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Molecular Mechanisms Associated With PTHrP-Induced Proliferation of Colon Cancer Cells

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ABSTRACT

Parathyroid Hormone-related Protein (PTHrP) is normally produced in many tissues and is recognized for its endocrine, paracrine, autocrine and intracrine modes of action. PTHrP is also implicated in different types of cancer and its expression correlates with the severity of colon carcinoma. Using the human colon cell line Caco-2 we recently obtained evidence that PTHrP, through a paracrine pathway, exerts a protective effect under apoptotic conditions. However, if exogenous PTHrP is able or not to induce the proliferation of these intestinal tumor cells is not known. We found that PTHrP treatment increases the number of live Caco-2 cells. The hormone induces the phosphorylation and nuclear translocation of ERK 1/2, α p38 MAPK, and Akt, without affecting JNK phosphorylation. In addition, PTHrP-dependent ERK phosphorylation is reverted when PI3K activity was inhibited. Following MAPKs nuclear translocation, the transcription factors ATF-1 and CREB were activated in a biphasic manner. In addition PTHrP induces the translocation into the nucleus of β-catenin, protein that plays key role in maintaining the growth and proliferation of colorectal cancer, and increases the amount of both positive cell cycle regulators c-Myc and Cyclin D. Studies with ERK1/2, α p38 MAPK, and PI3K specific inhibitors showed that PTHrP regulates Caco-2 cell proliferation via these signaling pathways. In conclusion, the results obtained in this work expand our knowledge on the role of exogenous PTHrP in intestinal tumor cells and identify the signaling pathways that are involved in the mitogenic effect of the hormone on Caco-2 cells. J. Cell. Biochem. 115: 2133–2145, 2014.

KEY WORDS: PTHrP; CACO-2 CELLS; PROLIFERATION; SIGNAL TRANSDUCTION

The parathyroid hormone-related protein (PTHrP), originally identified as the factor responsible for malignant hypercalcemia, was later found to be widely expressed in fetal and adult tissues. Today it is recognized for its endocrine, paracrine, and autocrine modes of action [Maioli and Fortino, 2004; McCauley and Martin, 2012]. Full-length PTHrP undergoes posttranslational processing, which gives rise to a family of mature secretory peptides [Wysolmerski, 2012]. Peptides with the N-terminal PTH-like region, like PTHrP (1–34), bind to and activate the PTH/PTHrP receptor (PTH1R), whereas midregion and C-terminal peptides are functionally active through yet uncharacterized receptors [Wysolmerski, 2012]. Cytosolic PTHrP can also use a bipartite multibasic nuclear localization signal to translocate to the nucleus and act through an intracrine pathway [McCauley and Martin, 2012]. Widespread expression of PTHrP and the PTH/PTHrP receptor genes and proteins has been found in gut villus epithelium [Li et al., 1995] suggesting that PTHrP exerts a local regulatory role via an autocrine/paracrine pathway.

Colorectal cancer is one of the most common tumors and is a major cause of cancer death worldwide. Colorectal carcinogenesis is a complex multistep process involving progressive disruption of intestinal epithelial cell proliferation, apoptosis, differentiation, and survival mechanisms [Fang and Richardson, 2005]. The molecular processes of tumor progression are mediated via inherent tumor cell characteristics and growth factors, matrix molecules, and cytokines in the tumor environment [Kopfstein and Christofori, 2006]. One of these factors is PTHrP, whose expression correlates with the severity of colon carcinoma, specifically, with cell differentiation, depth of invasion, lymphatic invasion, lymph node, and hepatic metastasis [Nishihara et al., 1999].

Conflict of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Mitogen-activated protein kinases (MAPK), which belong to a large family of serine-threonine kinases, form major cell-proliferation signaling pathways from the cell surface to the nucleus. There are three major subfamilies of MAPK: the extracellular-signal-regulated kinases (ERK1/2); the c-Jun N-terminal or stress-activated protein kinases (JNK or SAPK) and p38 MAPK. All MAPKs are activated via a unique dual phosphorylation mechanism, on a Thr-X-Tyr motif, located in the phosphorylation loop. The ERK1/2 pathway is one of the most important for cell proliferation and several key growth factors and proto-oncogenes transduce the signals that promote growth and differentiation through this cascade [Cargnello and Roux, 2011]. There is growing evidence that activation of the ERK1/2 pathway is involved in the pathogenesis, progression, and oncogenic behaviour of human colorectal cancer [Fang and Richardson, 2005]. Another important regulatory protein is phosphatidylinositol-3-kinase (PI3K) involved in the regulation of cell growth, proliferation, survival, and differentiation. An important downstream effector of PI3K is the serine-threonine kinase Akt or protein kinase B (PKB); its activation is mainly induced by the phosphorylation of residue Ser-473 and Thr-308 and once activated Akt plays a pivotal role in fundamental cellular functions such as cell proliferation by phosphorylating a variety of substrates. It is known that deregulation of PI3K/Akt signal-transduction pathway plays a significant role for the progression of the human colorectal cancer [Roy et al., 2002; Samuels et al., 2004].

Several studies report discordant effects of PTHrP in different cell types and/or under varying experimental conditions [Maioli and Fortino, 2004]. Thus, the hormone can either promote or suppress cell growth or apoptosis [Chen et al., 2002; Fortino et al., 2002; Hastings et al., 2003; Datta et al., 2005, 2007]. Using the human colon cell line Caco-2 cultured in a serum-deprived medium we recently obtained evidence that exogenous PTHrP exerts a protective effect under apoptotic conditions [Lezcano et al., 2013]. However, the effect of PTHrP treatment on these intestinal tumor cells grown in a medium containing serum is still unknown. Therefore, the objectives of this study were to investigate if exogenous PTHrP is able or not to induce the proliferation of Caco-2 cells and if so, to identify the underlying mechanism of neoplastic proliferation induced by PTHrP.

MATERIALS AND METHODS

MATERIALS

Human PTHrP (1–34), High glucose Dubelcco's modified Eagle's medium (DMEM), DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride), and Trypan blue dye were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina). Antibodies were from the following sources: anti-phospho JNK, anti-phopho ERK1/2, anti-ERK1/2, anti-phospho (Ser473) Akt, anti-Akt, anti-phospho CREB/ATF-1, anti-Cyclin D1, anti- lamin B, and anti-cytochrome c were from Cell Signaling Technology (Beverly, MA). Anti-human full length PTHrP, anti-α p38 MAPK, anti-phospho α p38 MAPK, anti-JNK, anti-c-Myc, goat anti-rabbit peroxidase-conjugated secondary antibody, and goat anti-mouse peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). IgG1 monoclonal mouse antibody was from DAKO (Glostrup, Denmark). Anti-actin antibody was from Sigma (Sigma Chemical Co., St. Louis, MO). Anti-β-catenin antibody was from Lab Vision Corporation (Fremont, California). Alexa Fluor 488 conjugated-anti-rabbit antibody was from Molecular Probes. PD 98059, SB 203580, LY 294002 were from Calbiochem (San Diego, CA). Crystal violet was from MERCK (Buenos Aires, Argentina). Protein size markers were from Amersham Biosciences (Piscataway, NJ), 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 UI/ml penicillin, 100 mg/ml streptomycin and 50 mg/ml gentamycin in a humid atmosphere of 5% CO_2 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} M 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 10^{-8} M of both hormones, PTH (1–34) and PTHrP (1–34) in Caco-2 cells expressing PTH1R [Calvo et al., 2009; Lezcano et al., 2013]. 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 PI3-kinase). 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]. In the experiments where Caco-2 cells were incubated with anti-PTHrP antibody for 5 days with the purpose to neutralize PTHrP effects, its dose was selected based on previous studies performed by Ahmed et al. [2006].

CELL PROLIFERATION ASSAYS

CellTiter-Blue™ assay. Cell viability was evaluated by CellTiter-blue™ Assay (Promega, Madison, WI). Cells were plated for triplicate in 96- well plates. After each treatment, 20 μl of the reagent was added on each well followed by 4 hours of incubation at 37 °C. Viable cells retain the ability to reduce resazurin into resorufin. After the incubation step, the cells were lysed in 30% SDS, the medium was aspirated and the maximum absorbance of resazurin was measured at 605 nm. To determine background absorbance, wells without cells served as negative control.

Crystal violet assay. After each treatment, the cells were fixed with methanol for 15 min at −20 °C and stained with 0.1% crystal violet for 30 min at room temperature. The dye that absorbed onto the cells was solubilized with 0.2% Triton X-100. The absorbance obtained in solution, which correlates with the cell number, was measured at 590 nm.

Trypan blue dye exclusion test. Cells were washed with PBS buffer, released from the cultured dish using trypsin-EDTA, incubated with 0.4% of Trypan Blue stain and counted in a Neubauer chamber observed in a microscope. Cells were counted per field and the number of cells that excluded the stain (viable cells) was determined in each condition.

WESTERN BLOT ANALYSIS

Caco-2 cells were washed with PBS buffer plus 25 mM NaF and 1 mM Na_3VO_4, and lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 3 mM KCl, 1 mM EDTA, 1% Tween-20, 1% Nonidet P-40, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM NaF, and 1 mM Na_3VO_4. The lysates were incubated on ice for 10 min, vortexed for 45 s and maintained on ice for another 10 min. After centrifugation at 14,000 g and 4 °C during 15 min the supernatant was collected and proteins were quantified by the Bradford method [Bradford, 1976]. Lysate proteins dissolved in 6× Laemmli sample buffer were separated (25 μg/lane) using SDS–polyacrylamide gels (10% acrylamide) and electrotransfered to PVDF membranes. After blocking with 5% non-fat milk in TBST buffer (50 mM Tris pH 7.2–7.4, 200 mM NaCl, 0.1% Tween-20), the membranes were incubated overnight with the appropriate dilution of primary antibody in TBST with 1% non-fat milk. After washing, membranes were incubated with the appropriate dilution of horseradish peroxidase-conjugated secondary antibody in TBST with 1% non-fat milk. Finally, the blots were developed by ECL with the use of Kodak BioMax Light film and digitalized with a GS-700 Imaging Densitomer (Bio-Rad, Hercules, CA).

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 mM Tris-HCl pH 6.8, 2% SDS and 50 mM 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.

EVALUATION OF PTHrP PROTEIN LEVELS IN A PROTIEN-FREE MEDIUM

To determine the release of endogenously produced PTHrP to the medium where Caco-2 cells were cultured, the cells were washed twice with PBS buffer and then incubated for 24 h with a protein-free medium (NaCl 120 mM; $NaHCO_3$ 24 mM, KCl 2.7 mM, NaH_2PO_4 0.4 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.8 mM, glucose 5 mM; pH 7.4). At the end of the incubation period the supernatant was collected, 100 μl desoxycholate (5 mM) was added and the sample was placed on ice for 15 min. Thereafter trichloroacetic acid (100%) was added for another 30 min and the sample was placed again on ice. Finally, the protein was pelleted by centrifugation, resolved in 35 μl Laemmli buffer and 10 μl Tris-HCl (pH 9.5) and heated for 5 min at 95 °C. The sample was subjected to SDS-PAGE and then immunoblotted with an antibody against full length PTHrP.

IMMUNOCYTOCHEMISTRY

Caco-2 cells grown onto glass cover slips were fixed in methanol at −20 °C for 15 min. After washing with PBS, non-specific sites were blocked with 5% BSA in PBS. Samples were then incubated overnight with the appropriate primary antibody prepared in PBS, 2% BSA (1:50). After washing with PBS, the samples were incubated for 1 h at room temperature with secondary Alexa Fluor 488 conjugated antibody (1:200) and were stained with DAPI (1:500), a fluorescent dye that binds to DNA to visualize cell nuclei, during the last 30 min of incubation with the secondary antibody. Then, cells were washed with PBS and mounted. The samples were examined using a fluorescence microscope (NIKON Eclipse Ti-S) equipped with standard filter sets to capture fluorescent signals, and images were collected using a digital camera.

SUBCELLULAR FRACTIONATION

Cells were washed with PBS, resuspended in ice-cold TES buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 250 mM sucrose containing protease inhibitors) homogenized in a Teflon-glass hand homogenizer (30 stokes), and then centrifuged at 4500 rpm for 20 min at 4 °C to pellet the nuclei. The resulting supernatant is the cytosolic fraction. Nuclear fraction was washed twice in TES buffer. The purity of each isolated fraction was assessed by assaying for proteins known to be associated with cellular components. Routinely we included anti-lamin B and anti-cytochrome c antibodies to check the purity of nuclear and cytosolic fractions. Proteins from each fraction were quantified by the Bradford method [Bradford, 1976]. Equal proteins from each fraction were subjected to SDS-PAGE.

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

EXOGENOUS PTHrP FRAGMENT (1–34) STIMULATES CACO-2 CELLS PROLIFERATION

We first investigated the effects of exogenous PTHrP (1–34) (10^{-8} M) on Caco-2 cells proliferation. To that end, cells treated with the peptide for 4–6 days were incubated with resazurin and, upon treatment, the metabolic capacity of these intestinal cells was measured as an indicator of cell viability. We found that PTHrP increased the bioreduction of resazurin in a time-dependent fashion with the maximum response achieved at 5 days. As shown in Figure 1A, in Caco-2 cells treated with PTHrP for 5 days the quantity of resazurin was 44% below control (black bars). We also estimated the cell number in PTHrP treated culture using crystal violet, a basic dye which stains cell nuclei and we observed that PTHrP treatment for 5 days increased the absorbance by 27% with respect to untreated cells (Fig. 1A, grey bars). Previous studies evidenced the expression of PTHrP by immunohistochemical staining in tissue samples from normal colorectal mucosa, polyps, and colorectal carcinoma [Malakouti et al., 1996]. Therefore, we examined PTHrP protein

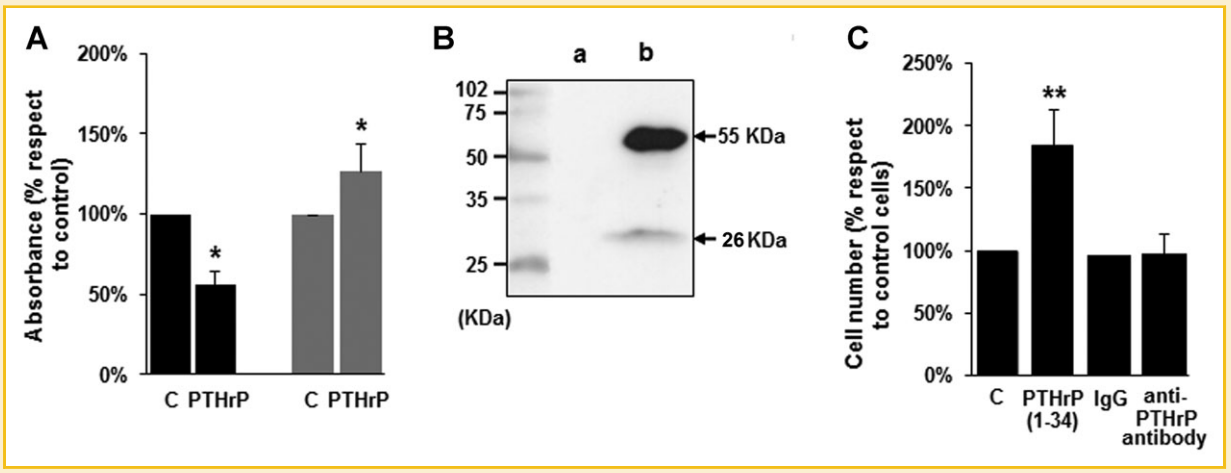

Fig. 1. Exogenous PTHrP (1–34) stimulates Caco-2 cells proliferation. (A) Caco-2 cells were exposed to PTHrP (1–34) 10^{-8} M (5 days). The number of viable cells was determined by Resazurin staining and Crystal violet staining as described in materials and methods. Results were expressed as percentage relative to control of three independent measurements performed in triplicate. $^{*}P < 0.05$ with respect to the corresponding control. (B) PTHrP protein expression levels. Proteins from Caco-2 cell lysate (a, left lane) and from the medium where the cells were incubated (b, right lane) were obtained as described under methods and then were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by immunoblotting using a monoclonal antibody against full length PTHrP. A representative immunoblot from two independent experiments is shown. (C) Endogenous PTHrP is not required to stimulate the cell growth. After Caco-2 cells were exposed for 5 days to an antibody that neutralizes full-length PTHrP (0.1 μg/ml) or to PTHrP (1–34), cell counts were performed in a Neubauer chamber by means of trypan blue dye exclusion. IgG, non-specific IgG, 0.5 μl/ml. The effects of each treatment have been compared with the control (untreated cells). The control has been set to 100%. The data shown are the average of cell number of three independent experiments. $^{**}P < 0.01$ with respect to the corresponding control.

expression in colonic Caco-2 cells with the purpose to evaluate if endogenously produced PTHrP also increases cell proliferation. As shown in Figure 1 B, Western blot analysis with a specific monoclonal anti-PTHrP antibody revealed the presence of a 26 KDa protein, the size expected for the full length PTHrP [Rizk-Rabin et al., 2008], in the medium where the cells were incubated. Other 55 KDa band (corresponding to a nonspecific protein but with immunoreactivity against anti-PTHrP antibody) also was seen. However, the protein expression of the 26 KDa PTHrP was not observed in the whole cell lysate from Caco-2 cells, suggesting that these tumor intestinal cells synthesize PTHrP that is totally secreted to the medium. Based in this finding, we choose to expose Caco-2 cells for 5 days to an antibody that neutralizes full-length PTHrP (0.1 μg/ml) or to PTHrP (1–34) to investigate the influence of endogenous PTHrP on cell proliferation. Counting live cells in a Neubauer chamber by means of trypan blue dye exclusion revealed that the cells treated with PTHrP (1–34) showed an increase in cell number at 5 days (83% respect to control cells) (Fig. 1C). However, the antibody did not decrease the cell number, suggesting that endogenous PTHrP is not required to stimulate the cell growth.

PTHrP INDUCES MAPK PHOSPHORYLATION IN CACO-2 CELLS

MAPK activity is essential for many cell functions and these kinases mediate Caco-2 cells proliferation induced by serum [Buzzi et al., 2009]. Based on our findings and with the aim to elucidate whether exogenous PTHrP is able to activate MAP kinase cascades in Caco-2 cells, we investigated changes in the phosphorylation of ERK1/2, p38 MAPKs, and JNKs. To that end, Caco-2 cells were exposed for different times to PTHrP, whole cell lysates were subjected to SDS-PAGE and then immunoblotted with an antibody that specifically recognizes the active form of the MAPKs as detailed in materials and methods. As shown in Figures 2A, B, and C, PTHrP markedly increased ERK 1/2-tyrosine phosphorylation in a time-dependent manner. The maximal stimulation was achieved at 60 min and declined at 150 min of treatment with the peptide (Fig. 2B, bottom panel). No effects were observed when Caco-2 cells were exposed to PTHrP for longer periods (3–24 h; Fig. 2C, bottom panel). The protein levels of total ERK1/2 were no different in the absence or presence of PTHrP demonstrating a true increase in their phosphorylation status (Fig. 2A, top panel; B and C, bottom panels). Western blot analysis with an antibody that reacts with the phosphorylated form of α p38 MAPK revealed that PTHrP rapidly induced an increase of α p38 phosphorylation. The effect was significant for the time interval between 10 and 60 min (Fig. 2D, left panel). No effects were observed when Caco-2 cells were exposed to PTHrP for longer periods (3–24 h; Fig. 2E, right panel). PTH analog did not modify the expression of α p38 (Figs. 2D and E) and this result excluded the possibility that an increase in α p38 expression upon treatment may contribute to changes in α p38 phosphorylation. We also evaluated the effect of PTHrP on JNK. Its phosphorylation was measured in cell lysates by Western blot analysis using anti-phospho JNK antibody. We found that the levels of both isoforms (46 and 54 KDa) of activated JNK were similar to those detected in control cells in all times of PTHrP treatment evaluated (10–60 min and 3–24 h, data not shown).

PTHrP STIMULATES AKT PHOSPHORYLATION AND MODULATES ERK 1/2 ACTIVATION THROUGH PI3K-Akt SIGNALING PATHWAY IN CACO-2 CELLS

As different studies suggest that the PI3K-Akt pathway is vital to the growth of colon cancer cells [Pandurangan, 2013], then we investigated whether exogenous PTHrP activates Akt in Caco-2 cells. After PTH analog treatment for different times, Akt

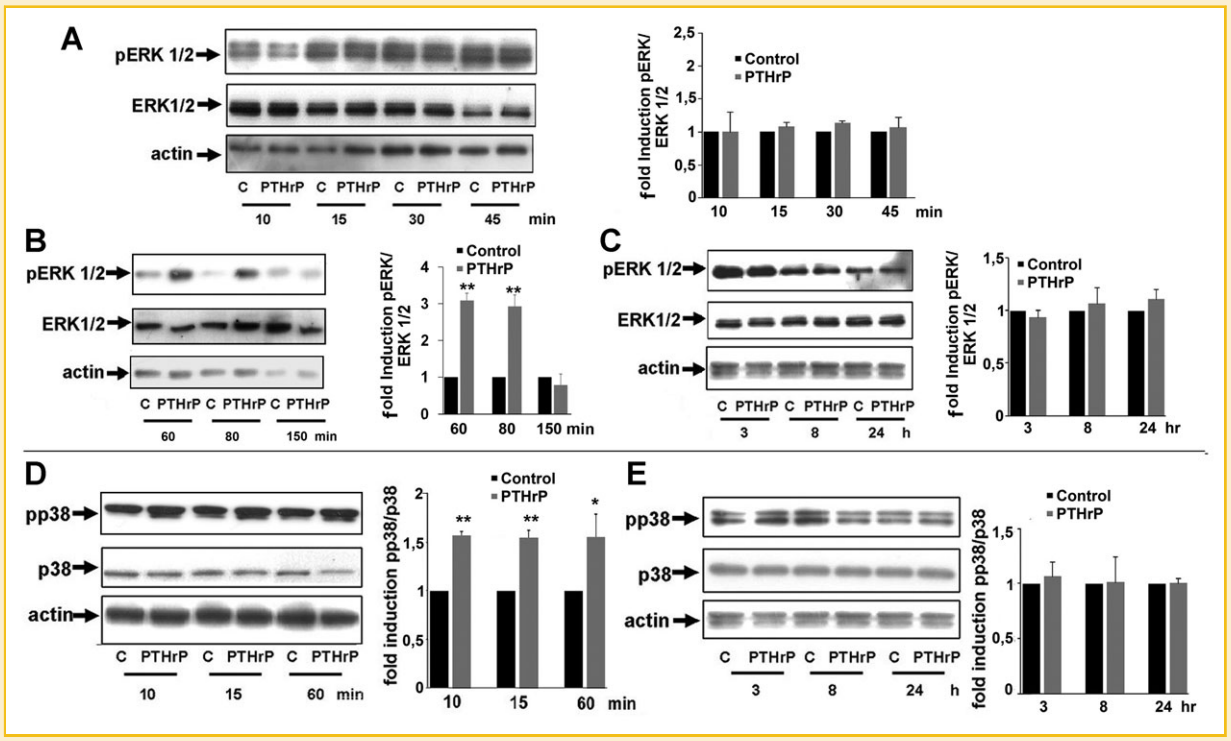

Fig. 2. (A), (B) and (C): Time course of PTHrP- induced phosphorylation of ERK 1/2 in colon cancer Caco-2 cells. Caco-2 cells were treated with PTHrP (1–34) 10^{-8} M for different time intervals (10– 150 min and 3–24 hours) as shown in the top and bottom panels. Whole cell proteins were extracted and Western blot was done using specific anti-MAPKs antibodies. The membranes were stripped and re-blotted with anti-β-actin antibody to ensure the equivalence of protein content among the different experimental conditions. A representative immunoblot and the quantification by scanning densitometry of three independent experiments are shown; means ± S.D. are given. **$P<0.01$ with respect to the control. (D) and (E): Time course of PTHrP- induced phosphorylation of α p38 MAPK in colon cancer Caco-2 cells. Caco-2 cells were treated with PTHrP (1–34) 10^{-8} M for different time intervals (10–60 min and 3–24 hours) as shown in the left and right panels, respectively. Whole cell proteins were extracted and Western blot was done using specific anti-MAPKs antibodies. The membranes were stripped and re-blotted with anti-β-actin antibody to ensure the equivalence of protein content among the different experimental conditions. A representative immunoblot and the quantification by scanning densitometry of three independent experiments are shown; means ± S.D. are given. *$P<0.05$, **$P<0.01$ with respect to the control.

phosphorylation was determined by Western blot analysis using a phospho-specific antibody against the Ser-473 residue. Figure 3A (top panel) shows that the phosphorylation of Akt significantly increased at 1 h of PTHrP exposure and returned to near basal levels by 3–24 h (Fig. 3A, bottom panel) and, in addition, there was no change in the protein expression of Akt during the time-profile examined (Fig. 3A, top and bottom panels). It is known that PI3-kinase/Akt pathway is required for the activation of ERK1/2 in many cell types [Wennstrom and Downward, 1999; Perkinton et al., 2002]. Based on our results showing that in Caco-2 cells the phosphorylation of both kinases, Akt and ERK, is seen at 1 h of peptide exposure, we further investigated if ERK1/2 is activated by PTHrP through PI3-kinase/Akt pathway. To that end Caco-2 cells were pre-incubated with a specific inhibitor of PI3K, LY294002 (50 μM), and then treated for 1 h with PTHrP. Western blot analysis using specific anti-phospho-ERK 1/2 antibody revealed that PI3K inhibitor totally suppressed hormone-dependent MAPK phosphorylation (Fig. 3B).

PTHrP INDUCES ERKS, α p38 MAPK AND Akt TRANSLOCATION TO THE NUCLEUS

As active MAPKs translocate from the cytoplasm to the nucleus [Cargnello and Roux, 2011] to control cell proliferation and also in a later step to its activation Akt translocates to the nucleus where many of its substrates are located [Osaki et al., 2004], therefore we performed fluorescence microscopy studies to examine the localization of ERK1/2, α p38 MAPK, and Akt in Caco-2 cells after PTHrP treatment. Figures 4A, B, and C (top panels) show that in resting cells, both MAPKs and Akt were distributed into the cytoplasm and nucleus of Caco-2 cells. However, after PTHrP exposure nuclear fluorescence was more intense than that observed in control cells whereas the immunoreactivity of all these kinases was diffuse in cytoplasm. We then performed subcellular fractionation followed by Western blot analysis employing anti-phospho Akt antibody and confirmed its subcellular distribution in cells exposed to PTHrP for 5 h (Fig. 4D, bottom and right panel).

INVOLVEMENT OF MAP KINASES IN PTHrP-DEPENDENT CREB/ATF-1 PHOSPHORYLATION

Activated MAPK into the nucleus induces phosphorylation and activation of transcription factors, such as CREB and ATF-1 [Cargnello and Roux, 2011]. To study changes in CREB/ATF-1 phosphorylation, Caco-2 cells were exposed to PTHrP followed by western blot analysis using an antibody that recognizes phospho-CREB and

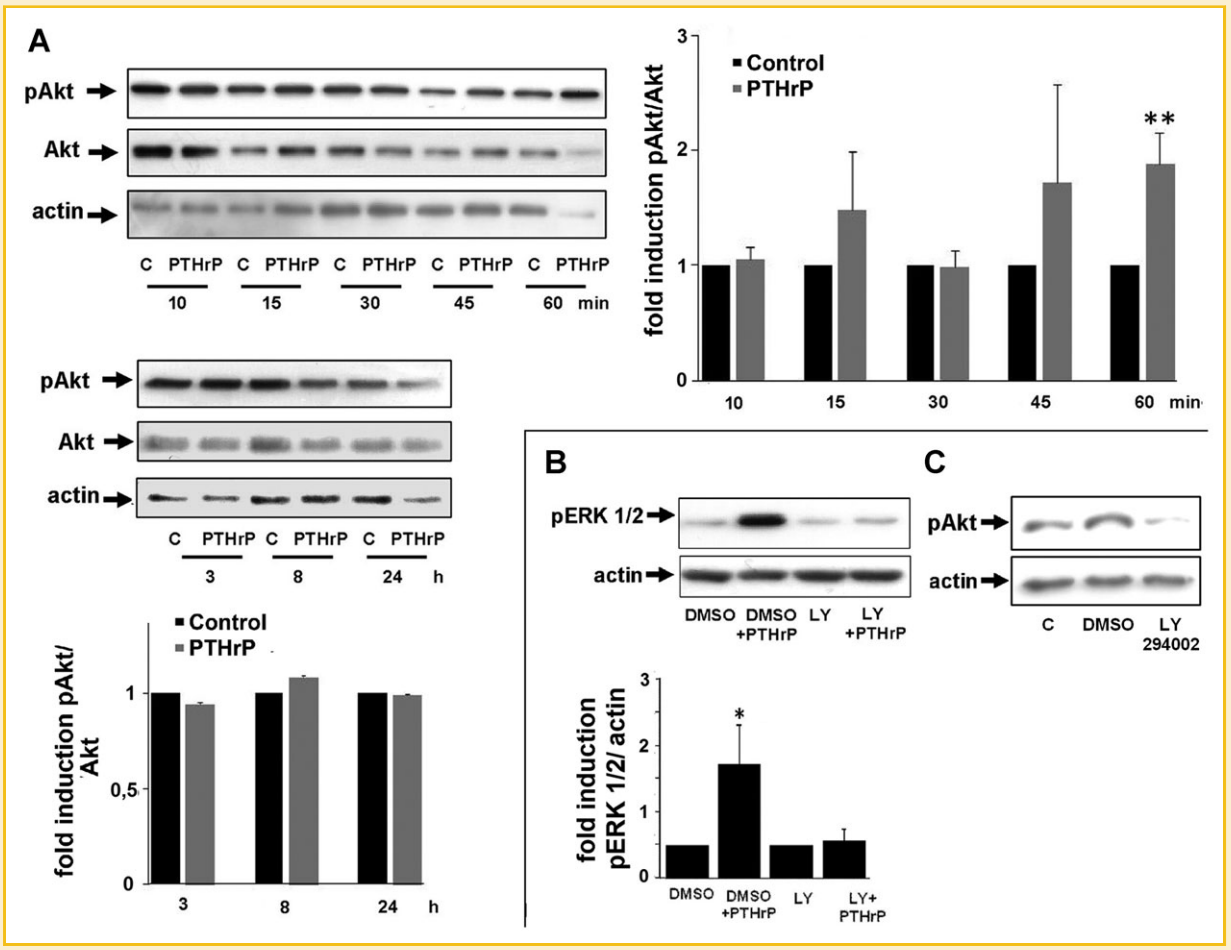

Fig. 3. (A) Time course of Akt/PKB phosphorylation in PTHrP- stimulated Caco-2 cells. Caco-2 cells were treated with PTHrP (1–34) 10^{-8} M for the indicated times. Proteins of cell lysate were separated by SDS-PAGE followed by Western blotting with anti-phospho-Ser 473 Akt/PKB antibody as described under materials and methods. Total Akt was measured in the same immunoblot by stripping the membrane and re-incubating with anti-Akt antibody. (B) The PI3K inhibitor affect MAPK phosphorylation induced by PTHrP. Caco-2 cells were treated with PTHrP during 1 h in the presence or absence of LY294002 (50 μM). Western blot analysis of cell lysates was carried out using an anti-phospho ERK 1/2 antibody. To ensure the equivalence of protein content among the different experimental conditions all membranes were stripped and re-blotted with anti-β-actin antibody. Representative images and bar graphs of phospho Akt (A) or phospho ERK1/2 (B) quantified by scanning densitometry of blots from three independent experiments are shown. $^{*}P < 0.05$ with respect to the control. (C) The effectiveness of the inhibitor LY294002 was confirmed by determining phosphorylated protein levels of Akt by Western blot analysis. Control conditions were performed in the absence or presence of DMSO (vehicle).

phospho-ATF-1. As shown in Figure 5A (left and right panels), phosphorylation of both transcription factors was biphasic, with an early phase (10–15 min) and a second phase peaking at 1 h and reaching basal values at 3 h of treatment with the hormone. In order to investigate the involvement of MAP kinases in PTHrP-dependent CREB/ATF-1 phosphorylation, Caco-2 cells were pre-incubated in the presence or absence of PD98059 (20 μM), a specific inhibitor of MEK1/2 (upstream kinases of ERK1/2) or SB203580 (20 μM), an α p38, and β p38 inhibitor, and then treated for 15 or 60 min with the hormone. Figure 5B (left panel) showed that p38 inhibitor totally blocked the early phase of CREB/ATF-1 phosphorylation induced by PTHrP whereas ERK1/2 inhibitor did not reverse PTHrP effect confirming that the treatment with the hormone for 15 min did not affect ERK1/2 phosphorylation status (Fig. 5B, right panel). Both MAPK inhibitors partially suppressed the second phase of phosphorylation (Fig. 5C, left and right panels).

PTHrP PROMOTES β-CATENIN NUCLEAR TRANSLOCATION AND INCREASES THE EXPRESSION OF c-Myc AND CYCLIN D1 IN CACO-2 CELLS

β-catenin is the major component of a key signaling pathway that plays key role in maintaining the growth and proliferation of colorectal cancer [Saif and Chu, 2010]. Activated Akt indirectly promotes β-catenin translocation to the nucleus and this protein is involved in the consequences of Akt signaling that are related to the regulation of cell proliferation [Osaki et al., 2004]. It is well established that once in the nucleus, β-catenin combines with different transcription factors to induce the expression of several genes, such as c-Myc and Cyclin D [Saif and Chu, 2010]. As PTHrP activates Akt in Caco-2 cells, we then investigate if the hormone also modulates the nuclear localization of β-catenin and the expression of c-Myc and Cyclin D1. As shown in Figure 6A, the images obtained by fluorescence microscopy suggest that

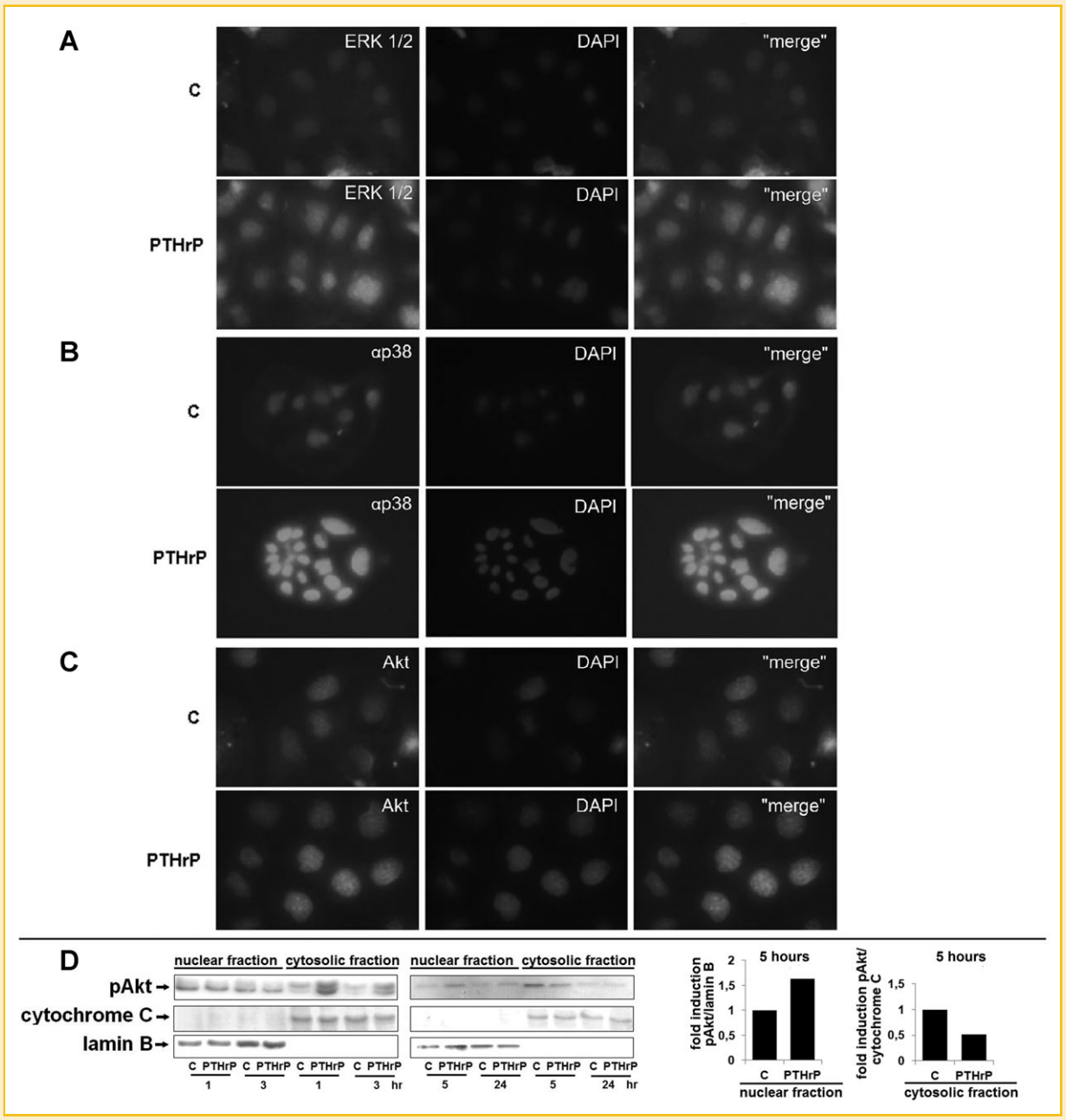

Fig. 4. Subcellular localization of ERKs, α p38 MAPK and Akt in PTHrP-stimulated Caco-2 cells. Top panel: Caco-2 cells were stimulated with PTH analog (1–34) 10^{-8} M for 1–6 h. After treatment the cells were fixed in methanol, washed with PBS, blocked in PBS plus 5% BSA, and incubated with (A) anti-ERK1/2 antibody, (B) anti-α p38 MAPK antibody, and (C) anti-Akt antibody. Samples were examined using a fluorescence microscope. Representative photographs of two independent experiments are shown. (D), bottom panel: Subcellular fractionation followed by Western blot analysis employing anti-phospho Akt antibody in cells exposed to PTHrP for 1, 3, 5, and 24 h. Anti-lamin B and anti-cytochrome c antibodies were included to check the purity of nuclear and cytosolic fractions, respectively. A representative immunoblot and the quantification by scanning densitometry of two independent experiments are shown.

PTHrP stimulated β-catenin translocation into the nucleus of Caco-2 cells. Furthermore, western blot analysis using specific anti-c-Myc and anti-Cyclin D1 antibodies revealed that Cyclin D1 and c-Myc protein expression was increased in PTHrP- treated cells for 1–3 h (Fig. 6B, top panel). No effects were observed when the cells were exposed to the peptide for 8–24 h (Fig. 6B, bottom panel).

PTHrP STIMULATES CACO-2 CELLS PROLIFERATION THROUGH PI3K/Akt AND MAPKs SIGNALING PATHWAYS

In view of the role of MAPK and PI3K/Akt pathways in the regulation of cellular proliferation, studies were carried out to test whether exogenous PTHrP increases the number of Caco-2 cells through the activation of these enzymes. To that end, cells were pre-incubated with PD98059 (20 μM), SB203580 (20 μM), or with LY294002

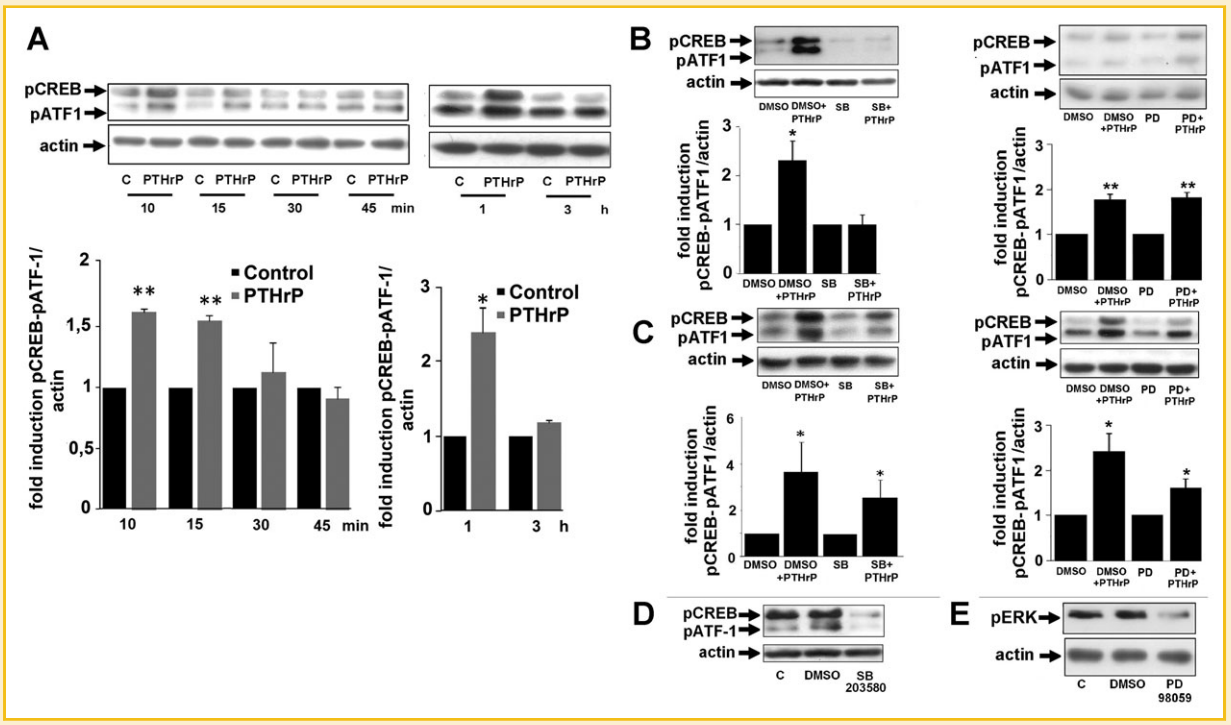

Fig. 5. (A): Effects of PTHrP on CREB/ATF-1 phosphorylation. Caco-2 cells were treated with PTHrP (1–34) 10^{-8} M for different time intervals (10–45 min and 1–3 hours) as shown in left and right panels, respectively. Western blot analysis of cell lysates was carried out using an anti-phospho CREB/ATF-1 antibody. The membranes were stripped and reblotted with anti-β-actin antibody to ensure the equivalence of protein loading. Representative immunoblots and the quantification by scanning densitometry of three independent experiments are shown; means ± S.D. are given. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) and (C): Involvement of MAP kinases in PTHrP-dependent CREB/ATF-1 phosphorylation. Caco-2 cells were pre-incubated with p38 MAPK inhibitor SB203580 (20 μM) and then treated with PTHrP (1–34) 10^{-8} M for 15 min or 1 h (B and C, left panels) or pre-incubated with ERK specific inhibitor PD98059 (20 μM) and then treated with the hormone for 15 min or 1 h (B and C, right panels). Western blot analysis of cell lysates was carried out using an anti-phospho CREB/ATF-1 antibody. The membranes were stripped and reblotted with anti-β-actin antibody to ensure the equivalence of protein loading. Representative immunoblots and the quantification by scanning densitometry of three independent experiments are shown; means ± S.D. are given. $^{*}P < 0.05$. (D) and (E): The effectiveness of the inhibitors SB203580 and PD98059 were confirmed by determining phosphorylated protein levels of CREB/ATF1 and ERK, respectively, by Western blot analysis. Control conditions were performed in the absence or presence of DMSO (vehicle).

(50 μM) and then treated for 5 days with PTHrP. As shown in Figure 7, Caco-2 cells response to PTHrP was completely abolished in the presence of MAPKs and PI3K inhibitors.

DISCUSSION

We have previously demonstrated that parathyroid hormone (PTH) at a dose of 10^{-8} M induces apoptosis and G0/G1 phase arrest of human colon adenocarcinoma Caco-2 cells [Calvo et al., 2008, 2009, 2011]. Recently we found that, differently to PTH, its tumoral analog (PTHrP) (10^{-8} M) exerts protective effects under apoptotic conditions in this cell line [Lezcano et al., 2013]. Herein we demonstrate, for the first time, that PTH analog (10^{-8} M) increases the number of live Caco-2 cells. PTH and PTHrP share PTH receptor type 1 (PTH1R), being this receptor a class b of G-protein coupled receptor [Mannstadt et al., 1999]. The fact that PTH and PTHrP exert opposing effects in Caco-2 cells, even though they interact with the same receptor, could be explained because of cell-specific responses elicited by either ligand that are dependent of the complement of available G proteins and also, of cytosolic factors that are not G proteins but are able to bind and regulate the activity of the PTH1R.

It is known that the vast majority of PTHrP actions are paracrine in nature [Maioli and Fortino, 2004; McCauley and Martin, 2012] for this reason Caco-2 cells were treated with the N-terminal 1–34 synthetic fragment of PTHrP since this region of the hormone contains a signal sequence that directs PTHrP to the secretory pathway, where it exerts paracrine effects. There are a number of reports showing that exogenously added amino-terminal PTHrP fragment induces the proliferation of normal cells such as human β cells [Guthalu Kondegowda et al., 2010] and human transformed cells such as prostate [Asadi et al., 2001; Tovar Sepulveda and Falzon, 2002] and adenocortical tumor cell lines [Rizk-Rabin et al., 2008]. However, the treatment with the peptide decrease cell proliferation in human breast and lung cancer cell lines [Luparello et al., 1997; Hastings et al., 2001]. Therefore, the growth-related effects of the amino-terminal PTHrP fragment are complex, and can be both stimulatory and inhibitory; these opposite actions of the peptide further support the observations described by many researchers respect that the hormone exerts discordant effects depending on cell type and/or experimental conditions.

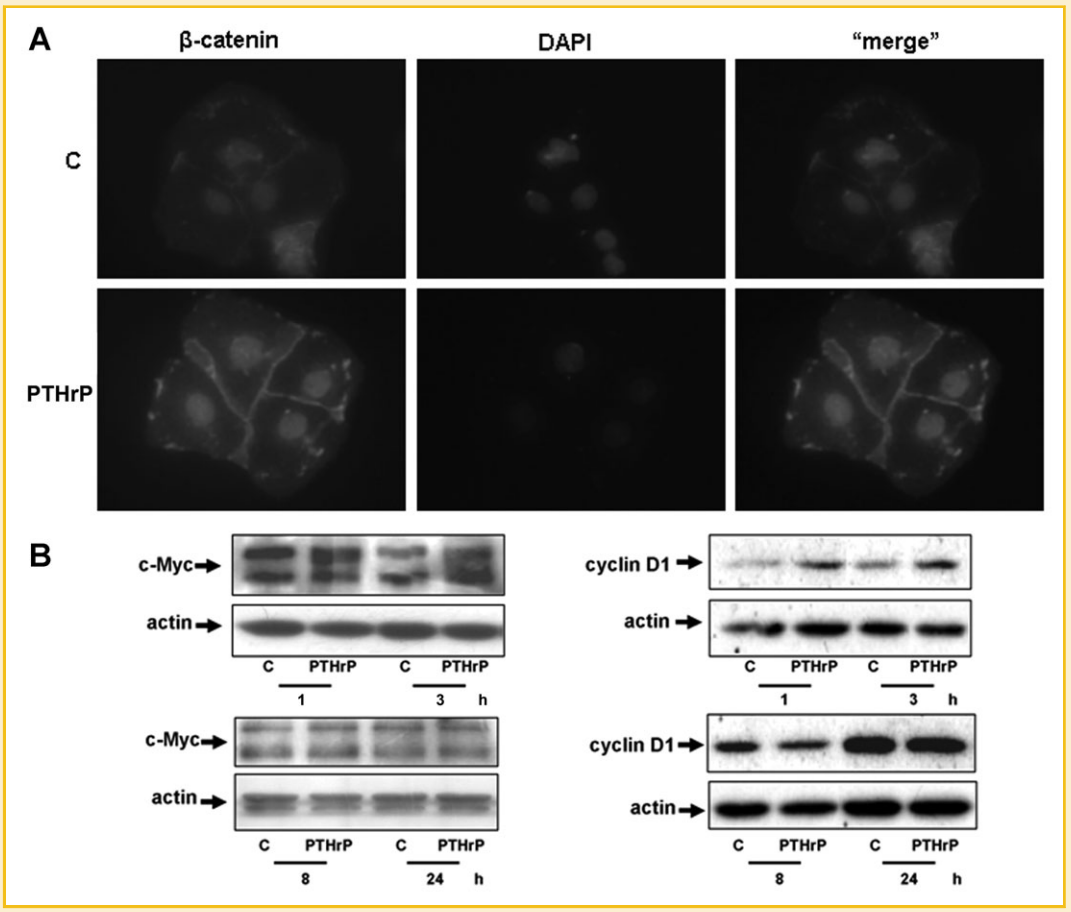

Fig. 6. (A) Subcellular localization of β-catenin in PTHrP-stimulated Caco-2 cells. Caco-2 cells were stimulated with PTH analog (1–34) at a dose of 10^{-8} M during 1 h. After treatment the cells were fixed in methanol, washed with PBS, blocked in PBS plus 5% BSA and incubated with an anti-β-catenin antibody. Samples were examined using a fluorescence microscope. Representative photographs of two independent experiments are shown. (B) Time-course of c-Myc and Cyclin D1 expression induced by PTHrP in Caco-2 cells. Caco-2 cells were treated with PTHrP (1–34) 10^{-8} M for different time intervals (1–3 h and 8–24 h) as shown in the top and bottom panels, respectively. Cell lysates were immunoblotted with specific antibodies against c-Myc and Cyclin D1 proteins as described in materials and methods. Anti-β-actin antibody was used as the loading control. A representative immunoblot of two independent experiments are shown.

In view of our initial result then we studied what signaling pathways related to proliferative events were stimulated by exogenous PTHrP in these intestinal cells. We observed that PTHrP stimulates the phosphorylation of ERKs as well as α isoform of p38 MAPK without affecting their protein expression. However, activated JNK levels did not change when Caco-2 cells were treated with PTHrP. Signals transmitted through protein kinase C (PKC) or Ras trigger ERK pathway signaling, which then activate Raf1, initiating a cascade involving MEK and then ERK activation [Cargnello and Roux, 2011]. The Ras/Raf/MEK/ERK cascade is involved in the control of growth signals, cell survival, and invasion in cancer. During the process of oncogenic transformation, colorectal cancer cells escape from normal growth and differentiation control and acquire the ability to invade surrounding tissues and organs [Fang and Richardson, 2005]. Mutations of Ras or BRAF, a serine-threonine kinase of the Raf family, and the up-regulation of the EGF receptor are the major mechanisms that activates ERK/MAPK signaling in colorectal cancer [Fang and Richardson, 2005]. However, in this type of cancer, the regulation of cell proliferation is also affected by external signals such as several growth factors that trigger signal transduction cascades through binding to membrane receptors [Fang and Richardson, 2005]. In this regard, our results suggest that PTHrP is an external factor that increased ERK-MAPK signaling in human colorectal adenocarcinoma. p38 MAPK pathway is involved in proliferation, differentiation, metabolism and cell death [Cargnello and Roux, 2011]. This cascade regulates the activity of several transcription factors in a signal-dependent and tissue-specific manner [Chiacchiera and Simone, 2008]. In mammals, four genes encode for different p38 isoforms, p38 α, β, γ, y, and δ. Comes et al. [2007] evaluated the impact of p38 signaling on colorectal cancer cell fate using the inhibitor SB202190 (specific for p38 α/β kinases) and they concluded that α p38 is required for colorectal cancer cell homeostasis. Based on this previous finding, the fact that PTHrP phosphorylates α p38 MAPK in Caco-2 cells emphasizes the significance of the mechanisms activated by PTH analog in human colon adenocarcinoma cells. JNKs are activated in response to

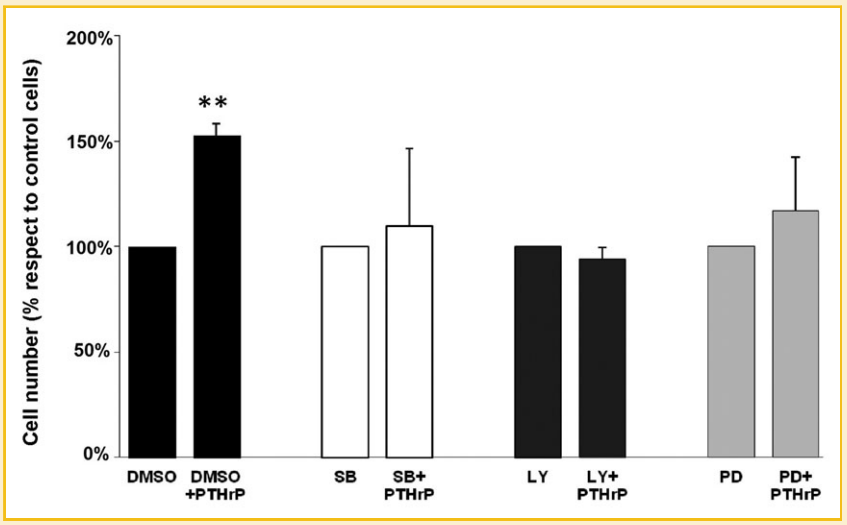

Fig. 7. Involvement of MAPK and PI3K signaling pathways in Caco-2 cells proliferation induced by PTHrP. Cells were pre-incubated with or without MEK inhibitor PD98059 (20 μM), p38 MAPK inhibitor SB203580 (20 μM), or PI3K inhibitor LY294002 (50 μM) for 30 min and then treated with PTHrP for 5 days. Cells were counted using Trypan Blue dye as described in materials and methods. Results are the average of four independent experiments. $^{**}P < 0.01$ where *P*-values refer to differences in proliferation between PTHrP treated cells (in the presence or absence of the inhibitor) with respect to its control.

inflammatory cytokines, environmental stresses, such as UV radiation, osmotic and heat shock, DNA and protein synthesis inhibition, and growth factors [Cargnello and Roux, 2011]. Several lines of evidence suggest that JNK pathway is not a regulator of cell proliferation in colorectal cancer [Fang and Richardson, 2005]. The observation that PTHrP does not activate JNK supports the idea that the peptide has proliferative effects on Caco-2 cells.

We found that exogenous PTHrP increases the levels of phosphorylated (active) Akt in Caco-2 cells and, in addition, PI3K-Akt pathway is involved on PTHrP- induced ERK 1/2 activation. Like MAPKs, there is increasing evidence that the activation of PI3K/Akt is associated with colorectal carcinoma, and can convert differentiated human gastric or colonic mucosa to a less differentiated and more malignant phenotype. The effects of PI3K on tumor growth and progression are thought to be mediated mainly by Akt [Pandurangan, 2013]. Akt is over-expressed in several cancers, including those of the colon, pancreas, ovary, and breast. Moreover, Akt phosphorylation in human colon carcinomas correlates with cell proliferation and inhibition of apoptosis, as well as different clinic-pathological parameters such as invasive grade, vessel infiltration, lymph node metastasis, and tumor stage [Khaleghpour et al., 2004].

Our fluorescence microscopy and subcellular fractionation studies suggest that extracellular PTHrP stimulated the translocation of ERK1/2, α p38, and Akt into the nucleus of colonic Caco-2 cells. It is known that once activated, MAPKs can translocate from the cytoplasm to the nucleus, leading to phosphorylation of several transcription factors and regulation of gene expression [Cargnello and Roux, 2011]. Recently, Zehorai et al. [2010] describe a novel nuclear translocation mechanism for ERKs, which is based on a nuclear translocation sequence (NTS) within their kinase insert domain (KID). The regulatory mechanisms of subcellular localization and nucleocytoplasmic trafficking of p38 MAPK still remain unclear because p38 MAPK have not an obvious signal sequence for nuclear import or nuclear export as ERK1/2 has [Zehorai et al., 2010]. Nevertheless, it has been shown that p38 MAPK is translocated to the nucleus by different stimuli in different cell types [Blanco-Aparicio et al., 1999]. Both Akt/PKB itself and several of its upstream regulators and downstream targets have been shown to partition between the nucleus and the cytoplasm in a manner that suggests a high degree of regulation of this process [Ferrigno and Silver, 1999]. Thus, following its activation, Akt has been implicated, either directly or indirectly, in the phosphorylation and subsequent regulation of several transcription factors [Kops et al., 1999].

The ATF/CREB family plays important roles in the regulation of a number of cell functions, including proliferation and apoptosis. Members of the ATF/CREB family dimerize with themselves or other family members and bind to the cyclic AMP response element (CRE) on target genes (van Dam and Castellazzi, 2001). We obtained evidence that PTHrP induces the phosphorylation of ATF-1 and CREB transcription factors in a biphasic manner. Our results suggest that p38 MAPK contribute to the changes observed in the early (first) phase of ATF/CREB phosphorylation, whereas the second phase is dependent of both p38 MAP kinase and ERK1/2. In agreement with these findings, Buzzi et al. [2009] showed that in Caco-2 cells and in presence of serum, CREB phosphorylation is dependent of MAPK activation.

Our experimental data indicates that exogenous PTHrP stimulates the translocation of β-catenin into the nucleus of Caco-2 cells and also increases the amount of both proteins c-Myc and Cyclin D. It is known that upon proliferative stimulus, β-catenin translocates to the nucleus [Osaki et al., 2004]; once in the nucleus, it combines with different transcription factors to induce the expression of several genes, such as c-Myc and Cyclin D [Saif and Chu, 2010]. Deregulation of the β-catenin signaling pathway has been observed

in various cancer types [Polakis, 2007]. For example, defective β-catenin destruction in cytosol has been reported in colorectal cancer which then leads to decreased degradation of β-catenin and its abnormal accumulation in the nucleus with subsequent constitutive activation of target genes. Mutations in the β-catenin gene have also been identified in colorectal cancer and several other solid tumors [Saif and Chu, 2010]. Myc is a pleiotropic basic helix-loop-helix leucine zipper transcription factor that coordinates expression of the diverse intracellular and extracellular programs that together are necessary for growth and expansion of somatic cells [Soucek et al., 2008]. The elevated expression of c-Myc has been observed as a frequent genetic abnormality in colon cancer and its depletion inhibits human colon cancer colo 320 cells growth [Hongxing et al., 2008]. With respect of Cyclin D1, this and other cell cycle-regulatory proteins are essential for cell cycle progression from G1 to S phase. There is evidence, under experimental conditions that lead to cell proliferation, that c-Myc expression was increased by serum in Caco-2 cells [Buzzi et al., 2009] whereas PTHrP stimulated the progression of cell cycle inducing Cyclin D1 expression in early osteoblastic cells [Datta et al., 2007] and, in chondrocytes and chondrosarcoma cells, an activating mutation of the PTH receptor (PTH1R) caused an increase in Cyclin D1 and Cyclin A expression [Beier and LuValle, 2002]. Our results showing that PTHrP up-regulates both c-Myc and Cyclin D1 synthesis provide additional information supporting the idea that PTHrP has proliferative effects on Caco-2 cells. More studies are necessary to evaluate if other cell cycle-regulatory proteins modulate cell cycle progression in Caco-2 cells exposed to PTHrP.

Finally, we showed that ERK1/2, p38 MAPK, and PI3-kinase inhibitors completely abolished the effect on PTHrP-induced colonic cell proliferation. Several evidences demonstrate that these signaling pathways are involved in the anti-apoptotic effects exerted by PTHrP through a paracrine pathway [Hochane et al., 2013; Lezcano et al., 2013]. Herein we prove the first evidence, to our knowledge, that ERK1/2, p38 MAPK, and PI3-kinase are also involved in the mitogenic action of exogenous PTHrP (1–34) fragment on Caco-2 cells. The fact that p38 MAPK pathway is required for the activation of Akt in human cancer cells [Kim et al., 2008] and taking into account that in Caco-2 cells Akt phosphorylation is seen at 1 h and that of p38 MAPK as early as 10 min of PTHrP exposure, suggest that p38 MAPK plays a role upstream in the signaling mechanism leading to Akt activation and subsequent ERK 1/2 activation in PTHrP-treated Caco-2 cells. Additional studies are necessary to further elucidate the molecular mechanisms that mediate MAPK and PI3K activation by PTHrP.

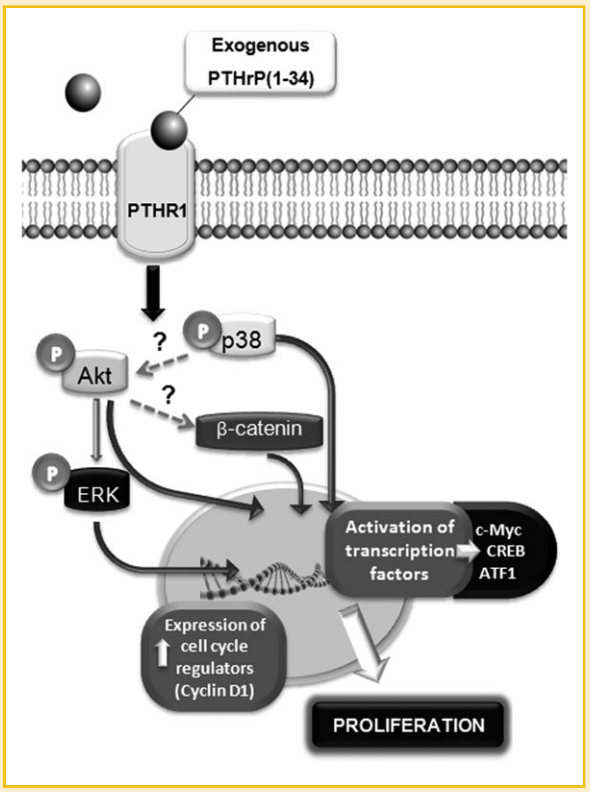

Fig. 8. Signal transduction pathways associated with the proliferation of human colon adenocarcinoma cells induced by exogenous PTHrP (this figure is original for this manuscript).

CONCLUSIONS

The present investigation provide, to our knowledge, the first direct evidence demonstrating that exogenous PTHrP induces the proliferation of Caco-2 cells, a cell line derived from human colon adenocarcinoma. The results obtained in this work expand our knowledge on the modes of action of PTHrP in intestinal tumor cells and identify the signaling pathways that are involved in the mitogenic effect of exogenous PTHrP on colon cancer cells (Fig. 8). These findings might be relevant for understanding tumor cell development, resistance to treatment regimens and the design of new therapeutic strategies aimed at blocking PTHrP action in colon cancer.

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