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PTHrP promotes colon cancer cell migration and invasion in an integrin $\alpha 6 \beta 4$ -dependent manner through activation of Rac1

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Abstract

Parathyroid hormone-related protein (PTHrP) is expressed by human colon cancer tissue and cell lines. Rac1 GTPase enhances colon cancer cell migration and invasion. Here we report a positive correlation between PTHrP expression and Rac1 activity in LoVo (human colon cancer) cells. The positive effects of PTHrP on Rac1 activity and on cell migration and invasion are mediated via the guanine nucleotide exchange factor Tiam1. Knockdown of integrin $\alpha 6 \beta 4$, which is upregulated by PTHrP, negates the PTHrP-mediated increase in Rac1 activation. Integrin $\alpha 6 \beta 4$ signals synergistically with growth factor receptors to activate the phosphatidylinositol 3-kinase (PI3-K) pathway. Chemical inhibition of PI3-K negates the PTHrP-mediated effects on Tiam1 and Rac1 activity. Tumors from PTHrP-overexpressing LoVo cells also show increased expression of Tiam1. Taken together, these observations provide evidence of a link between PTHrP and Rac1 activity through integrin $\alpha 6 \beta 4$, resulting in enhanced cell migration and invasion. Targeting PTHrP production in colon cancer may thus prove therapeutically beneficial.

Keywords

PTHrP; integrin $\alpha 6 \beta 4$; phosphatidylinositol 3-kinase; Tiam1; Rac1

1. Introduction

Colorectal adenocarcinoma accounts for over 90% of the malignant tumors of the large bowel, and is the second most common cause of death from malignant disease in the Western world [1,2]. While the overall 5-year survival rate for patients with colorectal carcinoma is 64%, the rate drops to 10% or less in patients with metastatic disease [1]. Cellular migration and invasion are critical for the ability of tumor cells to metastasize locally and to distant sites [3]. These properties are dependent on inherent tumor cell characteristics [4–6], and on the presence of several growth factors in the metastatic microenvironment. One of these factors is parathyroid hormone-related protein (PTHrP). Overexpression of PTHrP has been linked to increased colon cancer cell migration and invasion both *in vitro* and *in vivo* [7,8]. PTHrP expression correlates

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Conflict of Interest Statement

We declare that none of the authors have a financial interest related to this work.

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with the severity of colon carcinoma, including depth of invasion, lymphatic invasion, lymph node and hepatic metastasis, and Dukes' classification [9].

Rac1 GTPase is a member of the Rho family of small GTPases which play critical roles in the regulation of various cellular processes, including reorganization of the actin cytoskeleton, cell cycle progression, cell migration and cell survival [10]. The dysregulation of Rac1 activity has been strongly implicated in cancer development. Elevated expression of Rac1 is seen in colon, breast, and lung tumors, among others [11]. Rho family proteins act as molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state. This cycling is tightly regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins. Overexpression of the Rac1-specific GEF, T-cell lymphoma invasion and metastasis 1 (Tiam1), has been reported in colon carcinomas and in highly invasive breast tumours, and contributes to elevated Rac1 signaling in these cancers [12,13].

Integrins regulate many cellular functions including cell adhesion, survival, proliferation, gene transcription, protein translation, cell migration and invasion and tumor development [14]. Integrins are comprised of $\alpha\beta$ heterodimers; different combinations of the α and β subunits produce receptors with different ligand specificities [15]. Integrin $\alpha6\beta4$ expression is upregulated in primary colonic tumors [16]. Integrin $\alpha6\beta4$ expression correlates with colon cancer invasiveness, and stable transfection of integrin $\alpha6\beta4$ in $\beta4$ -deficient colon cancer cells increases their migratory and invasive potential [17–20]. Numerous groups have established a role for Rho GTPases, including Rac1, in integrin-mediated motility [21,22]. One of the pathways via which integrins such as the $\alpha6\beta4$ integrin activate Rac1 is through upregulation of GEFs such as Tiam1.

A positive correlation exists between PTHrP and integrin $\alpha6\beta4$ expression in LoVo cells, and PTHrP upregulates the expression of both subunits at the mRNA and protein levels [7,8,23]. Moreover, immunohistochemical analysis shows increased integrin $\alpha6$ and $\beta4$ levels in tumor xenografts from PTHrP-overexpressing LoVo cells [8]. Given the association between PTHrP, integrin $\alpha6\beta4$ signaling and in turn Rac1 activity, here we asked whether PTHrP increases Rac1 activity through upregulation of integrin $\alpha6\beta4$, resulting in increased cell migration and invasion. As a model system, we used the human colon cancer cell line LoVo, which is derived from a left supraclavicular region metastasis of a Dukes' type C, grade IV colorectal carcinoma [24]. The mechanisms through which PTHrP exerts its effects in colon cancer are not fully understood. Since the gastrointestinal epithelium is prone to cancer development, particularly in the colon, understanding the role of PTHrP in this system may provide important information for the diagnosis and treatment of colon cancer.

2. Materials and Methods

2.1. Materials

Fetal bovine serum (FBS) and NuSerum were obtained from Atlanta Biologicals (Norcross, GA) and BD Biosciences (San Diego, CA) respectively. Tissue culture supplies were purchased from Life Technologies, Inc. (Gaithersburg, MD). Antibodies for Western blot analysis and immunohistochemistry were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Cell Signaling Technology (Danvers, MA), and Bethyl Laboratories (Montgomery, TX). The FluoroBlok inserts for analysis of migration and invasion were purchased from BD Pharmingen (San Diego, CA). Matrigel was obtained from BD Biosciences and Calcein-AM was obtained from Molecular Probes (Eugene, OR). The Rac Activation Assay system was purchased from Cell Biolabs (San Diego, CA). The small interfering RNAs (siRNAs) targeting PTHrP, Tiam1, the integrin $\alpha6$ and $\beta4$ subunits, and the corresponding non-target control (NTC) siRNA sequences were purchased from Dharmacon (Lafayette, CO).

2.2. Cell culture and transfection

LoVo cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were grown at 37°C in a humidified 95% air/5% CO₂ atmosphere in Ham's F12 medium supplemented with 10% FBS and L-glutamine.

For overexpression of PTHrP, cells were plated in 6-well plates at 2×10^5 cells/well in medium containing 10% FBS (Atlanta Biologicals). After 48 h, the cells were transiently transfected by electroporation with a construct encoding human PTHrP (from Genentech, Inc., South San Francisco, CA) cloned into the expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA). Cells transfected with the empty vector were used as controls. Cells were analyzed 48 h after transfection, as described below.

For siRNA transfections, cells were plated as described above. After 48 h, the cells were transfected by electroporation with ON-Target plus siRNAs directed against PTHrP, the integrin $\alpha 6$ or integrin $\beta 4$ subunits, or Tiam1 (100 nM; Dharmacon). In some experiments, the cells were transfected with the integrin $\alpha 6$ plus integrin $\beta 4$ siRNAs. In other experiments, the cells were transfected with the PTHrP-expressing construct plus the siRNA targeting Tiam1. 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. In some experiments, cells were treated for 4 h with LY49002 (25 μ M or 50 μ M) 24 h after transfection.

2.3. Analysis of PTHrP, Tiam1, integrin $\alpha 6$, and integrin $\beta 4$ levels

Total RNA was extracted using the RNAqueous[®] isolation kit (Ambion Inc., Austin, TX), per the manufacturer's protocol. RNA concentrations were determined by spectrophotometry. PTHrP, Tiam1, integrin $\alpha 6$, and integrin $\beta 4$ mRNA levels were analyzed by reverse transcription/real-time PCR as described [7]. The following TaqMan inventoried products were used: PTHrP, Hs00174969_m1; integrin $\alpha 6$, Hs00173952_m1; integrin $\beta 4$, Hs00173995_m1; Tiam1, Hs00180075_m1 and the pre-developed 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). Western blot analyses were performed as previously described [7].

2.4. Cell migration and invasion

Cells were transfected with the PTHrP-expressing construct as described in Section 2.2. In some experiments, the cells were co-transfected with the PTHrP-expressing construct plus Tiam1 siRNA. After 48 h, the cells were trypsinized and 0.5×10^6 cells were loaded with Calcein-AM (10 μ M; Molecular Probes) and plated onto FluoroBlok inserts (BD Pharmingen) in the absence of FBS. To measure invasion, the inserts were coated with Matrigel (Becton Dickinson) [7]. In some experiments, migration and invasion were measured in the presence of W56 (250 μ M; Sigma, St. Louis, MO). FBS (10%) was used as the chemoattractant. Cell migration and invasion were measured 4 h after plating onto the inserts [7]. Data are presented as the percentage of cells that migrated through the membrane or invaded the Matrigel barrier.

2.5. Monolayer scratch assay

Cells were grown to confluence in 6-well dishes in medium containing 5% NuSerum. NuSerum was used to decrease the exposure of cells to the extracellular matrix (ECM) proteins present in FBS. The confluent monolayer was scraped with a P200 pipette tip, and then rinsed with PBS to dislodge cellular debris. The cells were then further incubated under standard conditions for 2 days. Pictures were taken at time zero, and at 2 days after wounding, using a Nikon phase contrast microscope. The extent of migration was analyzed using the NIH image software (<http://rsb.info.nih.gov/nihi-image/Default.html>).

2.6 Measurement of Rac activity

Cells were transfected as described in Section 2.2. The Rac1-GTPase activity assays were performed using commercially prepared reagents according to the manufacturer's instructions (Cell Biolabs, Cat. # STA-401-1). Briefly, at 48 h after transfection, the cell monolayer was washed twice with ice-cold PBS, and the cells were lysed with assay/lysis buffer containing protease inhibitors for 15 min on ice. The cell lysates were then cleared by centrifugation and protein content was estimated using the Bio-Rad protein assay. These lysates were used to analyze for total Rac content by Western blot analysis.

To analyze for active Rac levels, the volume of each sample (equivalent to 1 mg protein) was adjusted to 1 ml with 1X assay/lysis buffer, and 40 μ l of resuspended PAK PBD agarose bead slurry was added to each tube. After incubating at 4 °C for 1 h with gentle agitation, the beads were pelleted by centrifugation and washed 3 $\times$ with assay/lysis buffer. The beads were then analyzed by Western blot analysis using anti-Rac1 antibody.

2.7. Western blot analysis

Cells were grown to 70–80% confluence in 100 mm dishes. For whole cell extracts, 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). To enrich for membrane fractions, the cells were washed and lysed as described for whole cells extracts. The cell lysates were centrifuged twice at 1000 $\times$ g for 5 min at 4 °C. The supernatants from each step were pooled and centrifuged at 13,000 $\times$ g for 30 min at 4 °C. The membrane material in the pellet was resuspended in 1:1 RIPA buffer and MB-EGTA (210 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 10 mM HEPES). Total protein was estimated using the Bio-Rad protein assay. Protein levels were analyzed by Western blot analysis as previously described [7]. The signals were detected using the SuperSignal West Pico Substrate kit (Pierce Biotechnology Inc., Rockford, IL).

2.8. Nude mouse tumor studies

For these experiments, LoVo cells stably transfected with a PTHrP-expressing construct were used. The establishment and characterization of these clones has been described [23]. The cells were cultured in medium containing 10% FBS. At 70% confluency, the cells were trypsinized and the cell pellet was washed once with FBS-containing medium and three times with PBS. The cells were then resuspended in PBS at 3 $\times$ 10⁶ cells/100 μ l.

Male athymic nude mice, ~6 weeks of age (Harlan Sprague Dawley, Indianapolis, IN), received subcutaneous injections on the dorsal surface with 100 μ l of the cell suspension. The following LoVo cell clones were used (6 mice/group): four independent PTHrP-overexpressing clones, three independent empty vector-transfected clones (control), and parental cells. Mice were sacrificed on day 35 after injection. All animal experiments were carried out under an Institutional Animal Care and Use Committee-approved protocol.

2.9. Immunohistochemistry

Portions of the dissected mouse tumors were fixed immediately in 10% neutral buffered formalin for 24 h at room temperature after harvesting, and then placed in 70% ethanol. Formalin-fixed tissues were embedded in paraffin, and sections (5 μ m) were cut from the paraffin blocks. The sections were deparaffinized in xylene, and rehydrated in descending ethanol series. Protein staining was performed using the DAKO EnVision Kit (Dako Corporation, Carpinteria, CA), as previously described [8]. All sections were counterstained with haematoxylin and observed by light microscopy. For negative controls, sections were

incubated with rabbit IgG (Santa Cruz Biotechnology) in place of primary antibody. Images were recorded using a Nikon microscope at 40 × magnification.

2.10. 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.

3. Results

3.1. Direct correlation between PTHrP and Tiam1 mRNA and protein levels

Tiam1 mRNA and protein levels were measured in LoVo cells with suppressed or enhanced PTHrP expression. To suppress PTHrP expression, cells were transfected with a PTHrP siRNA. To eliminate the potential for off-target effects, two independent PTHrP-specific siRNAs were used. Transfection with either of the two siRNAs caused an ~ 85% decrease in PTHrP mRNA levels (Fig. 1A), and an ~ 75% decrease in Tiam1 mRNA levels (Fig. 1B), compared to parental cells. Overexpression of PTHrP by transfection with a PTHrP-expressing construct resulted in an ~ 15-fold increase in PTHrP mRNA levels (Fig. 1A) and an ~7-fold increase in Tiam1 mRNA levels (Fig. 1B). Suppressing or elevating PTHrP expression also produced a corresponding change in Tiam1 protein levels (Fig. 1C). Transfection with the NTC siRNA or the empty vector control had no effect on PTHrP or Tiam1 mRNA and protein levels (Fig. 1, A to C).

We previously showed that overexpression of PTHrP increases LoVo xenograft growth [8]. Here we show that Tiam1 levels are elevated in xenografts from PTHrP-overexpressing cells (Fig. 1D). No staining was observed when IgG was used as the primary antibody (Fig. 1D). There were no differences in staining between sections obtained from the tumors derived from parental cells and empty vector transfectants (data not shown).

3.2. The effects of PTHrP on Rac1 activity are mediated via Tiam1

Upregulation of Tiam1 is associated with Rac1 activation [12]. We therefore asked whether there is a correlation between PTHrP levels and Rac1 activity. We report lower Rac1 activity in cells transfected with the PTHrP siRNA (Fig. 2A). Conversely, active Rac1 levels were higher in cells overexpressing PTHrP (Fig. 2A). Total Rac1 levels were unchanged in cells with suppressed or elevated PTHrP expression (Fig. 2A).

To further link PTHrP expression with Rac1 activity through regulation of Tiam1, cells overexpressing PTHrP were co-transfected with an siRNA targeting Tiam1. Co-transfection with the Tiam1 siRNA suppressed Tiam1 protein levels by ~ 75% (Fig. 2B) and negated the effects of PTHrP on Rac1 activity, such that after suppressing Tiam1 expression in PTHrP-overexpressing cells, Rac1 activity was not higher than that in empty vector-transfected cells co-transfected with a NTC siRNA (Fig. 2B).

3.3. Tiam1 and Rac1 contribute to the pro-migratory and pro-invasive effects of PTHrP

We observed a direct correlation between PTHrP expression and the migration of LoVo cells, as measured using the FluoroBlok system. Thus, ~ 12% of control (parental and empty vector-transfected) cells migrated through the FluoroBlok membrane, compared to ~ 20% of cells overexpressing PTHrP (Fig. 3A). Transfection with the Tiam1 siRNA decreased cell migration in both the control and PTHrP-overexpressing cells. The decrease in migration was more pronounced in PTHrP-overexpressing cells, such that the migration of these cells was now not significantly higher than that of the control cells transfected with Tiam1 siRNA (Fig. 3A). To

confirm that Rac1 is involved in the pro-migratory effects of PTHrP, migration was measured in the presence of the Rac1 inhibitor W56. Treatment with W56 abolished the PTHrP-mediated increase in cell migration, such that the migration of W56-treated PTHrP-overexpressing cells was not significantly higher than that of control cells (Fig. 3A).

LoVo cell invasion also correlated directly with levels of PTHrP expression, such that ~ 12% of control cells invaded the Matrigel barrier, vs. 18% of PTHrP-overexpressing cells (Fig. 3B). Suppression of Tiam1 expression or treatment with W56 negated the difference in Matrigel invasion of PTHrP-overexpressing cells vs. controls (Fig. 4B). There was no difference in the migration and invasion of parental cells and cells transfected with the empty vector or the NTC siRNA (Fig. 3, A and B).

Cell migration was also assessed using the monolayer scratch assay, which measures the ability of cells to migrate and bridge a gap induced in a cell monolayer by scratching. Repair of the cell monolayer was significantly faster in PTHrP overexpressing cells compared to parental and empty vector-transfected (control) cells. Thus, repair was complete at 48 h after wounding the monolayer of PTHrP-overexpressing cells transfected with the NTC siRNA but not in the control (parental and empty vector-transfected) cells similarly transfected with the NTC siRNA (Fig. 3C). Transfection with Tiam1 siRNA slowed cell migration in both the control and PTHrP-overexpressing cells (Fig. 3C). After suppression of Tiam1 levels, there was no significant difference in the migration of cells overexpressing PTHrP vs. that in the control cells (Fig. 3D). There was no significant difference in cell monolayer repair between NTC siRNA-transfected and non-transfected cells (data not shown).

PTHrP also increases LoVo cell proliferation and survival [7]. Therefore, the observed differences in cell migration, invasion, and wound healing in PTHrP-overexpressing vs. control cells may be partially attributed to the proliferative and anti-apoptotic effects of PTHrP. However, measurement of cell migration and invasion using the FluoroBlok system was performed 4 h after plating the cells; the contribution of cell proliferation at this time point is expected to be minimal.

3.4. The effects of PTHrP on Rac1 activity are mediated via integrin $\alpha 6 \beta 4$

The expression of integrin $\alpha 6 \beta 4$ is positively correlated with LoVo cell migration and invasion [7]. In this and previous studies, we show that PTHrP upregulates integrin $\alpha 6 \beta 4$ expression (Fig. 4C) [7,8]. Integrin $\alpha 6 \beta 4$ has been shown to activate Rac1 [25]. To ask if the effects of PTHrP on Rac1 activity are mediated via integrin $\alpha 6 \beta 4$, LoVo cells overexpressing PTHrP were transfected with siRNAs targeting the integrin $\alpha 6$ and/or $\beta 4$ subunit. After transfection with the integrin $\alpha 6$ - or $\beta 4$ -specific siRNAs, there was a significant decrease in the mRNA levels for the corresponding subunit (Fig. 4, A and B). The mRNA levels of both the integrin $\alpha 6$ and $\beta 4$ subunits were suppressed in cells co-transfected with siRNAs targeting both subunits (Fig. 4, A and B).

Whole cell integrin $\alpha 6$ and $\beta 4$ protein levels were also significantly higher after transfection with the PTHrP-expressing construct (Fig. 4C). Since the integrin $\beta 4$ subunit undergoes proteolytic processing [26,27], multiple bands are evident (Fig. 4C). These multiple bands may also be evidence of proteolysis of the integrin $\beta 4$ subunit within the whole cell lysates. Transfection with the integrin $\alpha 6$ or $\beta 4$ siRNA decreased whole cell protein levels of the respective integrin subunit (Fig. 4C). We observed a difference in the whole cell isoform expression profile for the $\beta 4$ subunit in cells transfected with NTC vs. those transfected with the integrin $\alpha 6$ siRNA (Fig. 4C). Cell surface protein expression of integrin $\alpha 6 \beta 4$ requires dimerization of the $\alpha 6$ and $\beta 4$ subunits. Therefore, this difference in the isoform expression profile may be explained by the fact that cells transfected with the integrin $\alpha 6$ siRNA lack cell surface integrin $\beta 4$ expression. The protein levels of both subunits were suppressed in cells

transfected with both siRNAs (data not shown). PTHrP-overexpressing cells also had elevated p-Akt levels, compared to control cells (Fig. 4C). Total Akt levels were unchanged (Fig. 4C). Transfections with the integrin $\alpha 6$ and/or $\beta 4$ siRNA negated the PTHrP-mediated increase in p-Akt levels (Fig. 4C).

Transfection of PTHrP-overexpressing cells with the siRNA targeting the integrin $\alpha 6$ and/or $\beta 4$ subunit negated the PTHrP-mediated increase in Rac1 activity (Fig. 4C and data not shown). Rac1 activity was higher in PTHrP-overexpressing cells transfected with NTC (Fig. 4C). Transfection with the integrin $\alpha 6$ and/or $\beta 4$ siRNAs had no effect on total Rac1 levels (Fig. 4C and data not shown).

3.5. PI3-K plays a role in the integrin $\alpha 6\beta 4$ -mediated activation of Rac1

Integrin $\alpha 6\beta 4$ signals synergistically with growth factor receptors, such as ErbB2, ErbB-3 and c-Met [28–30], resulting in activation of the PI3-K pathway [19]. PTHrP activates the PI3-K/Akt pathway in LoVo cells [7,8]. Here we asked whether PI3-K acts as an intermediate in the PTHrP-mediated increase in Tiam1 and Rac1 activity. Since Tiam1 binds to the products of PI3-K at the cell membrane, we measured Tiam1 levels in isolated cell membrane fractions from PTHrP-overexpressing and control cells treated with the PI3-K inhibitor LY49002 (25 μ M or 50 μ M). Treatment with LY49002 significantly decreased p-Akt levels and membrane Tiam1 levels (Fig. 5). LY49002 also inhibited the PTHrP-mediated Rac1 activation by ~4-fold, such that active Rac1 levels were comparable in PTHrP-overexpressing and control cells treated with LY49002 (Fig. 5).

4. Discussion

PTHrP plays a major role in bone development, and targeted disruption of the PTHrP gene in mice results in skeletal dysplasia [reviewed in 31]. However, PTHrP exerts a much broader spectrum of effects in both normal physiology and disease states. The protein regulates cell growth, cell survival, smooth muscle relaxation and development [31,32]. Multiple studies demonstrate that PTHrP plays a major role in tumors that metastasize to the bone, such as breast and prostate cancer [33,34]. These effects of PTHrP are mediated via its calciotropic effects. There is now increasing evidence that PTHrP also plays a role in cancers that metastasize to other regions of the body, such as colon tumors which show a preference for liver metastasis [2]. The ability of tumor 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. Therefore, determining the factors involved in promoting colon cancer invasion and metastasis is crucial for understanding the pathogenesis of the disease. We previously showed a positive correlation between PTHrP expression and LoVo cell migration and invasion [7,8]. Our main findings in this study are that activation of the Rho GTPase Rac1 by PTHrP plays a major role in the pro-migratory and pro-invasive effects of PTHrP. This effect of PTHrP is mediated via the Rac-specific GEF Tiam1 and is dependent on upregulation of integrin $\alpha 6\beta 4$ expression by PTHrP.

Cytoskeletal rearrangements as a result of activation of Rho GTPases play a key role in cell motility, a pre-requisite for tumor cell migration and invasion. The coordinated and polarized movement of a cell involves adhesion, subsequent loss of attachment, and re-adhesion of lamellipodia and filopodia at a cell's leading edge to the substratum [35–37]. Work from a large number of laboratories has shown that the Rho family members are essential for cell transformation, tumor cell invasion and metastasis [reviewed in 38]. The first evidence of a role for Rac1 in invasion was obtained when the Rac-specific GEF Tiam1 was identified in a retroviral insertional mutagenesis screen using T-lymphoma cells [39]. Subsequently, Rac1 was shown to confer an invasive potential to these cells [40,41]. Further studies showed that Tiam1 regulates cell migration, invasion, and metastasis not only in T-lymphoma cells, but

also in colorectal, breast, and lung cancers, among others [12]. Various studies demonstrate a decisive role for Tiam1 and Rac1 in colorectal tumorigenesis. For example, siRNA-mediated suppression of Rac1 strongly inhibited lamellipodia formation and cell migration and invasion in LoVo cells [42], and forced expression of Tiam1 in Tiam1-deficient cells induced Rac1 activation [43]. In an *in vivo* orthotopic model that closely reproduces the clinical behavior of human colorectal adenocarcinoma, overexpression of Rac1 resulted in the acceleration of the tumorigenic process. In contrast, specific inhibition of Rac1 expression by RNA interference completely suppressed tumor formation [44]. Moreover, a Rac1 splice variant, Rac1b, which functions in accelerated GDP–GTP exchange and thereby mimics the phenotype of a constitutively active mutant, is highly expressed in colon and breast tumors [45]. Given the correlation between colon cancer aggressiveness and Tiam1 levels and Rac1 activity, our demonstration of an increase in Tiam1 levels in tumor xenografts from PTHrP-overexpressing cells further underlines the critical role of PTHrP in colon cancer. In line with these studies, we previously showed that PTHrP overexpression in LoVo cells increases tumor growth [8].

Integrin $\alpha 6 \beta 4$ regulates many cellular functions, including cell migration, invasion and tumor development and progression [14]. The levels of this integrin are elevated in several types of carcinomas, and a positive correlation between integrin $\alpha 6 \beta 4$ expression and a motile and invasive phenotype have been reported [20]. Numerous groups have established a role for Rho GTPases, including Rac1, in integrin-mediated motility [21,22]. Here we report that integrin $\alpha 6 \beta 4$ provides the link between PTHrP expression and Rac1 activity, in that suppressing the expression of these integrin subunits in PTHrP-overexpressing cells negates the increase in active Rac1 levels in PTHrP-overexpressing vs. control cells. Similar results have been reported in pancreatic cancer cells, where Rac1 activity was stimulated by $\alpha 6 \beta 4$ expression [46].

PI3-K functions upstream of Tiam1, linking integrins to Rac1 [21]. Regulation of Tiam1 involves both PI3-K-dependent and -independent pathways [47–49]. Tiam1 becomes activated in a PI3-K-dependent manner upon localization to the plasma membrane via binding of its pleckstrin homology (PH) domain to the lipid products of PI3-K [50,51]. Synergistic signaling between integrin $\alpha 6 \beta 4$ and growth factor receptors, such as ErbB2, ErbB-3 and c-Met [27–29], results in PI3-K activation [19]. We previously showed that the PTHrP-mediated increase in integrin $\alpha 6 \beta 4$ expression is accompanied by activation of the PI3-K/Akt pathway [7]. Here we directly demonstrate a link between PTHrP and PI3-K/Akt activation through integrin $\alpha 6 \beta 4$ in LoVo cells. Similar effects have been observed in the human prostate cancer cell line C4-2 [52]. Moreover, here we show that PI3-K plays a major role in the PTHrP-mediated activation of Rac1, and that integrin $\alpha 6 \beta 4$ is involved in this effect. We therefore conclude that one of the pathways via which PTHrP activates Rac1 is through Tiam1 activation via the PI3-K pathway. The PI3-K-independent regulation of Tiam1 involves Ras activation [47,48]. PTHrP has been reported to stimulate Ras activity in a PI3-K-independent pathway [53]. This pathway may also contribute to Rac1 activation by PTHrP. We also observed increased Tiam1 mRNA levels in PTHrP overexpressing cells. Therefore, PTHrP may also increase Tiam1 levels via a transcriptional and/or post-transcriptional pathway.

We therefore propose the following model for Rac1 activation by PTHrP (Fig. 6). PTHrP upregulates integrin $\alpha 6 \beta 4$ expression, which in turn leads to activation of the PI3-K/Akt pathway. This results in upregulation of Tiam1 and therefore Rac1 activation. PTHrP may also regulate Tiam1 levels via a transcriptional/post-transcriptional pathway. The net result is increased cell migration and invasion. Our data support a role for Rac1 activation by PTHrP in the observed pro-migratory and pro-invasive effects of PTHrP, since suppression of Tiam1 expression using siRNAs reverses the pro-migratory and pro-invasive effects of PTHrP.

In conclusion, the results presented here demonstrate that PTHrP increases cell migration and invasion in LoVo cells, and that this effect is mediated via the Rho GTPase Rac1. These results establish PTHrP signaling as a mediator of increased colon cancer cell migration and invasion, with integrin $\alpha 6 \beta 4$ providing the link between PTHrP and Rac1. Strategies aimed at decreasing PTHrP production in colon cancer may thus provide therapeutic benefits.

Acknowledgments

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Figure 1. Correlation between PTHrP and Tiam1 levels in LoVo cells

(A and B) PTHrP and Tiam1 mRNA levels. PTHrP (A) and Tiam1 (B) mRNA levels were measured by reverse transcription/real-time PCR. Values are expressed relative to the corresponding parental (P) value, set arbitrarily at 1.0. Each bar is the mean $\pm$ SEM of three independent experiments. For the NTC and PTHrP si values, each bar within the group represents transfection with two independent siRNAs (PTHrP si) or non-target control siRNAs (NTC). * = Significantly different from the control (P) value ($P < 0.001$). (C) Western blot analysis for Tiam1. Equal loading was confirmed by re-probing with GAPDH. P = parental cells. Transfected with: NTC = non-targeting control siRNA; V = empty vector; PTHrP si = PTHrP siRNA; PTHrP+ = PTHrP-expressing construct. Within each group of transfectants, each lane represents two independent transfections (V and PTHrP+) or transfection with two independent siRNAs (NTC and PTHrP si). (D) **Tiam1 levels in xenografts from PTHrP-overexpressing LoVo cells.** IgG = sections stained with an anti-rabbit IgG antibody (negative control). + = xenograft sections from PTHrP-overexpressing cells; V = xenograft sections from control (empty vector-transfected) cells. Each panel is representative of five sections for each of six tumors from mice injected with one of four independent PTHrP-overexpressing clones or three empty vector-transfected clones. Magnification, $\times 40$.

Figure 2.

(A) Correlation between PTHrP levels and Rac1 activity in LoVo cells. Active and total Rac1 levels were analyzed in cell lysates from LoVo cells transfected with a PTHrP-expressing construct (PTHrP+) or with an siRNA targeting PTHrP (PTHrP si). Empty vector transfectants (V) and non-target control siRNA transfectants (NTC) were used as controls. P = parental cells. Within each group, each lane represents two independent transfections (V and PTHrP+) or transfection with two independent siRNAs (NTC and PTHrP si). **(B) Suppression of Tiam1 expression negates the increase in Rac1 activity in PTHrP-overexpressing cells.** Tiam1 expression was suppressed in PTHrP-overexpressing (PTHrP+) and control (empty vector-transfected, V) cells. Cell lysates were analyzed for active and total Rac1 levels. + = transfected

with Tiam1-directed siRNA; - = transfected with NTC siRNA. PTHrP+ = transfected with PTHrP-expressing construct; V = transfected with empty vector control.

Figure 3. The pro-migratory and pro-invasive effects of PTHrP are dependent on Tiam1 expression, and are suppressed upon inhibition of the Tiam1/Rac1 interaction
 (A and B) Migration and invasion of PTHrP-overexpressing and control LoVo cells transfected with an siRNA targeting Tiam1 or treated with W56. For the NTC and PTHrP si values, each bar represents transfection with two independent siRNAs (PTHrP si) or non-target control siRNAs (NTC). P = parental cells; V = empty vector-transfected controls; PTHrP+ = transfected with PTHrP-expressing construct. Each bar is the mean $\pm$ SEM of three independent experiments. * = Significantly different from the NTC-transfected V values ($P < 0.001$); # = significantly different from the PTHrP+ value transfected with NTC siRNA ($P < 0.001$). (C) Migration of PTHrP-overexpressing (PTHrP+) and control (parental and empty vector-transfected) cells transfected with an siRNA targeting Tiam1 or the NTC control siRNA was analyzed using the monolayer scratch assay. Images at 0 and 2 days after wounding are shown. The lines mark the edges of the monolayer. Magnification, $\times 10$. (D) Quantitation of cell migration 48 h after wounding the cell monolayer. Each bar is the mean $\pm$ SEM of three independent experiments for each of the two independent Tiam1 siRNAs or NTC siRNAs. * = Significantly different from the respective NTC value ($P < 0.001$); # = significantly different from the parental cells (P) value transfected with NTC siRNA ($P < 0.001$).

Figure 4. Suppression of integrin $\alpha 6$ and $\beta 4$ expression in PTHrP-overexpressing LoVo cells inhibits Rac1 activation

(A and B) Reverse transcription/real-time PCR analysis for integrin $\alpha 6$ or $\beta 4$ mRNA levels in cells transfected with an siRNA targeting the integrin $\alpha 6$ and/or $\beta 4$ subunit. Each bar is the mean $\pm$ SEM of three experiments. Data for two independent siRNAs are presented. * = Significantly different from the respective NTC control ($P < 0.001$). (C) Western blot analysis for the integrin $\alpha 6$ and $\beta 4$ subunits, p-Akt and total Akt, and active and total Rac1 in cells transfected with siRNAs targeting the integrin $\alpha 6$ or $\beta 4$ subunit. Within each group of transfectants (V and PTHrP+), each lane represents two independent transfections. Transfected with: V = empty vector control; PTHrP+ = PTHrP-expressing construct; $\alpha 6$ si, siRNA targeting integrin $\alpha 6$; $\beta 4$ si, siRNA targeting integrin $\beta 4$; NTC = non-target control siRNA.

Figure 5. Suppressing PI3-K activity in PTHrP-overexpressing cells downregulates Rac1 activity and membrane Tiam1 levels

Rac1 and membrane Tiam1 (mTiam1) levels were measured in PTHrP-overexpressing (PTHrP+) and empty vector transfected (V) LoVo cells treated with the PI3-K inhibitor LY49002. + = treated with LY49002; - = vehicle controls. p-Akt levels were analyzed to confirm suppression of PI3-K activity.

Figure 6.

Proposed mechanism of the regulation of cell migration and invasion by PTHrP, involving activation of Rac1. PTHrP increases cell surface levels of integrin $\alpha 6\beta 4$, which signals synergistically with growth factor receptors to activate the PI3-K pathway. This results in activation of the GTPase Rac1 through the Rac1-specific guanine nucleotide exchange factor Tiam1. Rac1 activation in turn increases cell migration and invasion. PTHrP may also increase Tiam1 protein levels through upregulation of Tiam1 mRNA levels via a transcriptional/post-transcriptional pathway.