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Parathyroid Hormone Uses Multiple Mechanisms to Arrest the Cell Cycle Progression of Osteoblastic Cells from G₁ to S Phase*

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Parathyroid hormone (PTH) plays a major role in bone remodeling and has the ability to increase bone mass if administered daily. *In vitro*, PTH inhibits the growth of osteoblastic cell lines, arresting them in G₁ phase. Here, we demonstrate that PTH regulates the expression of at least three genes to achieve the following: inducing expression of MAPK phosphatase 1 (MKP-1) and p21^{Cip1} and decreasing expression of cyclin D1 at both mRNA and protein levels. The induction of MKP-1 causes the dephosphorylation of extracellular signal-regulated kinase and therefore the decrease in cyclin D1. Overexpression of MKP-1 arrests UMR cells in G₁ phase. The mechanisms involved in PTH regulation of these genes were studied. Most importantly, PTH administration produces similar effects on expression of these genes in rat femoral metaphyseal primary spongiosa. Analyses of p21^{Cip1} expression levels in bone indicate that repeated daily PTH injections make the osteoblast more sensitive to successive PTH treatments, and this might be an important feature for the anabolic functions of PTH. In summary, our data suggest that one mechanism for PTH to exert its anabolic effect is to arrest the cell cycle progression of the osteoblast and hence increase its differentiation.

The adult human skeleton is continuously resorbed and renewed by the actions of osteoclasts and osteoblasts. The maintenance of the skeleton requires the coordinated activities of these cells. Disruption of this coordination underlies many bone diseases, including osteoporosis. Parathyroid hormone (PTH)¹ is released by the parathyroid gland in response to slightly lowered serum ionized calcium concentrations and directly acts on the osteoblast but not the osteoclast. It plays a major role in the balance between bone formation and bone resorption. Although the physiological role of PTH is to mobilize calcium from bone into blood by stimulating bone resorption, current interest has focused on its ability to increase bone

mass in humans when administered once daily.

Several mechanisms have been proposed to explain this anabolic effect (for a review, see Ref. 1). Among them, a prevailing hypothesis proposes that PTH promotes osteoblast differentiation. Osteoblasts originate from bone marrow stromal stem cells. These cells undergo proliferation and differentiation into preosteoblasts and then into a final stage of differentiation concomitant with extracellular matrix mineralization. These cells are considered to be terminally differentiated mature osteoblasts that must exit the cell cycle and end their differentiation as osteocytes embedded in a mineralized matrix. Therefore, arresting cell cycle progression of the osteoblast could be part of the mechanism elicited by PTH to facilitate differentiation. *In vitro* studies demonstrated PTH could stimulate the proliferation of osteoblastic cells under some circumstances, such as low concentration of PTH (2), high cell density (3), short exposure to PTH (4), or in a particular cell line (TE-85) (5), through a possible mechanism of increasing Cdc2 expression (6). In chondrocytes and chondrosarcoma cells, an activating mutation of the PTH receptor (PTH1R) causes an increase in cyclin D1 and cyclin A expression (7). However, a high PTH concentration (10⁻⁸ M) consistently inhibits the growth of several well established osteoblastic cell lines (UMR 106–01 (8), MC3T3-E1 (9), SaOS-2 (10), and rat calvarial primary cultures).²

Mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1), originally named Erp/3CH134 or CL100, is a protein capable of regulating cell cycle progression. MKP-1 is an immediate early gene induced by growth factors and stress (11, 12). It belongs to a family of dual specificity phosphatases capable of removing both phosphotyrosine and phosphothreonine from the Thr-X-Tyr motif, whose dual phosphorylation is critical for the activation of MAPKs (reviewed in Refs. 13 and 14). Evidence strongly suggests that one of the MAPKs, extracellular signal-regulated kinases (ERK1/2, p42/p44^{MAPK}), whose dual phosphorylation is connected to cell proliferation in most cell types, are substrates for MKP-1 (15). Whereas constitutive overexpression or microinjection of MKP-1 blocks S phase entry in fibroblasts (16) and suppresses normal and oncogene-driven proliferation (17, 18), antisense oligonucleotides targeted to MKP-1 prolong the activation state of MAPK (19).

In mammalian cells, the cell cycle is governed by the activities of the cyclin-dependent kinases (CDKs) and their regulatory cyclin partners. Progression from G₁ to S phase requires cyclin D-Cdk4(6) and cyclin E-Cdk2. These complexes phosphorylate retinoblastoma protein (Rb), resulting in the dissociation of Rb and E2F. Released E2F is essential for cells to enter S phase. CDK inhibitors (CDKIs) are of two types: INK4 proteins

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¹ The abbreviations used are: PTH, parathyroid hormone; hPTH, human PTH; MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; PMA, phorbol 12-myristate 13-acetate; RT, reverse transcription; EGFP-F, farnesylated enhanced green fluorescent protein; PKA, protein kinase A; PKC, protein kinase C; Rb, retinoblastoma protein; ERK, extracellular signal-regulated kinase.

² Qin, L., Tamasi, J., Raggatt, L., Li, X., Feyen, J. H. M., Lee, D. C., DiCicco-Bloom, E., and Partridge, N. C. (2005) *J. Biol. Chem.* **280**, in press.

TABLE I
Sequences of primers for real time RT-PCR

Gene	5' primer	3' primer
MKP-1	TGGAGGACAACCACAAGGCA	TGGCAGTGCACAAACACCT
Cyclin D1	TGGAAGTCTTCTGGTGAACAA	TTGTTCTCATCCGCTCTGG
p21 ^{Cip1}	GCAAAGTATGCCGTCGTCTGTT	GCGAAGTCAAAGTCCACCGT
p27 ^{Kip1}	TCCGCTGCAGAAACCTCTT	AATCCACTTGGCGTGGCTC
β -Actin	TCCTGAGCGCAAGTACTCTGTG	CGGACTCATCGTACTCTGCTT
Glyceraldehyde-3-phosphate dehydrogenase	AACCCATCACCATCTCCAGG	GCCTTCTCCATGGTGGTAA

interfere with cyclin D-Cdk4(6), and CIP/KIP members (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}) bind to both complexes. Whereas it acts as an assembly factor for cyclin D-Cdk4(6), CIP/KIP inhibits the activity of cyclin E-Cdk2. Upon cell cycle arrest, the levels of CIP/KIP proteins increase, saturate D-type cyclins, and then bind to cyclin E-Cdk2 to block the catalytic activity of that kinase (20).

Previous studies demonstrated that 10^{-8} M PTH arrested UMR 106 cells, a rat osteoblastic osteosarcoma cell line, in G₁ phase possibly by up-regulation of p27^{Kip1} (21). However, the detailed mechanism of how PTH inhibits cell cycle progression and the relationship between this feature and the anabolic effects that PTH has on bone are not fully understood. Here we report that PTH employs several mechanisms to inhibit cell cycle progression: inducing expression of MKP-1 and p21^{Cip1} and decreasing expression of cyclin D1. This is true not only in osteoblastic cells (UMR 106-01 and rat calvarial primary culture) but also in PTH-treated osteoblast-rich bone samples, suggesting that arresting osteoblastic cells in G₁ could be an important part of the anabolic effect of PTH.

EXPERIMENTAL PROCEDURES

Chemicals—Synthetic human PTH-(1–38), human PTH-(1–34), human PTH-(1–31), and human PTH-(13–34) were purchased from Bachem (Torrance, CA). Inhibitors H-89 and GF109203X were purchased from Calbiochem. Rat PTH-(1–34), 8-bromo-cyclic AMP, PMA, sodium orthovanadate, cycloheximide, and antibody for MKP-1 were obtained from Sigma. Antibodies for ERK1/2, phospho-ERK1/2, and cyclin D1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for p21^{Cip1}, p27^{Kip1}, and cAMP-response element-binding protein were purchased from Cell Signaling (Beverly, MA).

Cell Culture—UMR 106–01 cells were maintained in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, non-essential amino acids, 25 mM HEPES (pH 7.5), 100 units/ml penicillin, and 100 μ g/ml streptomycin. For experiments, cells were seeded in 100-mm dishes at a density of 1.2×10^4 cells/cm² overnight and then switched to serum-free minimal essential medium for 1 day before the addition of PTH. Rat primary calvarial osteoblastic cells were obtained and cultured as described previously (22). Before PTH treatment, these cells were serum starved for 1 day.

In Vivo Injection of PTH into Rats—Sprague-Dawley rats (4-week-old male, about 75 g, and 3-month old female, about 250 g) were purchased from Hilltop (Scottsdale, PA). Rats were injected subcutaneously with vehicle (0.9% saline solution) or hPTH (1–38 for 4-week-old and 1–34 for 3-month-old; 8 μ g/100 g) and euthanized using CO₂ at the indicated time after injection. The animal protocols were approved by the Robert Wood Johnson Medical School Institutional Animal Care and Use Committee. The primary spongiosa samples from the distal femur were harvested immediately after sacrifice as described previously (23).

Analysis of mRNA Abundance by Real Time RT-PCR—Cells or tissues were harvested at the indicated time points after PTH treatment. Total RNA was isolated using Tri Reagent (Sigma) followed by purification with the RNeasy kit (Qiagen). A TaqMan® Reverse Transcription kit (Applied Biosystems) was used to reverse transcribe mRNA into cDNA. Following this, PCR was performed on Opticon (MJ Research) using a SYBR® Green PCR Core kit (Applied Biosystems). Each analysis was performed three to four times with independent sets of cells or tissues from PTH treatment to RT-PCR to obtain mean values and S.E. shown in the figures. The primers used for the RT-PCR are summarized in Table I. For UMR 106-01 cells, β -actin was used as an internal control. For femoral samples and primary osteoblastic cells, glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

Cell Cycle Analysis—UMR 106-01 cells were seeded into 6-well plates

at 5×10^5 cells/well and grown overnight. Cells were then transfected with pCMV-hMKP-1 (a generous gift from Dr. Laura Mauro) or pCMV control vector along with the transfection marker pEGFP-F (Clontech), a plasmid containing enhanced green fluorescent protein with a farne-sylation sequence. The next day, cells were trypsinized, washed once with PBS, and fixed in 70% ethanol for at least 1 h on ice. Fixed cells were washed with PBS and incubated with propidium iodide solution containing ribonuclease A. Cell cycle analyses were performed on a Beckman Coulter XL.

Immunoblotting—Preparation of cell lysates and Western blot analyses were performed as described previously (24).

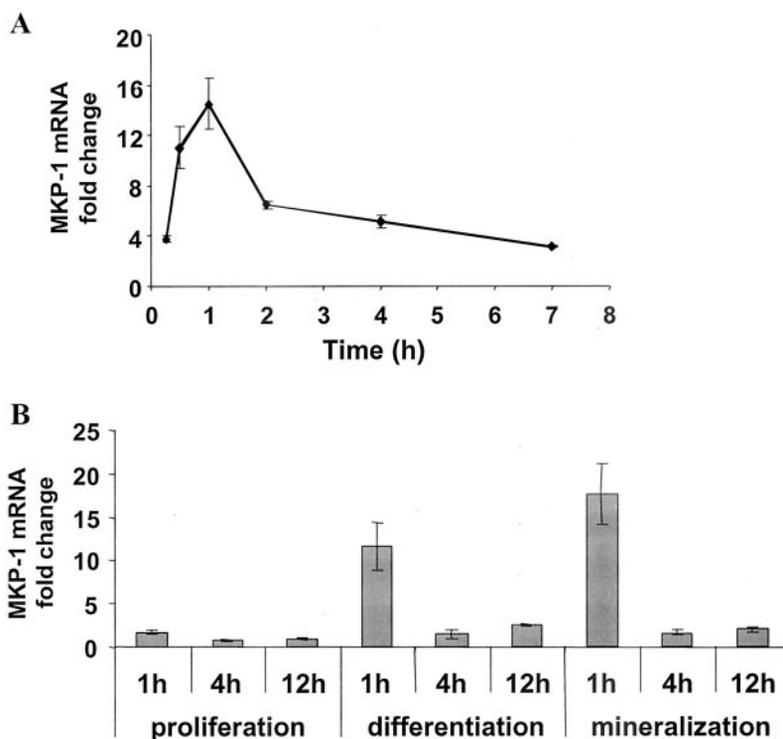
RESULTS

PTH Regulates MKP-1 mRNA Abundance in Osteoblastic Cells—Recent microarray studies from our laboratory identified MKP-1 as a target gene for rat PTH-(1–34) in UMR 106-01 cells (25). We confirmed this result by using real time RT-PCR. As shown in Fig. 1A, 10^{-8} M rPTH-(1–34) greatly induced MKP-1 mRNA expression in UMR 106-01 cells, with a peak of 15-fold after 1 h of treatment. This induction occurs very quickly, since there is a 4-fold increase of MKP-1 expression after just 15 min of PTH treatment. Western blot demonstrates that PTH also regulates MKP-1 expression at the protein level (Fig. 2A). In untreated UMR cells, there is a very low detectable amount of MKP-1. Similar to the mRNA induction, MKP-1 protein peaks after 1 h of treatment, indicating that MKP-1 mRNA translates rapidly. A similar phenomenon is also observed in another osteoblastic cell preparation, rat primary calvarial osteoblastic cells (Fig. 1B). These cells mimic the development of osteoblasts in bone and go through three stages (proliferation, differentiation, and mineralization) sequentially when cultured in dishes. Cells at different stages were treated with rPTH-(1–34) (10^{-8} M) for different time periods, and the level of MKP-1 mRNA was measured by real time RT-PCR. At all developmental stages, MKP-1 levels were stimulated to the greatest level at 1 h and then decreased to base line at 4 h. The highest induction (18-fold) is observed in the mineralization stage, and the lowest (2-fold) is seen in the proliferation stage.

The Induction of MKP-1 Expression Correlates with a Decrease in ERK Phosphorylation in Osteoblastic Cells—PTH- or cAMP-generating agents such as forskolin have been shown to inhibit the basal activity of ERK1/2 or their activation by epidermal growth factor in osteoblastic cells (26, 27). Consistent with those results, we found that in UMR 106-01 cells rPTH-(1–34) (10^{-8} M) strongly but not completely inhibits the basal phosphorylation of ERK1/2 using Western blot analysis (Fig. 2A). The maximum inhibition occurs 1 h after the addition of PTH. Since phosphorylated ERKs are substrates for MKP-1 activity, we compared the pattern of MKP-1 protein induction and ERK1/2 dephosphorylation after PTH treatment (Fig. 2A, top two panels). It is interesting to note that both patterns correlate well with maximal effects at 1 h. This result implies that the induction of MKP-1 may be responsible for the decrease in ERK1/2 phosphorylation.

Next we used two approaches to test this hypothesis. First, we pretreated UMR cells with cycloheximide to inhibit *de novo* protein synthesis. As shown in Fig. 2B, cycloheximide completely abolished the MKP-1 protein synthesis induced by PTH.

FIG. 1. PTH rapidly stimulates MKP-1 mRNA expression in osteoblastic cells. Cells were serum-starved for 1 day and then treated with 10^{-8} M rPTH-(1–34). RNAs were collected at the indicated time points from both control and PTH-treated samples to assess the -fold change in MKP-1 mRNA using real time RT-PCR analysis. **A**, UMR 106-01 cells. **B**, rat primary calvarial osteoblastic cells at day 6 (proliferation phase), day 14 (differentiation phase), and day 21 (mineralization phase) in culture.



Concurrently, it also eliminated the effect of PTH on ERK dephosphorylation, suggesting that this effect requires new protein synthesis that would include MKP-1. Second, we pre-treated UMR cells with orthovanadate, a tyrosine phosphatase inhibitor, to inhibit MKP-1 activity. Whereas the addition of orthovanadate alone had no effect on ERK phosphorylation in UMR cells, it totally abolished the dephosphorylation of ERK caused by PTH (Fig. 2C), providing more evidence that MKP-1 probably mediates the effect of PTH on the dephosphorylation of ERK.

Overexpression of MKP-1 Arrests UMR 106-01 Cells in G₁ Phase—MKP-1 plays an important role in cell cycle regulation. To address its role in the osteoblast, we transfected a human MKP-1 plasmid into UMR cells and measured the percentage of cells in each phase of the cell cycle by flow cytometry. Since the transfection efficiency of UMR 106-01 cells is about 20% (data not shown), we used farnesylated enhanced green fluorescent protein (EGFP-F) as a cotransfection marker to do this experiment. Cell cycle analyses were performed on EGFP-F-expressing cells, which should also have the MKP-1 vector. As shown in Table II, flow cytometry revealed that about 47% of UMR cells cotransfected with EGFP-F, and various amounts of pCMV vector controls are in G₁ phase, and 29 and 23% of cells are in S phase and G₂ + M phases, respectively. Interestingly, in cells cotransfected with EGFP-F and 1 μ g/well MKP-1, there is a definite increase in the percentage of cells in G₁ phase (56%) as well as a decrease in the percentage of cells in S phase (20%) and in G₂ + M phases (20%). More importantly, as the amount of transfected MKP-1 plasmid increased to 2 μ g/well, there were more cells in G₁ phase (65%) and fewer cells in S phase (16%) and in G₂ + M phase (17%). These results clearly show that overexpression of MKP-1 prevents UMR cells progressing from G₁ to S phase of the cell cycle.

PTH Inhibits Cyclin D1 Expression Possibly through MKP-1 Regulation—The proliferative effect of phosphorylated ERK is partly mediated by its ability to induce cyclin D1 expression (28). Since the above data suggest that the up-regulation of MKP-1 by PTH causes the dephosphorylation of ERKs and overexpression of MKP-1 inhibits cell cycle progression, we

hypothesized that similar PTH treatment will decrease cyclin D1 expression. Indeed, our previous microarray analysis suggested that cyclin D1 expression decreased to about 43% in UMR cells after 4 h of rPTH-(1–34) (10^{-8} M) treatment (25). We confirmed this result using real time RT-PCR. As shown in Fig. 3A, PTH treatment significantly decreased cyclin D1 expression to about 60% of base line at 4 and 8 h ($p < 0.05$). This effect is more evident at the protein level. Densitometric measurement of Western blots revealed that cyclin D1 protein was 51, 36, 28, and 42% of control after 2, 4, 8, and 24 h, respectively, of PTH treatment (Fig. 3B). It is interesting to note that the effect of PTH on cyclin D1 starts at 2 h and peaks around 4–8 h, just after the maximal effect (1 h) on MKP-1 expression and dephosphorylation of ERK.

Cyclin D1 expression is also inhibited by rPTH-(1–34) (10^{-8} M) in rat primary calvarial osteoblastic cells in the mineralization phase (Fig. 3C). After 12 h of treatment, the level of cyclin D1 mRNA significantly decreased to 65% in PTH-treated cells compared with that in control cells ($p < 0.05$). A 1.8-fold increase of cyclin D1 mRNA was observed after 1 h of PTH treatment. However, this increase is not statistically significant ($p = 0.2$). There was no effect of PTH on cyclin D1 in primary osteoblastic cells in the differentiation phase.

If PTH regulation of cyclin D1 is through MKP-1 and ERK pathways, then cyclin D1 must be a secondary response gene for PTH, and this response will be eliminated when MKP-1 synthesis or activity is blocked. In fact, cycloheximide abolished the PTH effect on cyclin D1 expression in UMR cells (Fig. 3D), suggesting that this effect also requires new protein synthesis. As shown in Fig. 3E, orthovanadate had no effect on the basal expression of cyclin D1 protein (compare lanes 1 and 3). However, this MKP-1 activity inhibitor completely eliminated the inhibition of cyclin D1 by PTH treatment (compare lanes 2 and 4). Therefore, it is reasonable to propose that the effect of PTH on cyclin D1 is downstream of MKP-1 and ERK.

PTH Stimulates p21^{Cip1}, but Not p27^{Kip1}, in UMR 106-01 and Rat Calvarial Osteoblastic Cells—To further understand the mechanism of how PTH arrests cell cycle progression of osteoblastic cells, we performed real time RT-PCR to study the

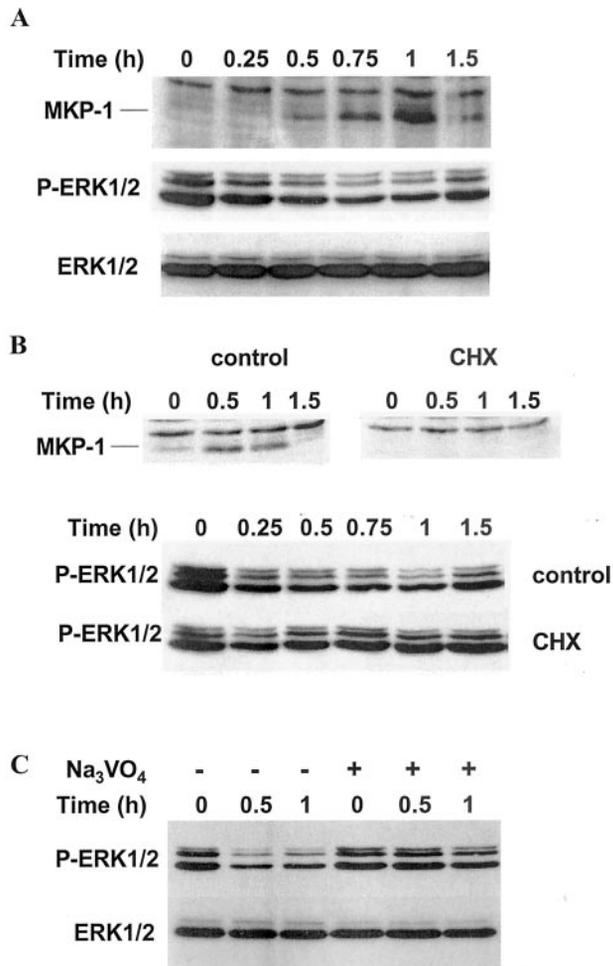


FIG. 2. PTH inhibits ERK phosphorylation through an increase in MKP-1 expression. *A*, UMR 106-01 cells were serum-starved for 1 day and then treated with 10^{-8} M rPTH-(1-34). Protein lysates were collected at the indicated time points, and the same amounts of protein (35 μ g/well) were analyzed by immunoblotting using various antibodies. Western blots with total ERK1/2 antibody were used as an internal control. *B*, the dephosphorylation of ERK1/2 by PTH requires new protein synthesis. Control (ethanol) or 30 μ g/ml cycloheximide (CHX) was added to the medium 30 min before the addition of 10^{-8} M rPTH-(1-34). *C*, blockage of MKP-1 activity by orthovanadate abolishes the effect of PTH on ERK1/2 dephosphorylation. 50 μ M Na₃VO₄ was added to the medium 30 min before the addition of 10^{-8} M rPTH-(1-34).

TABLE II
Overexpression of MKP-1 in UMR 106-01 cells inhibits cell cycle progression from G₁ to S phase

Plasmid	G ₁	S	G ₂ + M
	%	%	%
EGFP-F + 1 μ g of vector	46.5	29.6	22.8
EGFP-F + 2 μ g of vector	47.0	28.3	23.3
EGFP-F + 1 μ g of MKP-1	56.2	20.0	20.4
EGFP-F + 2 μ g of MKP-1	64.8	16.1	16.9

expression levels of other cyclins (cyclin D2, D3, E, A, and B), CDKs (Cdk2, -4, and -6), and CDKIs (p21^{Cip1}, p27^{Kip1}, p57^{Kip2}, p15, and p16) in UMR 106-01 cells after PTH treatment. Only p21^{Cip1}, and not the other aforementioned genes, was found to be regulated by PTH. The mRNA level of p21^{Cip1} was quickly stimulated by PTH, with a maximal 8-fold increase at 1 h (Fig. 4A). Western blot analysis confirmed this result. Whereas there was no detectable level of p21^{Cip1} in untreated cells, p21^{Cip1} protein became obvious at 1 h and peaked between 2 and 4 h after treatment (Fig. 4B). However, there was no

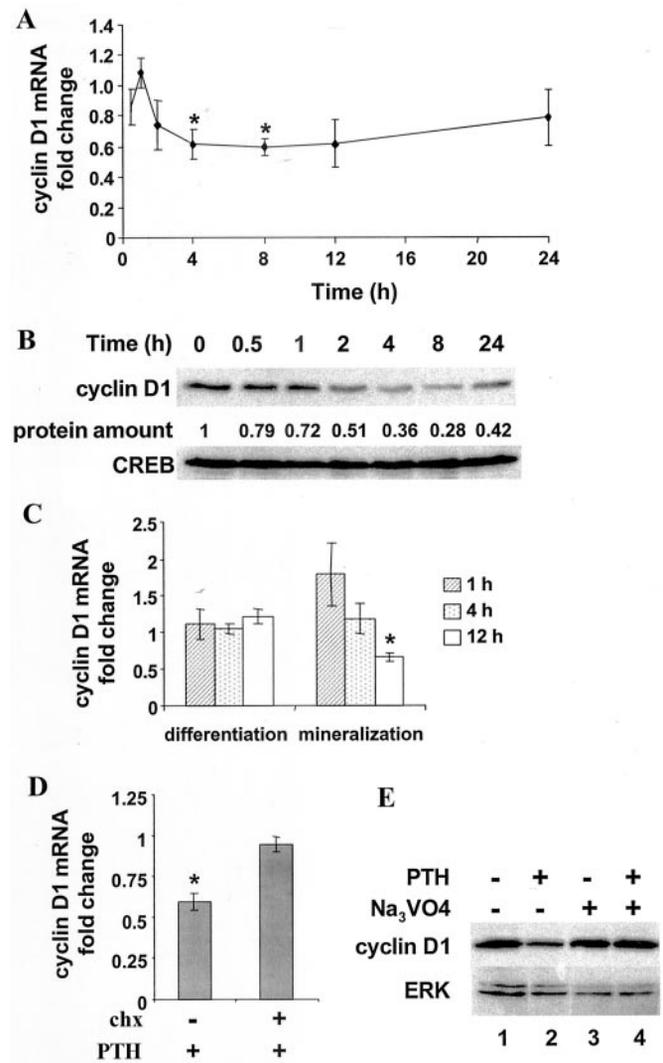


FIG. 3. PTH decreases cyclin D1 expression by up-regulation of MKP-1. UMR 106-01 cells were serum-starved for 1 day and then treated with 10^{-8} M rPTH-(1-34). RNA and protein lysates were collected at the indicated time points and were analyzed by real time RT-PCR (A) and immunoblotting (B), respectively. The cyclin D1 protein amounts shown at the bottom of the gel were obtained by densitometric measurement. The amount in untreated cells is set as 1. C, the effect of PTH on cyclin D1 mRNA in primary calvarial osteoblastic cultures in the differentiation (day 14) and mineralization phases (day 21) as measured by real time RT-PCR. D, PTH regulation of cyclin D1 is a secondary response. Control (ethanol) or 30 μ g/ml cycloheximide (CHX) was added to the medium 30 min before 8 h of 10^{-8} M rPTH-(1-34) treatment. The expression levels of cyclin D1 were assayed by real time RT-PCR. E, immunoblotting demonstrates that inhibition of MKP-1 activity eliminates the PTH effect on cyclin D1 expression. 50 μ M Na₃VO₄ were added to the medium 30 min before 8 h of 10^{-8} M rPTH-(1-34) treatment. Western blot with total ERK antibody was used as an internal control. The asterisks in C and D represent $p < 0.05$ compared with control. CREB, cAMP-response element-binding protein.

significant change observed in both mRNA and protein levels of p27^{Kip1} by PTH throughout the time course (Fig. 4, A and B). This is inconsistent with a previous study indicating that PTH stimulates the protein level of p27^{Kip1} (3-fold), but not p21^{Cip1}, in UMR 106 after 12 and 24 h (21). The reason for the discrepancy of the PTH effect on p27^{Kip1} is not known, but it could be due to the strain difference between UMR 106 used previously and UMR 106-01 used in the present report.

Furthermore, a similar effect of PTH on p21^{Cip1} and p27^{Kip1} was also observed in primary calvarial osteoblastic cells. In cells in the differentiation phase, PTH stimulated p21^{Cip1} mRNA 3.8-fold at 4 h, and in cells in the mineralization phase,

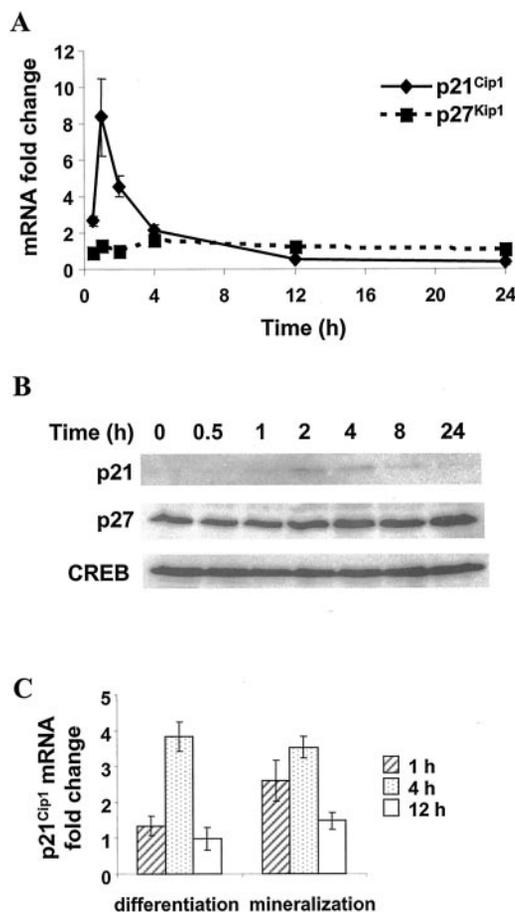


FIG. 4. CDK inhibitor p21^{Cip1}, but not p27^{Kip1}, is an early response gene for PTH. UMR 106-01 cells (A and B) and primary calvarial osteoblastic cells in differentiation (day 14) and mineralization (day 21) phases (C) were serum-starved for 1 day and then treated with 10^{-8} M rPTH-(1–34). RNA and protein lysates were collected at the indicated time points and were analyzed by real time RT-PCR (A and C) and immunoblotting (B), respectively. Western blot with cAMP-response element-binding protein antibody (CREB) was used as an internal control.

the induction was 2.6- and 3.5-fold at 1 and 4 h, respectively (Fig. 4C). Consistent with the above results from the UMR 106-01 cells, we did not detect a change in p27^{Kip1} mRNA level after PTH treatment (data not shown).

Expression of MKP-1, Cyclin D1, p21^{Cip1}, and p27^{Kip1} during Osteoblast Differentiation—During its differentiation, the osteoblast gradually ceases proliferation and expresses various bone-specific markers at different differentiation stages. For example, in primary calvarial cultures, alkaline phosphatase has its highest expression level during the differentiation phase, whereas osteocalcin is a marker of mineralization. To further investigate the role of the above PTH-regulated cell cycle genes, we studied their expression in primary calvarial cells during differentiation using real time RT-PCR (Fig. 5). MKP-1 mRNA increased 2-fold in both differentiation and mineralization phases compared with the proliferation phase. p21^{Cip1}, along with another CDKI p27^{Kip1}, was expressed to a greater extent in the later two phases, since the mRNA levels of p21^{Cip1} in the differentiation and mineralization phases were 6- and 5-fold that in the proliferation phase. For p27^{Kip1}, it is 3- and 4-fold increased. These results demonstrate why the differentiated osteoblast ceases proliferation. Surprisingly, we observed that cyclin D1 had its highest expression in the differentiation phase (5-fold compared with that in the proliferation phase). As cells mineralized, its expression decreased

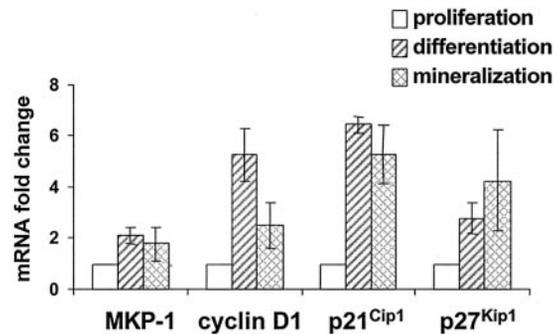


FIG. 5. The expression of MKP-1, cyclin D1, p21^{Cip1} and p27^{Kip1} during osteoblast differentiation. Real time RT-PCR was used to analyze RNA extracted from rat calvarial primary osteoblastic cells at three stages of differentiation: day 6 (proliferation), day 14 (differentiation), and day 21 (mineralization) in culture.

about 50%, probably adding another mechanism preventing mineralized osteoblastic cells from proliferating.

PTH Regulation of MKP-1 and p21^{Cip1} Is PKA-dependent and Is a Primary Response—PTH signals through both PKA and PKC pathways after binding with its receptor, PTH1R, on the osteoblast membrane (29, 30). To study which pathway PTH uses to regulate MKP-1 and p21^{Cip1}, we took advantage of different peptide fragments of PTH that activate different pathways and inhibitors and activators of these pathways. As shown in Fig. 6A, hPTH-(1–31) (which activates PKA but not PKC) retained the ability to stimulate MKP-1 and p21^{Cip1} mRNA levels (21- and 8-fold, respectively) after a 1-h treatment of UMR 106-01 cells. However, hPTH-(13–34) (which activates PKC but not PKA) had no effect on the expression of those genes. In comparison, 8-bromo-cyclic AMP, a cell-permeable cAMP analog, stimulated the expression of MKP-1 and p21^{Cip1} about 13- and 8-fold, respectively. PMA, an activator of the PKC pathway, had very little effect on MKP-1 expression (3-fold increase compared with 22-fold increase by rPTH-(1–34)). In contrast, PMA stimulated p21^{Cip1} expression by 5-fold, the same level as rPTH-(1–34) achieved, indicating that p21^{Cip1} could be regulated by both PKA and PKC pathways. Nevertheless, in the presence of the PKA inhibitor H-89, but not in the presence of the PKC inhibitor GF109203X, rPTH-(1–34) lost its ability to induce both MKP-1 and p21^{Cip1}, suggesting that this regulation is PKA-dependent but not PKC-dependent in osteoblastic cells.

To determine whether PTH induction of MKP-1 and p21^{Cip1} is a primary response, UMR 106-01 cells were treated with rPTH-(1–34) (10^{-8} M) in the presence or the absence of cycloheximide. Fig. 6B shows that cycloheximide had no effect on the PTH induction of MKP-1 and p21^{Cip1}, suggesting that this process does not require *de novo* protein synthesis (*i.e.* this effect is a primary response).

MKP-1, Cyclin D1, and p21^{Cip1} Expression Are Similarly Regulated by PTH in Bone—A classic animal model for PTH anabolic function is repeated daily injection of PTH in rats. A single injection of hPTH-(1–34) (8 μ g/100g) is sufficient to modify the expression of genes involved in the actions of PTH in rat osteoblast-enriched femoral metaphyseal primary spongiosa (23). We injected human PTH-(1–38) (8 μ g/100 g) or saline vehicle into young male rats (4-week-old) and harvested RNA from the femoral metaphyses at various time points. Real time RT-PCR was performed to study the expression levels of MKP-1, cyclin D1, and p21^{Cip1} in these animals. Fig. 7 demonstrates that one injection of PTH greatly stimulated MKP-1 expression with a 7-fold stimulation over control after 30 min (A), decreased cyclin D1 expression significantly to 60% of control levels 1 h after PTH injection (B), and increased p21^{Cip1}

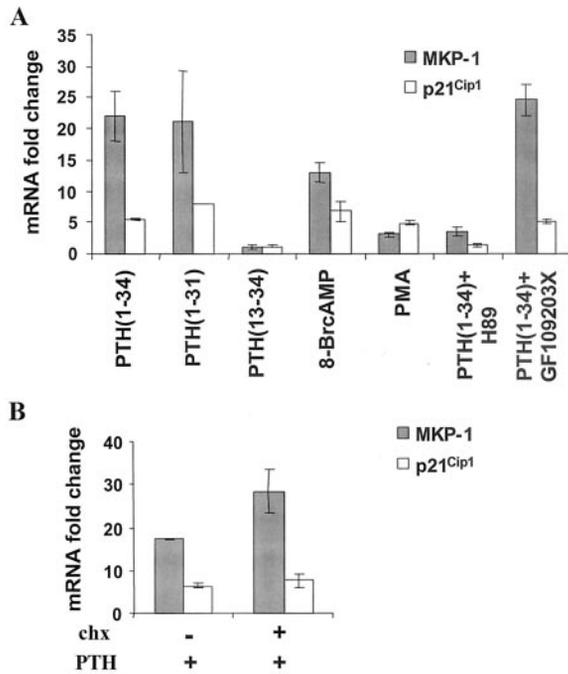


FIG. 6. The stimulation of MKP-1 and p21^{Cip1} mRNA expression by PTH is PKA-dependent (A) and is a primary response (B). A, after serum starvation, UMR 106-01 cells were pretreated with the following for 30 min: Me₂SO (0.1%, v/v), 40 μ M H-89, or 5 μ M GF109203X. Cells were then treated with 10⁻⁸ M rPTH-(1-34), 10⁻⁸ M hPTH-(1-31), 10⁻⁸ M hPTH-(13-34), 1 mM 8-bromo-cyclic AMP, or 3 μ M PMA as indicated for 1 h before harvesting RNA for real time RT-PCR analysis to determine the -fold changes in MKP-1 and p21^{Cip1}. B, UMR 106-01 cells were treated with ethanol (0.1%, v/v) or cycloheximide (chx; 30 μ g/ml) for 1 h. Cells were then treated with 10⁻⁸ M rPTH-(1-34) for another 1 h before harvesting RNA for real time RT-PCR analyses.

expression more than 2-fold at both 30 min and 1 h (C). These results are consistent with our results on the effects of PTH in UMR and primary osteoblastic cells, strongly suggesting that the inhibitory effect of PTH on cell cycle progression observed in several osteoblastic cell lines is not confined only to *in vitro* cell culture experiments but is indeed related to the anabolic function of PTH *in vivo*.

Similar to previous reports (31), we observed a significant anabolic effect of PTH on trabecular bone, including an increase in bone mineral density, bone formation rate, and osteoblast numbers, in 3-month-old female rats after 14 days of daily hPTH-(1-34) (8 μ g/100 g) administration (data not shown). After 2 weeks of injections, RNA was harvested from femoral metaphyseal primary spongiosa, and the levels of the genes of interest were compared with that of vehicle-treated samples using real-time RT-PCR. Surprisingly, we found that whereas one injection of PTH only increased the p21^{Cip1} mRNA level 2-fold at 1 h, 14 days of daily injections substantially increased the same transcript 8-fold compared with control 1 h after the last injection (Fig. 7D). Note that the p21^{Cip1} mRNA level returns to basal 4 h after one PTH injection (Fig. 7C). Therefore, the basal level of p21^{Cip1} mRNA before PTH injection at 14 days should be similar to that of vehicle-treated animals. It appears that repeated PTH injections make the osteoblast more sensitive to the subsequent injections, giving a reason why the anabolic effect of PTH requires multiple administrations. The stimulation of MKP-1 and inhibition of cyclin D1 after 1 h of PTH injection were unchanged between one injection and 14 days of injections.

DISCUSSION

There are contradictory reports regarding whether PTH stimulates or inhibits osteoblast cell proliferation. It seems

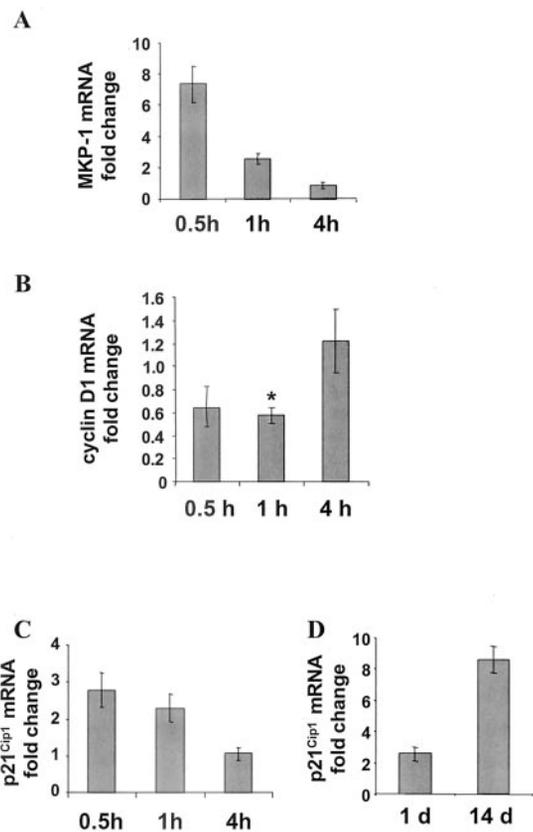


FIG. 7. PTH regulates MKP-1, cyclin D1, and p21^{Cip1} similarly in bone as it does in osteoblastic cells. A-C, mRNAs were extracted from distal femur metaphyses 0.5, 1, and 4 h after one subcutaneous injection of hPTH-(1-38) (8 μ g/100 g) or saline control into 4-week-old male rats, and the levels of MKP-1 (A), cyclin D1 (B), and p21^{Cip1} (C) in those mRNAs were analyzed by real time RT-PCR. The asterisk in B represents $p < 0.05$ compared with vehicle-treated rats. D, 3-month-old female rats were subcutaneously injected with hPTH-(1-34) (8 μ g/100 g) or saline control either with one injection or daily for 14 days. Messenger RNA was extracted from the distal femur metaphyses 1 h after the last injection and subjected to real-time RT-PCR to determine the -fold change in p21^{Cip1}.

that the outcome depends on the cells used, the length of treatment, PTH concentration, etc. However, how this effect is related to the anabolic function of PTH in bone is largely unknown. This is the first report demonstrating that PTH up-regulates MKP-1 and p21^{Cip1} and down-regulates cyclin D1 not only in osteoblastic cells, such as UMR 106-01 and primary calvarial cultures, but also in bone cells *in vivo* after intermittent injection, strongly suggesting that the ability of PTH to inhibit cell proliferation *in vitro* is relevant to its physiological role.

Regulation of all the above three genes results in preventing cell cycle transition from G₁ to S phase. In most cases, exit from the cell cycle at G₁ is required for terminal cell differentiation. Moreover, cell cycle arrest actually stimulates differentiation. The current model of cell cycle regulation indicates that Rb is inhibited by CDKs. Therefore, the stimulation of p21^{Cip1} and inhibition of cyclin D1 would lead to increased activity of Rb. Rb belongs to the pocket protein family, which also includes p107 and p130. An essential role for Rb in all cell types is proliferation control. It acts as a generic corepressor of the E2F family of transcription factors. These proteins, with their partners DP1 and DP2, transactivate genes important for G₁ to S phase transition (32). In addition, Rb also plays important roles in the differentiation of mesenchymal lineages, including myogenesis (33), adipogenesis (34), and osteogenesis (35). Osteosarcoma is the second most common tumor after retinoblas-

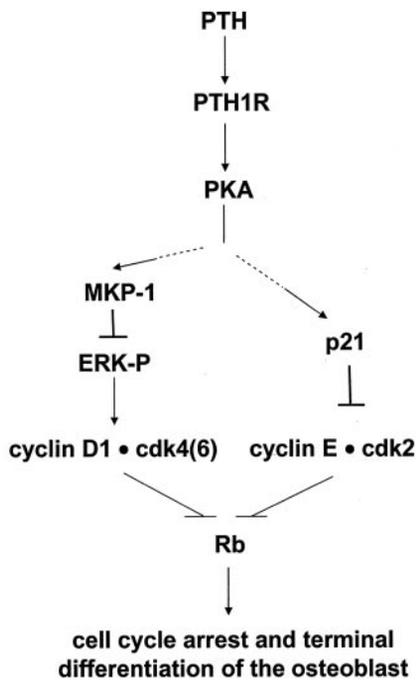


FIG. 8. A model to describe how PTH regulates cell cycle exit in the osteoblast and hence stimulates the cell's terminal differentiation.

toma, occurring in individuals with inherited heterozygous loss of the Rb gene (36). In addition, up to 60% of sporadic osteosarcomas have loss of Rb (37, 38). It turns out that Rb plays a key role in facilitating expression of late markers of osteoblast differentiation through physical association with Runx2, a transcriptional regulator of osteoblast differentiation. Loss of Rb in osteoblastic cells results in blockage of late osteoblast differentiation (35). In addition to Rb, Cip/Kip CDKs support differentiation of a variety of cells (39), possibly through increasing Rb activity. A well studied system is MyoD, a skeletal muscle-specific transcription factor that induces terminal cell cycle arrest associated with skeletal muscle differentiation, which has been shown to induce p21^{Cip1} (33, 40). Our data also suggest that both p21^{Cip1} and p27^{Kip1} levels increase as osteoblastic cells differentiate. These data are consistent with a previous report on p27^{Kip1} but not p21^{Cip1} (41). In that report, compared with the proliferative phase, p21^{Cip1} protein levels decreased in the differentiation phase and then returned to comparable levels in the mineralization phase.

Accordingly, a model is proposed to explain how PTH arrests osteoblastic cells in G₁ and stimulates terminal differentiation (Fig. 8). PTH binds to its receptor PTH1R on the mature osteoblast and activates the PKA pathway. Subsequently, posttranslational modification of some transcription factors, such as cAMP-response element-binding protein, quickly induces the expression of MKP-1 and p21^{Cip1}. The increase in MKP-1 protein results in dephosphorylation of its substrate, phosphorylated ERKs, and hence decreases the expression of cyclin D1 and subsequently the activity of the cyclin D1-Cdk4(6) complex. The increase in p21^{Cip1} inhibits the activity of the cyclin E-Cdk2 complex. Both result in greater activity of Rb, which would suppress proliferation and promote differentiation through interacting with Runx2.

Why is a two-pronged approach needed for the growth-inhibitory function of PTH? We can speculate that the p21^{Cip1} pathway is used to arrest the intrinsic growth of the osteoblast, whereas the MKP-1/cyclin D1 pathway is used to prevent the osteoblast from responding to external growth signals. Osteoblasts reside in an environment containing a variety of cells,

such as bone marrow stromal cells, hematopoietic stem cells, blood cells, adipocytes, osteoblast progenitors, osteoclasts, and many more. These cells communicate and influence each other to form a balanced microenvironment. Many growth factors, produced locally from surrounding cells or systemically, can act on the osteoblast and simulate proliferation by increasing ERK phosphorylation. These factors include FGF-2 (42), platelet-derived growth factor BB (43), insulin-like growth factor-I (44), epidermal growth factor (24), amphiregulin,² etc. The increase in MKP-1 after PTH treatment could antagonize those signals. In fact, this explains a previous report that PTH inhibited the activation of ERK by epidermal growth factor (27). It was also reported that glucocorticoids rapidly up-regulate MKP-1 in osteoblastic cell lines, resulting in dephosphorylation of PMA-stimulated ERKs and impairment of osteoblast proliferation (45). Together with our data, it seems that MKP-1 is used as a common mechanism to prevent cells from responding to growth signals.

The anabolic function of PTH requires repeated daily administration. It takes a minimum of 2 weeks to observe a bone mineral density increase in rats and mice, whereas the PTH treatment for osteoporosis patients takes months to be effective. A very recent paper using ovariectomized mice comparing PTH and estradiol treatment showed only a small overlap of the genes expressed, and notably estradiol stimulated both p21^{Cip1} and cyclin D1 (46). Interestingly, we found that the stimulation of p21^{Cip1} by PTH in rats is higher after 14 days of injection than that after one injection (8-fold *versus* 2-fold). It seems that repeated injections modify the bone cells in a way that they become more sensitive to the next PTH treatment. However, we did not detect the same change in cyclin D1 and MKP-1 expression. This could be due to the fact that we did not harvest samples at the optimum time point after 14 days of treatment or that only a fraction of PTH response genes behave similarly to p21^{Cip1}. Nevertheless, this feature may be important for the anabolic function of PTH, and we are currently studying whether PTH has the same effect on other PTH-regulated genes.

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