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Parathyroid hormone inhibits phosphorylation of mitogen-activated protein kinase (MAPK) ERK1/2 through inhibition of c-Raf and activation of MKP-1 in osteoblastic cells

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Parathyroid hormone (PTH) regulation of mitogen-activated protein kinases (MAPK) ERK1/2 contributes to PTH regulation of osteoblast growth and apoptosis. We investigated the mechanisms by which PTH inhibits ERK1/2 activity in osteoblastic UMR 106-01 cells. Treatment with PTH significantly inhibited phosphorylated ERK1/2 between 5 and 60 min. Transient transfection of cells with a cDNA encoding MAPK phosphatase-1 (MKP-1) resulted in 30–40% inhibition of pERK1/2; however MKP-1 protein levels were only significantly stimulated by PTH after 30 mins, suggesting another mechanism for the early phase of pERK1/2 inhibition. The active upstream kinase c-Raf phosphorylation at serine 338 (ser^{338}) was significantly inhibited by PTH treatment within 5 min and transfection of the cells with constitutively-active c-Raf blocked PTH inhibition of pERK1/2. Inhibition of pERK1/2 and phosphor-c-Raf were seen when cells were treated with PTH(1-34) or PTH(1-31) analogues that stimulate cAMP, but not with PTH(3-34), PTH(7-34) or PTH(18-48) that do not stimulate cAMP. Stimulation of the cells with forskolin or 8BrcAMP also inhibited pERK1/2 and c-Raf.p338. Our results suggest that rapid PTH inhibition of ERK1/2 activity is mediated by PKA dependent inhibition of c-Raf activity and that stimulation of MKP-1 may contribute to maintaining pERK1/2 inhibition over prolonged time.

KEY WORDS — parathyroid hormone; MAP-kinase; ERK1/2; MKP-1; c-Raf; osteoblast; PKA

INTRODUCTION

Parathyroid hormone (PTH) is an 84 amino acid peptide hormone secreted from the parathyroid glands in response to decreased plasma calcium concentrations. PTH restores ambient calcium levels through a combination of effects on the kidney to increase calcium reabsorption from the glomerula filtrate as well as mobilizing calcium and phosphate release from the stores of mineral in bone. PTH also indirectly increases calcium uptake from the intestines through its stimulation of the 1-hydroxylase enzyme in the kidney, Cyp27b1, that is responsible for the final activation of vitamin D to its active form of $1,25(OH_2)$vitamin D.

Depending on the duration of exposure, PTH can be either anabolic or catabolic to bone mass. Under normal physiological conditions PTH causes bone resorption and consequently releases calcium and phosphate from the bone mineral in order to maintain serum calcium homeostasis. This bone resorption is balanced by later deposition of new bone. When bone is exposed to PTH for prolonged times on the other hand bone degradation predominates and bone mass and strength are diminished. As a therapeutic agent, repeated transient PTH treatments can increase bone mass and decrease fracture incidence in patients with osteoporosis.[1]

The signaling pathways that mediate the effects of PTH in both kidney and bone have been studied by many groups and found to be mediated by the PTH receptor (PTH1R).[2] The PTH1R is a G-protein coupled receptor, which couples primarily to the $G\alpha_s$ and $G\alpha_{q/11}$ proteins.[2–4] These two G proteins mediate adenylyl cyclase regulation of PKA and PLC-β regulation of PKC, respectively. The effects of PTH on osteoblasts are primarily the result of PKA signaling, with PKC activation enhancing some effects such as PTH activation of insulin-like growth factor binding protein and matrix metalloproteinase 13.[5–7] A variety of PTH analogues have been tested for their effects on stimulation of PKA and PKC pathways through the PTH1R. In general, an intact amino terminus is required for PTH to stimulate cAMP levels in cells whereas PTH analogues that are N-terminally truncated, such as PTH(3-34), and PTH(13-34) and mid-region fragments such as PTH(28-48) can stimulate PKC but

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not PKA in many cells including osteoblasts.[8–10] PTH(1-31) has been reported to selectively stimulate adenylyl cyclase in osteoblasts with no stimulation of PKC.[8]

Mitogen-activated protein (MAP) kinases are members of distinct signaling cascades, and they have been shown to regulate multiple cellular activities in osteoblasts. The mammalian MAP kinase family has been divided into several groups: extracellular signal-regulated kinases (ERK1/2), p38 MAP kinases (p38α-δ), the c-jun N-terminal kinases (JNK1-3), and the ERK5 protein.[11–13] Different MAP kinases are activated by dual phosphorylation (on threonine and tyrosine residues) by similar but distinct MAP kinase kinases, which are likewise phosphorylated and stimulated by upstream kinases activated by different extracellular stimuli.[14] For example, ERK1/2 is activated when Ras is stimulated by a variety of cell surface receptors. Activated Ras stimulates c-Raf, which in turn phosphorylates MEK1, the primary upstream phosphorylator of ERK1/2. Activated MAP kinases can subsequently phosphorylate downstream substrates including other protein kinases and transcription factors to regulate cell proliferation, differentiation, and apoptosis. MAP kinases are inactivated by phosphatases known as MAP kinase phosphatases, or MKPs, that belong to the dual-specificity phosphatase family.[15] MKP-1 is a highly inducible member of the MKP family that is found primarily in the nucleus. MKP-1 can be induced by a variety of stimuli such as heat shock, oxidative stress and dexamethasone.[15]

In osteoblasts, ERK1/2 has been shown to regulate osteoblast proliferation, apoptosis and differentiation by regulating the expression of cell-cycle regulators[16–18] as well as the activity of the skeletal specific transcription factor Runx2.[19,20] PTH has been shown to either activate or inactivate ERK1/2 depending on the stage of differentiation of the cells in which the receptor is expressed and the concentration of PTH to which it is exposed.[21] The mechanism by which the PTH1R stimulated ERK1/2 was investigated in HEK293 cells transfected with PTH1R in which they found PTH stimulation of ERK1/2 was mediated by a combination of second messenger stimulated pathways and arrestin-mediated pathways.[22] The mechanisms by which PTH1R can inhibit ERK1/2 activity on the other hand are less well known. In this study we have used the osteosarcoma cell line UMR 106-01 in which PTH rapidly inhibits ERK1/2 within 5 min to investigate the mechanism.

METHODS

Cell culture and cell treatments

UMR 106-01 osteoblastic cells (from Dr N. Partridge, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ, USA) were grown in 50% Dulbecco's modified Eagle's medium and 50% F-12 medium containing 1 U/ml penicillin, 1 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, and supplemented with 5% fetal bovine serum. All culture reagents were purchased from Gibco BRL/Invitrogen (Burlington, ON, Canada). Cell treatments were performed 2 days after cell plating or 24 h after transfection. Recombinant rat PTH (1-34) human PTH (1-31), bovine PTH (3-34) bovine (D-Trp^{12}, Tyr^{34}) PTH (7-34) amide and human PTH (18-48) were purchased from Bachem (King of Prussia, PA, USA). Adenosine-3′,5′-cyclic monophosphate 8-bromo (8BrcAMP) and forskolin (fsk) were purchased from EMD Biosciences (San Diego, CA). Primary antibodies (pERK1/2: #9101, ERK1/2: #9102, p338 c-Raf: #9427, c-Raf: #9422) were purchased from Cell Signaling Technology, antibody to MKP-1 was purchased from Santa Cruz Technology (#sc370) (Santa Cruz, CA, USA).

Transient transfection

UMR 106-01 cells were grown to 60–70% confluence in 6-well plates over 24 h, and were transfected with the following expression vectors: pEGFP-C1 (Clontech, Mountain View, CA, USA), pSV-β-galactosidase (Promega, Madison, WI, USA), pSG5-MKP-1 (a generous gift from Dr N. Tonks, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA) or BXB, a constitutively-active c-Raf mutant (a generous gift from Dr U.R. Rapp, University of Wuerzburg, Wuerzburg, Germany), using the Lipofectamine^{TM} reagent (Invitrogen). For each well, one ug of DNA and 4 μl of transfection reagent were incubated in the culture medium mentioned above with the serum removed. The transfection efficiency was determined by transfecting the pSV-β-galactosidase vector. It was found to be approximately 30% based on the percentage of blue-stained cells after staining with X-gal.

Cell lysate preparation and western blotting

Cells were washed with ice-cold PBS and then lysed in buffer: 50 μM Tris, pH7.4, 150 μM NaCl, 1 μM EDTA, 1 μM NaF, 1.5 μM Na3VO4, 1 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1% Nonidet P-40, on ice for 30 min. Lysates were cleared by centrifugation at 12 000 *g* for 15 min at 4°C. Protein concentrations of the cleared lysates were determined by the Amido Black protein assay.[23] Cell lysates were separated on 11% SDS-polyacrylamide gels, transferred to nitrocellulose membranes (GE Healthcare, Baie d'Urfe', QC, Canada), and blocked with Tris-based buffer containing 0.1% Tween (TBST) with 5% milk powder. Nitrocellulose membranes were then probed with specific primary polyclonal antibodies diluted in TBST with 1% BSA overnight (pERK1/2, ERK1/2, p338 c-Raf, and c-Raf: 1:1000; MKP-1: 1:500) followed by incubation with HRP-conjugated anti-rabbit secondary antibody (Cell signaling Technology, Danvers, MA, USA) diluted (1:2 000) in TBST with 5% milk. All nitrocellulose membranes were finally incubated with ECL enhanced chemiluminesence solution (GE Healthcare), and exposed to Kodak X-OMAT films. Protein band intensities were quantitated by scanning

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densitometry using ImageQuant™ software (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis

All data shown are representative of at least three independent experiments. Data from each independent experiment were normalized to the control sample in that particular experiment. Quantitative data were plotted as mean ± SD of the values obtained from three individual experiments, and analyzed either by Student's *t*-test or one-way ANOVA followed by Bonferroni's multiple comparison post-hoc test. Analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Dephosphorylation of ERK1/2 by PTH in UMR 106-01 osteoblastic cells

The effect of PTH on the ERK1/2 activity was determined by measuring the phosphorylation level of ERK1/2 in UMR 106-01 cells treated with 10 nM PTH (1-34). An antibody specific for the ERK1/2 protein dual-phosphorylated at threonine 202 and tyrosine 204 was used in the western blot. PTH was able to dephosphorylate ERK1/2 rapidly, which was observed within 5 min after the PTH treatment, and sustained for at least 1 h (Figure 1A). PTH dephosphorylated ERK1/2 in a concentration-dependent manner with an IC_{50} of approximately 3 nM (Figure 1B).

MKP-1 expression is stimulated by PTH

Several previous studies *in vivo* and *in vitro* have found that the mRNA encoding MAPK phosphatase MKP-1 is stimulated by PTH. Since ERK1/2 is a known substrate for MKP-1 we examined the effect of PTH stimulation on the MKP-1 protein levels in UMR 106-01 cells. Expression of the MKP-1 protein in UMR 106-01 cells treated with 10 nM PTH (1-34) was determined by western blots with a specific antibody against the MKP-1 protein. Increased MKP-1 expression was observed after 30 min of PTH treatment. PTH increased the MKP-1 protein level by more than 10-fold, with the maximum level seen at 60 min. The MKP-1 protein level remained elevated for at least 3 h (Figure 2A). PTH stimulated the MKP-1 protein expression showed a similar concentration-dependence as that seen for MKP-1 mRNA with an EC_{50} of approximately 6 nM (Figure 2B). These data showed that PTH stimulates the MKP-1 protein levels but with a slower time course than seen for the inhibition of ERK1/2.

Overexpression of MKP-1 decreases the ERK1/2 phosphorylation levels

In order to examine the possibility that PTH-induced dephosphorylation of the ERK1/2 protein could be mediated by stimulation of the MKP-1 protein, the ERK1/2 phosphorylation levels in UMR 106-01 cells transiently transfected with a MKP-1 expression vector was determined. UMR 106-01 cells expressing MKP-1 cDNA had an elevated MKP-1 protein level similar to that found with PTH treatment (Figure 3). Overexpressing MKP-1 or PTH treatment caused similar levels of dephosphorylation of ERK1/2 proteins (Figures 1A and 3). These results support the hypothesis that PTH stimulation of MKP-1 contributes to PTH dephosphorylation of ERK1/2 in UMR 106-01 cells at least after 30 min of stimulation.

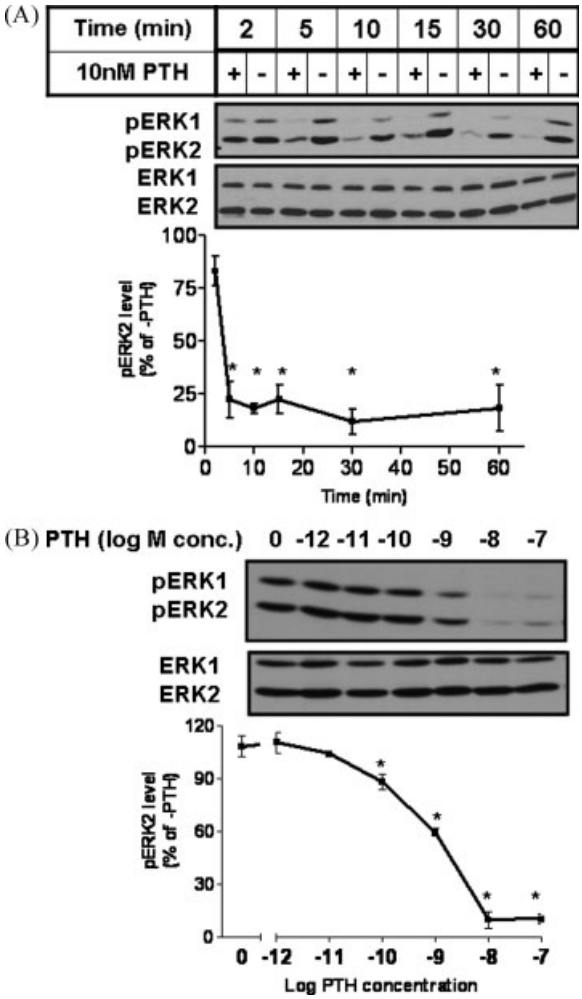

Figure 1. PTH dephosphorylation of ERK1/2 in osteoblastic cells. UMR 106-01 cells were cultured with 10 nM PTH (1-34) for the indicated time periods (A), or with the indicated concentrations of PTH for 30 min (B). Total cell lysates (10 μg) were analyzed by SDS-PAGE, and western blots using specific antibodies against phosphorylated ERK1/2 (pERK1/2) or total ERK1/2. Quantitations of the pERK2 level from three blots were averaged and plotted as mean ± SD. * Significantly different from cells not treated with PTH ($p < 0.05$). The protein levels of total ERK1/2 were used as a loading control. Representative blots are shown

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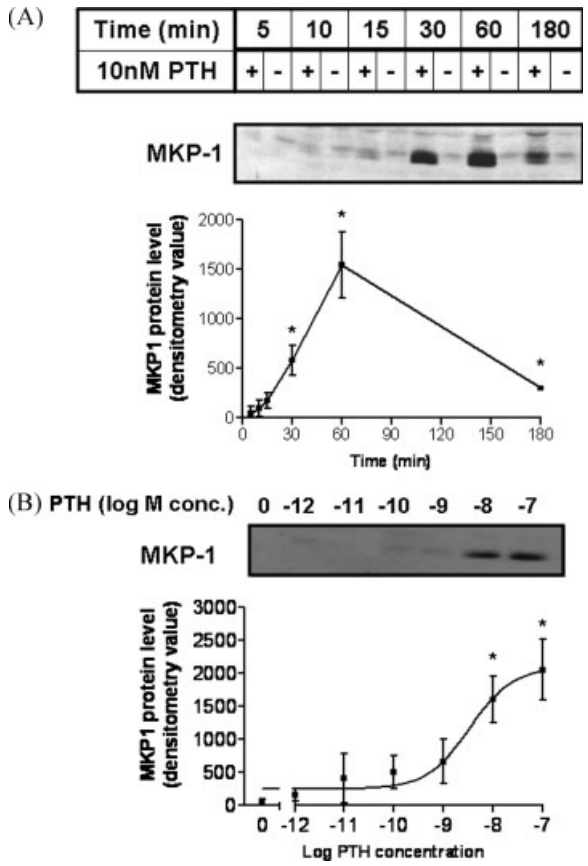

Figure 2. PTH stimulation of the MKP-1 protein in osteoblastic cells. UMR 106-01 cells were cultured with 10nM PTH (1-34) for the indicated time periods (A), or for 30 min with the indicated PTH (1-34) concentrations (B). Total cell lysates (30 μg) were analyzed by SDS-PAGE, and western blots using specific antibodies against the MKP-1 protein. Quantitations from three different blots were averaged and plotted, and representative blots are shown. * Significantly different from cells not treated with PTH ($p < 0.001$)

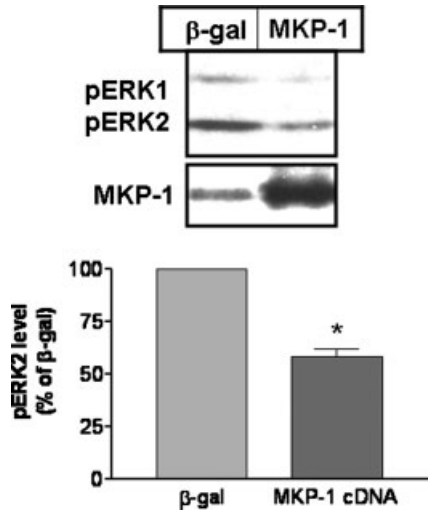

Figure 3. Overexpression of the MKP-1 protein dephosphorylates ERK1/2. UMR 106-01 cells were transiently transfected with the vector expressing the MKP-1 protein or β-galactosidase (β-gal). Total cell lysates (10 μg for pERK1/2 or 30 μg for MKP-1) were analyzed by SDS-PAGE, and western blots using specific antibodies against phosphorylated ERK1/2 (pERK1/2) or MKP-1. Quantitations of the pERK2 level from three blots were averaged and plotted as mean ± SD. A representative blot is shown. * Significantly different from β-gal transfected cells ($p < 0.05$)

Inactivation of c-Raf by PTH in UMR 106-01 osteoblastic cells

Inhibition of pERK1/2 levels in UMR 106-01 cells appeared to precede increases in MKP-1 protein in these cells therefore we examined the effect of PTH on the activated c-Raf levels by measuring the phosphorylation level of the c-Raf protein at serine 338 (ser^{338}) in UMR 106-01 cells treated with 10 nM PTH (1-34). An antibody specific for the ser^{338}-phosphorylated c-Raf was used in the western blots. PTH stimulation resulted in rapid dephosphorylation of c-Raf observed within 5 min after the PTH treatment, and sustained for at least 1 h (Figure 4A). PTH stimulation of c-Raf dephosphorylation occurred at very low PTH concentrations with a maximal effect seen with 1 nM PTH (Figure 4B). PTH had no effect on the expression of c-Raf protein (Figure 4).

Constitutively-active c-Raf alleviates PTH dephosphorylation of ERK1/2

In order to examine the role of c-Raf inhibition in mediating PTH dephosphorylation of the ERK1/2 protein, the ERK1/2 phosphorylation level was determined in UMR-106 cells transiently transfected with a vector expressing the constitutively-active c-Raf mutant (BXB). The BXB mutant is truncated at the carboxyl terminus removing regulatory sites including ser^{338}. UMR-106 cells transiently transfected with BXB had an elevated ERK1/2 phosphorylation level (Figure 5). Furthermore, PTH was not able to significantly dephosphorylate ERK1/2 in the BXB-transfected cells (Figure 5). These results support the hypothesis that PTH inactivation of c-Raf mediates PTH dephosphorylation of ERK1/2 in UMR 106-01 cells.

Stimulation of PKA mediates inhibition of pERK and c-Rafp338

To determine which signal transduction pathway stimulated by PTH mediated inhibition of ERK1/2 phosphorylation and c-Raf phosphorylation UMR106-01 cells were incubated with various PTH analogues. Stimulation with PTH (1-31) and PTH (1-34) as well as forskolin and a cell-permeable analogue of cAMP, 8BrcAMP all had similar inhibitory effects on pERK1/2 phosphorylation (Figure 6A) as well as inhibition of c-Rafp338 (Figure 6B). Amino-terminally truncated PTH analogues PTH (3-34) and PTH (7-34) as well as the mid-region fragment PTH (18-48) each had no significant effect on either pERK1/2 or c-Rafp338 in the

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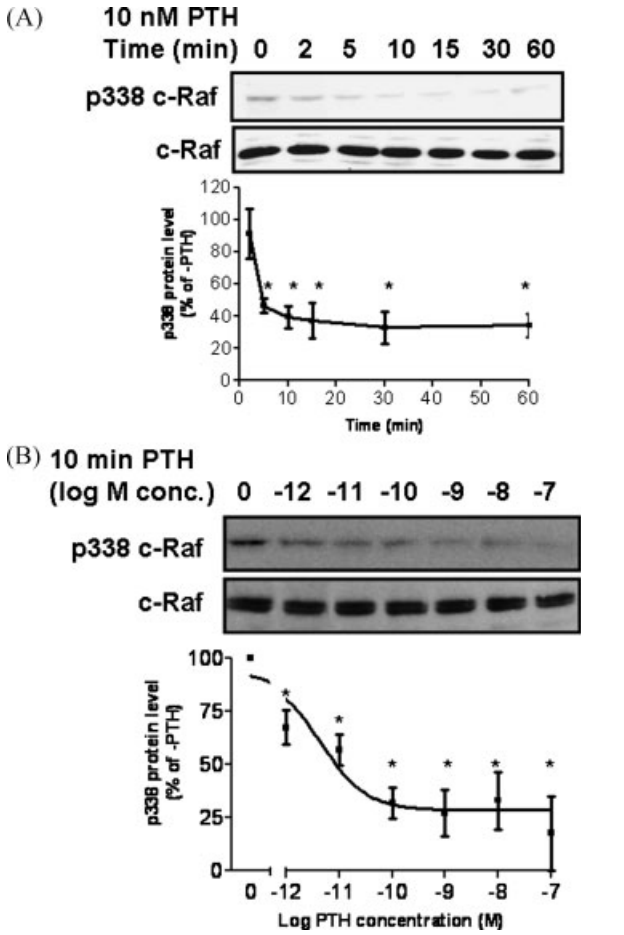

Figure 4. PTH inactivation of c-Raf in osteoblastic cells. UMR 106-01 cells were cultured with 10 nM PTH (1-34) for the indicated time periods (A), or for 30 min with the indicated PTH (1-34) concentrations (B). Total cell lysates (5 μg) were analyzed by SDS-PAGE, and western blots using specific antibodies against the c-Raf protein phosphorylated at serine 338 (p338 c-Raf) or total c-Raf. Quantitations from three different blots were averaged and plotted as mean ± SD. * Significantly different from cells not treated with PTH ($p < 0.05$). Representative blots are shown. The protein level of c-Raf was used as a loading control

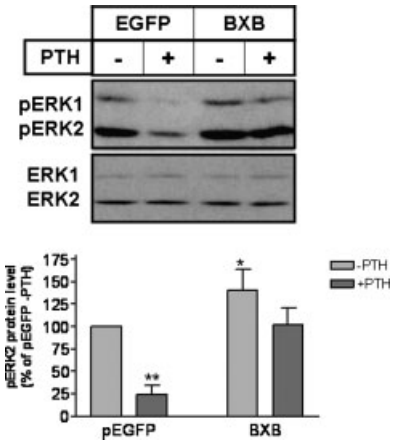

Figure 5. The constitutively-active c-Raf alleviates PTH dephosphorylation of ERK1/2 in osteoblastic cells. UMR 106-01 cells transiently transfected with the vector expressing the constitutively-active c-Raf protein (BXB) or EGFP, were treated with 10 nM PTH (1-34) for 30 min. Total cell lysates (10 μg) were analyzed by SDS-PAGE, and western blots using specific antibodies against phosphorylated ERK1/2 (pERK1/2) or total ERK1/2. Quantitations of the pERK2 level from three blots were averaged and plotted as mean ± SD. The protein levels of total ERK1/2 were used as a loading control. Representative blots are shown. * BXB-PTH significantly different from EGFP-PTH and ** EGFP + PTH significantly different from EGFP-PTH ($p < 0.05$)

cells (Figure 6A and B, respectively). These results suggest that PTH regulated both c-Raf and ERK1/2 activity through a cAMP-dependent pathway.

DISCUSSION

The aim of this study was to identify the signaling pathways that mediate PTH inhibition of ERK1/2 in osteoblastic cells. Using an osteosarcoma cell line, UMR 106-01, we showed that PTH decreases ERK1/2 activity rapidly with a concomitant decrease in c-Raf activity and stimulation of MKP-1 levels. Transient transfection experiments showed that both c-Raf and MKP-1 can contribute to PTH inhibition of ERK1/2. However, the slower time course of PTH stimulation of MKP-1 suggested that dephosphorylation of pERK1/2 by this enzyme probably mediates the late phase of PTH inactivation of ERK1/2, while inhibition of c-Raf activity mediates the early phase.

MKP-1 belongs to the dual-specificity protein phosphatase family, which can specifically dephosphorylate both the threonine and tyrosine residues of MAP kinases in order to inactivate them. There are ten members in this family, and they can be broadly divided into two groups, based on their subcellular localization and how they are regulated at the transcriptional level.[15] MKP-1 is encoded by an immediate-early gene, and it can be regulated at the transcriptional level by the PKA or PKC signaling pathway. MKP-1 has been identified as a PTH-regulated gene in microarray analysis of UMR 106-01 cells,[5] and we demonstrate here a profound increase in MKP-1 protein levels seen within 30 min of PTH treatment. These data are in agreement with previous studies demonstrating that the regulation of MKP-1 by PTH is mainly mediated by the PKA signaling pathway, and MKP-1 can mediate PTH regulation of ERK1/2 in osteoblasts.[18,24] Previous studies found that a phosphatase can contribute to PTH regulation of ERK1/2 in UMR 106-01 cells and that PTH stimulated MKP-1 expression in these cells after 30 min.[25] We have shown that overexpression of the MKP-1 protein was able to decrease the ERK1/2 activity, however, when examined at earlier times than those assessed by Qin *et al.*[18] it is clear that the slower effect of PTH on MKP-1 expression, compared to the rapid inactivation of ERK1/2 by PTH in UMR106-01 cells suggested another signaling pathway mediating the early phase of ERK1/2 inactivation. It is possible that PTH also regulates other members of the MKP family of proteins that can dephosphorylate ERK1/2

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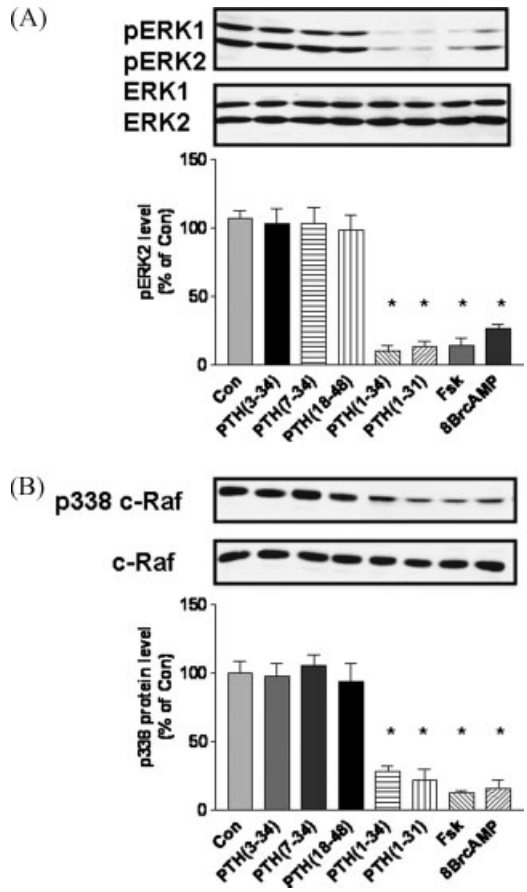

Figure 6. Inactivation of c-Raf in osteoblastic cells by PTH analogues and activators of PKA. UMR 106-01 cells were cultured with 10 nM PTH (1-34) or PTH (3-34) or 100 nM PTH (3-34), PTH (7-34) or PTH (18-48) or 10 μM Fsk, 1 mM 8BrcAMP for 30 min (A). Total cell lysates (10 μg) were analyzed by SDS-PAGE, and western blots using specific antibodies against phosphorylated ERK1/2 (pERK1/2) or total ERK1/2. Quantitations of the pERK2 level from three blots were averaged and plotted as mean + SD. The protein levels of total ERK1/2 were used as a loading control. Representative blots are shown (B). Total cell lysates (10 μg) were analyzed by SDS-PAGE, and western blots using specific antibodies c-Raf protein phosphorylated at serine 338 (p338 c-Raf) or total c-Raf. Quantitations from three different blots were averaged and plotted as mean ± SD. Representative blots are shown. The protein level of c-Raf was used as a loading control. * Significantly different from cells not treated with PTH ($p < 0.001$)

and that this could contribute to the early phase of ERK1/2 dephosphorylation seen in the UMR cells. However other MKP family members were not found to be regulated in a study of PTH regulation of UMR106-01 cells using microarray analysis,[5] and therefore they were not investigated here.

c-Raf is one of the key upstream kinases that phosphorylate MEK1/2 and subsequently activate the ERK1/2 signaling pathway. It is activated by phosphorylation when it is recruited to the plasma membrane after binding to activated Ras protein. However, the regulation of c-Raf activity is more complicated than that of MEK1/2 or ERK1/2, which are regulated simply by phosphorylation or dephosphorylation. There are at least 13 phosphorylation sites (mainly serines) in the c-Raf protein. Phosphorylation of the amino acids on the N-terminal end usually results in inactivation, while phosphorylation of the C-terminal end usually results in activation. In particular, serine[338], tyrosine[341], threonine[491] and serine[495] are crucial for c-Raf activity, and they have been shown to be phosphorylated as a result of c-Raf recruitment to the plasma membrane.[25–28] We showed that PTH treatment resulted in rapid dephosphorylation of c-Raf at ser[338], suggesting that PTH is able to inhibit the c-Raf activity in osteoblasts. In addition, we showed that PTH was able to decrease both ERK1/2 and c-Raf activity with the same time course and similar efficacy. Overexpression of BXB, a C-terminally truncated constitutively active c-Raf, in UMR 106-01 cells alleviated the inhibitory effect of PTH on ERK1/2, further suggesting that c-Raf plays an important role in mediating PTH regulation of ERK1/2.

The molecular mechanism by which PTH inactivates the c-Raf activity was examined in this study and only PTH analogues that stimulate cAMP levels in the cells, PTH (1-34) and PTH (1-31) inhibited ERK1/2 and c-Raf phosphorylations. Stimulation by forskolin or 8BrcAMP also resulted in ERK1/2 and c-Raf dephosphorylation. Thus it would be reasonable to propose that the PKA signaling pathway plays an important role in mediating PTH effect on these two proteins and that activation of this pathway is sufficient to cause these effects. PKA has been shown to be able to directly phosphorylate three serine residues on c-Raf; serine[43], serine[233], and serine[259]. Phosphorylation of serine[43] disrupts c-Raf interaction with the Ras protein, while phosphorylation of serine[233] and serine[259] promotes 14-3-3 interaction with c-Raf, which disrupts recruitment of c-Raf to the plasma membrane.[29] Overall, phosphorylation of c-Raf by PKA results in c-Raf inactivation.

The Raf/MEK/ERK1/2 signalling pathway has been shown to be a key regulator of osteoblast apoptosis and differentiation.[17–19] Overexpression of an active-form of the c-Raf protein decreased β1-integrin-mediated osteoblast-extracellular matrix interaction, which indirectly increased osteoblast apoptosis.[16] Furthermore, c-Raf was also shown to mediate mechanical stress stimulation of Runx2 expression in osteoblasts.[20] Our studies further demonstrated the important role of c-Raf signaling pathway in mediating PTH regulation of ERK1/2 in osteoblasts and provide a mechanism by which PTH at high concentrations inhibits the growth of osteoblasts. The molecular mechanisms that mediate both the anabolic and catabolic effects of PTH on bone remain to be elucidated. Future studies examining the differential effects of prolonged and transient PTH1R activation on MKP-1, c-Raf and ERK1/2 in osteoblasts will likely provide new insights into the effect of PTH on bone turnover.

In summary, we show here for the first time that PTH stimulation of osteoblastic UMR106-01 cells can regulate c-Raf phosphorylation through a cAMP-mediated pathway and that this inhibition of c-Raf plays a role in the rapid dephosphorylation of ERK.

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REFERENCES

1. Hodsman AB, Bauer DC, Dempster DW, *et al.* Parathyroid hormone and teriparatide for the treatment of osteoporosis: a review of the evidence and suggested guidelines for its use. *Endocr Rev* 2005; **26**: 688–703.
2. Abou-Samra AB, Juppner H, Force T, *et al.* Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc Natl Acad Sci U S A* 1992; **89**: 2732–2736.
3. Demay M, Mitchell J, Goltzman D. Comparison of renal and osseous binding of parathyroid hormone and hormonal fragments. *Am J Physiol* 1985; **249**: E437–E446.
4. Mitchell J, Bansal A. Dexamethasone increases G alpha q-11 expression and hormone-stimulated phospholipase C activity in UMR-106-0 cells. *Am J Physiol* 1997; **273**: E528–535.
5. Qin L, Qiu P, Wang L, *et al.* Gene expression profiles and transcription factors involved in parathyroid hormone signaling in osteoblasts revealed by microarray and bioinformatics. *J Biol Chem* 2003; **278**: 19723–19731.
6. Erclik MS, Mitchell J. The role of protein kinase C-delta in PTH stimulation of IGF-binding protein-5 mRNA in UMR-106-01 cells. *Am J Physiol Endocrinol Metab* 2002; **282**: E534–541.
7. Cheung R, Erclik MS, Mitchell J. Increased expression of G11alpha in osteoblastic cells enhances parathyroid hormone activation of phospholipase C and AP-1 regulation of matrix metalloproteinase-13 mRNA. *J Cell Physiol* 2005; **204**: 336–343.
8. Whitfield JF, Isaacs RJ, Chakravarthy B, *et al.* Stimulation of protein kinase C activity in cells expressing human parathyroid hormone receptors by C- and N-terminally truncated fragments of parathyroid hormone 1-34. *J Bone Miner Res* 2001; **16**: 441–447.
9. Azarani A, Goltzman D, Orlowski J. Parathyroid hormone and parathyroid hormone-related peptide inhibit the apical Na+/H+ exchanger NHE-3 isoform in renal cells (OK) via a dual signaling cascade involving protein kinase A and C. *J Biol Chem* 1995; **270**: 20004–20010.
10. Shimoyama M, Ogino K, Uchida K, *et al.* Fragment-specific actions of parathyroid hormone in isolated perfused rat hearts. *Calcif Tissue Int* 2001; **69**: 88–93.
11. Cobb MH. MAP kinase pathways. *Prog Biophys Mol Biol* 1999; **71**: 479–500.
12. Pearson G, Robinson F, Beers Gibson T, *et al.* Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 2001; **22**: 153–183.
13. Davis RJ. MAPKs: new JNK expands the group. *Trends Biochem Sci* 1994; **19**: 470–473.
14. Cobb MH, Goldsmith EJ. How MAP kinases are regulated. *J Biol Chem* 1995; **270**: 14843–14846.
15. Farooq A, Zhou MM. Structure and regulation of MAPK phosphatases. *Cell Signal* 2004; **16**: 769–779.
16. Tanaka Y, Nakayamada S, Fujimoto H, *et al.* H-Ras/mitogen-activated protein kinase pathway inhibits integrin-mediated adhesion and induces apoptosis in osteoblasts. *J Biol Chem* 2002; **277**: 21446–21452.
17. Datta NS, Chen C, Berry JE, McCauley LK. PTHrP signaling targets cyclin D1 and induces osteoblastic cell growth arrest. *J Bone Miner Res* 2005; **20**: 1051–1064.
18. Qin L, Li X, Ko JK, Partridge NC. Parathyroid hormone uses multiple mechanisms to arrest the cell cycle progression of osteoblastic cells from G1 to S phase. *J Biol Chem* 2005; **280**: 3104–3111.
19. Ge C, Xiao G, Jiang D, Franceschi RT. Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. *J Cell Biol* 2007; **176**: 709–718.
20. Kanno T, Takahashi T, Tsujisawa T, Ariyoshi W, Nishihara T. Mechanical stress-mediated Runx2 activation is dependent on Ras/ERK1/2 MAPK signaling in osteoblasts. *J Cell Biochem* 2007; **101**: 1266–1277.
21. Chen C, Koh AJ, Datta NS, *et al.* Impact of the mitogen-activated protein kinase pathway on parathyroid hormone-related protein actions in osteoblasts. *J Biol Chem* 2004; **279**: 29121–29129.
22. Gesty-Palmer D, Chen M, Reiter E, *et al.* Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *J Biol Chem* 2006; **281**: 10856–10864.
23. Schaffner W, Weissmann C. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal Biochem* 1973; **56**: 502–514.
24. Homme M, Schmitt CP, Mehls O, Schaefer F. Mechanisms of mitogen-activated protein kinase inhibition by parathyroid hormone in osteoblast-like cells. *J Am Soc Nephrol* 2004; **15**: 2844–2850.
25. Chong H, Lee J, Guan KL. Positive and negative regulation of Raf kinase activity and function by phosphorylation. *EMBO J* 2001; **20**: 3716–3727.
26. Fabian JR, Daar IO, Morrison DK. Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. *Mol Cell Biol* 1993; **13**: 7170–7179.
27. King AJ, Sun H, Diaz B, *et al.* The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. *Nature* 1998; **396**: 180–183.
28. Mason CS, Springer CJ, Cooper RG, Superti-Furga G, Marshall CJ, Marais R. Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. *EMBO J* 1999; **18**: 2137–2148.
29. Dumaz N, Marais R. Protein kinase A blocks Raf-1 activity by stimulating 14-3-3 binding and blocking Raf-1 interaction with Ras. *J Biol Chem* 2003; **278**: 29819–29823.