Involvement of ERK1/2, p38 MAPK, and PI3K/Akt signaling pathways in the regulation of cell cycle progression by PTHrP in colon adenocarcinoma cells

Natalia Calvo, María Julia Martín, Ana Russo de Boland, and Claudia Gentili

Abstract: Parathyroid hormone-related peptide (PTHrP) is distributed in most fetal and adult tissues, and its expression correlates with the severity of colon carcinoma. Recently we obtained evidence that in Caco-2 cells, a cell line from human colorectal adenocarcinoma, exogenous PTHrP increases the number of live cells, via ERK1/2, p38 MAPK, and PI3-kinase and induces the expression of cyclin D1, a cell cycle regulatory protein. In this study, we further investigated the role of PTHrP in the regulation of the cell cycle progression in these intestinal cells. Flow cytometry analysis revealed that PTHrP treatment diminishes the number of cells in the G0/G1 phase and increases the number in both S and G2/M phases. The hormone increases the expression of CDK6 and diminishes the amount of negative cell cycle regulators p27Kip1, p53, and p35. However, PTHrP does not modify the expression of cyclin D3, CDK4, and p16INK4A. In addition, inhibitors of ERK1/2 (PD98059), p38 MAPK (SB203580), and PI3-kinase (LY294002) reversed PTHrP response in Caco-2 cells. Taken together, our results suggest that PTHrP positively modulates cell cycle progression and changes the expression of proteins involved in cell cycle regulation via ERK1/2, p38 MAPK, and PI3K/Akt signaling pathways in Caco-2 cells.

Key words: PTHrP, Caco-2 cells, cell cycle, MAPKs, PI3K/AKT.

Mots-clés : PTHrP, cellules Caco-2, cycle cellulaire, MAPKs, PI3K/AKT.

Introduction
Parathyroid hormone-related protein (PTHrP) was originally identified as the causative agent of humoral hypercalcemia of malignancy, one of the most frequent paraneoplastic syndromes (Strewler 2000). The PTHrP molecule is synthesized as a “prepro” isoform. PTHrP can function either as a secreted protein that associates with the nuclei of target cells due to a nuclear localization sequence (de Papp et al. 1995; Mannstadt et al. 1999). PTHrP was found to be widely distributed in most fetal and adult tissues, including the gut mucosal epithelium (Wysolmerski and Stewart 1998; Li et al. 1995). There is evidence that the hormone is implicated in different cancers, such as colon carcinoma (Nishihara et al. 1999). Colorectal carcinogenesis, a major cause of cancer death worldwide, is a complex multistep process involving progressive disruption of intestinal epithelial-cell proliferation, apoptosis, differentiation, and survival mechanisms (Calvert and Frucht 2002). In colorectal cancer and other malignant disorders, deregulation of cell cycle is common and contributes to tumorigenesis.

Cell cycle control is achieved through the actions of a family of cyclin-dependent protein kinases (CDKs) and cyclins that initiate phosphorylation events to allow progression through checkpoints in the cell cycle. In mammalian cells, progression through the G1 phase requires, early in G1, the activity of the cyclin D-dependent kinases CDK4 and (or) CDK6 and, later in G1, the cyclin E-dependent kinase CDK2 (Sherr 1994). Two families of CDK inhibitors (CDKIs) are critical mediators of anti-proliferative signals that arrest the cell cycle. Cip/Kip family includes p21Cip1 (also known as WAF1, Sdi1, and CAP20), p27Kip1, and p57Kip2, whereas INK4 family includes p16INK4A, p15INK4B, p18INK4C, and p19INK4D. These inhibitors negatively regulate G1 phase progression by forming complexes with CDKs and thus preventing S phase entry (Besson et al. 2008). Another regulator of cell cycle progression is p53 protein, which induces the transcription of genes that negatively regulate the
Table 1. Sequences of primers (forward, fw; reverse, rv), primer sequences size, and PCR products size of real-time quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Product</th>
<th>Primer</th>
<th>Primers</th>
<th>size (bp)</th>
<th>size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>452</td>
<td>fw:19</td>
<td>rv:20</td>
<td>5′−3′</td>
<td></td>
</tr>
<tr>
<td>p53NK4B</td>
<td>93</td>
<td>fw:21</td>
<td>rv:19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p27Kip1</td>
<td>154</td>
<td>fw:20</td>
<td>rv:25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdk6</td>
<td>70</td>
<td>fw:25</td>
<td>rv:20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p15INK4B</td>
<td>93</td>
<td>fw:21</td>
<td>rv:20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

progression of the cell cycle in response to DNA damage or other cellular stressors and thus participates in maintaining genome stability (Costanzo et al. 2000). The regulatory system of the cell cycle is affected by external signals and internal cell signaling pathways. PTHrP is an external factor that, depending on the experimental conditions, can inhibit or promote proliferation and induce alterations in cell cycle regulation thereby contributing to tumorigenesis (Datta et al. 2007; MacLean et al. 2004; Guthalu Kondegowda et al. 2010).

Recently we obtained evidence that in Caco-2 cells, a cell line from human colorectal adenocarcinoma, exogenous PTHrP increases the number of live cells via ERK1/2, p38 MAPK, and PI3-kinase signaling pathways and induces the expression of cyclin D1, a cell cycle-regulatory protein, which is essential for cell cycle progression from G1 to S phase (Martín MJ, Calvo N, Russo de Boland A, Gentili C, submitted). However, it remains to be elucidated if other cell cycle regulatory proteins are involved in PTHrP modulation of cell cycle progression in Caco-2 cells. Therefore, the present study was designed to explore if PTHrP also regulates cell cycle progression in these colon cancer cells and, if so, to investigate the mechanisms that are involved in this process.

Materials and methods

Materials

Human PTHrP (1-34) and high glucose Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina). Antibodies were from the following sources: anti-cyclin D3, anti-CDK4, anti-CDK6, anti-p27Kip1, anti-p15INK4B, anti-p16INK4A, and anti-p53 were from Cell Signaling Technologies (Beverly, Massachusetts, USA). Goat anti-rabbit peroxidase-conjugated secondary antibody and goat anti-mouse peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, California, USA). Anti-actin antibody was from Sigma-Aldrich. PD 98059, SB 203580, LY 294002 were from Calbiochem (San Diego, California, USA). RNase Cocktail™ Enzyme Mix was from Applied Biosystems (Carlsbad, California, USA). Propidium iodide (PI) was from Invitrogen (Carlsbad, California, USA). Protein size markers were from Amersham Biosciences (Piscataway, New Jersey, USA), and PVDF (Immobilon polyvinylidene difluoride) membranes and ECL chemiluminescence detection kit were from Amersham (Little Chalfont, Buckinghamshire, England). All other reagents used were of analytical grade.

Cell culture and treatment

The human colon cell line Caco-2 (from the American Type Culture Collection, Manassas, Virginia) was cultured at 37 °C in DMEM containing 20% FBS, 1% non-essential acids, 100 U/mL penicillin, 100 mg/mL streptomycin and 50 mg/mL gentamycin in a humid atmosphere of 5% CO2 in air. Cultures were passaged every 2 days with fresh medium. Experimental cultures were grown to 50%–70% confluence in serum-containing medium, and then cells were serum-deprived 24 h before the addition of 10−8 mol/L PTHrP (1-34) for different times in DMEM containing 5% FBS. This dose of PTHrP exposure was selected because we previously studied the effects of both hormones, PTH (1-34) and PTHrP (1-34) 10−8 mol/L, in Caco-2 cells expressing PTH/PTHrP receptor (Calvo et al. 2011; Lezcano et al. 2013, Martín et al. submitted). Where indicated, cells were pretreated for 30 min with PD 98059 (an inhibitor of ERK1/2), SB 203580 (an inhibitor of p38 MAPK), or LY 294002 (an inhibitor of PI3kinase). Control conditions were performed by addition of an equivalent volume of DMSO (the vehicle of the inhibitors). In previous work we confirmed the effectiveness of the kinases inhibitors employed by determining phosphorylated protein levels of Akt and MAPKs by Western blot analysis (Lezcano et al. 2013).
Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry. Cells were incubated with or without PTHrP for 24 h, trypsinized, washed once with PBS, and fixed in 70% ethanol for at least 1 h at −20 °C. Fixed cells were washed with PBS and incubated with propidium iodide (PI) staining solution (69 μmol/L IP, 38 mmol/L sodium citrate, and 0.7 mg/mL ribonuclease A, pH7.4) for 30 min at 37 °C in the dark. The stained cells were analyzed by FACScalibur flow cytometer (Becton Dickinson). The program used for the acquisition and analysis of the samples was CellQuest.

Western blot analysis

Caco-2 cells were washed with PBS buffer plus 25 mmol/L NaF and 1 mmol/L Na3VO4, and lysed in buffer containing 50 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L EDTA, 1% Tween-20, 1% Nonidet P-40, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 25 mmol/L NaF, and 1 mmol/L Na3VO4. The lysates were incubated on ice for 10 min, vortexed for 45 s, and maintained on ice for another 10 min. After centrifugation at 14 000g and 4 °C for 15 min, the supernatant was collected and proteins were quantified by the Bradford method (Bradford 1976). Lysate proteins dissolved in 6x Laemmli sample buffer were separated (25 μg/lane) using SDS-polyacrylamide gels (12% acrylamide) and electrotransferred to PVDF membranes. After blocking with 5% nonfat milk in TBST buffer (50 mmol/L Tris pH 7.2–7.4, 200 mmol/L NaCl, 0.1% Tween-20), the membranes

Table 2. Quantitative real-time RT-PCR analysis was performed, as described in Materials and methods, to measure mRNA levels of CDK6, p15INK4B, and p27Kip1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>PTHrP (10−8 mol/L)</th>
<th>SEMa</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p15b</td>
<td>1</td>
<td>1,15</td>
<td>0.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Cdk6c</td>
<td>1</td>
<td>1,7</td>
<td>0.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>p27c</td>
<td>1</td>
<td>0.65</td>
<td>0.23</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Note: Data represents mean ± SEM of three independent experiments.
aSEM, standard error of mean. mRNA levels quantified in PTHrP-treated cells for *45 minutes or †10 hours.
were incubated overnight with the appropriate dilution of primary antibody in TBST with 1% nonfat milk. After washing, membranes were incubated with the appropriate dilution of horseradish peroxidase-conjugated secondary antibody in TBST with 1% nonfat milk. Finally, the blots were developed by ECL with the use of Kodak BioMax Light film and digitalized with a GS-700 Imaging Densitometer (Bio-Rad, Hercules, California, USA).

**Stripping and reprobing membranes**

The complete removal of primary and secondary antibodies from the membranes was achieved by incubating the membranes in stripping buffer (62.5 mmol/L Tris-HCl pH 6.8, 2% SDS and 50 mmol/L β-mercaptoethanol) at 55 °C for 30 min with agitation. Then, membranes were washed for 10 min in TBST (1% Tween-20) and blocked, as indicated above, for 1 h at room temperature. After that, membranes were ready to reprobe with the corresponding antibodies.

**RNA Isolation and cDNA Synthesis**

Caco-2 cells were incubated in serum-free DMEM for 24 h and then treated with or without PTHrP (10^{-8} mol/L, in DMEM 5% FBS) for 45 min or 10 h. Total RNA from all samples was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. The concentration of isolated total RNA was quantified with a Beckman DU 530 UV–vis spectrophotometer. Then, first-strand DNA was synthesized from 1 μg total RNA with oligo(dT) primer (Gibco/BRL, Life Technologies, Paisley, UK) and superscript reverse transcriptase (Gibco/BRL). The cDNA product was quantified and then was stored at −20 °C for real-time quantitative RT-PCR (qRT-PCR).

**Real-Time Quantitative RT-PCR**

The PCR reaction was carried out in a real-time PCR system (Applied Biosystems, model 7500). The details of the oligonucleotide primer sequences, primer lengths, and predicted amplified product lengths are listed in Table 1. The PCR reactions were prepared using SYBR green master mix (No. 4309159, Applied Biosystems). On ice, the following were added: 5 μL of 2x SYBR green, 0.8 μL of forward primer (400 nmol/L), 0.8 μL of reverse primer (400 nmol/L), 2 μL of template cDNA (12 ng), 1.4 μL of sterile water. GAPDH cDNA was amplified in parallel for all genes to provide an appropriate internal PCR control. For each experiment, a nontemplate reaction, where water was added as sample, served as a negative control. Mean \( C_T \) of the gene of interest (CDK6, p27Kip1, or p15INK4B) was calculated from triplicate measurements. \( \Delta C_T \) was calculated according the following equation: 

\[
\Delta C_T = C_T - C_{GAPDH}
\]

mRNA levels were calculated according to the \( 2^{-\Delta C_T} \) method and later each control group was set to 1.

**Statistical analysis**

The statistical significance of the data was evaluated using Student’s t test (Snedecor and Cochran 1989), and probability values below 0.050 (\( p < 0.050 \)) were considered significant. Quantitative data are expressed as means ± SD from the indicated set of experiments.
Results

PTHRP diminishes the number of cells in the G0/G1 phase and increases the number in both S and G2/M phases

Initial experiments were performed to investigate the effect of PTHrP (10−8 mol/L) on the progression of the cell cycle in Caco-2 cells. To that end, cells were incubated with or without PTHrP for 24 h, and the percentages of cells in the G0/G1, S, and G2/M phases were determined by flow cytometric analysis of propidium iodide stained cells, as described in the Materials and methods section above. As shown in Fig. 1, the hormone decreased the percentage of cells in G0/G1 from 73.4% to 53.3% (p < 0.01), which was accompanied by a corresponding increment in the percentages of cells in S and G2/M phases from 19.7% to 25.3% and from 7.2% to 21.9% (p < 0.01), respectively. These data indicate that PTHrP increased the percentage of cells in S and G2/M phases in Caco-2 cells.

PTHRP regulates the expression of CDK6, p27Kip1, and p15INK4B but does not modify the expression of CDK4, p16INK4A, and cyclin D3

Based on our findings and with the aim to explore the effect of PTHrP in the regulation of cell cycle-regulatory proteins expression, Caco-2 cells were treated with or without PTHrP for 1−24 h, followed by immunoblot analysis using specific antibodies to evaluate the protein expression of cyclin D3, CDK6, and CDK4 and the specific CDK inhibitors, p15INK4B, p16INK4A, and p27Kip1. As demonstrated in Fig. 2A, PTHrP markedly increased the protein expression of CDK6, which is responsible for cell cycle progression in the early G1 phase, after 24 h of treatment. The hormone also diminished the expression of the inhibitory protein p27Kip1 at 12 h with a maximal effect at 24 h, while the expression of p15INK4B inhibitor was only down-regulated at 1 h. However, the protein levels of cyclin D3, CDK4, and p16INK4A were no different in the absence or presence of PTHrP (Fig. 2B). Taken together, these
results reveal that PTHrP up-regulates CDK6 and down-regulates p15INK4B and p27Kip1 protein expressions. In addition, and consistent with the changes observed on the protein expression, the treatment with the hormone (10 h) increases CDK6 and decreases p27Kip1 mRNA expression, while p15INK4B mARN levels in PTHrP-treated cells for 45 min were similar to those quantified in control cells (Table 2). These results indicate that the effects on the regulation of CDK6 and p27Kip1 protein expression are due to transcriptional regulation.

**PTHrP modulates cell cycle regulatory proteins expression through ERK1/2 and p38 MAPK**

We previously reported that PTHrP activates ERKs as well as the α isoform of p38 MAPK, and that both kinases participate in PTHrP-induced Caco-2 cell proliferation (Martín et al. submitted). As it has been demonstrated that MAPK activity is essential for many cell functions such as cell cycle regulation (Chambard et al. 2007), to gain insight into the signaling events that link PTHrP to the cell cycle regulatory machinery, Caco-2 cells were pre-incubated with MAPK inhibitors for 30 min and treated with PTHrP for 1 and 24 h, followed by immunoblot analysis using specific antibodies. As shown in Fig. 3, PD98059 (20 μmol/L), a specific inhibitor of ERK1/2, prevented the inhibitory effect of PTHrP on p15INK4B and p27Kip1 expression. Moreover, PTHrP-dependent CDK6 protein level significantly decreased when Caco-2 cells were incubated with the inhibitor. Furthermore, when Caco-2 cells were exposed to SB203580 (20 μmol/L), a p38 inhibitor, the PTH analog response is reversed (Fig. 4). Taken together, these findings indicate that the effect of PTHrP on the expression of CDK6, p15INK4B, and p27Kip1 is dependent on ERK 1/2 and p38 MAPK pathways.

**The effect of PTHrP on the expression of CDK6, p15INK4B, and p27Kip1 is dependent on the PI3K/Akt pathway**

It has been shown that PI3K/Akt signal-transduction pathway is involved in the regulation of cell cycle progression (Sears and
Since we previously reported that PTHrP induced the phosphorylation of Akt in Caco-2 cells (Martín et al. submitted), we also investigated the role of PI3K/Akt in the regulation of the cell cycle progression by PTHrP. To that end, cells were pre-treated with a specific inhibitor of PI3K, LY294002 (50 μmol/L), and then incubated with PTHrP for 1 and 24 h. Western blot analysis revealed that the effect of PTH analog on p15INK4B, p27Kip1, and CDK6 protein levels is reversed by the PI3K inhibitor, suggesting that PTHrP-mediated expression of these cell cycle regulatory proteins is also dependent on the PI3K/Akt pathway in Caco-2 cells (Fig. 5).

**PTHrP diminishes the expression of p53**

An important target for checkpoint signaling in cells that are in G1/S transition before the restriction point is p53 tumor suppressor protein (Costanzo et al. 2000; Reisman et al. 2012). To further clarify the mechanism involved in PTHrP regulation of cell cycle progression in Caco-2 cells, we evaluated the expression of p53 after PTHrP treatment for 1 to 24 h. In Fig. 6, Western blot analysis shows that PTHrP diminished the expression of the inhibitory protein p53. Preliminary observations suggest that PTHrP also down-regulated its protein expression, as well as p15INK4B and p27Kip1 protein levels, in HCT116 cell line from human colon carcinoma. To investigate the involvement of MAP kinases and PI3K/Akt in the down-regulation of p53 expression induced by PTHrP, Caco-2 cells were pre-incubated in the presence or absence of the inhibitors PD98059 (20 μmol/L), SB203580 (20 μmol/L), or LY294002 (50 μmol/L) and then treated for 1 or 24 h with PTHrP. Figs. 7A and 7B show that the inhibitors reversed PTH analog-mediated down-regulation of p53, suggesting that PTHrP diminished p53 protein expression via ERK1/2, p38 MAPK, and PI3K/Akt signaling pathways.

**Discussion**

PTHrP is recognized for its widespread distribution, its endocrine, paracrine, and intracrine modes of action driving numer-

---

Fig. 5. Effects of LY294002 on the expression of cell cycle regulatory proteins. Caco-2 cells were pre-incubated for 30 min with LY294002 (50 μmol/L), an inhibitor of PI3K, and then exposed to PTHrP (10−8 mol/L, in DMEM 5% FBS) for 1 or 24 h followed by western blot analysis of proteins from cell lysates using anti-CDK6, anti-p15INK4B, or anti-p27Kip1 antibodies. Blotted membranes were reprobed with anti-β-actin antibody. A representative immunoblot and the quantification by scanning densitometry of 3 independent experiments are shown; means ± SD are given. *P < 0.05 with respect to the control.

Calvo et al. 311

Biochem. Cell Biol. Downloaded from www.nrcresearchpress.com by CSP Staff on 10/17/14 For personal use only.
Fig. 6. PTHrP diminishes the expression of p53. Caco-2 cells were incubated in serum-free DMEM for 24 h and then treated with or without PTHrP (10−8 mol/L, in DMEM 5% FBS) for 1, 3, 6 and 24 h. Proteins from lysates were prepared as described in Materials and methods, separated on 12% SDS-PAGE, and immunoblotted using anti-p53 antibody. To evaluate the equivalence of protein content among the different experimental conditions, blotted membranes were reprobed with anti-β-actin antibody. Densitometric analyses were performed on the anti-p53 immunoblot from 3 independent experiments; means ± SD are given. *P < 0.025, with respect to the corresponding control.

A recent study (Calvo et al. 2011; herein we showed the opposite effects of its occurrence) revealed that PTHrP (1-36) is sufficient to induce proliferation, causing an increase in expression at the posttranscriptional level of two activators of the late phase of the G1/S cell cycle checkpoint, Cdk2, and cyclin E. It has been well documented that cell cycle is primarily regulated by complexes containing CDKs and cyclins, including cyclin D-CDK4/6 and cyclin E-CDK2 (Sherr and Roberts 2004), which are critical for its progression (van den Heuvel and Harlow 1993). Aberrant proliferation of cancer cells involves the deregulation of key G1 phase cell cycle regulators, and overexpression of cyclins and CDKs provides a selective growth advantage to tumor cells (Sherr 1996). The fact that PTHrP treatment of Caco-2 cells caused an increase in mRNA and protein levels of CDK6 and a decrease in p27Kip1 suggests a potential role of these regulatory proteins in the progression of the cell cycle. p27Kip1, a negative regulator of the cell cycle as well as a promoter of apoptosis (Thomas et al. 1998; Lloyd et al. 1999), plays important role in the regulation of both quiescence and G1 progression. Reduced expression of p27Kip1 is known as an independent prognostic marker in a large variety of cancers and is associated with unfavorable prognosis (Li et al. 2006). In agreement with our results, it was reported that in smooth-muscle cells PTHrP stimulates the progression of cell cycle from G1 to S phase by decreasing the protein level of p27Kip1 (Fiaschi-Taesch et al. 2006). p15INK4b, a member of the INK4 family, is an inducer of G1 cell cycle arrest, negatively regulates cell cycle progression by inhibition of cyclin-dependent kinase (CDK) 4/6, and prevents its interaction with cyclin D. p15INK4b is an essential mediator in cell cycle arrest in response to cytotactic signals, and its inactivation is involved in abnormalities leading to malignancies (Roussel 1999). Our data suggest that in Caco-2 cells, PTHrP-induced cell cycle progression is also mediated by down-regulation of p15INK4b protein expression. This down-regulation may not be a transcriptional effect, but perhaps may be a post-transcriptional regulation, since the protein changes observed for this cell cycle regulator occurs at 1 h of PTHrP treatment. In line with our findings, a PTHrP mutant that lacks of the nuclear localization signal (NLS) inhibits the proliferation of vascular smooth muscle cells, increasing the level of p15INK4b and p27Kip1 expression (Fiaschi-Taesch et al. 2009).

The regulatory system of the cell cycle is affected by external signals and internal cell signaling pathways. As external signals, several growth factors trigger signal transduction cascades through binding to membrane receptors. Mitogen-activated protein kinases (MAPKs), which belong to a large family of serine-threonine kinases, form major cell-proliferation signaling pathways from the cell surface to the nucleus (Raman et al. 2007). The best known are the extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK1-3), and p38 (α, β, γ, and δ) families (Pai et al. 2002). Several lines of evidence suggest that the ERK1/2 pathway is a major regulator of cell proliferation in colorectal cancer (Pai et al. 2002). The present study shows that in Caco-2 cells, ERK inhibition up-regulated the levels of p27Kip1 and p15INK4b but down-regulated CDK6 protein, indicating that PTHrP regulates the expression of these proteins through an ERK-dependent pathway. According to our results, activation of the ERK cascade usually promotes cell cycle progression (Chambard et al. 2007), playing a role in the inhibition of CDKIs. Several studies revealed that the inhibition of ERK pathway up-regulates p15INK4b and p27Kip1 and down-regulates cyclin D1, cyclin E, and cyclin A, which in turn leads to reactivation of retinoblastoma gene (RB) and repression of a number of cell cycle-promoting molecules, with subsequent arrest of cell cycle at the G1 phase (Gysin et al. 2005; Koyama et al. 2007). p38 MAPK pathway is involved in proliferation, differentiation, metabolism and cell death (Hui et al. 2005; Koyama et al. 2007). p38 MAPK pathway is involved in proliferation, differentiation, metabolism and cell death (Hui et al. 2005; Koyama et al. 2007).
in the regulation of cyclin D1 levels (Page et al. 2001). Moreover, Pillai et al. (2011) suggest that p27 induction in hypoxic cardiac fibroblasts may be due to down-regulation of Skp2, a potential downstream target of p38 MAPK.

Like MAPKs, there is increasing evidence that the activation of PI3K/Akt signaling pathways is associated with colorectal carcinoma and can convert differentiated human gastric or colonic mucosa to a less differentiated and more malignant phenotype (Semba et al. 2002). The effects of PI3K on tumor growth and progression are thought to be mediated mainly by Akt. PI3K/Akt signaling is also involved in the regulation of cell cycle progression (Fresno Vara et al. 2004). Our results suggest that PI3K/Akt pathway is also involved in PTHrP modulation of CDK6, p15INK4B, and p27Kip1 expression. In line with our observations, Akt down-regulates p27Kip1 expression via the inhibition of FOXO transcription factors and through the inhibition of glycogen synthase kinase 3-β (GSK3-β) phosphorylation (Wymann and Marone 2005). The fact that PI3K/Akt pathway is involved in PTHrP-induced ERK 1/2 activation in Caco-2 cells (Martín et al. submitted) supports the idea that PI3K/Akt signaling can act cooperatively with ERK signaling in regulating cell cycle progression.

Activated p53 participates in a program that includes cell cycle arrest, DNA repair, apoptosis, senescence, or autophagy ( Vousden and Prives 2005). Loss of p53 function is a critical event in tumorigenesis and resistance to therapies. Functional wild type p53 is often inactivated by altered upstream pathways (Strano et al. 2007). It has been reported that the Caco-2 cell line is deficient in functional p53 protein: one allele is deleted, whereas the other contains a nonsense E204X mutation (Djelloul et al. 1997). We found that PTHrP diminished p53 protein expression via ERK1/2, p38 MAPK, and PI3K/Akt signaling pathways in these intestinal cells. It has become clear that p53 protein can functionally interact with the MAPK pathways, including the p38 MAPK and ERK1/2 (She et al. 2002). Recent studies have suggested a role for p53 as an upstream activator to regulate MAPKs signaling via the transcriptional activation of members of the dual specificity phosphatase family (Li et al. 2003). Because both p53 and MAPKs signaling pathways are altered in the majority of human tumors, understanding

---

**Fig. 7.** Effects of PD98059, SB203580, and LY294002 on the expression of p53. Caco-2 cells were pre-incubated for 30 min with PD98059 (20 μmol/L), SB203580 (20 μmol/L), or LY294002 (50 μmol/L) and then exposed to PTHrP (10−8 mol/L, in DMEM 5% FBS) for 1 h (A) or 24 h (B) followed by western blot analysis of proteins from cell lysates using anti-p53 antibody. Blotted membranes were reprobed with anti-β-actin antibody. A representative immunoblot and the quantification by scanning densitometry of 3 independent experiments are shown; means ± SD are given. *P < 0.025 with respect to the control.
their functional interaction may provide new insights into the deregulated cell proliferation and survival that is characteristic of cancer (Wu 2004).

In conclusion, the present investigation provides, to our knowledge, the first direct evidence demonstrating that exogenous PTHrP increases the number of cells in S and G2/M phases and promotes cell cycle progression of Caco-2 cells, a cell line derived from human colon adenocarcinoma. The hormone also changes the expression of proteins involved in cell cycle regulation via ERK1/2, p38 MAPK, and PI3K/Akt signaling pathways. These findings suggest that PTHrP contributes to colonic tumoral cell proliferation by regulating cell cycle progression, and that the hormone may constitute a novel target in the treatment of intestinal tumors.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Instituto Nacional del Cáncer and Universidad Nacional del Sur, Argentina.

References


Li, M., Zhou, J.Y., Ge, Y., Matherly, L.H., and Wu, G.S. 2003. The phosphatase
Li, H., Seitz, P.K., Thomas, M.L., Selvanayagam, P., Rajaraman, S., and
MacLean, H.E., Guo, J., Knight, M.C., Zhang, P., Cobrinik, D., and
Lloyd, R.V., Erickson, L.A., Jin, L., Kulig, E., Qian, X., Cheville, J.C., and
Mannstadt, M., Blomme, E.A., Koh, A.J., Henderson, J.E., Pienta, K.J., Rosol, T.J.,
Pai, R., Soreghan, B., Szabo, L.I., Pavelka, M., Baatar, D., and
Pilili, M.S., Sapna, S., and Shikumakura, K. 2011. p38 MAPK regulates G1-S transition
PMID:17496099.
Roussel, M.F. 1999. The INK4 family of cell cycle inhibitors in cancer. Oncogene,
Sears, R.C., and Nevin, J.J. 2002. Signaling networks that link cell proliferation and
Down-regulation of PIK3CG, a catalytic subunit of phosphatidylinositol 3-kinase
complex I, by CpG hypermethylation in human colorectal carcinoma. Cancer
She, Q.B., Ma, W.Y., and Dong, Z. 2002. Role of MAP kinases in UVB-induced 
Shen, X., and Falzon, M. 2005. PTH-related protein enhances LoVo colon cancer 
cell proliferation, adhesion, and integrin expression. Regul. Pept. 125(1–3):
science.274.5293.1672. PMID:8398498.
Sherr, C.J., and Roberts, J.M. 2004. Living with or without cyclins and cyclin-
PMID:15545627.
University Press.
Strano, S., Dell’Orso, S., Di Agostino, S., Fontemaggi, G., Sacchi, A., and
Blandino, G. 2007. Mutation of p53 as an oncogenic transcription factor. Oncogene,
NEJM200002033420306. PMID:10753944.
Thomas, G.V., Szigeti, K., Murphy, M., Draetta, G., Pagano, M., and Loda, M.
3108(98)70061-0. PMID:9736017.
2566003. PMID:2566010.
Wu, G.S. 2004. The functional interactions between the p53 and MAPK signaling
Wymann, M.P., and Marone, R. 2005. Phosphoinositide-3 kinase in disease: 
Ye, Y., Falzon, M., Seitz, P.K., and Cooper, C.W. 2001. Overexpression of parathyroid hormone-related protein promotes cell growth in the rat intestinal cell line