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Parathyroid Hormone and Parathyroid Hormone-related Protein Exert Both Pro- and Anti-apoptotic Effects in Mesenchymal Cells*

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During bone formation, multipotential mesenchymal cells proliferate and differentiate into osteoblasts, and subsequently many die because of apoptosis. Evidence suggests that the receptor for parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP), the PTH-1 receptor (PTH-1R), plays an important role in this process. Multipotential mesenchymal cells (C3H10T1/2) transfected with normal or mutant PTH-1Rs and MC3T3-E1 osteoblastic cells were used to explore the roles of PTH, PTHrP, and the PTH-1R in cell viability relative to osteoblastic differentiation. Overexpression of wild-type PTH-1R increased cell numbers and promoted osteocalcin gene expression *versus* inactivated mutant receptors. Furthermore, the effects of PTH and PTHrP on apoptosis were dramatically dependent on cell status. In preconfluent C3H10T1/2 and MC3T3-E1 cells, PTH and PTHrP protected against dexamethasone-induced reduction in cell viability, which was dependent on cAMP activation. Conversely, PTH and PTHrP resulted in reduced cell viability in postconfluent cells, which was also dependent on cAMP activation. Further, the proapoptotic-like effects were associated with an inhibition of Akt phosphorylation. These data suggest that parathyroid hormones accelerate turnover of osteoblasts by promoting cell viability early and promoting cell departure from the differentiation program later in their developmental scheme. Both of these actions occur at least in part via the protein kinase A pathway.

The classical skeletal action of parathyroid hormone (PTH)[1] is stimulation of osteoclastic bone resorption to maintain calcium levels in blood. However, daily subcutaneous PTH injection increases bone mass and shows great potential in treating osteoporosis (1). The mechanisms underlying the PTH anabolic effect in bone are not fully understood. The increased bone formation has been attributed to activation of growth factors (2, 3), osteoblast precursor cell proliferation (2, 4), and mature osteoblast function (2). Recent studies suggest that suppression of osteoblast apoptosis might play a major role in PTH anabolic action (5); however, the impact of cell differentiation and the pathways operating in this process have not been well characterized.

The PTH-1 receptor (PTH/PTHrP receptor; PTH-1R) was the first PTH receptor isolated, cloned, and sequenced and binds both PTH and PTHrP with similar affinity (6, 7). The PTH-1R responds to binding of PTH or PTHrP by activation of the protein kinase A (PKA) and protein kinase C (PKC) pathways (8) and clearly plays an important role in bone. Although other PTH receptors have been identified (9, 10), the PTH-1R is the major receptor responsible for skeletal actions of PTH and PTHrP, as evidenced by similar phenotypes in the PTHrP and PTH-1R null mouse models (11). Ablation of the PTH-1R gene in mice results in a neonatal-lethal phenotype with severe abnormalities in development of cartilage and bone (12).

To form bone, multipotential mesenchymal cells proliferate and differentiate into osteoblasts, which secrete an extracellular matrix that becomes mineralized. Mature osteoblasts eventually become osteocytes or lining cells or vanish because of apoptosis. Evidence suggests that the PTH-1R plays an important role in proliferation, differentiation, and apoptosis that occurs during bone formation. Expression of the PTH-1R is considered a hallmark for osteoblastic differentiation, because the PTH-1R is primarily associated with active collagen-producing osteoblasts compared with osteoblasts in earlier or later stages of differentiation (13). Further, osteocalcin mRNA, a specific osteoblastic marker, is undetectable in osteoblasts lacking the PTH-1R but becomes detectable after transfecting PTH-1R null cells with wild-type PTH-1R (14). In addition, transgenic mice overexpressing a constitutively active PTH-1R (H223R) display increased osteoblastic proliferation and decreased apoptosis in trabecular bone leading to an increase in trabecular bone volume (15).

In humans, genetic abnormalities of the PTH-1R also result in profound skeletal defects. Constitutively active mutant PTH-1 receptors H223R (16), T410P (17), and I458R (18) have been identified as the cause for Jansen's metaphyseal chondrodysplasia, a disease characterized by short limbed dwarfism as a result of retarded chondrocyte differentiation. The expression of H223R, T410P, and I458R in COS-7 cells results in a 4–8-fold increase in basal cAMP accumulation when compared with the wild-type PTH-1R control (16–18). In contrast, inactivated mutant PTH-1 receptors Δ373–383 (19) and P132L (20) result

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[1] The abbreviations used are: PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; PTH-1R, PTH-1 receptor; PKA, protein kinase A; PKC, protein kinase C; CREB, cAMP response element-binding protein; BMP, bone morphogenetic protein; PE, phycoerythrin; 7-AAD, 7-amino-actinomycin; ELISA, enzyme-linked immunosorbent assay.

in Blomstrand chondrodysplasia, which is a lethal genetic disorder with extremely advanced endochondral bone formation. The mutant Δ373–383 expressed in COS-7 cells does not bind PTH or PTHrP and fails to induce detectable stimulation of either cAMP or inositol phosphate production (19). P132L, a milder inactivated mutant receptor, results in severely reduced PTH-induced cAMP accumulation and undetectable PTH-induced inositol phosphate accumulation (20).

The role of the PTH-1R during bone formation has not been thoroughly and mechanistically investigated. Therefore, this study takes advantage of the different functional activities of wild-type and mutant PTH-1 receptors to explore the impact of the PTH-1R in a mesenchymal cell differentiation system. Hence, the purpose of this study was to determine the effects of overexpressing mutant and wild-type PTH-1 receptors on the cellular growth program of mesenchymal cells.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The plasmid constructs utilized for the PTH-1R transfection experiments included pEGFP-N2 (pE), (CLONTECH, Palo Alto, CA) and pEGFP-N2 containing cDNA for the wild-type PTH-1 receptor (WT) or mutant PTH-1 receptors Δ373–383 (Δ), P132L (P), and H223R (H) (16, 19, 20). The pCMV-CREB (constitutively expresses the wild-type CREB) (CLONTECH), pCMV-CREB133 (a dominant-negative mutant vector preventing Ser^{133} phosphorylation of CREB) (CLONTECH), TAM-67 (a c-*jun* dominant-negative vector) (21), and pcDNA (control plasmid for TAM-67) (Invitrogen) constructs were used to determine the role of CREB and AP-1 complex in signal transduction of apoptosis pathway.

Cell Culture and Transfection—C3H10T1/2 is an undifferentiated murine mesenchymal cell line (22) with the potential to become myoblasts, adipocytes, chondroblasts, and osteoblasts (23, 24). Their induction into the osteoblastic lineage can be stimulated with bone morphogenetic proteins (BMPs) (25, 26). C3H10T1/2 cells (American Type Culture Collection, Manassas, VA) were cultured in basal medium Eagle's (Invitrogen) with 10% fetal bovine serum (Hyclone, Logan, UT) and 100 units/ml of penicillin and streptomycin (Invitrogen). The cells were passaged every 4–5 days. C3H10T1/2 cells were transfected with different plasmid constructs (pE, WT, Δ, or P) using PerFect-2 (Invitrogen) or LipofectAMINE™ Plus (Invitrogen). The transfected cells were selected in 700 μg/ml Geneticin (Invitrogen) for 3 weeks to establish stable cell lines. The subclones were generated by limiting dilution. Geneticin (350 μg/ml) was added to cultures once a week to maintain the stability of transfectants. For transient transfection experiments, C3H10T1/2 cells were transfected with different plasmid constructs (pE, Δ, WT, H, pCMV-CREB, CREB133, TAM-67, or pcDNA) using LipofectAMINE™ Plus.

MC3T3-E1 cells were obtained through Dr. Renny Franceschi from Dr. M. Kumegawa (Meikai University, Sakado, Japan) and maintained as described previously (27). Briefly, cells were grown in minimum essential medium alpha medium (Invitrogen) with 10% fetal bovine serum and 100 units/ml penicillin and streptomycin. The cells were passaged every 4–5 days.

Adenylyl Cyclase Stimulation Assay—The adenylyl cyclase stimulation and cAMP-binding protein assay were performed as described previously with minor modification (28). The cells were plated in triplicate at 20,000 cells/cm^2 into 24-well plates, and medium containing ascorbic acid (Fisher) (50 μg/ml) was changed every other day. BMP-4 (R & D Systems Inc., Minneapolis, MN) (50 ng/ml) was added on day 3, and the adenylyl cyclase stimulation assay was performed at day 8. Briefly, the cells in triplicate wells were stimulated with 10^{-7} M hPTH (1–34) (Bachem, Inc., Torrance, CA) or vehicle control (0.1% bovine serum albumin with 4 mM HCl) in calcium- and magnesium-free Hanks' balanced salt solution (Invitrogen) containing 0.1% bovine serum albumin and 1 mM isobutylmethylxanthine at 37 °C for 10 min. After aspirating the medium, cAMP was extracted by adding 250 μl/well ice-cold 5% perchloric acid and incubating overnight at −20 °C. After thawing, the pH was adjusted to 7.5 with 4 N KOH, and the neutralized extract was then assayed for cAMP using a cAMP-binding protein assay. The cAMP-binding protein assay was performed by incubating [^{3}H]cAMP (ICN, Irvine, CA) with standards or unknowns and a cAMP-binding protein sufficient to bind ~30% of radioactivity for 90 min on ice. The samples were then incubated with dextran-coated charcoal for 20 min and centrifuged to remove unbound from bound cAMP-binding protein-[^{3}H]cAMP complexes. The radioactivity of the supernatants was determined with a liquid scintillation spectrophotometer, and cAMP levels were calculated by the log-logit method using the GraphPad Prism 3 program (GraphPad Software, San Diego, CA). Triplicate wells were analyzed for DNA content by fluorometric analysis as described previously to standardize cAMP levels (13).

Viable Cell Enumeration Assay—The cells were plated in triplicate in 24-well plates and treated with experimental reagents at designated times, and viable cell numbers were determined by the trypan blue dye exclusion method using a hemacytometer. To determine the effects of PTH/PTHrP on apoptosis, caspase inhibitors 2×10^{-7} M YVAD-cmk (inhibits caspase-1) and DEVD-fmk (inhibits caspase-3 and caspase-8) (CLONTECH) were used to inhibit apoptosis (29) and dexamethasone (10^{-7} M) was used to induce apoptosis (5) as described previously.

Flow Cytometry Assay—During apoptosis, the cell membrane phospholipid phosphatidylserine is translocated from the inner to the outer leaflet of the plasma membrane. Annexin V binds with high affinity to cells with exposed phosphatidylserine. Annexin V conjugated to fluorochromes such as phycoerythrin (PE) can be used in flow cytometry for apoptosis analysis (30, 31). Annexin V-PE staining is used in conjunction with a vital dye, 7-amino-actinomycin (7-AAD), for detection of early apoptotic cells (Annexin V-PE-positive and 7-AAD-negative). C3H10T1/2 cells were plated in duplicate at 20,000 cells/cm^2 in 12-well plates and transfected with plasmid DNA using LipofectAMINE™ Plus on the second day. Treatment with or without serum deprivation was performed by incubation in basal medium Eagle's with or without 10% fetal bovine serum for 16 h after washing cells. The floating cells were collected, and the adherent cells were trypsinized with trypsin/EDTA (Invitrogen). Floating and adherent cells were combined, rinsed with cold phosphate-buffered saline, and then resuspended in 1× annexin V binding buffer. Annexin V-PE and 7-AAD (PharMingen, San Diego, CA) staining were performed by adding 5 μl of both agents to all treatment groups. The controls included annexin V-PE only, 7-AAD only, and no staining. The cells were incubated in the dark for 15 min, and flow cytometry was performed within 1 h at the University of Michigan Flow Cytometry Core. Annexin V-PE-positive and 7-AAD-negative cells were defined as apoptotic cells.

Cell Death ELISA—Apoptosis cleaves cellular DNA into histone-associated fragments. The cell death detection ELISA Plus (Roche Molecular Biochemicals) is a quantitative sandwich enzyme immunoassay utilized to measure nucleosomal particles in cytoplasmic fractions. To measure apoptosis in postconfluent cells, WT subclone cells were plated at 5,000 cells/cm^2 at day 0. On day 7, after a 3-h treatment with caspase inhibitors (2×10^{-7} M YVAD-cmk + 2×10^{-7} M DEVD-fmk) or vehicle (0.04% Me_2SO), the cells were exposed to PTH (1–34) (10^{-8} M) or vehicle treatment, and apoptosis ELISA was performed at day 8 as described previously (32). The cells were lysed, and 20 μl of supernatant was added to each well in a streptavidin-coated microtiter plate. Subsequently, a mixture of anti-histone-biotin and anti-DNA-peroxidase monoclonal antibodies was added and incubated with shaking for 2 h. Following several washing steps, the nucleosomes were quantified by adding 2,2′-azino-di-[3-ethylbenzthiazolin sulfonate] diammonium salt to the peroxidase retained in the immunocomplex. The subsequent color reaction was measured in an ELISA plate reader at 405 nm against a 2,2′-azino-di-[3-ethylbenzthiazolin sulfonate] diammonium salt blank (reference wavelength at 490 nm).

Northern Blot Analysis—The cells were plated in duplicate at 50,000 cells/cm^2 in 60-mm dishes with medium containing ascorbic acid (50 μg/ml) for each cell type. After the cells reached confluence, the culture medium was changed into medium containing BMP-4 (50 ng/ml) and ascorbic acid (50 μg/ml) for 2 days, and then the total RNA was isolated 4 days after confluence. The steady state expression of osteocalcin was determined by Northern blot analysis. RNA isolation and Northern blot analysis were performed as described (33). Briefly, total RNA (10 μg) was electrophoresed on 1.2% agarose-formaldehyde gels. The RNA was transferred to nylon membranes (Duralon UV) (Stratagene, La Jolla, CA) via passive transfer and cross-linked with UV transillumination (Stratalinker 2400, Stratagene). The membranes were hybridized with a cDNA probe for osteocalcin labeled with [α-^{32}P]dCTP (Amersham Biosciences) using the Rediprime labeling system (Amersham Biosciences). After hybridization and washing, radioactivity counts were measured using an Instant Imager (Packard Instrument Co., San Diego, CA) and blots exposed to Biomax MR film (Eastman Kodak Co.) at −70 °C for 24–72 h. The blots were stripped and probed with an 18 S ribosomal RNA cDNA to control for RNA loading.

Western Blot Analysis—MC3T3-E1 cells were plated at 5,000 cells/cm^2 in 60-mm dishes. The culture medium was changed at days 1, 3, 5, and 7. At day 8, the cells received PTHrP (1–34) (10^{-7} M) (0–120 min),

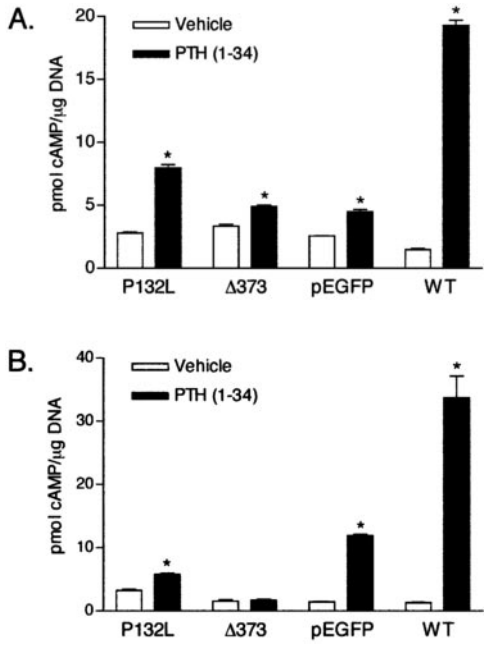

FIG. 1. **Determination of biologic activity of PTH-1R in transfectants of stable mixed populations (*A*) and stable subclones (*B*).** The cells were plated at 20,000 cells/cm^2 and induced to differentiate with the addition of ascorbic acid (50 μg/ml) and BMP-4 (50 ng/ml). The cells in triplicate wells were stimulated with PTH (1–34) (10^{-7} M) or vehicle. The cAMP levels were determined by cAMP-binding protein assay, standardized to DNA levels, and expressed as the means ± S.E. *, $p < 0.005$ *versus* respective control groups.

and the proteins were isolated for Western blot analysis as described (34). After resolving by 10% SDS-PAGE, the proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad) and blocked in TBST (10 mM Tris, pH 8.0, 0.85% NaCl, 0.1% Tween 20) with 5% nonfat dry milk for 1 h. The membranes were then incubated overnight at 4 °C in a 1:500 dilution of Akt antibody (Cell Signaling, Beverly, MA) or 1:1,000 dilution of phosphorylated Akt antibody (Cell Signaling) in TBST. After three washes with TBST and incubation for 1 h with secondary antibody, the membranes were washed five times with TBST and developed by chemiluminescent detection according to protocols supplied by the manufacturer (ECL; Amersham Biosciences).

Statistical Analysis—All of the assays were repeated at least three times with similar results unless otherwise noted. The data were analyzed using either analysis of variance or a Student's *t* test with the Instat 2.1 biostatistics program (GraphPad Software).

RESULTS

Cyclic AMP Response to PTH Stimulation Confirms the Biologic Activity of the Transfected PTH-1Rs—PTH binds to the PTH-1R and activates adenylyl cyclase, which leads to increased cAMP. To verify the existence and function of the transfected PTH-1Rs, adenylyl cyclase stimulation assays were performed after cells were induced to differentiate with the addition of BMP-4 and standardized to DNA levels in all samples. BMPs are widely used to induce osteoblastic differentiation in C3H10T1/2 cells (25, 26). In stable mixed populations (Fig. 1*A*), all groups had elevated cAMP after PTH (1–34) stimulation reflecting the biologic activity of the PTH-1R. WT transfectants had more biologically active PTH-1Rs, as demonstrated by the greatest cAMP response. In contrast, the groups with mild (P132L) and severely (Δ373) inactivated mutant PTH-1Rs showed a lower cAMP response and the least cAMP response, respectively. The cAMP response of the plasmid-only group (pEGFP) indicated the existence of functional endogenous wild-type PTH-1Rs in C3H10T1/2 cells. Representative subclones were also utilized in experiments to get homogeneous populations of each type of stable transfectant. The result of adenylyl cyclase stimulation of selected subclones (Fig. 1*B*)

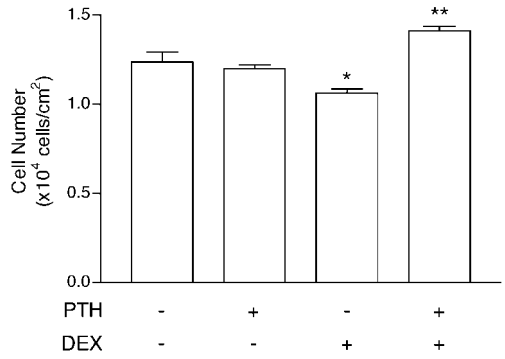

FIG. 2. **PTH blocks the dexamethasone-induced cell number reduction in preconfluent cells.** WT subclone cells were plated overnight in triplicate, treated for 1 h with PTH (1–34) (10^{-8} M) or vehicle, and then treated with dexamethasone (*DEX*; 10^{-7} M) or vehicle for 6 h. Viable cell numbers were determined by trypan blue dye exclusion and hemacytometer enumeration. The results are expressed as the means ± S.E. *, $p < 0.05$ *versus* PTH(−)Dex(−). **, $p < 0.01$ *versus* PTH(−)Dex(+).

demonstrated significant cAMP responses in all groups except Δ373. The cAMP response after PTH stimulation was the highest in the WT subclone and the lowest in Δ373 similar to their respective mixed populations. Interestingly, we noted that WT mixed populations and subclones demonstrated a 65 and 60% increase in DNA content when compared with the respective Δ373 groups, suggesting that the expression of functional PTH-1Rs resulted in increased numbers of cells. The assays were also performed in subclones without BMP induction of differentiation. Basal cAMP levels were not altered in the absence of BMP induction (P132L, 2.24 ± 1.26 pmol cAMP/μg DNA; Δ373–383, 1.27 ± 1.07 pmol cAMP/μg DNA; pEGFP, 0.95 ± 0.66 pmol cAMP/μg DNA; and WT, 1.02 ± 0.81 pmol cAMP/μg DNA). Without BMP treatment, no PTH-induced cAMP levels were found in P132L and Δ373 subclones. WT and pEGFP had 2.7- and 3.3-fold elevated cAMP levels, which demonstrated a PTH response, but significantly lower than when BMP-induced. This suggests that BMP treatment may induce PTH receptor expression as previously demonstrated (25, 26, 35).

PTH and PTHrP Inhibit Apoptosis of Preconfluent Cells through the PKA Pathway—The anabolic action of PTH has been attributed to its antiapoptotic effects. Thus, viable cell enumeration assays were performed to determine the effect of PTH on dexamethasone-induced apoptosis in preconfluent WT subclone cells (Fig. 2). PTH (1–34) demonstrated an antiapoptotic-like effect by blocking the dexamethasone-induced decrease in viable cell number. In this assay system, dexamethasone has previously been reported to stimulate apoptosis, and PTH was found to prevent the dexamethasone-induced effect (5).

Cyclic AMP is an important intracellular secondary messenger in the PTH-1R signal transduction pathway. To explore the mechanism of PTH on apoptosis in preconfluent cells, flow cytometry detection of apoptosis with parallel adenylyl cyclase stimulation assays were performed on transient transfectants of pEGFP, WT, and H223R. WT had a higher PTH-induced cAMP response than H223R as reported in COS-7 cells (17). In addition, H223R demonstrated the highest basal cAMP levels among all groups indicative of its constitutive activity (Fig. 3*A*). The H223R transfected cells had a significantly reduced percentage of apoptotic cells (annexin V-PE-positive and 7-AAD-negative cells) after serum withdrawal (Fig. 3*B*). Likewise, treatment with forskolin (10^{-6} M), an activator of cAMP, prevented the dexamethasone-induced reduction in viable cell numbers in preconfluent C3H10T1/2 cells (data not shown). These data suggest that cAMP is responsible for the antiapoptotic effect of PTH in preconfluent undifferentiated cells.

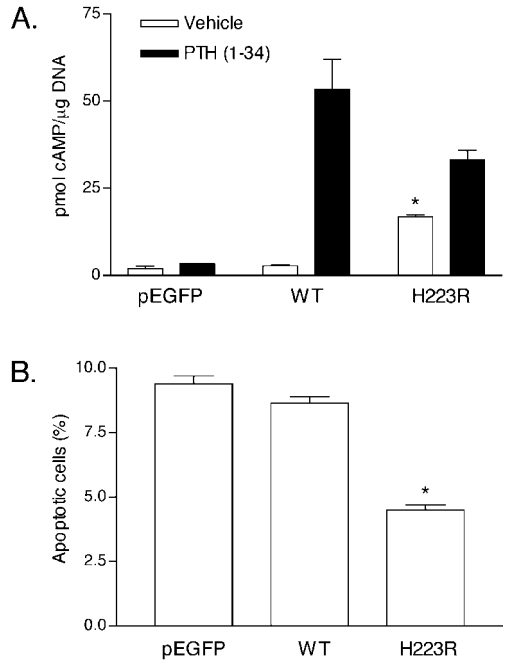

FIG. 3. **High basal cAMP levels reduce the apoptosis induced by serum withdrawal in preconfluent cells.** C3H10T1/2 cells were plated in triplicate, transfected the next day, serum-deprived for 16 h, and then stained with annexin V-PE and 7-AAD. Apoptosis was determined by flow cytometry. Parallel adenylyl cyclase stimulation assays were performed. *A*, H223R transient transfected cells demonstrated higher basal cAMP levels than other transfectants. *, $p < 0.0001$ *versus* other vehicle-treated groups. *B*, H223R transient transfectants had reduced apoptosis induced by serum withdrawal. *, $p < 0.05$ *versus* other groups. The results are expressed as the means ± S.E.

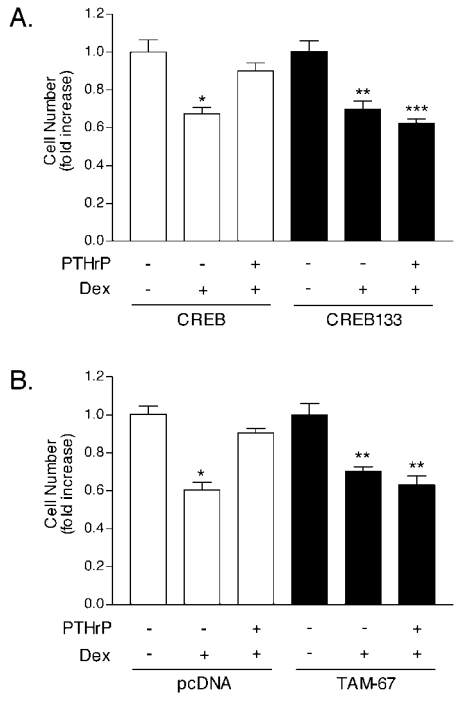

FIG. 4. **PTHrP protection from apoptosis via CREB and AP-1 complex in preconfluent cells.** WT subclone cells were plated overnight in triplicate, transfected with different plasmids for 3 h, treated for 1 h with PTHrP (1–34) (10^{-7} M) or vehicle, and then treated with dexamethasone (*Dex*; 10^{-7} M) or vehicle for 6 h. Viable cell numbers were determined by trypan blue dye exclusion and hemacytometer enumeration. *A*, cells transfected with CREB or CREB133 (a dominant-negative mutant vector preventing Ser^{133} phosphorylation of CREB). *, $p < 0.05$ *versus* control and PTHrP(+)Dex(+). **, $p < 0.05$ *versus* control. ***, $p < 0.01$ *versus* control. *B*, cells transfected with pcDNA or TAM-67 (a c-*jun* dominant-negative vector). *, $p < 0.05$ *versus* control and PTHrP(+)Dex(+). **, $p < 0.05$ *versus* control. Transient transfection of CREB133 or TAM-67 abolished the PTHrP protective effect on dexamethasone-induced reduction in cell numbers. The representative results from two assays are expressed as the means ± S.E.

CREB and the AP-1 complex are two downstream mediators of the PKA pathway (36, 37). To further characterize the PTH/PTHrP antiapoptotic effect in preconfluent cells, WT subclone cells were transiently transfected with specific constructs that inhibit either CREB (CREB133) or the AP-1 complex (TAM-67) and evaluated in viable cell enumeration assays (Fig. 4). PTHrP prevented the apoptosis induced by dexamethasone in the plasmid-only group but not in the CREB133 or TAM-67 transfection groups. This indicates that the PKA downstream mediators, CREB and AP-1 complex, play important roles in mediating the antiapoptotic effect of PTH/PTHrP in preconfluent mesenchymal cells.

Overexpression of Functional PTH-1R Increases Viable Cell Numbers—Viable cell enumeration assays were performed on stable mixed populations (Fig. 5*A*) and stable subclones (Fig. 5*B*) to determine the effects of the PTH-1R transfection on cell numbers over time. Viable cell numbers after an 8-day culture in both assays were the lowest in Δ373 and increased respectively in pEGFP, P132L, and WT. The WT cells demonstrated a more than 2-fold increase in viable cell numbers compared with Δ373 cells in both mixed populations and representative subclones at day 8. This suggests that overexpression of functional wild-type PTH-1 receptor leads to an increase in cell numbers over time but does not distinguish between effects on proliferation *versus* cell death.

PTH and PTHrP Reduce Numbers of Postconfluent Cells—The adenylyl cyclase stimulation assays indicated differential responses to PTH among groups. Therefore, viable cell enumeration assays were performed to explore the effect of PTH on stable subclones during an 8-day culture (Fig. 6*A*). Interestingly, PTH treatment significantly decreased WT cell numbers at day 8 but not earlier. The cells becoming confluent at day 4 or 5 in the assay suggested that the PTH effect was restricted to postconfluent cells. In addition, no significant reduction in cell numbers was observed in other groups, suggesting that this phenomenon was associated with biologically active PTH-1Rs. Furthermore, viable cell enumeration assays were performed to determine whether the PKA pathway was responsible for the effect on viable cell numbers of WT subclones at day 8 (Fig. 6*B*). When cells were cultured with activators of PKA, PTH (1–34), and forskolin, there was a reduction in viable cell numbers at day 8. In contrast, the antagonist, PTH (7–34), which binds the PTH-1R without activation of cAMP, had no effect. These data suggest that the PKA pathway mediates the PTH-induced reduction in viable cell numbers in postconfluent cells.

The reduction in numbers of postconfluent WT subclone cells by PTH or forskolin might be due to either a decrease in proliferation or an increase in apoptosis and was contrary to the PTH protection against apoptosis found in preconfluent cells. Therefore, the effect of PTH on apoptosis of day 8 WT subclone was explored using additional viable cell enumeration assays (Fig. 7*A*). Caspase inhibitors, which prevent apoptosis, significantly inhibited the PTH-induced decrease in viable cell numbers. This suggests that the reduction in cell numbers in response to PTH in more mature cells is due to a proapoptotic mechanism. To further confirm this hypothesis, cell death ELISAs were performed on day 8 WT subclone cells (Fig. 7*B*). PTH treatment increased DNA fragmentation, a characteristic of apoptotic cell death, whereas caspase inhibitors prevented it.

These data suggest a biphasic response where early during a

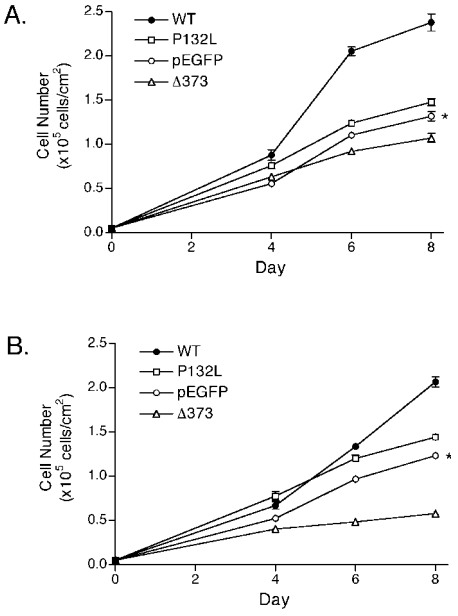

FIG. 5. **Overexpression of functional PTH-1R increases viable cell numbers over time.** The cells were plated at 5,000 cells/cm^2 into triplicate wells, and viable cell numbers were determined at days 4, 6, and 8 using the trypan blue dye exclusion method. *A*, stable mixed populations. *, $p < 0.05$ *versus* WT and Δ373. *B*, stable subclones. *, $p < 0.01$ *versus* all other groups. Representative viable cell numbers from two assays are expressed as the means ± S.E. Viable cell numbers at day 8 were the highest in WT and the lowest in Δ373.

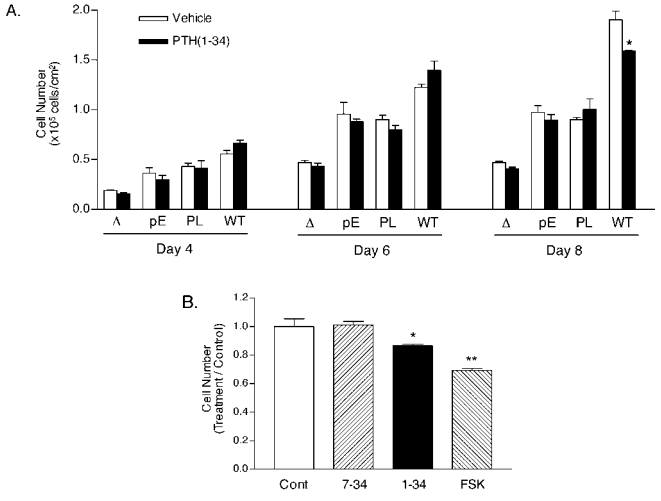

FIG. 6. **PTH reduces viable cell numbers in postconfluent cells.** *A*, stable subclones were plated at 5,000 cells/cm^2 into triplicate wells. PTH (1–34) (10^{-8} M) or vehicle was added at days 1, 3, 5, and 7. Viable cell numbers were determined at days 4, 6, and 8. PTH decreased viable cell numbers in WT group at day 8. *, $p < 0.05$ *versus* day 8 WT vehicle group. *B*, WT subclone cells were plated at 5,000 cells/cm^2 into triplicate wells. PTH (7–34) (10^{-8} M), PTH (1–34) (10^{-8} M), forskolin (10^{-6} M), or vehicle were added at days 1, 3, 5, and 7, and viable cell numbers were determined at day 8. PTH (1–34) and forskolin (*FSK*), but not PTH (7–34), decreased viable cell numbers. *, $p < 0.05$ *versus* control. **, $p < 0.01$ *versus* control. The results are expressed as the means ± S.E.

proliferative phase PTH protects against apoptosis, whereas later after confluence it stimulates apoptosis. These effects may be associated with the differentiation program of the cell. Hence, the impact of the PTH-1R expression on a marker of osteoblast differentiation was further evaluated.

Expression of Functional PTH-1R Increases Osteocalcin Gene Expression—Osteocalcin is a specific osteoblastic marker. Thus, the expression of osteocalcin was used to determine the effects of the PTH-1R on osteoblastic differentiation in mesenchymal cells. Northern blot analyses were performed on total RNA isolated from differentiated stable mixed populations and subclones. WT had the highest and Δ373 had the lowest expression levels of osteocalcin in both stable mixed populations (data not shown) and subclones (Fig. 8). These data suggest that the expression of functional PTH-1R promotes mesenchymal cell differentiation toward the osteoblastic lineage. In addition, PTH reduced the numbers of cells after 8 days of culture in WT, but not in other transfectants (Fig. 7*A*), further supporting the role of PTH in inducing apoptosis in more differentiated mesenchymal cell types.

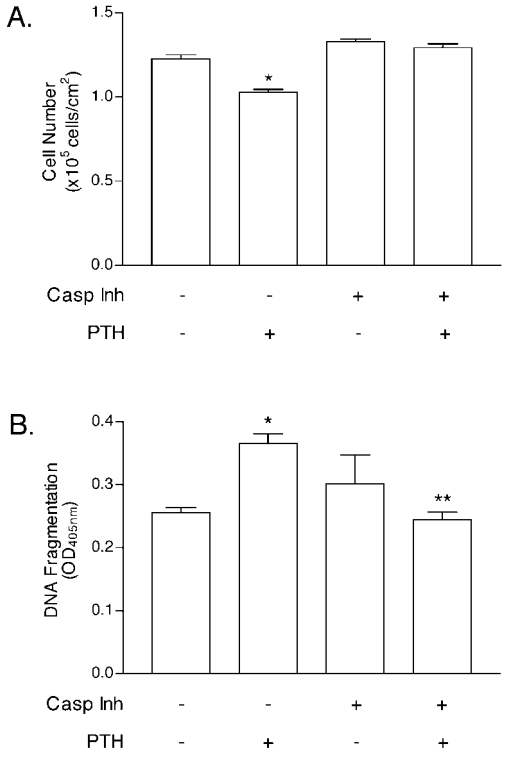

FIG. 7. **PTH induces apoptosis in postconfluent cells.** WT subclone cells were plated at 5,000 cells/cm^2 into triplicate wells. *A*, PTH (1–34) (10^{-8} M) or vehicle was added at days 1, 3, 5, and 7. In addition, caspase inhibitors (combination of 2×10^{-7} M YVAD-cmk and DEVD-fmk) or vehicle 3-h pretreatment were performed at days 5 and 7. Viable cell numbers were determined at day 8 using the trypan blue dye exclusion method. Caspase inhibitors (*Casp Inh*) reversed the PTH-induced reduction in viable cell numbers at day 8. *, $p < 0.005$ *versus* other three groups. *B*, cells were treated with PTH (1–34) (10^{-7} M) or vehicle control after a 3-h pretreatment with caspase inhibitors (combination of 2×10^{-7} M YVAD-cmk and DEVD-fmk) or vehicle control at day 7. Cell death ELISA was performed at day 8. *, $p < 0.005$ *versus* caspase inhibitor(−)PTH(−). **, $p < 0.005$ *versus* caspase inhibitor(−)PTH(+). All of the data are expressed as the means ± S.E.

The PTH/PTHrP Bi-directional Effects on Apoptosis Are Dependent on the Differentiation State in MC3T3-E1 Cells—In C3H10T1/2 transfectants, PTH (1–34) was antiapoptotic in preconfluent cells and proapoptotic in more differentiated postconfluent cells. This was further tested in a well characterized MC3T3-E1 osteoblastic differentiation system. Caspase inhibitors prevented the dexamethasone-induced reduction in viable cell numbers in preconfluent cells (Fig. 9*A*). This suggests that dexamethasone decreased the viable cell numbers via apoptosis similar to other reports (5). As with the C3H10T1/2 cells, PTHrP prevented the reduction in cell viability induced by dexamethasone, and forskolin mimicked the antiapoptotic effect of PTHrP in preconfluent cells (Fig. 9*B*). Forskolin alone slightly but not significantly reduced cell numbers in preconfluent cells. Conversely, PTH (1–34) and PTHrP (1–34) both significantly decreased the viable cell numbers in differenti-

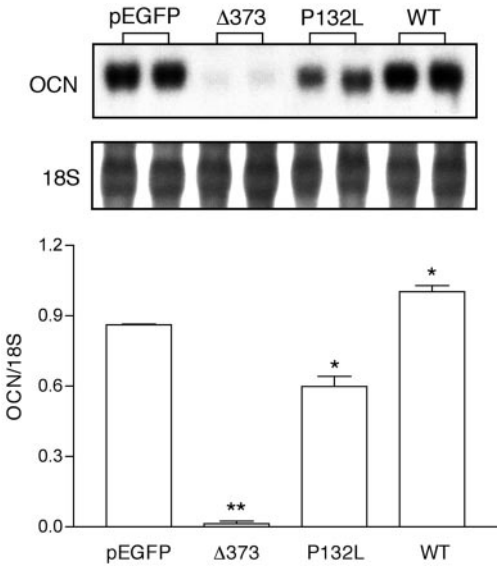

FIG. 8. **Expression of functional PTH-1R increases osteocalcin gene expression.** *A*, autoradiograph of Northern blot analysis of osteocalcin mRNA and 18 S rRNA. *B*, plot of osteocalcin expression after adjusted to 18 S rRNA expression. The results are expressed as the means ± S.E. from duplicate samples. WT had the highest and Δ373 had the lowest, level of osteocalcin (*OCN*) expression. *, $p < 0.05$ *versus* pEGFP. **, $p < 0.001$ *versus* pEGFP.

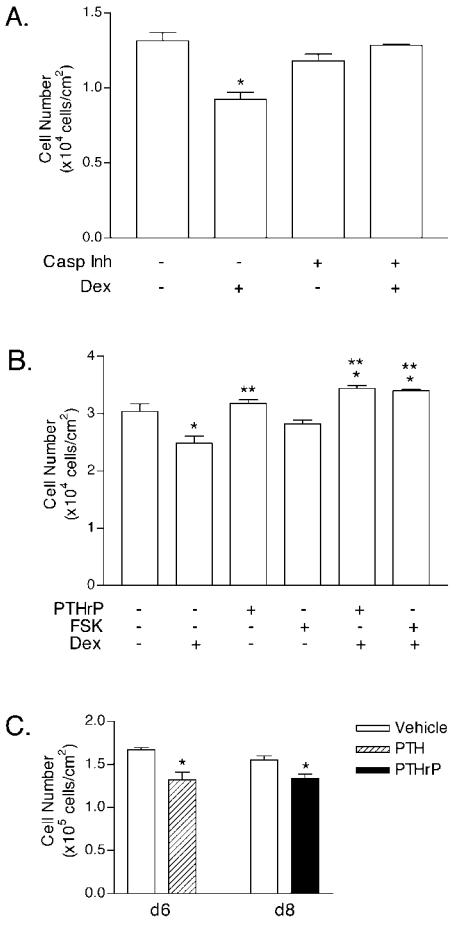

FIG. 9. **PTH/PTHrP effects on apoptosis are dependent on the differentiation state of the MC3T3-E1 cells.** *A*, cells were plated overnight in triplicate at 10,000 cells/cm^2, after a 3-h pretreatment with caspase inhibitors (*Casp Inh*; combination of 2×10^{-7} M YVAD-cmk and DEVD-fmk) or vehicle, dexamethasone (*Dex*; 10^{-7} M), or vehicle was added, and viable cells were enumerated 6 h later. *, $p < 0.05$ *versus* other three groups. *B*, cells were plated overnight in triplicate at 30,000 cells/cm^2, treated for 1 h with PTHrP (1–34) (10^{-7} M), forskolin (*FSK*; 10^{-7} M) or vehicle, and then treated with dexamethasone (10^{-7} M) or vehicle for 6 h. *, $p < 0.05$ *versus* control. **, $p < 0.01$ *versus* dexamethasone only group. *C*, cells were plated in triplicate at 5,000 cells/cm^2 and cultured in differentiation medium (50 μg/ml ascorbic acid) for either 6 or 8 days before treatment. Viable cell numbers were determined after 1 h of PTH (1–34) (10^{-8} M) (6-d group), PTHrP (1–34) (10^{-7} M) (8-d group), or vehicle treatment followed by 6 h of dexamethasone vehicle treatment (to simulate similar conditions as *A*, *B*) by trypan blue dye exclusion and hemacytometer enumeration. *, $p < 0.05$ *versus* control. In preconfluent cells, caspase inhibitors, PTHrP, and forskolin prevented the dexamethasone-induced reduction in viable cell numbers. In contrast, PTH and PTHrP treatment decreased viable cell numbers in the postconfluent differentiated cells. The results are expressed as the means ± S.E. of triplicate samples.

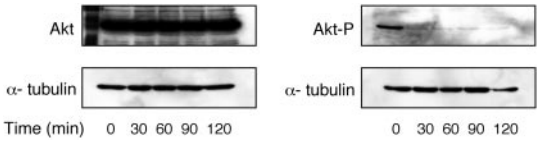

FIG. 10. **PTHrP-mediated inhibition of Akt phosphorylation in postconfluent differentiated cells.** MC3T3-E1 cells were cultured in differentiation medium (50 μg/ml ascorbic acid) for 8 days and treated with PTHrP (1–34) (10^{-7} M) for 0–120 min followed by protein isolation and Western blot analysis. Akt and phosphorylated Akt were analyzed. PTHrP inhibited Akt phosphorylation but did not alter Akt levels.

ated MC3T3-E1 cells (Fig. 9*C*). These data confirm that the effects of PTH/PTHrP on apoptosis are dependent on the differentiation state of the cells.

PTH Prevents Akt Phosphorylation in Postconfluent Cells—Akt is an antiapoptotic signaling molecule in multiple cell types when challenged with cell death inducers (38, 39). Phosphorylation is critical for its activation by upstream kinases and for the maintenance of its activity. To explore the possible mechanism of the PTH proapoptotic effect in more mature cells, postconfluent differentiated MC3T3-E1 cells were used in time course Western blot analyses for Akt and phosphorylated Akt (Fig. 10). PTHrP (1–34) decreased the Akt phosphorylation within 30 min. This suggests that the reduction in phosphorylated Akt is a mechanism by which PTH exerts its proapoptotic effect on more mature cells. In contrast, preconfluent cells express Akt protein, but phosphorylation was not detected at steady state nor with PTHrP nor with dexamethasone treatment for 1 or 2 h (data not shown). Because the untreated groups did not have phosphorylated Akt, the inhibition of apoptosis in preconfluent osteoblast precursors is unlikely via mechanisms relative to Akt phosphorylation.

DISCUSSION

To form bone, multipotential mesenchymal cells proliferate and differentiate into osteoblasts. Once mature osteoblasts have finished bone formation, the majority (50–70%) originally located at the remodeling site die because of apoptosis, and the rest become osteocytes or lining cells (40). In this study we report that PTH and its receptor play important roles in this process. Expression of functional PTH receptors increases viable cell numbers and promotes osteoblastic differentiation. Furthermore, PTH treatment protects against apoptosis in less mature cells and promotes apoptosis in more mature cells.

C3H10T1/2 cells stably transfected with various PTH receptors had different levels of osteocalcin and altered cell numbers. Both PTH (1–34) and PTHrP (1–34) bind to the PTH-1 receptors with similar affinity (7), activate the same signal transduction pathway with similar potency (41, 42), and exert anabolic actions in bone (43). In our study, overexpression of wild-type PTH receptors increased cell number after 8 days in culture, whereas overexpression of inactivated receptors resulted in lower cell numbers compared with controls. Our data suggest that functional PTH receptors, likely through affecting

proliferation or apoptosis, contribute to the increase of mesenchymal cell numbers. These results agree with studies reporting that PTH stimulates osteoblast proliferation (2, 4) and increases osteoblast number (44) and that a constitutively active PTH-1R increases osteoblast number in trabecular bone in transgenic mice (15). Additionally, Watson *et al.* (45) suggest that the PTH-1R localizes to the nucleus and may be associated with ligand-independent proliferation. Further, our data may explain the disparity in the results from many other cell systems (46, 47), because the effect of PTH, PTHrP, and the PTH-1R likely depends on the differentiation state of the cell.

The effect of the PTH receptor expression on osteoblastic differentiation of mesenchymal cells was reflected by osteocalcin expression levels. After BMP-4 treatment, overexpression of functional PTH receptors resulted in the highest level of osteocalcin expression, whereas overexpression of two types of inactivated mutant PTH receptors resulted in lower levels of osteocalcin expression. Our results agree with previous reports indicating that the presence of the PTH receptor is essential for detection of osteocalcin mRNA expression by Northern blot analysis (14). BMPs are well known to induce the differentiation of C3H10T1/2 cells into PTH-responsive osteoblasts (25). Still, C3H10T1/2 cells produce endogenous BMP-1, BMP-2, and BMP-4 (48, 49); thus they may become more differentiated after their confluence even without exogenous BMP. It is likely that the postconfluent WT cells used in our study represent a more differentiated cell stage in contrast to preconfluent cells. The specific details of the interactions between BMP and PTH signaling pathway are as yet unclear. However, BMP may play a role in the anabolic effects of intermittent PTH treatment *in vivo* through the following mechanisms: 1) The biologically active MH2 domain of Smad1 leads to the immediate up-regulation of the PTH-1R gene in mesenchymal progenitors C3H10T1/2 (35). Thus, BMP induces osteoblastic differentiation, which leads to expression of PTH-1R and potentially enhances PTH anabolic effects. 2) Runx2 is a transcription factor regulating both differentiation and function of osteoblasts (50). BMPs increase the expression of Runx2, and PTH induces PKA-mediated post-transcriptional modification of Runx2 (26, 51, 52). Runx2 and c-Fos·c-Jun physically interact and cooperatively bind the AP-1- and runt domain-binding sites in the collagenase-3 promoter (53). This process may also be crucial for activation of other osteoblastic genes, including osteocalcin and COL1A1 and COL1A2, which also contain AP-1- and runt domain-binding sites in their promoters (54, 55). Thus, BMP may aid in PTH anabolic actions *in vivo* through Runx2.

The reported effects of PTH on apoptosis appear to be dependent on the cell culture system. PTH is antiapoptotic in osteoblasts (5) and chick embryo hypertrophic chondrocytes (56), whereas it promotes apoptosis in 293 cells, a transformed primary embryonal kidney cell line (29). Interestingly, our findings of a bi-directional effect of PTH on cells of differing maturity stage are similar to those reported for another important factor in skeletal development, fibroblast growth factor (57–59). Fibroblast growth factor treatment increases proliferation in immature osteoblasts but promotes apoptosis in differentiating osteoblasts (59). Our study indicates that the effects of PTH on apoptosis are similarly dependent on cell status. PTH is antiapoptotic in preconfluent cells and proapoptotic in more differentiated postconfluent cells. The underlying mechanisms of this dual effect are not totally clear at present but appear to be dependent on the PKA pathway. This is in contrast to the proapoptotic effects of PTH reported in kidney cells that were found to be dependent on the PKC pathway (29).

The PTH-1R responds to binding of PTH or PTHrP by activation of PKA and PKC pathways. The PKA pathway is mediated by the G_s protein that activates adenylyl cyclase and leads to cAMP production and protein kinase A activation. The PKC pathway is mediated by the G_q protein that activates phospholipase C, resulting in an increase in intracellular Ca^{2+} levels and activation of protein kinase C. In previous work, PTH induced apoptosis in 293 cells through PKC pathway rather than PKA pathway (29). However, other studies indicate that cAMP suppresses apoptosis in rat periosteal cells (60) and human osteoblasts (61). Transgenic mice overexpressing a constitutively active PTH-1R (H223R), which results in higher basal cAMP levels, also had decreased apoptosis in trabecular bone leading to an increase in osteoblast number in trabecular bone (15). In our study, the transient transfection of H223R resulted in high basal cAMP levels and suppressed apoptosis as determined by flow cytometry assays in preconfluent mesenchymal cells. In addition, forskolin, an activator of cAMP, mimicked the antiapoptotic effect of PTHrP in preconfluent MC3T3-E1 cells. Furthermore, CREB and AP-1 complex are two mediators downstream of cAMP. We found that inhibition of either the CREB or the AP-1 complex abolished the PTHrP protective effect on viable cell numbers in preconfluent C3H10T1/2 transfectants. CREB has been found to be anti-apoptotic in other cell types (62, 63), whereas AP-1 family members have been found to be both anti- and proapoptotic (64, 65). The c-*jun* N-terminal kinase can be activated by stimuli such as mitogenic signals and growth factors (66) and is responsible for phosphorylating transcription factors such as c-Jun and the activating transcription factor-2, which may be important in the PTHrP signaling cascade. Reports indicate that early expression of the c-*jun* N-terminal kinase is associated with a protection from apoptosis, whereas later expression is associated with a stimulation of apoptosis (67, 68). Dissecting out the specific AP-1 family members involved in the regulation of apoptosis is complex as evidenced by findings that JunB is pro-apoptotic and c-Jun is anti-apoptotic (65). Interestingly, in contrast to the early protective effect, cAMP likely promotes apoptosis in postconfluent cells as evidenced by reduction of viable cell numbers after induction of cAMP production. In addition, in the more differentiated cells, the proapoptotic effect is likely due to the loss of Akt phosphorylation that has a well known protective role in cell survival (69, 70). Akt was not found to be a mediator of the protective effects of PTH in the less differentiated cells because there was no alteration in Akt or its phosphorylation with PTH treatment of undifferentiated cells. Interestingly, reports of cAMP inhibition of Akt activity in 3T3, COS, and HEK293 cells corroborate our data and indicate that the inhibition is mediated through an upstream regulation of Akt phosphoinositide-dependent kinase (71).

In summary, we show that PTH, PTHrP, and the PTH-1 receptor play important roles at multiple stages during bone formation through their effects on cell survival. PTH prevents the apoptosis of more immature preconfluent mesenchymal cells, increases their numbers and promotes their differentiation into osteoblasts for bone formation. In more mature postconfluent cells, PTH induces apoptosis of osteoblasts. Finally, one may speculate that this promotion of turnover of more mature cells may help to clear the way for the less differentiated cells to produce extracellular matrix and hence contribute to the anabolic actions of PTH in bone.

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