

Plasmid constructs

A cDNA encoding human PTHrP (obtained from Genentech, Inc., South San Francisco, CA) was cloned into the *EcoR*I and *Hind*III sites of the expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA). This construct has been described [19]. This construct, as well as the empty vector control pcDNA3.1(+), were transfected into C4-2 and PC-3 cells by electroporation. The constructs expressing the integrin α_6 and β_4 subunits were obtained from Dr. L. M. Shaw [20]. The integrin β_4 promoter construct (L5.5K) was obtained from Dr. S. Hirohashi [21]. A region extending from –308 to +215 was amplified by PCR from this construct, and cloned into the *Xho*I and *Hind*III sites of pGL3-basic (Promega). For

integrin β_6 , a region extending from -812 to +299 [22] was amplified using genomic DNA as starting material. The PCR product was then cloned into the *XhoI* and *HindIII* sites of pGL3-basic. These constructs were used to prepare the constructs mutated over the putative NF- κ B sites (Fig. 3B). 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 [16].

Cell culture and transfection

C4-2 cells were purchased from UroCor (Oklahoma City, OK) and were grown at 37 °C in humidified 95% air/5% CO₂ in RPMI 1640 medium containing 10% FBS and L-glutamine. PC-3 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and grown at 37 °C in humidified 95% air/5% CO₂ in RPMI 1640 medium supplemented with 8% FBS and L-glutamine. The cells were stably transfected with the PTHrP construct or with the empty vector by electroporation. Individual clones overexpressing PTHrP were tested for PTHrP mRNA levels by reverse transcription/real-time PCR and for PTHrP secretion using an immunoradiometric (IRMA) assay (Diagnostics Systems Laboratories, Webster, TX). These C4-2 and PC-3 clones have been described [23,24].

For assays to measure NF- κ B and integrin β_6 and β_4 promoter activity, cells (1×10^5) were plated into 24-well dishes. After 48 h, the cells were transfected with 200 ng of DNA using Lipofectamine Plus (Invitrogen, Carlsbad, CA). After 4 h, the medium was replaced with complete medium. Luciferase activity was measured after 24 h using the Luciferase Dual-assay kit (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

Specific gene silencing using siRNA

Cells were plated in 6-well dishes at 2×10^5 cells per well in medium containing FBS. After 48 h, the cells were transfected with ON-Target plus siRNAs directed against PTHrP (Dharmacon L00369800; 100nmol/l) or the integrin β_4 subunit (Dharmacon LU00801100; 100nmol/l). To eliminate the potential for off-target effects, two independent siRNAs were used. As a control, cells were transfected with ON-Target plus non-target control (NTC; Dharmacon, D001206140) siRNA. Cells were transfected using the DharmaFECT 3 transfection reagent (Dharmacon) following the manufacturer's protocol. In some experiments, cells were treated with 50 μ M of the broad spectrum caspase inhibitor zVAD-fmk (Promega) 24 h after transfection. Z-FA-fmk (50 μ M, Promega) was used as negative control. In other experiments, cells were treated with zVAD-fmk or Z-FA-fmk plus doxorubicin. 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 analysis.

Western blot analysis

Cells were washed twice with cold PBS on ice and lysed in RIPA buffer containing a Protease Inhibitor cocktail and Phosphatase Inhibitor cocktails A and B (Santa Cruz Biotechnology). Protein concentrations were estimated using the Bio-Rad protein assay. Protein levels were analyzed by Western blot analysis as previously described [24]. GAPDH was used as loading control. The signals were detected using the SuperSignal West Pico Substrate kit (Pierce Biotechnology Inc., Rockford, IL). Densito-metric analysis was performed using the Alpha Innotech Image Analysis system (Alpha Innotech Corporation, San Leandro, CA).

Determination of integrin $\alpha 6$ and $\beta 4$ protein half-life

To ask whether PTHrP regulates integrin $\alpha 6$ and $\beta 4$ protein levels via a post-translational pathway, the protein $t_{1/2}$ were measured in PTHrP-overexpressing and control PC-3 and C4-2 cells. Cells were plated in 6 well dishes at 2×10^5 cells/dish. When the cells were ~70% confluent, they were treated with cycloheximide (CHX) to a final concentration of 150 μ g/ml. The cells were harvested at intervals ranging from 0 to 72 h. A portion of the cell suspension was used to measure cell viability using trypan blue exclusion. The rest of the cell suspension was used to prepare cell extracts for Western blot analysis. To detect integrin $\alpha 6$, an anti-integrin $\alpha 6$ antibody (Santa Cruz Biotechnology, sc-10730) was used. Since integrin $\beta 4$ undergoes proteolysis by cleavage of the cytoplasmic domain [25], an antibody that recognizes the cytoplasmic domain (BD Biosciences, clone 11A, 555722) was used. An anti-GAPDH antibody (Santa Cruz Biotechnology, sc-25778), was used to detect GAPDH (loading control).

Analysis of integrin $\beta 4$ profile

To determine the cleavage pattern of the integrin $\beta 4$ subunit in PTHrP-overexpressing and control cells, and the role of the PTHrP-mediated caspase inhibition on this pattern, cells were treated for 16 h with 10 μ M of the 26 S proteasome inhibitor *N*-carboben-zoxyl-Leu-Leu-leucinal (MG132; Sigma) plus 50 μ M of the caspase inhibitor zVAD-fmk. Z-FA-fmk was used as negative control. Cell extracts were processed for Western blot analysis.

To measure ubiquitinated levels of the integrin $\beta 4$ subunit, PTHrP-overexpressing and control cells were transfected with constructs expressing the integrin $\beta 4$ subunit and HA-Ub. Twenty-four hour after transfection, the cells were co-treated with 10 μ M MG132 and 50 μ M zVAD-fmk or Z-FA-fmk for 16 h. The cells were washed twice with 1x PBS and lysed with immunoprecipitation buffer containing Protease Inhibitor cocktail and Phosphatase Inhibitor cocktails A and B (Santa Cruz Biotechnology) plus 5 μ M *N*-ethylmaleimide and 0.01 μ M ubiquitin aldehyde. Protein concentrations in the cell lysates were estimated using the Bio-Rad protein assay. Equal amounts of cell lysate protein were then incubated with the anti-integrin $\beta 4$ antibody and Protein A/G agarose at 4 °C for 3 h. After washing, bound proteins were analyzed by SDS-PAGE and Western blotting using an anti-HA antibody (Santa Cruz Biotechnology, sc-805). The blots were then stripped and re-probed with the anti-integrin $\beta 4$ antibody.

Induction of cell apoptosis and measurement of apoptosis and caspase activity

Cells were plated in 96-well plates (1×10^4 cells/well) in medium containing FBS. In some experiments, the cells were pre-treated with 50 μ M zVAD-fmk or Z-FA-fmk (negative control) for 1 h. They were then treated with doxorubicin (Calbiochem) at 0.1 or 0.3 μ g/ml for 16 h (C4-2 cells) or for 72 h (PC-3 cells) to induce apoptosis. In some experiments, the cells were transfected with 100 nmol/l of the PTHrP or integrin $\beta 4$ siRNA or with the NTC 48 h after plating. zVAD-fmk (or Z-FA-fmk) and doxorubicin were then added. Apoptosis was measured 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 μ l of lysis buffer. After centrifugation (10 min at $200 \times g$), 20 μ l of the supernatant was transferred onto a streptavidin-coated microplate for quantitation at 405 nm per the manufacturer's protocol.

To assess the effect of PTHrP on caspase activity, cells plated in 96-well dishes were treated with zVAD-fmk or Z-FA-fmk (negative control) and doxorubicin as described above. Caspase activity was measured using the Caspase-Glo 3/7 kit (Promega). Caspase-GloR 3/7 Reagent (100 μ l) was added to the wells, and the plate was shaken at 500 rpm for 30 s using

a plate shaker. After incubation at 25 °C for 1 h, the luminescence was measured using a Synergy 2 Microplate reader (BioTek).

Active caspase levels were also determined by Western blot analysis. Cells (2×10^5) were plated in 6-well dishes until ~70% confluent. They were then treated with doxorubicin as described above. Active caspase-3 levels were detected using an antibody that detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175 (Cell Signaling, 9661). This antibody does not recognize full length caspase-3 or other cleaved caspases.

Measurement of cell migration

Cell migration was measured using the FluoroBlok system (BD Pharmingen) as previously described [26]. Briefly, cells were grown to ~70% confluence. They were then pre-treated for 1 h with zVAD-fmk as described above. In some experiments, cells were transfected with the PTHrP or integrin $\alpha 6$ siRNA or NTC (100 nmol/l; Dharmacon), then treated with zVAD-fmk or Z-FA-fmk 24 h after transfection. Pre-treated and untreated cells were then trypsinized, washed once with medium containing 10% FBS, then once in serum-free medium, and resuspended in PBS. After counting, 0.5×10^6 cells were loaded with Calcein-AM (10 μ mol/l; Molecular Probes), and incubated for 45 min in a humidified incubator at 37 °C in 95% air/5% CO₂. After centrifugation, the cells were washed once in FBS-containing medium to quench the Calcein-AM, and once in serum-free medium to wash out the FBS. After resuspension in serum-free medium, the cells were plated onto FluoroBlok inserts (BD Pharmingen). As a control, cells were plated onto clear inserts. Medium containing 10% FBS was added to the lower chamber as a chemoattractant. As a negative control, medium containing 0.1% BSA was placed in the lower chamber. After incubation for 4 h in a humidified incubator at 37 °C in 95% air/5% CO₂, the plates were scanned with a bottom-reading Fluroscan Ascent CF Microplate Fluorometer (Thermo Labsystems, Franklin, MA), using an excitation wavelength of 485 nm, and an emission wavelength of 530 nm. Cell migration was calculated as follows: cell migration (%) = (total fluorescence intensity in the presence of FluoroBlok insert / total fluorescence intensity in the presence of clear insert) $\times$ 100. Pilot studies were performed to verify a linear and reproducible relationship between cell number and fluorescence intensity (data not shown).

Statistics

Numerical data are presented as the mean + SEM. The data were analyzed by ANOVA, followed by a Bonferroni post-test to determine the statistical significance of differences. All statistical analyses were performed using Instat Software (GraphPad Software, Inc, San Diego, CA). $P < 0.05$ was considered significant.

Results

Positive correlation between levels of PTHrP and the integrin $\alpha 6$ and $\beta 4$ subunits

C4-2 and PC-3 cells were engineered to overexpress PTHrP (1–139) by stable transfection with a construct encoding PTHrP (1–139). Empty vector transfectants were used as controls. These cells have been described [23,24]. Suppression of PTHrP expression was achieved by transfection with a PTHrP siRNA [16]. Non-target control (NTC) siRNA was used as control. Since C4-2 cells express low levels of PTHrP, this approach was only used in PC-3 cells. RT/real-time PCR and Western blot analysis confirmed the expected alterations in PTHrP levels in these clones (Fig. 1A – D). We report a positive correlation between levels of PTHrP and those of the integrin $\alpha 6$ and $\beta 4$ subunits at both the mRNA and protein levels (Fig. 1A – D). Moreover, we show that the transfection of PTHrP-overexpressing PC-3 cells

with a PTHrP-targeting siRNA significantly decreases PTHrP mRNA levels; this decrease was accompanied by decreases in integrin $\alpha 6$ and $\beta 4$ mRNA levels (Fig. 1A).

PTHrP increases the promoter activity of the integrin $\alpha 6$ and $\beta 4$ subunits

To ask if the effects of PTHrP on integrin $\alpha 6$ and $\beta 4$ levels are mediated via a transcriptional pathway, we compared integrin $\alpha 6$ and $\beta 4$ promoter activity in PTHrP-overexpressing and control C4-2 and PC-3 cells. Since promoter activity was low in control cells, we did not measure activity in cells with suppressed PTHrP levels.

In both C4-2 and PC-3 cells, promoter activity of the integrin $\beta 4$ subunit was elevated by ~3-fold in PTHrP-overexpressing vs. control cells (Fig. 2A and B). Integrin $\alpha 6$ promoter activity was elevated by ~2.5-fold in PTHrP-overexpressing vs. control PC-3 cells (Fig. 2A). In contrast, PTHrP had no effect on integrin $\alpha 6$ promoter activity in C4-2 cells (Fig. 2B).

Role of NF- κ B in transcriptional activation of the integrin $\alpha 6$ and $\beta 4$ promoters by PTHrP

Putative NF- κ B sites are present within the promoter regions of both the integrin $\alpha 6$ and $\beta 4$ subunits [21,22,27]. Using a reporter construct containing the NF- κ B site in the context of the -162/+44 human IL-8 promoter, here we show a direct correlation between PTHrP levels and NF- κ B transcriptional activity (Fig. 3A). Only PC-3 cells were used to measure NF- κ B activity after PTHrP suppression, since parental C4-2 cells express low levels of PTHrP.

The integrin $\beta 4$ promoter has two putative NF- κ B sites located at -208 bp and +86 bp with reference to the transcription start site [21]. The integrin $\alpha 6$ promoter has one putative NF- κ B site located at position -171 bp with reference to the transcription start site [22,27]. To ask if regulation of integrin $\alpha 6$ and/or $\beta 4$ promoter activity by PTHrP involves NF- κ B, these putative sites within the promoters were mutated as shown in Fig. 3B. Wild-type and mutated integrin $\beta 4$ promoter constructs were transfected into PTHrP-overexpressing and control PC-3 and C4-2 cells. For the integrin $\alpha 6$ promoter, only PC-3 cells were used, since PTHrP does not regulate integrin $\alpha 6$ promoter activity in C4-2 cells (Fig. 2B).

In control C4-2 and PC-3 cells, mutation of either the upstream or downstream NF- κ B site within the integrin $\beta 4$ promoter had no significant effect on promoter activity (Fig. 3D and E). However, mutating both the upstream and downstream NF- κ B sites resulted in an ~2-fold decrease in promoter activity (Fig. 3D and E). In PTHrP-overexpressing cells, individual mutation of the NF- κ B sites within the integrin $\beta 4$ promoter significantly decreased promoter activity (Fig. 3D and E). Mutating both NF- κ B sites resulted in a significantly greater response than mutation of either site alone (3–5-fold decrease; Fig. 3D and E). These data indicate that the NF- κ B pathway plays a role in regulation of integrin $\beta 4$ promoter activity. The greater response observed in the presence of high PTHrP levels may be attributed to the fact that NF- κ B activity is significantly higher in PTHrP-overexpressing vs. control cells [16]. The lack of response in control C4-2 and PC-3 cells may be due to a low basal level of NF- κ B activity in these cells, whereby other pathways independent of NF- κ B may regulate integrin $\beta 4$ promoter activity.

Since PTHrP only regulates integrin $\alpha 6$ promoter activity in PC-3 cells (Fig. 2), the effect of mutating the putative NF- κ B site on promoter activity was only investigated in this cell line. In contrast to the data obtained with the integrin $\beta 4$ promoter, here we observed that mutating this site had no effect on integrin $\alpha 6$ promoter activity (Fig. 3C). Thus, regulation of integrin $\alpha 6$ mRNA levels by PTHrP appears to be mediated via pathways independent of NF- κ B.

PTHrP increases the protein half-life of the integrin $\alpha 6$ and $\beta 4$ subunits

PTHrP may also regulate cell-surface integrin $\alpha 6$ and $\beta 4$ protein levels via a post-translational pathway. To ask if PTHrP regulates the integrin $\alpha 6$ and $\beta 4$ protein half-life ($t_{1/2}$), this parameter was measured in PTHrP-overexpressing PC-3 and C4-2 cells. The integrin $\beta 4$ subunit undergoes proteolytic processing and multiple bands are typically evident by Western blot analysis [28]. Since proteolytic degradation involves cleavage of the cytoplasmic domain, Western blot analyses to determine whether PTHrP alters the integrin $\beta 4$ half life were performed using an antibody that recognizes this domain [25].

We show that the protein $t_{1/2}$ of both the integrin $\alpha 6$ and $\beta 4$ subunits is significantly higher (~2-fold increase) in PC-3 cells overexpressing PTHrP vs. that in control cells (Fig. 4A to F). Similar results were obtained in C4-2 cells (data not shown). No significant change in cell viability after cycloheximide treatment (measured by trypan blue exclusion) was observed at any of the time-points measured (data not shown). Pulse-chase experiments performed using PC-3 cells confirmed the data obtained using cycloheximide (supplemental data).

PTHrP decreases active caspase-3 and caspase-7 levels

Post-translational regulation of the integrin $\beta 4$ subunit by PTHrP may involve altered degradation. Since the integrin $\beta 4$ subunit is cleaved by caspase-3 and -7 [25], here we asked if PTHrP decreases proteolytic cleavage of the integrin $\beta 4$ subunit through an effect on levels of caspase-3 and caspase-7. Caspase activities were significantly lower in untreated PTHrP-overexpressing vs. control PC-3 and C4-2 cells (Fig. 5A and B). Transfection with the PTHrP siRNA resulted in an increase in caspase activity in PC-3 cells (Fig. 5A). In PC-3 cells, doxorubicin treatment increased levels of these caspases in PTHrP-overexpressing and control cells, as well as in cells with suppressed PTHrP expression (Fig. 5A). However, even after doxorubicin treatment, caspase levels were significantly lower in PTHrP-overexpressing cells (Fig. 5A). In C4-2 cells, doxorubicin also increased caspase levels in both PTHrP-overexpressing and control cells (Fig. 5B). Again, caspase levels were significantly lower in PTHrP-overexpressing cells vs. control cells (Fig. 5B).

Levels of active caspase-3 were also measured by Western blotting. Activation of caspase-3 requires cleavage of the procaspase-3 form, giving rise to the active caspase-3 moieties of 17–19 kDa [29]. Active caspase-3 levels were higher in control vs. PTHrP-overexpressing PC-3 and C4-2 cells (Fig. 5C and D). Suppressing PTHrP expression in PC-3 cells resulted in enhanced levels of active caspase-3 (Fig. 5C). Treatment with doxorubicin increased active caspase-3 levels to a significantly higher extent in control vs. PTHrP-overexpressing cells (Fig. 5C and D).

Inhibition of caspase activity results in elevated full-length integrin $\beta 4$ levels

The integrin $\beta 4$ subunit has a large cytoplasmic tail spanning ~10³ amino acids [20,30]. Integrin $\beta 4$ is cleaved by caspase-3 and -7 at Asp¹¹⁰⁹, generating an unstable carboxyl-terminal product that is rapidly degraded by the proteasome [25]. Here we investigated the relationship between caspase activity and integrin $\beta 4$ levels in whole-cell extracts of PTHrP-overexpressing and control PC-3 and C4-2 cells. Data in Fig. 6A are shown for PC-3 cells. A similar profile was obtained for C4-2 cells (data not shown). Levels of both the integrin $\alpha 6$ and $\beta 4$ subunits are higher in PTHrP-overexpressing vs. control cells (Fig. 1C and D). Here we show that the relative levels of full-length integrin $\beta 4$ (~220 kD) is higher in PTHrP-overexpressing vs. control cells (Fig. 6A). Proteolysis products (mol. wt.<220kD) are evident in both PTHrP-overexpressing and control cells. However, the relative level of lysed integrin $\beta 4$ products (bands of mol. wt.<220 kD compared to ~220 kD band) is greater in control cells (Fig. 6A). Inhibition of caspase activity using the broad spectrum caspase

inhibitor zVAD-fmk resulted in a decrease in cleaved integrin $\alpha 4$ products in both PTHrP-overexpressing and control cells (Fig. 6A). Z-FA-fmk (negative control) had no effect on the integrin $\alpha 4$ lyses pattern spectrum (data not shown).

Inhibiting caspase activity with zVAD-fmk also resulted in an increase in integrin $\beta 4$ levels. However, no change in the profile of this subunit was observed in the presence or absence of zVAD-fmk (Fig. 6A).

PTHrP overexpression decreases ubiquitinated levels of the integrin $\beta 4$ subunit

Caspase-mediated proteolysis of the integrin $\alpha 4$ subunit generates a product that is rapidly degraded by the proteasome pathway. Proteasomal degradation requires conjugation to ubiquitin [31]. Here we measured ubiquitinated integrin $\alpha 4$ levels in PTHrP-overexpressing and control PC-3 and C4-2 cells transiently transfected with a construct expressing integrin $\alpha 4$ [20]. We show that ubiquitinated integrin $\alpha 4$ levels are significantly higher in control vs. PTHrP-overexpressing PC-3 cells (Fig. 6B). Incubation in the presence of z-VAD-fmk resulted in a decrease in ubiquitinated integrin $\alpha 4$ levels (Fig. 6B). A similar profile was obtained in C4-2 cells (data not shown). These results confirm that PTHrP plays a role in degradation of the integrin $\alpha 4$ subunit in a caspase-dependent pathway.

Regulation of integrin $\beta 4$ levels by PTHrP as a result of modulation of caspase activity results in alterations in cell survival and migration

An inverse correlation was observed between PTHrP levels and basal levels of apoptosis in PC-3 and C4-2 cells (Fig. 7A and B). Thus, apoptosis was lower in PTHrP-overexpressing PC-3 and C4-2 cells (Fig. 7A and B) and suppressing PTHrP expression in PC-3 cells resulted in an increase in apoptosis (Fig. 7A). Moreover, PTHrP decreased the sensitivity of PC-3 and C4-2 cells to doxorubicin-induced apoptosis. Thus, apoptosis was lower in PTHrP-overexpressing PC-3 and C4-2 cells treated with doxorubicin (Fig. 7A and B). In contrast, PC-3 cells with suppressed PTHrP expression were more sensitive to doxorubicin.

A direct correlation between PTHrP levels and PC-3 and C4-2 cell migration was also observed, with migration being higher in PTHrP-overexpressing PC-3 and C4-2 cells (Fig. 7C and D). PTHrP-siRNA-transfected PC-3 cells had a lower migration (Fig. 7C).

Proteolysis of the integrin $\alpha 4$ subunit sensitizes cells to apoptosis and inhibits cell migration [25]. Here we investigated the link between PTHrP and integrin $\beta 4$ levels, caspase activity, and cell apoptosis and migration. PTHrP-overexpressing and control PC-3 and C4-2 cells were transfected with siRNAs directed against the integrin $\alpha 4$ subunit or with NTC siRNA [16]. As previously reported [16], integrin $\alpha 4$ levels were higher in PTHrP-overexpressing vs. control cells (Fig. 8A). Transfection with the integrin $\alpha 4$ -directed siRNA resulted in an ~75% decrease in integrin $\alpha 4$ levels (Fig. 8A). PTHrP overexpression caused a significant decrease in apoptosis and a significant increase in cell migration (Fig. 8B and C). Inhibiting caspase activity decreased apoptosis and increased cell migration in both PTHrP-overexpressing and control cells (Fig. 8B and C). However, after transfection with the integrin $\alpha 4$ siRNA, the effect of caspase inhibition on apoptosis and cell migration was significantly attenuated. Thus, these parameters were not significantly different in integrin $\alpha 4$ siRNA-transfected cells treated or untreated with zVAD-fmk (Fig. 8B and C). Z-FA-fmk had no effect on apoptosis or migration (data not shown). Data are shown for PC-3 cells; similar effects were obtained in C4-2 cells (data not shown). These data indicate that integrin $\alpha 4$ acts as an intermediate linking the effects of PTHrP on caspase activity to those on cell survival and migration.

Discussion

Multiple studies have shown that PTHrP expression is increased in CaP cells compared to normal prostate epithelium and BPH [5,6,32]. PTHrP also plays a role in the development of the osteoblastic/osteolytic lesions that accompany prostate cancer [11]. However, the molecular pathways via which PTHrP exerts its effects have not yet been fully elucidated. Overexpression of PTHrP is accompanied by increased expression of the pro-invasive integrin $\alpha_6\beta_4$ [16]. These effects of PTHrP are mediated via an intracrine pathway, in that deletion of the nuclear localization signal within PTHrP suppresses its effects on integrin α_6 and β_4 levels and on apoptosis [23]. Integrin $\alpha_6\beta_4$ signals synergistically with growth factor receptors, such as ErbB2, ErbB-3 and c-Met [33–35] to activate the PI3-K pathway [20]. Regulation of integrin $\alpha_6\beta_4$ levels by PTHrP functions as an intermediate linking PTHrP to activation of PI3-K, and it is via activation of this pathway that PTHrP increases cell survival and migration [16]. However, the mechanisms via which PTHrP regulates integrin $\alpha_6\beta_4$ levels have not been elucidated. Since this integrin plays a key role in diverse epithelial cell functions, including cell survival and migration [20,36], it is important to elucidate the pathways via which it is regulated. Here we show that PTHrP regulates integrin $\alpha_6\beta_4$ levels via both transcriptional and post-translational pathways.

PTHrP increases integrin α_6 and β_4 mRNA levels [16]. Here we show that, for the integrin α_6 subunit, this increase involves a transcriptional pathway in PC-3, but not in C4-2, cells. This cell type-specific regulation of integrin α_6 promoter activity may be dependent on levels of specific transcription factors involved in regulation of this promoter in the different cell lines. Regulation of integrin α_6 mRNA levels by PTHrP in C4-2 cells may involve post-transcriptional pathways. In contrast, PTHrP increases integrin β_4 promoter activity in both PC-3 and C4-2 cells.

Multiple putative transcriptional sites are present in the integrin α_6 and β_4 promoters, including NF- κ B, CREB/AP-1, SP-1, and c-myc [21,22,27]. Here we focused on NF- κ B since PI3-K signaling is known to activate this pathway, leading to increased cell survival [37]. Moreover, using an NF- κ B reporter construct, we have previously shown that PTHrP increases NF- κ B activity [16]. While both the integrin α_6 and β_4 promoters contain putative NF- κ B sites [21,22,27], here we show that this pathway is only involved in regulating integrin β_4 promoter activity. This promoter contains two putative NF- κ B sites [21]. The lack of effect of mutating individual sites in control (empty vector-transfected) PC-3 and C4-2 cells may be due to the fact that constitutive NF- κ B activity is low in these cells, thereby preventing an observable effect on promoter activity. In contrast, mutating individual NF- κ B sites in PTHrP-overexpressing cells significantly decreased integrin β_4 promoter activity. This effect was greater when both sites were mutated, indicating an additive or synergistic effect between the two NF- κ B sites. Since NF- κ B is not involved in regulation of the integrin α_6 subunit in PTHrP-overexpressing or control cells, alternate transcriptional pathways may be involved. A possible pathway via which PTHrP may regulate integrin α_6 levels is through CREB/AP1. This pathway is known to be activated by PTHrP [38] and a putative CREB/AP1 site is present within the integrin α_6 promoter [22,27]. Future studies will identify the pathways via which the integrin α_6 promoter is regulated by PTHrP.

PTHrP also increases the protein half-life of the integrin α_6 and β_4 subunits. The integrin β_4 subunit is unique among integrins in that, in contrast to the short intracellular domains of other integrins, it has a large cytoplasmic tail which spans approximately 1000 amino acids [30,39]. Werner et al. [25] have shown that this subunit is cleaved by caspase-3 and -7 at a conserved Asp residue (Asp1109), resulting in the removal of most of its cytoplasmic tail. The caspase cleavage products of integrin β_4 are an unstable carboxyl-terminal product that

is rapidly degraded by the proteasome pathway and an amino-terminal cleavage product (amino acids 1–1109) that is unable to assemble into mature hemidesmosomes [25]. Cleavage results in sensitization of the cells to apoptosis and inhibition of cell migration [25]. Here we show that PTHrP decreases both constitutive and doxorubicin-induced caspase-3 and -7 activities. Given that relative levels of full-length vs. cleaved integrin $\alpha 4$ are higher in PTHrP-overexpressing vs. control cells, and that inhibition of caspase activity protects against cleavage, we postulate that modulation of caspase activity by PTHrP contributes to the observed increase in integrin $\alpha 4$ levels.

We have previously shown that PTHrP protects C4-2 and PC-3 cells from doxorubicin-induced apoptosis and increases cell migration [16]. Integrin $\alpha 6 \beta 4$ plays a major role in mediating these pro-survival and pro-migratory effects of PTHrP [16,40]. Here we show a direct link between regulation of integrin $\alpha 4$ levels via the caspase pathway and modulation of apoptosis and cell migration, in that the effect of caspase inhibition on these parameters is attenuated in cells with suppressed integrin $\alpha 4$ expression. PTHrP also regulates members of the Bcl-2 family of proteins, and the anti-apoptotic effects of PTHrP are accompanied by increases in levels of anti-apoptotic proteins and decreases in pro-apoptotic proteins [16]. Increased caspase-3 activation is cell migration, in that the effect of caspase family, with increased expression of the proapoptotic protein Bax and decreased expression of the anti-apoptotic protein Bcl-2 [41]. The effect of PTHrP on levels of these proteins may also be mediated via regulation of caspase activity.

Integrin $\alpha 6$ is not a substrate for caspases. The integrin $\alpha 6$ and $\beta 4$ subunits heterodimerize intracellularly as precursors, prior to further processing in the Golgi from where the mature $\alpha 6 \beta 4$ heterodimer is transported to the cell surface [42]. Thus, we postulate that both the increased protein $t_{1/2}$ of the integrin $\alpha 6$ subunit and the increase in protein levels after caspase inhibition may be secondary to the effects on the $\beta 4$ subunit. Heterodimerization of the $\alpha 6$ subunit with the $\beta 4$ subunit may make it unavailable for cellular degradation.

In conclusion, the results presented here demonstrate that PTHrP regulates integrin $\alpha 6$ and $\beta 4$ levels via both transcriptional and post-translational pathways. Transcriptional regulation of the integrin $\beta 4$ subunit involves the NF- κ B pathway. PTHrP also inhibits caspase activity. Integrin $\beta 4$ is cleaved by caspase-3 and caspase-7, and post-translational regulation of this subunit by PTHrP is caspase-dependent. Since the integrin $\alpha 6$ subunit dimerizes with the integrin $\beta 4$ subunit, integrin $\beta 4$ stabilization in turn results in elevated integrin $\alpha 6$ levels. The net result is increased cell surface levels of pro-invasive integrin $\alpha 6 \beta 4$, resulting in increased cell survival and migration. These results establish integrin $\beta 4$ and integrin $\alpha 6 \beta 4$ signaling as a primary pathway mediating PTHrP-enhanced cell migration and cell survival under conditions of cellular stress. Strategies aimed at decreasing PTHrP production in prostate cancer may thus provide therapeutic benefits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Levels of PTHrP, and the integrin $\alpha 6$ and $\beta 4$ subunits in PC-3 and C4-2 cells transfected with a PTHrP-expressing construct or with PTHrP siRNA. (A) and (B) mRNA levels of PTHrP, and of the integrin $\alpha 6$ and $\beta 4$ subunits were measured by reverse transcription/real-time PCR. Values are expressed relative to the corresponding control (C) value, set arbitrarily at 1.0. For PC-3 cells, this C value represents the mean $\pm$ SEM of the values obtained for cells transfected with empty vector (control for PTHrP⁺), NTC siRNA (control for PTHrP siRNA-transfected cells), and empty vector+NTC siRNA (control for PTHrP⁺+PTHrP siRNA-transfected cells). For C4-2 cells, C is the mean $\pm$ SEM for cells transfected with empty vector (control for PTHrP⁺). Each bar is the mean $\pm$ SEM of three independent experiments. * = Significantly different from C value ($P < 0.001$). # = Significantly different from PTHrP⁺ value. (C) and (D) Western blot analysis for PTHrP, and integrins $\alpha 6$ and $\beta 4$. GAPDH was used as loading control. In (A)-(D), PTHrP⁺ = PTHrP-overexpressing cells; C = control cells; PTHrP si = cells transfected with PTHrP siRNA.

Fig. 2.

Integrin $\alpha 6$ and $\beta 4$ promoter activity in PTHrP-overexpressing and control PC-3 and C4-2 cells. Luciferase activity is expressed relative to that in empty vector-transfected control cells (C), set arbitrarily at 1.00. PTHrP⁺=PTHrP-overexpressing cells. Firefly luciferase activity was normalized to Renilla luciferase activity. Each bar is the mean $\pm$ SEM of three independent experiments. Values for two independent clones are shown. *=Significantly different from the control value ($P<0.001$).

Fig. 3.

(A) NF-κB activity as a function of PTHrP expression. Luciferase activity is expressed relative to that in control cells (C), set arbitrarily at 1.00 and defined as in Fig. 1. (B) Putative NF-κB sites in the α6 and β4 integrin promoter and site directed mutations of these sites. In the NF-κB consensus sequence, g=guanine, R=purine, Y=pyrimidine, and N=any nucleotide. Mutated nucleotides are underlined. (C)-(E) Promoter activity of the wild-type and mutated integrin α6 (C) and β4 (D) and (E) promoters in PTHrP-overexpressing and control PC-3 and C4-2 cells. Luciferase activity is expressed relative to that in wild-type empty vector-transfected control cells (C), set arbitrarily at 1.00. In (A) and (C)-(E), PTHrP⁺=PTHrP-overexpressing cells; C=control cells; PTHrP si=cells transfected with PTHrP siRNA. Firefly luciferase activity was normalized to Renilla luciferase activity. Each bar is the mean±SEM of three experiments for each of two independent clones. WT=wild-type, M=mutated (α6); M1=mutation of upstream NF-κB site (β4); M2=mutation of downstream NF-κB site (β4); M1+M2=mutated over both upstream and downstream NF-κB sites (β4). *=Significantly different from the respective empty vector-transfected value ($P < 0.001$); #=significantly different from the respective wild-type value ($P < 0.001$).

Fig. 4.

Protein half life ($t_{1/2}$) of the integrin $\alpha 6$ and $\beta 4$ subunits in PTHrP-overexpressing and control PC-3 cells. Cells were treated with cycloheximide for the indicated time periods. Empty vector-transfected cells were used as controls. PTHrP⁺=PTHrP-overexpressing cells. Integrin $\alpha 6$ and $\beta 4$ levels were analyzed by Western blot analysis at the indicated time points. (A and B) Representative Western blots showing decay of the integrin $\alpha 6$ (A) and $\beta 4$ (B) subunits. GAPDH was used as loading control. (C) and (D) Densitometric analysis of the decay of the integrin $\alpha 6$ and $\beta 4$. Each point is the mean $\pm$ SEM of data from three independent experiments. The 0 time-point (at the time of cycloheximide addition) is set at 100%. (E) and (F) Comparison of the half-life of the integrin $\alpha 6$ and $\beta 4$ subunits in PTHrP-overexpressing and control cells. The values were obtained from the data presented in (C) and (D). *=Significantly different from the vehicle control value ($P<0.001$).

Fig. 5.

Caspase activity in PC-3 and C4-2 cells transfected with a PTHrP-expressing construct or with PTHrP siRNA. (A) and (B) Caspase activity. Cells were treated with the indicated concentrations of doxorubicin (dox) for 16 h (C4-2) or 72 h (PC-3). Caspase activity was measured as described in Materials and methods. The control value (C) is defined as in Fig. 1. Each bar is the mean $\pm$ SEM of three independent experiments for each of two independent clones (PTHrP⁺) or PTHrP siRNAs. * = Significantly different from the respective no doxorubicin (dox) value ($P < 0.001$). # = Significantly different from the respective C value ($P < 0.001$). (C) and (D) Western blot analysis for active caspase-3 levels (17–19 kDa) in cells treated with doxorubicin (0.3 μ g/ml). Each figure is representative of three independent experiments. In (A)–(D), PTHrP⁺ = PTHrP-overexpressing cells; C = control cells; PTHrP si = cells transfected with PTHrP siRNA.

Fig. 6.

Effect of caspase inhibition on the integrin $\alpha 4$ and $\alpha 6$ subunit profiles in PTHrP-overexpressing and control PC-3 cells. Cells were treated with MG132 (10 μ M) in the presence (+) or absence (-) of zVAD-fmk. Z-FA-fmk was used as negative control (-). The cells were harvested after 16 h and lysates were prepared for Western blotting. Equal loading was confirmed by re-probing with GAPDH. (B) Ubiquitinated (Ub) integrin $\alpha 4$ levels in PTHrP-overexpressing and control PC-3 cells. PTHrP-overexpressing and control PC-3 cells were co-transfected with an integrin p4-expressing construct and a HA-Ub construct. After transfection, cells were treated with MG132 in the presence (+) or absence (-) of zVAD-fmk. Z-FA-fmk was used as negative control (-). Cells were harvested after 16 h and lysates immunoprecipitated with the anti-integrin $\alpha 4$ antibody. The ubiquitination of integrin $\alpha 4$ was detected using the anti-HA antibody. The blot was stripped and re-probed with the anti-integrin $\alpha 4$ antibody. In (A) and (B), each figure is representative of three independent experiments.

Fig. 7.

(A) and (B) Apoptosis and (C) and (D) migration of PC-3 and C4-2 cells transfected with a PTHrP-expressing construct or with PTHrP siRNA (A) and (B) Cells were treated with the indicated concentrations of doxorubicin (dox) for 16 h (C4-2) or 72 h (PC-3). Cell apoptosis and migration were measured as described in Materials and methods. The control value (C) is defined as in Fig. 1. Each bar is the mean $\pm$ SEM of three independent experiments for each of two independent clones (PTHrP⁺) or PTHrP siRNAs. (A) and (B) * = Significantly different from the respective no doxorubicin (dox) value ($P < 0.001$). # = Significantly different from the respective C value ($P < 0.001$). (C) and (D) * = Significantly different from the control value ($P < 0.001$).

Fig. 8.

Apoptosis and migration of PTHrP-overexpressing and control PC-3 cells with suppressed integrin 4 expression. Cells were transfected with an siRNA targeting the integrin 4 subunit. NTC siRNA was used as control. (A) Western blot analysis for integrin 4 levels in cells transfected with the integrin 4-targeting or NTC siRNA (B) Apoptosis and (C) cell migration were measured in the presence (+) or absence (-) of zVAD-fmk. Z-FA-fmk was used as negative control (-). Apoptosis was induced by treatment with doxorubicin (0.3 µg/ml). Each bar is the mean±SEM of three independent experiments for each of two independent integrin 4-targeting siRNAs. *—Significantly different from the respective NTC value ($P < 0.001$); #—significantly different from the -zVAD-fmk value ($P < 0.001$).