Impact of the Mitogen-activated Protein Kinase Pathway on Parathyroid Hormone-related Protein Actions in Osteoblasts*

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Parathyroid hormone-related protein (PTHrP) regulates proliferation and differentiation of osteoblastic cells via binding to the parathyroid hormone receptor (PTH-1R). The cAMP-dependent protein kinase A pathway governs the majority of these effects, but recent evidence also implicates the MAPK pathway. MC3T3-E1 subclone 4 cells (MC4) were treated with the MAPK inhibitor U0126 and PTHrP. In differentiated MC4 cells, osteocalcin and bone sialoprotein gene expression were both down-regulated by PTHrP and also by inhibition of the MAPK pathway. PTHrP-mediated down-regulation of PTH-1R mRNA and up-regulation of c-fos mRNA were MAPK-independent, whereas PTHrP stimulation of fra-2 and interleukin-6 (IL-6) mRNA was MAPK-dependent. Luciferase promoter assays revealed that regulation of IL-6 involved the cAMP-dependent protein kinase A and MAPK pathways with a potential minor role of the protein kinase C pathway, and a promoter region containing an activator protein-1 site was necessary for PTHrP-induced IL-6 gene transcription. An alternative pathway, through cAMP/Epac/Rap1/MAPK, mediated ERK phosphorylation but was not sufficient for IL-6 promoter activation. Phosphorylation of the transcription factor CREB was also necessary but not sufficient for PTHrP-mediated IL-6 promoter activity. Most interesting, a bidirectional effect was found with PTHrP increasing phosphorylated ERK in undifferentiated MC4 cells but decreasing phosphorylated ERK in differentiated cells. These data indicate that inactivation of the MAPK pathway shows differential regulation of PTHrP-stimulated activator protein-1 members, blocks PTHrP-stimulated IL-6, and synergistically down-regulates certain osteoblastic markers associated with differentiation. These novel findings indicate that the MAPK pathway plays a selective but important role in the actions of PTHrP.

Parathyroid hormone (PTH)† and parathyroid hormone-related protein (PTHrP) both have catabolic and anabolic actions in bone, dependent on their mode of administration. The structural homology of PTHrP to PTH enables it to mimic the actions of PTH through its ability to bind to the type I PTH/PTHrP receptor (PTH-1R) (1), but these two proteins play different roles in development and disease. Studies of the downstream signaling events of PTH-1R have focused mainly on the cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) pathways (2), and the impact of these signaling events has been partially elucidated. However, the dependence on these and other signaling pathways for the various skeletal effects of PTH and PTHrP is still unclear.

At the cellular level, the PKA pathway governs the majority of the effects induced by PTH and PTHrP on osteoblasts, but recent evidence (3, 4) also implicates the mitogen-activated protein kinase (MAPK) pathway. The MAPK cascade is also called the extracellular signal-regulated kinase (ERK) cascade and has been found to be involved in cell adhesion, proliferation, differentiation, viability, and apoptosis (5, 6). Many extracellular growth factors signal through GTPase Ras/MAPK pathways to activate gene expression (7, 8). Another GTPase of the Ras superfamily, Rap1, is activated by PKA phosphorylation in human neutrophils (9, 10) and further activates the MAPK pathway (11, 12). Activation of the MAPK cascade involves phosphorylation of MAPKKK (Raf-1), MAPKK, and MAPK (ERK). Active MAPK subsequently phosphorylates transcription factors, other downstream kinases, and substrates leading to biologic activity (13).

PTHrP has been reported to regulate MAPK in several systems. PTHrP represses adipocyte differentiation through the PKA and MAPK pathways (3). PTHrP stimulates rat bone marrow cell proliferation through PKC activation of Ras/MAPK (4). PTHrP induces indices of osteoblast differentiation in MG-63 osteosarcoma cells via multiple signaling pathways including PKA, PKC, and MAPK (14). Furthermore, normal osteoblast-specific gene expression and differentiation in vitro require MAPK (15, 16). Taken together, these studies suggest that MAPK plays an important role in the actions of PTH and PTHrP in bone, but the precise pathways are still unclear. We have shown previously that PTHrP induces apoptosis in post-confluent osteoblastic cells but plays a protective role against apoptosis in less differentiated osteoblasts suggesting that actions of PTH and PTHrP may be cell context-dependent (17).

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§ O-Me-cAMP, 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate; AP-1, activator protein-1.
* The abbreviations used are: PTH, parathyroid hormone; IL-6, interleukin-6; PTHrP, parathyroid hormone-related protein; MAPK, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; CREB, cAMP-response element-binding protein; OCN, osteocalcin; BSP, bone sialoprotein; ERK, extracellular signal-regulated kinase; PMA, phorbol 12-myristate 13-acetate; PTH-1R, parathyroid hormone receptor; CRE, cAMP-response element; SCPT-2′-O-Me-cAMP, 8-(4-chloro-phenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate; AP-1, activator protein-1.
EXPERIMENTAL PROCEDURES

Reagents—Human PTH-(1–34) and PTHrP-(1–34) were obtained from Bachem (Torrance, CA). U0126, a specific MEK-1 inhibitor, and the dual luciferase assay system were obtained from Promega (Madison, WI). The Epaq analogue 8CPT-2’-O-Me-cAMP was obtained from Axolora LLC (San Diego, CA). H-89 was purchased from EMD Biosciences (San Diego, CA), and GF109203X was purchased from Calbiochem. Enhanced chemiluminescence reagents were purchased from Amersham Biosciences, and antibodies for p44/42 MAPK and phosphorylated p44/42 MAPK were purchased from Cell Signaling (Beverly, MA).

DNA Constructs—The luciferase-encoding plasmids utilized for the luciferase reporter assay were pAP1 (5A/TA-Luc (Clontech, Palo Alto, CA), pGL2-Basic without a promoter, pGL2-IL6p-(–1200) containing full-length human IL-6 promoter, pGL2-IL6p-(–225) containing 225-bp IL-6 promoter, pGL2-IL6p-(–160) containing 160-bp IL-6 promoter, and pGL2-IL6p-(–225)-NF-E-Bmut containing a mutated NF-E-B site of pGL2-IL6p-(–225), as described previously (18, 19). The pRL-SV40 construct, containing a cDNA for Renilla repressilase, was used as a control for transfection efficiency (Promega). Other plasmids included the pcMV-CREB (constitutively expresses the wild type CREB) (Clontech), pcMV-CREB133 (a dominant-negative mutant vector preventing Ser133 phosphorylation of CREB) (Clontech), pcDNA3.1(+) (Invitrogen), and pcDNA3.1(+)-FLAG-Rap1GAP (a pcDNA3.1(+) vector containing Rap1-GTPase-activating protein gene converting the GTP to GDP) (a gift from Dr. Philip J. St. Oskor, Oregon Health and Science University, Portland, OR).

Cell Culture, Differentiation, and Treatments—MC3T3-E1 subclone 4 cells (MC4) with high osteoblast differentiation potential were maintained as described previously (20). Briefly, cells were cultured in minimum essential α-medium (Invitrogen) with 10% fetal bovine serum (Hyclone, Logan, UT) and 100 units/ml penicillin and streptomycin. After 24 h of incubation, cells were transfected with the luciferase reporter plasmid and 0.2 μg of pRL-SV40. Follow-up transfection, cells were allowed to recover in serum-containing media for 20 h. Cells were then treated as indicated and harvested, and luciferase levels were measured using a Monolight 3010 luminometer (BD Biosciences).

Northern Blot Analysis—Cells were plated at 25,000 cells/cm² in 60-mm dishes and induced to differentiate with the addition of ascorbic acid (50 μg/ml) for 5–7 days. Without differentiation, cells were plated at 25,000 cells/cm² in 100-mm dishes. After being treated with PTHrP-(1–34) (0.1 μM) for various time points with or without U0126 (20 μM) pretreatment, proteins were isolated for Western blot analysis as described previously (29). The proteins were separated by 12% SDS-PAGE, and transferred onto polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked in TBST (10 mM Tris, pH 8.0, 0.85% NaCl, 0.1% Tween 20) with 5% non-fat dry milk for 1 h and then incubated overnight at 4 °C with diluted primary antibodies in TBST. The dilution factor was 1:1000 for p44/42 MAPK antibody and phosphorylated p44/42 MAPK antibody (Th-922/Thr-224). After three washes with TBST, membranes were incubated for 1 h with secondary antibody, membranes were washed five times with TBST and developed by ECL chemiluminescent detection according to protocols supplied by the manufacturer.

Statistical Analysis—The results of experiments were analyzed using analysis of variance followed by a Tukey-Kramer multiple comparison test or Student’s t test, with the Instat 2.1 biostatistics program (GraphPad Software, San Diego, CA). All experiments were performed in duplicate or triplicate and repeated 2–3 times with consistent results.

RESULTS

Effects of PTHrP and U0126 on Osteoblast-related Gene Expression—PTH and PTHrP are known to regulate various genes pertinent to osteoblast function. Three down-regulated genes (OCN, BSP, and the type I PTH/PTHrP receptor, PTH-1R) and three up-regulated genes (c-fos, fra-2, and interleukin-6) in response to PTH and PTHrP were selected for evaluation. To determine whether the MAPK pathway has an impact in the PTHrP regulation of these genes, a specific ERK1/2 phosphorylation inhibitor, U0126, was employed by pretreating cells before PTHrP administration. PTHrP significantly down-regulated OCN (90%), BSP (40%), and PTH-1R (40%) compared with vehicle-treated cells (Fig. 1). U0126 alone also decreased OCN and BSP but not PTH-1R suggesting that basal expression of OCN and BSP is partially dependent on the MAPK pathway. The addition of U0126 did not alter PTHrP-mediated decreases in OCN and PTH-1R but did further decrease BSP expression compared with PTHrP treatment alone suggesting that the MAPK pathway is in part responsible for the PTHrP regulation of BSP in osteoblasts. However, at the time point evaluated for these three genes down-regulated by PTHrP, there could be other indirect effects of inhibiting MAPK. Gene expression for IL-6 and the AP-1 transcription factors fra-2 and c-fos were significantly induced by PTHrP 5-, 11-, and 2.5-fold, respectively (Fig. 2). The PTHrP increase in IL-6 expression was significantly blocked by U0126. The PTHrP induction of fra-2 was partially blocked by U0126, but U0126 had no effect on PTHrP-induced c-fos gene expression. These data suggest that in differentiated osteoblasts, basal gene expression for OCN and BSP is partially dependent on the MAPK pathway. Furthermore, the actions of PTHrP on BSP, IL-6, and fra-2 gene expression are modified by blocking the MAPK pathway indicating that the MAPK pathway selectively impacts some PTH-responsive genes in osteoblastic cells.

PTHrP Transcriptional Control of IL-6 Depends on MAPK—Because Northern blot analysis suggested that PTHrP up-regulation of IL-6 gene expression was dependent on MAPK, the IL-6 promoter was employed to analyze the regulatory role of the MAPK pathway for PTHrP actions. Effects at the transcriptional level were examined by using full-length pGL2-IL6p(–1200) luciferase reporter plasmids, which contain the proximal promoter of human IL-6 gene for transcription factor activity, and various truncated or mutant constructs derived from pGL2-IL6p(–1200) (Fig. 3A). PTHrP concentrations from 0.1 nM to 1 μM significantly increased the full-length IL-6 promoter activity, with 10 and 100 nM having the maximal effect (Fig. 3B). PTHrP treatment at 4, 6, and 8 h increased

Western Blot Analysis—MC4 cells were plated at 50,000 cells/cm² in 60-mm dishes and treated with 50 μg/ml ascorbic acid for 5–7 days for differentiation. Without differentiation, cells were plated at 25,000 cells/cm² in 100-mm dishes. After being treated with PTHrP-(1–34) (0.1 μM) for various time points with or without U0126 (20 μM) pretreatment, proteins were isolated for Western blot analysis as described previously (29). The proteins were separated by 12% SDS-PAGE, and transferred onto polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked in TBST (10 mM Tris, pH 8.0, 0.85% NaCl, 0.1% Tween 20) with 5% non-fat dry milk for 1 h and then incubated overnight at 4 °C with diluted primary antibodies in TBST. The dilution factor was 1:1000 for p44/42 MAPK antibody and phosphorylated p44/42 MAPK antibody (Th-922/Thr-224). After three washes with TBST, membranes were incubated for 1 h with secondary antibody, membranes were washed five times with TBST and developed by ECL chemiluminescent detection according to protocols supplied by the manufacturer.
full-length IL-6 promoter activity, and U0126 pretreatment significantly decreased PTHrP effects (Fig. 3C). Furthermore, to investigate how PTHrP regulates transcription factors important in IL-6 promoter activity, various IL-6 promoter constructs were used for analysis. Basal activity of truncated 225- and 160-bp IL-6 promoter fragments was increased 2-fold compared with the basal activity of the full-length promoter fragment. There was a 10-fold increase in IL-6 luciferase activity with PTHrP treatment of cells transfected with the 1200-bp promoter (Fig. 3D). PTHrP stimulation of the truncated 225- and 160-bp promoter fragments was reduced to a net 3.6-fold increase compared with the basal activity. Furthermore, losing the region between −225 and −160, which contains a cAMP-response element (CRE)-binding site, did not alter this effect. These results suggest that the region containing the activator protein-1 (AP-1) site is important for the PTHrP stimulation, and this same region is associated with repression of basal promoter activity. This result also suggests that the AP-1-binding site might have regulation priority over the CRE-binding site for IL-6 transcription activation. Furthermore, inhibition of MAPK with U0126 blocked 30% of the stimulatory effects of PTHrP on the truncated 225- and 160-bp promoter fragments. In contrast, promoter activity with the nuclear factor-κB (NFκB) mutant 225-bp promoter fragment was dramatically reduced. However, the basal level and PTHrP stimulation were both reduced (Fig. 3D, see inset with expanded scale). This suggests that NFκB is important for IL-6 basal expression but not for PTHrP stimulation. Taken together, these data
FIG. 3. PTHrP activates the IL-6 promoter in a MAPK-dependent manner. A, diagram of IL-6 promoter constructs. The 5'-region of the IL-6 promoter was progressively deleted by selective restriction digests of pGL2-IL6p(-1200) followed by religation. Several response elements are indicated. GRE, glucocorticoid receptor response element; AP-1, activator protein-1; CRE, cAMP-response element; C/EBP-β, CCAAT/enhancer-binding protein β. B, PTHrP induction of IL-6 promoter activity is dose-dependent. MC4 cells were cotransfected with pGL2-IL6p(-1200) and pRL-SV40 Renilla luciferase normalization plasmids. After 20 h, cells were treated with vehicle or PTHrP-(1–34) (1 pM to 1 μM) for 6 h. The cell extract was collected for measurement of luciferase activity. The results are expressed as the mean ± S.E. n = 3/group. *, p < 0.001 versus vehicle treated group. C, PTHrP induction of IL-6 promoter activity is time-dependent. MC4 cells were cotransfected with pGL2-IL6p(-1200) and pRL-SV40 Renilla luciferase normalization plasmids. After 20 h, cells were pretreated with U0126 (20 μM) for 2 h, followed by PTHrP-(1–34) (0.1...
suggest that the AP-1 site-containing region between −1200 and −225 is important for PTHrP-mediated activation of IL-6 promoter, and the NFκB site is important for basal IL-6 promoter activity. Because the AP-1 site of the IL-6 promoter was important for PTHrP-mediated stimulation of IL-6, the dependence of regulation of AP-1 on MAPK was evaluated. MC4 cells were transfected with pAP1(PMA)-TA-Luc luciferase vector containing an AP-1 enhancer element and a minimal TK promoter. PTHrP up-regulated the AP-1-driven luciferase activity, but inhibition of MAPK by U0126 did not block this effect (Fig. 3E). Thus, PTHrP regulation of the IL-6 promoter is dependent on AP-1, but the MAPK pathway does not specifically target AP-1 at the level of transcription. The promoter analyses require transfection of nonconfluent cells, which are not differentiated. Because the luciferase assay revealed that IL-6 promoter activity was up-regulated by PTHrP in undifferentiated cells, whereas IL-6 mRNA was up-regulated by PTHrP in differentiated cells (Fig. 2), IL-6 gene expression of undifferentiated MC4 cells was examined. Northern blot analysis showed that IL-6 mRNA gene expression in undifferentiated MC4 cells was also significantly increased by PTHrP and repressed by U0126 (42%) (Fig. 3F), following a similar pattern as found in differentiated cells (Fig. 2A). This suggests that PTHrP increases IL-6 gene expression both in undifferentiated and differentiated cells in a partially MAPK-dependent manner.

Pathways Involved in IL-6 Activation—Inhibitors were used to determine the involvement of PKA and PKC in the PTHrP actions at the IL-6 promoter. MC4 cells were transfected with either IL-6 promoter-driven luciferase reporter plasmids or promotorless pGL2-Basic and Renilla luciferase normalization plasmids. After 20 h of incubation, MC4 cells were pretreated with the selective inhibitor U0126 (MAPK), GF109203X (PKC), or H-89 (PKA) alone or with PTHrP. As shown in Fig. 4, inhibition of PKA by the addition of H-89 resulted in the most striking reduction in both basal and PTHrP-stimulated IL-6 promoter activity. GF109203X and U0126 resulted in less but still significant reductions. However, the 18% reduction by GF109203X could involve nonspecific effects of the inhibitor, because the PKC activator, H-89, alone, did not activate the IL-6 promoter. These results suggest that PKC may only have a minor role in IL-6 promoter activity. This study with inhibitors suggests that PTHrP-induced IL-6 transcription requires PKA and MAPK pathways, and PKC activation alone cannot replicate the PTHrP activation of the IL-6 promoter.

Epac and Rap1 Impact on IL-6 Activation—The cAMP-PKA pathway is involved in a variety of cellular processes, and it is well known that PTH and PTHrP signal via the cAMP-PKA pathway to mediate gene expression in bone cells (30, 31). The cAMP pathway is also known to interact with the MAPK pathway via PKA-independent means through direct regulation of Epac1 and Epac2, which are guanine nucleotide-exchange factors for the small GTPases Rap1 and Rap2 (32, 33). It is reported that stimulation of the Epac/Rap1/B-Raf pathway in MC4 cells can activate MAPK (34). To investigate the PKA-independent pathway of PTHrP action, we used an Epac-selective cAMP analogue, 8CPT-2′-O-Me-cAMP, to activate Epac1 in a PKA-independent manner (35). Epac-selective cAMP analogue induced ERK1/2 (MAPK p44/p42) phosphorylation in differentiated MC4 cells (Fig. 5A). Furthermore, we investigated this effect on the IL-6 promoter. MC4 cells were transfected with full-length pGL2-IL-6p-(-1200) promoter constructs and treated with PTHrP or 8CPT-2′-O-Me-cAMP for 6 h. Selective Epac1 activation did not induce IL-6 promoter activity (Fig. 5B). This suggests that activation of MAPK via Epac1 independent of PKA cannot induce IL-6 promoter activity. In B-Raf-containing cells, Rap1 is also phosphorylated directly by PKA to activate MAPK (9–11). Because MC3T3-E1 and MC4 cells contain B-Raf (34), we further examined the role of Rap1. To further clarify the PKA-Rap1-MAPK pathway of IL-6 activation, a pcDNA3.1(+) plasmid containing the Rap1GAP gene was used. Transfection of FLAG-Rap1GAP plasmid increases the cellular Rap1GAP level, which hydrolyzes active Rap1GTP to inactive Rap1GDP and shuts down the signaling pathway (36). MC4 cells were co-transfected with the IL-6 luciferase promoter and the FLAG-Rap1GAP plasmid. After 20 h, cells were treated with PTHrP or vehicle for 6 h. Cells transfected with Rap1GAP did not achieve as high a level of PTHrP-induced IL-6 promoter activity, but taking basal levels into account, the fold increase (8-fold) was similar in pcDNA3.1(+) and Rap1GAP groups (Fig. 5C). This suggests that Rap1 may influence the PTHrP stimulation of IL-6 promoter activation but plays only a minor role.

Fig. 4. Stimulation of IL-6 promoter activity by PTHrP is PKA- and MAPK-dependent. MC4 cells were pretreated with the PKC inhibitor GF109203X (1 μM) for 30 min, MAPK inhibitor U0126 (20 μM) for 2 h, or PKA inhibitor H-89 (20 μM) for 30 min and then treated with PTHrP(1–34) (0.1 μM), vehicle, or PMA alone (1 μM) for 6 h. Cell extracts were collected and measured for luciferase activity. The results are expressed as the mean ± S.E. n = 3/group. *, p < 0.001 versus PTHrP/IL6p-(-1200); **, p < 0.05 versus vehicle/IL6p-(-1200).

µM) treatment for the indicated times. The cell extract was collected for measurement of luciferase activity. *, p < 0.05 versus PTHrP-treated groups. D, PTHrP regulation of IL-6 promoter. MC4 cells were transfected with different IL-6 promoter constructs or pGL2 Basic luciferase vectors. Cells were then treated with U0126 (2 h) and PTHrP (6 h). The results are expressed as the mean ± S.E. n = 3/group. *, p < 0.01 versus respective control; **, p < 0.01 versus PTHrP/IL6p-(-1200); ***, p < 0.001 versus PTHrP with respective promoters. Inset shows NFκB mutant and plasmid controls on an expanded scale. Loss of the IL-6 promoter that contains an AP-1 site resulted in a significant but not total reduction of PTHrP activation of the IL-6 promoter. Inhibition of MAPK reduced PTHrP stimulation of IL-6 by nearly 50% regardless of the promoter length. E, impact of MAPK on PTH-stimulated AP-1. MC4 cells were transfected with AP-1 luciferase vectors, pretreated with U0126 (20 μM) or control for 2 h, and then treated with PTH(1–34) (0.1 μM) for 6 h. The representative results are expressed as the mean ± S.E. n = 3/group. *, p < 0.01 versus control or U0126. PTHrP increased AP-1 activity, but inhibition of MAPK did not alter the PTHrP stimulation of AP-1. F, undifferentiated MC4 cells were pretreated with U0126 (20 μM) for 2 h and treated with PTHrP(1–34) (0.1 μM) for another 2 h. Total RNA was extracted for Northern analysis using cDNA probes for IL-6 and 18 S rRNA. Results are expressed as IL-6 normalized to the 18 S. Representative autoradiographs are displayed from one experiment with graphic results containing data from two separate experiments and expressed as the mean ± S.E. with a total n = 4/group. *, p < 0.001 versus control; **, p < 0.005 versus PTHrP or U0126.
Fig. 5. Dependence on Epac and Rap1 for PTHrP regulation of IL-6. A, Epac-selective cAMP analogue activated ERK phosphorylation. MC4 cells were cotransfected with the Epac-selective cAMP analogue, 8CPT-2’-O-Me-cAMP (30 μM), or vehicle for 30 min. Western blot analysis showed that the Epac-selective cAMP analogue induced ERK1/2 phosphorylation. B, Epac regulation of IL-6. MC4 cells were cotransfected with pGL2-IL6p-(−1200) promoter constructs or pGL2 basic vectors. Cells were then treated with PTHrP-(1–34) (0.1 μM) and the Epac-selective cAMP analogue 8CPT-2’-O-Me-cAMP (30 μM), respectively, for 6 h. Cell extracts were measured for IL-6 promoter-driven luciferase activity.

C, Rap1 impact on PTHrP-regulated IL-6 transcription. MC4 cells were cotransfected with the pGL2-IL6p-(−1200) promoter plasmid and either pcDNA3.1(+) or pcDNA3.1(+)Rap1GAP plasmid (a negative regulator of Rap1). After 20 h, cells were treated with PTHrP or vehicle for 6 h. pcDNA, pcDNA3.1(+); p, p < 0.001 versus respective control; n = 3/group. The results are expressed as the means ± S.E. The overall activation of the IL-6 promoter was reduced by Rap1GAP, but the fold increase with PTHrP was not altered.

a partial role as it also affects the basal activity of this reporter construct.

CREB Is Necessary for IL-6 Promoter Activity—PKA mediates cAMP-regulated cellular gene expression via phosphorylation of its downstream target, the CREB, at Ser133 (37). Because the inhibitor experiment showed that IL-6 up-regulation by PTHrP was through PKA, and the IL-6 promoter construct experiment did not show the particular CREB effect (CREB binds to CRE site) in the AP-1-deleted IL6p-(−160) promoter fragment, we further investigated the role of CREB in the PTHrP mediation of IL-6 transcription. The pCMV-CREB plasmid containing wild type CREB gene and the pCMV-CREB333 plasmid containing a mutant CREB333 gene were used. The pCMV-CREB333 is a dominant-negative mutant preventing PKA-mediated Ser133 phosphorylation of CREB. MC4 cells were cotransfected with pGL2-IL6p-(−1200) and pCMV-CREB or pCMV-CREB333 and treated with PTHrP with or without U0126 pretreatment. PTHrP-stimulated IL-6 promoter activity was dramatically repressed to basal levels in CREB333-transfected cells (Fig. 6). This suggests that active phosphorylated CREB is important for the PTHrP regulation of IL-6 gene expression. Most interesting, transfection with wild type CREB did not further augment the PTHrP-mediated IL-6 promoter stimulation. Instead, the IL-6 activity was reduced but had the same fold increase as pcDNA3.1(+), and U0126 pretreatment did not alter the fold increase.

PTHRP Effect on MAPK Phosphorylation—Because of the logistics of the assays, gene expression studies were performed with differentiated MC4 cells highly expressing the PTH-1R, and promoter analysis assays were performed with pre-confluent cells for transient transfection. Because we had reported previously divergent effects in differentiated versus undifferentiated cells, phosphorylation of MAPK was measured in cultures of nonconfluent and confluent cells. MAPK p44 and p42 are also known as ERK1 and ERK2. To evaluate the PTHrP-induced MAPK response in undifferentiated and differentiated osteoblastic MC4 cells, ERK1/2 and phospho-ERK1/2 protein expression were measured by Western blot analysis. Most interesting, effects of PTHrP on ERK phosphorylation were opposite in these two cellular conditions. In undifferentiated MC4 cells, PTHrP rapidly induced ERK phosphorylation at 3 min (Fig. 7, A and B). By 24 h, cells reached confluence, and ERK phosphorylation was reduced (data not shown). In differentiated MC4 cells, PTHrP rapidly repressed ERK phosphorylation at 5 min (Fig. 7C). The effect of PTHrP reached maximal reduction at 2 and 24 h but returned to control by 48 h (Fig. 7D). These data suggest that PTHrP has bidirectional effects.
were treated with PTHrP-(1-34) (0.1 μM) for 0–20 min. Total cellular protein was harvested. Western blot analyses of ERK1/2 and phosphorylated ERK1/2 were performed. PTHrP rapidly induced ERK1/2 phosphorylation in undifferentiated MC4 cells. Undifferentiated MC4 cells were pretreated with U0126 (20 μM) or vehicle for 2 h and then PTHrP-(1-34) (0.1 μM) or vehicle for 20 min. Total cellular protein was harvested. Western blot analysis showed that PTHrP rapidly reduced ERK1/2 phosphorylation in differentiated MC4 cells, and Western blot analyses also showed that phospho-ERK1/2 was repressed. This indicates that the MAPK pathway is critical for basal OCN, and to a lesser extent BSP gene expression, and in agreement with a previous study from our group (16). Inhibition of the MAPK pathway appeared additive with the PTHrP effect on BSP gene expression but did not further down-regulate OCN mRNA, perhaps because down-regulation by PTHrP was already maximal. PTHrP decreased PTH-1R expression as well and others have reported previously (38, 39). However, inhibition of the MAPK pathway did not alter basal levels or the PTHrP-mediated down-regulation of the PTH-1R, which suggests that the PTH-1R promoter lacks elements that respond to mediators of the MAPK pathway. OCN, BSP, and PTH-1R are markers of osteoblastic differentiation. This study showed the diversity of regulation of these three genes and also suggested the complexity of differential pathway regulation. One caveat needs to be considered for the studies of MAPK inhibition in cells for 48 h as was the case for OCN, BSP, and PTH-1R. Our previous studies evaluated shorter time periods for MAPK inhibition of basal OCN and BSP with similar results (16). However, there are other confounding factors such as growth factor expression that may act as indirect mediators of the effects reported here. There was no alteration in the response of the PTH-1R to MAPK inhibition, and the PTH-1R is sensitive to growth factor regulation. This would suggest that these types of indirect effects may not be relevant, but it is important to acknowledge their potential impact in studies carried out for such duration.

The results here indicate that PTHrP signaling transiently stimulates mRNA expression of immediate-early genes, including c-fos, fra-2, and IL-6, which is in agreement with previous studies (27, 29, 40, 41). The present study further characterized the role of the MAPK pathway in the up-regulation of these genes. PTHrP stimulation of IL-6 was significantly but not totally blocked by inhibiting the MAPK pathway. PTHrP stimulation of fra-2 was also significantly blocked but not to as great an extent as IL-6. In contrast, up-regulation of c-fos by PTHrP was not altered by blocking MAPK. IL-6 is a prosresorptive cytokine suggesting that the MAPK pathway may be important in the catabolic actions of PTHrP; however, fra-2 has been reported to be associated with increased bone formation, so it is not likely that modification of PTHrP actions by the MAPK pathway is solely associated with anabolic or catabolic actions of PTH or PTHrP.

This study showed that the PTHrP effect on IL-6 mRNA expression was independent of cell differentiation state. Both undifferentiated and differentiated MC4 cells had PTHrP-mediated increases in IL-6 mRNA, but undifferentiated cells showed a greater fold increase than differentiated cells, and blocking MAPK reduced the PTHrP effect in both states. Western blot analyses showed that PTHrP increased ERK phosphorylation in undifferentiated cells and decreased ERK phosphorylation in differentiated cells. These two findings suggest that up-regulation of ERK phosphorylation by PTHrP results in on ERK phosphorylation dependent on the differentiation state of the osteoblasts.

**DISCUSSION**

The cAMP-activated PKA pathway has been found to govern most of the effects induced by PTH and PTHrP on osteoblasts, and more recently MAPK is also reported to be an important pathway for osteoblast-specific gene expression, cellular proliferation, and differentiation (15, 16). Therefore, the present study investigated the role of the MAPK pathway in PTHrP effects on osteoblasts. Northern blot analyses showed that both PTHrP and inhibition of the MAPK by U0126 resulted in decreased OCN and BSP mRNA expression in differentiated MC4 cells, and Western blot analyses also showed that phosphorylated ERK1/2 was repressed. This indicates that the MAPK pathway is critical for basal OCN, and to a lesser extent BSP gene expression, and in agreement with a previous study from our group (16). Inhibition of the MAPK pathway appeared additive with the PTHrP effect on BSP gene expression but did not further down-regulate OCN mRNA, perhaps because down-regulation by PTHrP was already maximal. PTHrP decreased PTH-1R expression as well and others have reported previously (38, 39). However, inhibition of the MAPK pathway did not alter basal levels or the PTHrP-mediated down-regulation of the PTH-1R, which suggests that the PTH-1R promoter lacks elements that respond to mediators of the MAPK pathway. OCN, BSP, and PTH-1R are markers of osteoblastic differentiation. This study showed the diversity of regulation of these three genes and also suggested the complexity of differential pathway regulation. One caveat needs to be considered for the studies of MAPK inhibition in cells for 48 h as was the case for OCN, BSP, and PTH-1R. Our previous studies evaluated shorter time periods for MAPK inhibition of basal OCN and BSP with similar results (16). However, there are other confounding factors such as growth factor expression that may act as indirect mediators of the effects reported here. There was no alteration in the response of the PTH-1R to MAPK inhibition, and the PTH-1R is sensitive to growth factor regulation. This would suggest that these types of indirect effects may not be relevant, but it is important to acknowledge their potential impact in studies carried out for such duration.

The results here indicate that PTHrP signaling transiently stimulates mRNA expression of immediate-early genes, including c-fos, fra-2, and IL-6, which is in agreement with previous studies (27, 29, 40, 41). The present study further characterized the role of the MAPK pathway in the up-regulation of these genes. PTHrP stimulation of IL-6 was significantly but not totally blocked by inhibiting the MAPK pathway. PTHrP stimulation of fra-2 was also significantly blocked but not to as great an extent as IL-6. In contrast, up-regulation of c-fos by PTHrP was not altered by blocking MAPK. IL-6 is a proresorptive cytokine suggesting that the MAPK pathway may be important in the catabolic actions of PTHrP; however, fra-2 has been reported to be associated with increased bone formation, so it is not likely that modification of PTHrP actions by the MAPK pathway is solely associated with anabolic or catabolic actions of PTH or PTHrP.

This study showed that the PTHrP effect on IL-6 mRNA expression was independent of cell differentiation state. Both undifferentiated and differentiated MC4 cells had PTHrP-mediated increases in IL-6 mRNA, but undifferentiated cells showed a greater fold increase than differentiated cells, and blocking MAPK reduced the PTHrP effect in both states. Western blot analyses showed that PTHrP increased ERK phosphorylation in undifferentiated cells and decreased ERK phosphorylation in differentiated cells. These two findings suggest that up-regulation of ERK phosphorylation by PTHrP results in

**FIG. 7. PTHrP effect on MAPK phosphorylation.** A. short term PTHrP effect on undifferentiated MC4 cells. Undifferentiated MC4 cells were treated with PTHrP-(1-34) (0.1 μM) for 0–20 min. Total cellular protein was harvested. Western blot analyses of ERK1/2 and phosphorylated ERK1/2 were performed. PTHrP rapidly induced ERK1/2 phosphorylation in undifferentiated MC4 cells. B. PTHrP and U0126 effects on undifferentiated MC4 cells. Undifferentiated MC4 cells were pretreated with U0126 (20 μM) or control for 2 h and then PTHrP-(1-34) (0.1 μM) or vehicle for 20 min. Total cellular protein was harvested. Western blot analysis showed that PTHrP rapidly increased ERK1/2 phosphorylation and U0126 blocked ERK1/2 phosphorylation. C, short term PTHrP effect on differentiated MC4 cells. MC4 cells were induced to differentiate with ascorbic acid for 5 days, and then treated with PTHrP-(1-34) (0.1 μM) for 0–60 min. Western blot analysis showed that PTHrP rapidly increased ERK1/2 phosphorylation and U0126 blocked ERK1/2 phosphorylation. D, long term PTHrP and U0126 effects on differentiated MC4. Differentiated MC4 cells were pretreated with U0126 (20 μM) or control for 2 h and then PTHrP-(1-34) (0.1 μM) or vehicle for 2, 24, or 48 h. Western blot analyses were performed. PTHrP treatment at 2 and 24 h resulted in reduced ERK1/2 phosphorylation; n = 2/group.
greater IL-6 gene expression in undifferentiated cells, and when ERK phosphorylation is down-regulated by PTHrP, the IL-6 increase is not as high.

Of the genes evaluated, because IL-6 was the most dependent on the MAPK pathway, we investigated its regulation in more detail. The transcriptional dependence on PTHrP and the MAPK pathway was confirmed using luciferase plasmids containing IL-6 promoter constructs, and regulatory elements were isolated. It was found that regions containing AP-1 and NFκB influence the basal activity of the IL-6 promoter with opposite effects, and the region containing the AP-1 site played an important role for PTHrP. Inhibition of MAPK resulted in an ∼50% reduction in the PTHrP stimulation of the IL-6 promoter in all the constructs tested, suggesting that the site critical for MAPK effects was not altered in the constructs that we evaluated. Furthermore, the specific promotion of AP-1 activity by PTHrP was not dependent on MAPK activity. Transcriptional regulation of the IL-6 gene in other cell systems has been reported to involve at least four different transcription factors, NFκB, AP-1, CCAAT/enhancer-binding protein β (C/EBP-β), and CREB (42). The role of these transcription factors varies between different cell types and may also vary within the same cells depending on the stimulus (19, 43–45). In this study, we evaluated the AP-1, NFκB, and CREB-binding sites of IL-6 promoter. The C/EBP-β site was not evaluated, which is in the area adjacent to −160-bp, but not deleted in the pGL2-IL6p(-160) constructs. Therefore, it is possible that this area is important for the MAPK effects, and most interesting, C/EBP-β has been found to be up-regulated by PTH in osteoblastic cells (46). Investigating IL-6 transcription by various stimuli has been performed in human intestine epithelial cells and human lung fibroblasts (47, 48). Results from those studies and the present study all suggest that activating IL-6 transcription by different stimuli requires the AP-1 transcription factor, and various requirements for CREB, C/EBP-β, and NFκB transcription factors are also operative.

Various signal transduction pathways mediated by PTH and PTHrP have been extensively investigated, and it is well known that PTHrP binds to the PTH-1R and activates PKA and PKC. The present study indicates that the PKA and MAPK pathways play roles in the PTHrP effects on the IL-6 promoter activity and that PKA is the most dominant pathway. A previous report (49) suggests that low dose PTH(-1–34) (1 and 10 pM) induced ERK activity via PKC in UMR 106-01 cells and primary rat osteoblasts, and some reports (50, 51) have shown that PKC is involved in PTH-induced IL-6 promoter activity in UMR-106 osteoblastic cells. However, in this study, PKC alone was not sufficient to activate IL-6 transcription. Recent studies (52–54) have suggested a PKA-independent but cAMP-dependent pathway that results in MAPK activation. The mediators of this pathway are Epac (guanine nucleotide-exchange factor) and the small GTPase Rap1. Epac is activated by cAMP, and Rap1 is a downstream molecule of Epac that mediates cAMP-induced regulation of the MAPK. A study showed that PTH activates ERK phosphorylation via PKA-independent and cAMP-activated Rap1 pathway in MC4 cells (34). Therefore, we suspected that the cAMP/Epac/Rap1 pathway might be involved in IL-6 activation. The results of this study show that activation of Epac alone induced ERK phosphorylation but did not activate the IL-6 promoter, and inactivation of Rap1GTP influenced both basal and PTHrP effect on IL-6. The results of the inhibitor experiments also indicated that PKA was essential. All of these results suggest that the PTHrP impact on the IL-6 promoter is cAMP/Epac/Rap1-independent but also that there might be cross-talk between PKA and Rap1. Recently, some studies found that MAPK activation and inhibition by PKA require members of the Src family (55, 56). The tyrosine kinase Src family is downstream of PKA, but its role is still unclear. Therefore, Src might be a player in the cross-talk of the PKA and MAPK pathways in PTHrP-mediated IL-6 transcription in osteoblasts.

The pGL2-IL6p(-160) plasmids lack both AP-1 and CREB-binding sites; therefore, the specific role of CREB alone in the PTHrP regulation of IL-6 was not evaluated with this construct. To investigate the particular role of CREB, the wild type and mutant CREB gene-inserted plasmids, pCMV-CREB and pCMV-CREB133, were used. Results showed that phosphorylated CREB is necessary but not sufficient for PTHrP-mediated IL-6 promoter activity. The IL-6 truncated promoter assays also suggested that phosphorylated CREB functions independently of its binding to the CRE site in the promoter region, and its regulatory priority may be lower than AP-1. Overexpression of wild type CREB reduced the basal level of IL-6 promoter activity, and this effect was also seen in NFκB mutant 225-hp promoter constructs. It is known that PKA induces CREB phosphorylation, and phosphorylated CREB binds to the transcriptional coactivator CBP to activate gene transcription (57). It is also known that NFκB requires the CBP coactivator to activate gene transcription (58). Hence, a possible explanation for the reduced IL-6 promoter activity in the transfectants that overexpressed CREB may be that both CREB and endogenous NFκB compete for CBP to activate IL-6 transcription. Because NFκB is essential for basal IL-6 expression, overexpressed CREB may take over most of the CBP and reduce the NFκB-CBP complex binding to the IL-6 promoter, hence decreasing IL-6 promoter activity. This hypothesis is also supported by the result of NFκB mutant promoter constructs, which had a similar effect in basal IL-6 reduction. The requirement for MAPK in CREB phosphorylation is not clear. However, because addition of the MAPK inhibitor to cultures transfected with wild type CREB resulted in further down-regulation of IL-6 promoter activity both for basal and PTHrP stimulation, this suggests that CREB and MAPK are acting independently for PTHrP activation of the IL-6 promoter.

The dual function of PTHrP in MAPK regulation is dependent on osteoblastic differentiation stages. This may explain in part the variations reported in the literature for MAPK pathway effects in osteoblasts and the impact of the MAPK pathway in PTH and PTHrP actions in bone. Studies report both effects of stimulation (49, 59, 60) and inhibition (61, 62) of the MAPK pathway by PTH or PTHrP in osteoblastic cells. Those opposite results may be due to the variations in the cell systems, e.g. osteosarcoma versus normal osteoblastic cells, and the differentiation state of the cells that have not been adequately addressed. This study suggests that differentiation stages may be responsible for variations that could impact the role of the MAPK pathway in the in vivo system where the complexities are magnified. Although the bi-directional effect was clearly demonstrated to be associated with the differentiation state, the individual cellular responses are likely cell context-dependent because there is still heterogeneity of differentiation states in the cultures.

Taken together, this study identifies PTHrP-responsive genes that are impacted by the MAPK pathway demonstrating that there are genes up-regulated by PTHrP (fra-2 and IL-6) and down-regulated by PTHrP (OCN and BSP) and genes whose PTHrP mediation is not dependent on the MAPK pathway (c-fos and PTH-1R). Furthermore, the regulation of IL-6 involves the PKA and MAPK pathways. Finally, the impact of PTHrP on osteoblastic lineage and the resulting downstream
alterations are clearly dependent on the differentiation state of the cells. This study emphasizes the complexity of interactions between signaling pathways and cell systems and the need for clearly delineated roles of mediators in vitro in order to better understand hormonal effects in vivo.

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REFERENCES
1. Jupner, H., Ahou-Samra, A. B., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kolakowski, L. F. J., Hock, J., Potts, J. T. J., Kronenberg, H. M., et al. (1991) Science 254, 1024–1026.
2. Ahou-Samra, A. B., Jupner, H., Force, T., Freeman, M. W., Kong, X. F., Schipani, E., Urena, P., Richards, J., Bouventre, J. Y., Potts, J. T. J., Kronenberg, H. M., and Segre, G. V. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2752–2756.
3. Chan, G. K., Deckelbaum, R. A., Belivar, I., Goltzman, D., and Karaplis, A. C. (2001) Endocrinology 142, 4900–4909.
4. Miao, D., Tong, X., Chan, G. K., Pandia, D., McPherson, P. S., and Goltzman, D. (2001) J. Biol. Chem. 276, 32204–32213.
5. Zhu, X., and Assoian, R. K. (1995) Mol. Cell. Biol. 15, 7379–7388.
6. Chen, Q., Knch, M. S., Lin, T. H., Burrige, K., and Juliano, R. L. (1994) J. Biol. Chem. 269, 26062–26066.
7. Cobb, M. H. (1999) Prog. Biophys. Mol. Biol. 71, 479–500.
8. Xiao, G., Jiang, D., Gopalakrishnan, R., and Franceschi, R. T. (2002) J. Biol. Chem. 277, 36181–36187.
9. Altschuler, D. L., Peterson, S. N., Ostrowski, M. C., and Lapeina, E. G. (1995) J. Biol. Chem. 270, 10337–10336.
10. Quilliam, L. A., Mueller, H., Bobi, B. P., Prossnitz, V., Sklar, L. A., Der, C. J., and Bokoch, G. M. (1991) J. Immunol. 147, 1628–1635.
11. Stork, P. J. S., and Schmitt, J. M. (2002) Trends Cell Biol. 12, 258–266.
12. Grewal, S. S., Fass, D. M., Yoo, H., Eilich, C. L., Goodman, R. H., and Stork, P. J. (2000) J Biol. Chem. 275, 34433–34441.
13. Hagemann, C., and Blank, J. L. (2001) Cell Signal. 13, 863–875.
14. Carpio, L., Glidt, J., Goltzman, D., and Rabanni, S. A. (2001) Am. J. Physiol. 281, E499–E509.
15. Lai, C. F., Chaudhary, L., Fausto, A., Halstead, L. R., Ory, D. S., Avioli, L. V., and Cheng, S. L. (2001) J. Biol. Chem. 276, 14443–14450.
16. Xiao, G., Gopalakrishnan, R., Jiang, D., Reith, E., Benson, M. D., and Franceschi, R. T. (2002) J. Bone Miner. Res. 17, 101–110.
17. Chen, H., Demiralp, B., Schneider, A., Koh, A. J., Silve, C., Wang, C. Y., and McIlvain, P. J. (2002) J. Biol. Chem. 277, 19374–19381.
18. Keller, E. T., Chang, C., and Eshrib, W. H. (1996) J. Biol. Chem. 271, 26267–26275.
19. Zhang, J., Johnston, G., Stebler, B., and Keller, E. T. (2001) Antioxid. Redox Signal. 3, 493–504.
20. McCaulay, L. K., Koh, A. J., Becker, C. A., Cui, Y., Decker, J. D., and Francheschini, R. T. (1995) J. Bone Miner. Res. 10, 1243–1255.
21. McCaulay, L. K., Koh, A. J., Becker, C. A., Cui, Y., Rosol, T. J., and Francheschini, R. T. (1996) J. Cell. Biochem. 61, 638–647.
22. McCaulay, L. K., Becker, C. A., Melton, M. E., Werkmeister, J. R., Jupner, H., Ahou-Samra, A. B., Segre, G. V., and Rosol, T. J. (1994) Mol. Cell. Endocrinol. 101, 331–336.
23. Celeste, A. J., Rosen, V., Buecker, J. L., Kri, R., Wang, E. A., and Wozney, J. M. (1986) EMBO J. 5, 1855–1869.
24. Young, M. P., Bharadi, K., Kerr, J. M., Lyu, M. S., and Kozak, C. A. (1994) Mamm. Genome 5, 108–111.
25. McCabe, L. R., Banerjee, C., Kundu, R., Harrisan, R. J., Dobner, P. R., Stein, L. J., Liu, J., and Stein, G. S. (1996) Endocrinology 137, 4396–4408.
26. Van Steenk, J., Caryphus, S., Zink, J. P., Renaud, J. C., Van Rooit, E., Boun, T., and Simpson, R. J. (1998) Eur. J. Immunol. 28, 193–197.
27. McCaulay, L. K., Koh, A. J., Becker, C. A., and Rosol, T. J. (1997) Endocrinology 138, 5427–5433.
28. Renkawitz, R., and Gerbi, S. A. (1979) Mol. Gen. Genet. 133, 1–13.
29. McCaulay, L. K., Koh-Paige, A. J., Chen, H., Cen, C., Ontiveros, C., Irwin, R., and McCabe, L. R. (2001) Endocrinology 142, 1975–1981.
30. Bokoch, G. M. (1995) Trends Cell Biol. 5, 378–384.
31. McCaulay, L. K., Koh, A. J., Buecker, J. L., and Stein, G. S. (1996) Endocrinology 137, 4396–4408.
32. Van Steenk, J., Caryphus, S., Zink, J. P., Renaud, J. C., Van Rooit, E., Boun, T., and Simpson, R. J. (1998) Eur. J. Immunol. 28, 193–197.
27. McCaulay, L. K., Koh, A. J., Becker, C. A., and Rosol, T. J. (1997) Endocrinology 138, 5427–5433.
33. Renkawitz, R., and Gerbi, S. A. (1979) Mol. Gen. Genet. 133, 1–13.
34. McCaulay, L. K., Koh-Paige, A. J., Chen, H., Cen, C., Ontiveros, C., Irwin, R., and McCabe, L. R. (2001) Endocrinology 142, 1975–1981.
35. Bokoch, G. M. (1995) Trends Cell Biol. 5, 378–384.
Impact of the Mitogen-activated Protein Kinase Pathway on Parathyroid Hormone-related Protein Actions in Osteoblasts
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