During the process of differentiation, chondrocytes integrate a complex array of signals from local or systemic factors like parathyroid hormone-related peptide (PTHrP), Indian hedgehog, bone morphogenetic proteins and transforming growth factor β. While PTHrP is known to be a critical regulator of chondrocyte proliferation and differentiation, the signaling pathways through which this factor acts remain to be elucidated. Here we show that both cAMP response element-binding protein (CREB) and AP-1 activation are critical to PTHrP signaling in chondrocytes. PTHrP treatment leads to rapid CREB phosphorylation and activation, while CREB DNA binding activity is constitutive. In contrast, PTHrP induces AP-1 DNA binding activity through induction of c-Fos protein expression. PTHrP activates CRE and TRE reporter constructs primarily through PKA-mediated signaling events. Both signaling pathways were found to be important mediators of PTHrP effects on chondrocyte phenotype. Alone, PTHrP suppresses maturation and stimulates proliferation of the chondrocyte cultures. However, in the presence of dominant negative inhibitors of CREB and c-Fos, these PTHrP effects were suppressed, and chondrocyte maturation was accelerated. Moreover, in combination, the effects of dominant negative c-Fos and CREB are synergistic, suggesting interaction between these signaling pathways during chondrocyte differentiation.

Endochondral ossification is regulated by local growth factors including PTHrP1, which has been defined as a critical regulator of the rate of endochondral ossification. Mice null for either PTHrP (7) or its receptor (8) display accelerated chondrocyte differentiation and therefore abnormal endochondral bone formation. In contrast, animals that overexpress PTHrP exhibit delay in chondrocyte terminal differentiation (9). Humans with an activating mutation in the PTH/PTHrP receptor have Jansen’s metaphyseal chondrodysplasia, characterized by disorganization of the growth plate and delayed chondrocyte terminal differentiation (10). Thus, PTHrP signaling is critical for normal growth plate morphology and function.

PTHRP receptor activation stimulates both protein kinase A (PKA) and protein kinase C (PKC) signaling. Activation of these signaling pathways mediates important effects and in chondrocytes PKA signaling is associated with proliferation, stimulation of proteoglycan synthesis, and inhibition of alkaline phosphatase activity (11). PTHrP also stimulates phospholipase C activity with metabolism of membrane phospholipids and production of diacyl glycerol and inositol phosphate (12, 13). Inositol phosphate stimulates the release of intracellular calcium stores while the regeneration of diacylglycerol leads to activation of protein kinase C. While much is known about the upstream signaling events immediately following receptor ligation, there is little information on the downstream transcription factors involved in mediating PTHrP effects in chondrocytes and their relationship with PKA and PKC signaling.

One of the potential downstream signaling targets of PKA is the cyclic AMP response element-binding protein (CREB) (14). This transcription factor is a CREB/ATF family member that is constitutively present in the nucleus. CREB binds to a DNA consensus cAMP response element (CRE) primarily as a homodimer, via a leucine zipper domain. CREB is activated by PKA-mediated phosphorylation within its P-box domain (15), which permits its interaction with p38/CBP and other coactivators, leading to gene transcription. Thymocytes and T cells from transgenic mice expressing a dominant-negative form of CREB specifically in these cells display a profound proliferative defect and G1 cell-cycle arrest in response to a number of different activation signals (16). ATF-2 deficiency, another member of the CREB/ATF family, has been shown to induce chondrodysplasia and neurological abnormalities in mutant mice (17). Although the role of CREB in cartilage development is well characterized, the specific functional implications of its regulation by PTHrP remain to be elucidated.

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ii The abbreviations used are: PTHrP, parathyroid hormone-related peptide; PKA, protein kinase A; CREB, cAMP response element-binding protein; CRE, cAMP response element; TRE, phorbol 12-myristate 13-acetate response element; USC, upper sternal chondrocytes; bp, base pairs; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.
or bone formation has not been investigated, the finding that CREB KO mouse has a dwarf phenotype (18) combined with the established important role of CREB in PKA mediated events in other cells, suggests that this transcription factor may be involved in regulating some of the critical events in chondrocyte differentiation.

PTHrP also activates the transcription factor AP-1, a complex formed through interactions between Fos and Jun family members (19, 20). These interactions are also mediated by leucine zipper domains to form Fos/Jun heterodimers or Jun/Jun homodimers (21). The protein complex binds to the phorbol 12-myristate 13-acetate response element (TRE), a specific cis-acting DNA consensus sequence in the promoter region of target genes. AP-1-regulated genes appear to have an important role in skeletal physiology. Transgenic mice overexpressing c-Fos develop bone and cartilaginous tumors (22), while Fos-Jun double transgenic mice develop osteosarcomas at a higher frequency (23). PTHrP stimulates c-Fos mRNA and protein synthesis in osteoblasts (24), and in chondrocytes PTHrP was found to increase c-Jun and JunD mRNA and protein levels (25). AP-1 activation has been shown to be mediated by several different signaling pathways including PKA and PKC (26, 27).

In this work we have characterized CREB and c-Fos as two important regulators of cartilage development and have investigated their activation by PKA and PKC signaling. We have shown that their activation occurs rapidly in response to PTHrP stimulation and that interference with their function results in alteration of cartilage phenotype produced by PTHrP. This represents the first report documenting a critical role for CREB in cartilage development.

MATERIALS AND METHODS

Chondrocyte Cell Culture—Embryonic cephalic sternal chondrocytes (day 13) were prepared and cultured as described (28). After isolation and primary culture for 5–7 days, floating cells were plated in secondary cultures at 2.5 × 10^5 cells/cm^2 in Dulbecco’s modified Eagle’s medium containing 10% NuSerum IV (Collaborative Biomedical, Bedford MA), 4 units/ml hyaluronidase (Sigma), and 2 mM L-glutamate (Sigma).

After 6 days, upper sternal chondrocytes were harvested and plated to nitrocellulose membrane (Schleicher and Schuell), the blots were washed twice for 15 min with 0.1% SSC and 0.1% SDS, followed by a 30-min wash with 0.1 M Na3PO4, 0.1% Triton X-100. Hybridization analysis was performed using denaturing formaldehyde/agarse gels as described (34). A synthetic type X oligonucleotide was end labeled as previously described (34). Prehybridization was performed in QuickHyb solution (Stratagene, La Jolla, CA) for 20 min at 68 °C. Hybridization was done at 73 °C for 1 h. The blot was washed twice for 15 min with 2 × SSC and 0.1% SDS, followed by a 30-min wash with 0.1 × SSC and 0.1% SDS. The blot was exposed to X-Omat AR film (Kodak, Rochester, NY) for autoradiography.

Alkaline Phosphatase Activity—Alkaline phosphatase activity was measured as previously described (35). Culture medium was aspirated from chondrocytes cultured in 6-well plates, which were then rinsed with 150 mM NaCl. One ml of reaction buffer containing 0.25 M 2-methoxy-2-amino-propional, 1 mM MgCl2, and 2.5 mg/ml p-nitrophenyl phosphate (Sigma) at pH 10.3 was added to the wells at 37 °C. The reaction was stopped after 30 min by the addition of 0.5 ml of 0.3 M Na2PO4 (pH 12.3). The alkaline phosphatase activity was determined spectrophotometrically at 410 nm by comparison with standard solutions of p-nitrophenol and an appropriate blank.

Fluorescence-activated Cell Sorter Analysis—Flow cytometry was performed using a fluorescence-activated cell sorter (Becton-Dickinson and the Cell Quest (Becton-Dickinson, Franklin Lakes, NJ) plotting program. 2–10^5 cells were resuspended in 70% EtOH at 4 °C for at least 12 h. The cells were then resuspended in 1 × phosphate-buffered saline with 1 mg/ml RNase and incubated for 30 min at room temperature. Finally cells were stained with propidium iodide (10 μg/ml in phosphate-buffered saline) and run through the flow cytometer.

Injection of Viral Vectors—Chick embryo fibroblasts grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 0.2% fetal chick serum and penicillin/streptomycin were transfected with the replication competent avian sarcoma virus RCASBP(A) alone and RCASBP(A) containing dominant negative c-Fos and CREB inserts. Cells were passaged three times to allow the spreading of the virus. At confluence, growth media was changed to low serum (Dulbecco’s modified Eagle’s medium with 10% NuSerum) for collection of virus. Viral supernatants were collected at 24-h intervals for 3 days. At the time of secondary plating, chondrocytes were incubated with fresh viral supernatant for 48 h followed by addition of PTHrP in some of the samples.

Northern Blot Analysis—Total RNA was extracted from cultures using the RNeasy kit (Qiagen, Valencia, CA). 5 μg of the total RNA was run on a 1.2% agarose gel containing 17.5% formaldehyde and transferred to a GeneScreen Plus membrane (PerkinElmer Life Sciences). The RNA was hybridized to the membrane (prehybridization was performed using denaturing formaldehyde/agarse gels as described (34)). A synthetic type X oligonucleotide was end labeled as previously described (34). Prehybridization was performed in QuickHyb solution (Stratagene, La Jolla, CA) for 20 min at 68 °C. Hybridization was done at 73 °C for 1 h. The blot was washed twice for 15 min with 2 × SSC and 0.1% SDS, followed by a 30-min wash with 0.1 × SSC and 0.1% SDS. The blot was exposed to X-Omat AR film (Kodak, Rochester, NY) for autoradiography.

Expression of ACREB and AFOUS using Retroviral Systems—Chick embryo fibroblasts grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 0.2% fetal chick serum and penicillin/streptomycin were transfected with the replication competent avian sarcoma virus RCASBP(A) alone and RCASBP(A) containing dominant negative c-Fos and CREB inserts. Cells were passaged three times to allow the spreading of the virus. At confluence, growth media was changed to low serum (Dulbecco’s modified Eagle’s medium with 10% NuSerum) for collection of virus. Viral supernatants were collected at 24-h intervals for 3 days. At the time of secondary plating, chondrocytes were incubated with fresh viral supernatant for 48 h followed by addition of PTHrP in some of the samples.

**Fluorescence-activated Cell Sorter Analysis**—Flow cytometry was performed using a fluorescence-activated cell sorter (Becton-Dickinson and the Cell Quest (Becton-Dickinson, Franklin Lakes, NJ) plotting program. 2–10^5 cells were resuspended in 70% EtOH at 4 °C for at least 12 h. The cells were then resuspended in 1 × phosphate-buffered saline with 1 mg/ml RNase and incubated for 30 min at room temperature. Finally cells were stained with propidium iodide (10 μg/ml in phosphate-buffered saline) and run through the flow cytometer.
Protein samples (25 μg) both phosphorylated and un-phosphorylated forms of CREB (α-pCREB), the other that recognizes both phosphorylated and un-phosphorylated forms of CREB (α-CREB) (A). PTHrP stimulation of c-Fos protein was assayed by Western blot using an antibody directed against c-Fos protein (B). A control for loading equal amounts of protein was performed by stripping the blot and reprobing it with an anti-β-actin antibody.

Statistics—Statistical comparisons were made between the groups using a way analysis of variance (ANOVA). Significance was considered present when the p value was less than 0.05 and is denoted in each of the figures.

RESULTS

Activation of Signaling Pathways by PTHrP—The effects of PTHrP stimulation on CREB/ATF activation were investigated in sternal chondrocytes at various times following treatment over a 2-h time course experiment. The level of CREB protein remained constant as determined by Western blotting (Fig. 1A). However, phosphorylated CREB (pCREB) levels were markedly increased following this treatment. The increase in pCREB was detectable within 5 min, maximal by 30 min, and returned to basal levels 2 h after PTHrP stimulation (Fig. 1A). Following PTHrP stimulation, there was also an increase in c-Fos protein levels that was observed as early as 30 min, was maximal by 1 h, and returned to basal levels by 4 h (Fig. 1B). To determine the effect of PTHrP stimulation on CREB DNA binding activity in chondrocytes, gel mobility shift assays were performed. These experiments revealed minimal differences over a 4-h time course following PTHrP stimulation (Fig. 2A). To characterize this protein-DNA complex, supershift experiments using specific antibodies to members of the CREB/ATF family were performed. In this experiment, only CREB binding to this consensus sequence was detected (Fig. 2B), although ATF-2 and CREM proteins could be detected in these extracts by Western blot (Fig. 2C). These findings suggest that CREB protein levels and DNA binding activity remain constant following PTHrP treatment. However, PTHrP may modulate gene expression in sternal chondrocytes by inducing CREB phosphorylation and therefore increasing its transcriptional activity.

To assess AP-1 activation following PTHrP stimulation, gel mobility shift assays were performed using a probe containing the TRE consensus sequence. PTHrP stimulated a marked increase in DNA binding (Fig. 2A). The increase was detectable at 30 min, peaked at 1 h, and remained elevated 4 h following PTHrP treatment. Addition of an antibody directed against c-Fos protein inhibited DNA binding (Fig. 2A), showing the presence of c-Fos protein in the gel mobility shift complex, consistent with the increase in c-Fos protein expression observed on Western blot. In contrast, incubation with antibodies directed against Fra1, ATF-2, and CREM did not affect DNA binding (Fig. 2B).

To determine whether this apparent stimulation of CREB and AP-1 signaling is associated with an increase in gene transcription, sternal chondrocytes were transfected with CRE and TRE luciferase reporter constructs and treated with PTHrP (Fig. 3). PTHrP (10^{-7} M) treatment resulted in transcriptional activation of both the TRE (23-fold) and CRE (103-fold) constructs. As a positive control we co-transfected the reporter constructs with either activated MAPK kinase kinase (MEKK) or PKA constructs. In these experiments the MEKK construct was a more potent stimulator of the TRE reporter, while the PKA construct caused about a 40-fold stimulation of both the CRE and the TRE reporters (Fig. 3A). These latter findings indicate the presence of cross-talk between these signaling pathways in chondrocytes following PTHrP stimulation. Furthermore, PKA signaling in these cells results in activation of both CRE and TRE mediated transcription.

To determine the relative effects of the PKA, PKC, mitogen-activated protein kinase, and calmodulin kinase signaling pathways on CRE activation, the CRE reporter was transfected...
into sternal chondrocytes and the cultures were treated with PTHrP in the presence and absence of pharmacological inhibitors of these various pathways (Fig. 3B). Inhibition of PKA signaling with H89 resulted in a 95% inhibition of CRE activation, consistent with the role of PKA as a potent inducer of CREB phosphorylation. Inhibitors of PKC (GO6976, 45% inhibition), mitogen-activated protein kinase (PD98059, 27% inhibition), and calmodulin kinase (KN93, 17% inhibition) all had less potent effects. PKA inhibition also had the greatest effect on the TRE reporter (80% inhibition), while inhibition of MAP kinase signaling resulted in a 37.7% decrease in luciferase activity. In contrast, inhibition of PKC and calmodulin kinase did not affect PTHrP-mediated activation of the CRE reporter (Fig. 3C).

To confirm our findings regarding CREB and AP-1 transcriptional activation following PTHrP treatment, constructs expressing dominant negative c-Fos (AFOS) and CREB (ACREB) were each co-transfected into sternal chondrocytes with reporter constructs for the CRE and TRE (Fig. 4). The ACREB construct completely inhibited CRE activation by PTHrP, while the AFOS construct did not inhibit PTHrP activation of the CRE (Fig. 4A). Moreover, the AFOS construct induced an increase in the PTHrP stimulation of CRE reporter. A possible explanation for this finding is that AP-1 inhibition may result in greater availability of the transcriptional coactivator, CBP (CREB-binding protein), for CREB-mediated signaling. In contrast, both ACREB and AFOS constructs inhibited activation of the TRE reporter following stimulation by PTHrP (Fig. 4B). Co-transfection of both constructs reduced the detectable luciferase activities to below basal levels. These findings further confirm PTHrP activation of PKA and phosphorylation of CREB, leading to CRE dependent transcription.

In contrast, activation of AP-1 by CREB is likely indirect and may be mediated by stimulation of c-Fos transcription. To test this, a c-Fos promoter-reporter construct was transfected into sternal chondrocytes. PTHrP treatment resulted in a 91-fold stimulation in luciferase activity (Fig. 4C). Co-transfection of these cultures with the ACREB construct blocked this PTHrP stimulation of the cFos promoter, while the AFOS construct had no effect, further supporting a model of PTHrP activation of AP-1 through CREB-dependent transcription of c-Fos.
through CREB or AP-1 signaling, we investigated the expression of markers of chondrocyte maturation, type X collagen (colX) mRNA expression, and alkaline phosphatase activity, in cell cultures following PTHrP treatment. Short-term experiments were performed using transiently transfected chondrocytes (2 days), while longer term experiments (7 days) were performed with chondrocytes infected with a replication-competent retrovirus that permitted sustained expression in a larger percentage of cells. In the absence of PTHrP, ACREB, and AFOS had minimal effects in both the transiently transfected and retrovirally infected cultures (Fig. 5A and B). However, in the presence of PTHrP, transient transfection of ACREB and AFOS constructs stimulated type X collagen (colX) mRNA expression, 1.6- and 1.3-fold, respectively (Fig. 5A), compared with vector-transfected control chondrocytes. These signaling pathways appeared to be synergistic, since co-transfection of both ACREB and AFOS resulted in even greater colX mRNA expression (5.2-fold). Effects of the dominant negative signaling molecules were much greater when the constructs were introduced by retroviral infection. Alone, both ACREB and AFOS infection resulted in a larger induction of colX in PTHrP-treated cultures compared with minimal increases in retroviral infected control samples (Fig. 5B).

In PTHrP-treated cultures, transient transfection with both ACREB and AFOS resulted in only small increases in alkaline phosphatase activity (data not shown). Much larger effects on alkaline phosphatase activity were observed when the cultures were infected with retroviral constructs, consistent with the findings observed with the other maturation marker, type X collagen. In the PTHrP-treated cultures, ACREB doubled and AFOS tripled alkaline phosphatase activity (Fig. 6) compared with smaller increases in untreated samples (1.3- and 1.7-fold increases). Collectively, these findings demonstrate that PTHrP effects on maturation are mediated by both CREB and AP-1 signaling. Furthermore, while the findings suggest basal AP-1 and CREB signaling, these transcription factors are much more relevant in the presence of PTHrP.

**CREB and AP-1 Effects on Chondrocyte Proliferation**—To evaluate the effects of PTHrP on proliferation, sternal chondrocytes were treated with PTHrP and analyzed by fluorescence-activated cell sorter 24 h later (Table I). PTHrP treatment resulted in more than a 2-fold increase in the number of cells entering the cell cycle and undergoing DNA synthesis (S
Since cyclin D1 is a required for the transition from the G1 to S phase, we next investigated the effect of PTHrP and downstream signals on the cyclin D1 promoter (Fig. 7). PTHrP treatment caused a 5.6-fold increase in luciferase activity by the full-length (1745 bp) cyclin D1 promoter (Fig. 7A). A point mutation at the CRE site (position 52 bp) results in a 30% reduction in PTHrP mediated stimulation. Similar experiments with a truncated 66-bp promoter construct that includes the CRE-binding site, results in a 2.1-fold stimulation following PTHrP treatment. This induction was lost in a mutant construct containing a point mutation that disrupts the CRE consensus sequence. Co-transfection of the cultures with the AFOS or ACRec constructs, either alone or in combination, further confirmed a role for the CRE site in PTHrP-mediated cyclin D1 promoter activation. Transfection of the dominant negative ACRec construct completely inhibited the PTHrP induction of both the full-length and truncated 66-bp cyclin D1 promoter. In contrast, transfection of the AFOS construct did not significantly alter the induction of the full-length construct and caused only a partial reduction in the truncated promoter (Fig. 7B). These findings confirm the effects of PTHrP on proliferation and

FIG. 5. PTHrP inhibits collagen type X collagen (col X) expression that is relieved by AFOS and ACRec expression. For the transient transfection experiments (A), USC were transfected in growth media with 2.5 μg of empty vector, AFOS, ACRec, or both, in a ratio 1:4 with Superfect transfection reagent (Qiagen). Twelve hours after transfection, cells were washed and treated with PTHrP (10⁻⁷ M) or left untreated for 48 h. Changes in colX were measured by Northern blot of mRNA. The ethidium bromide-stained 18 S RNA was used as a loading control. For the long-term experiments (B), USC were incubated for 2 days with fresh viral supernatant of: empty RCAS, or RCAS carrying either AFOS or ACRec. Chondrocytes were cultured for 7 days in the presence or absence of PTHrP.

FIG. 6. PTHrP inhibition of alkaline phosphatase activity is relieved by AFOS and ACRec expression. USC were incubated for 2 days with fresh viral supernatant of respective viral constructs: empty RCAS, or RCAS carrying either AFOS or ACRec. Chondrocytes were cultured for 7 days in the presence or absence of PTHrP (10⁻⁷ M). Each treatment group represents the mean alkaline phosphatase activity of triplicate samples normalized for protein concentration. (⁎ denotes statistical significance at p ≤ 0.025 and † denotes statistical significance at p ≤ 0.0001 when compared with control.)

FIG. 7. PTHrP activation of the cyclin D1 promoter is diminished by perturbation of CREB signaling. In A, USC were transfected in serum-free media with 1 μg of the following luciferase reporters: 1745 bp, 1745-bp mut, 66 bp, 66-bp mut. After transfection, cells were washed, and incubated with or without PTHrP (10⁻⁷ M) for 24 h. The relative luciferase activities (mean ± S.E.; n = 3) are presented. (⁎ denotes statistical significance at p ≤ 0.025 when compared with the PTHrP-treated sample for the 1745-bp reporter, ** denotes statistical significance at p ≤ 0.005 when compared with PTHrP-treated sample for the 66-bp reporter.) In B, USC were transfected in serum-free media with 1 μg of the luciferase reporters: 1745 bp, 1745-bp mut, 66 bp, 66-bp mut. After transfection, cells were washed, and incubated with or without PTHrP (10⁻⁷ M) for 24 h. The relative luciferase activities (mean ± S.E.; n = 3) are presented. (⁎ denotes statistical significance at p ≤ 0.025 when compared with the untreated control, ** denotes statistical significance at p ≤ 0.005 when compared with PTHrP-treated sample.)
suggest that the effects on cyclin D1 are primarily related to CREB signaling.

To further define the role of AP-1 and CREB on PTHrP mediated proliferative effects, chondrocytes were treated with PTHrP in cultures transfected with AFOS or ACREB alone or in combination (Fig. 8). ACREB resulted in a 20% reduction in thymidine incorporation and 25% AFOS reduction. A statistically significant 30% reduction in thymidine incorporation (p < 0.005 when compared with the untreated control. ** denotes statistical significance at p ≤ 0.005 when compared with PTHrP-treated sample.)

**Fig. 8.** PTHrP stimulation of chondrocyte proliferation is inhibited by AFOS and ACREB. USC were transfected in growth media with 1 μg of the empty vector, AFOS, ACREB, or together in a ratio 1:3. Twelve hours after transfection, cells were washed and treated with PTHrP (10⁻⁷ M) for 24 h. [³²P]Thymidine labeling was performed as described under “Experimental Procedures.” (*) denotes statistical significance at p ≤ 0.005 when compared with the untreated control. ** denotes statistical significance at p ≤ 0.005 when compared with PTHrP-treated sample.)

**DISCUSSION**

While PTHrP is known to be a critical regulator of chondrocyte proliferation and differentiation, the signaling pathways through which this factor acts remain to be elucidated. The current article demonstrates that both CREB and AP-1 activation are critical to PTHrP signaling in chondrocytes. In these chondrocytes, CREB DNA binding activity is constitutive. However, PTHrP treatment leads to CREB phosphorylation within 5 min. In contrast AP-1 DNA binding activity is induced by PTHrP, with effects initially observed 1 h following treatment. Consistent with these findings PTHrP activated CRE and TRE reporter constructs, and both signaling pathways were found to be important mediators of PTHrP effects on chondrocyte phenotype. Alone, PTHrP suppressed maturation and stimulated proliferation of the chondrocyte cultures. However, in the presence of dominant negative inhibitors of CREB and c-Fos, these PTHrP effects were suppressed and chondrocyte maturation was accelerated. Moreover, in combination, the effects of dominant negative cFos and CREB were synergistic, suggesting interaction between these signaling pathways during chondrocyte differentiation.

Prior studies have demonstrated that CREB binds constitutively to the CRE consensus sequence and is activated following phosphorylation by upstream kinases (36, 37). The cAMP-activated protein kinase A (PKA) dependent pathway is the classic activator of CREB and acts through phosphorylation of a critical serine residue located at position 133 (15, 38). However, other signals, including the calcium/calmodulin signaling (CaM kinases II and IV) (39), PKC signaling (40), and Ras signaling mediated by the serine/threonine kinase RSK2 also regulate CREB through phosphorylation at Ser133 or other sites (36, 41). In our pharmacological studies with inhibitors of these kinases, we found that the PKA pathway is the one through which the most potent signal is transduced.

Similarly, AP-1 activation is also stimulated by numerous pathways, but here we found that following PTHrP stimulation, PKA signaling is most critical for its transcriptional activity. Inhibition of PKA signaling with the drug H-89, or co-transfection of ACREB, resulted in nearly complete inhibition of AP-1 transcriptional activation, as measured by reporter assay. Maximal transcriptional activation of AP-1 regulated genes is mediated by c-Fos/c-Jun heterodimers, and is highly dependent upon c-Fos protein expression. Previously, it has been shown that c-Fos transcription is induced by CREB activation (42–44). Here we find evidence that this mechanism is also operative in chondrocytes following PTHrP stimulation. Our transient transfection experiments here demonstrated the induction of a c-Fos promoter by CREB as previously described (43, 45, 46). Consistent with these findings was the increase in c-Fos protein and the delay in AP-1 DNA binding activity, which was maximally increased at 1 h following PTHrP stimulation, while an increase in CREB phosphorylation could be detected within 5 min. Thus, our data support a mechanism whereby PTHrP rapidly activates CREB through PKA signaling, and results in the subsequent transcriptional activation of PKA/CREB-dependent genes. One of these genes is likely to be c-Fos, which following its de novo synthesis, enhances AP-1 signaling (43, 45, 46). These events result in a delayed enhancement of AP-1 signaling through c-Fos transcription, and are consistent with PTHrP effects reported in other cell systems (43, 44).

The primary role for cyclic AMP/CREB signals in the regulation of chondrocyte differentiation is supported by genetic experiments. CREB knockout mice has a dwarf phenotype and die in the neonatal period of respiratory distress, similar to PTHrP knockout animals. Recently, a mutant PTH receptor with normal phospholipase C signaling, but deficient Goα signaling has been expressed in chimeric mice (47). Cells with deficient Goα signaling underwent premature maturation in the growth plate, while wild type cells had a normal rate of differentiation. In contrast, mice that express a mutant PTHrP receptor with normal Goα signaling, but deficient phospholipase C signaling, have normal size, are fertile, and do not have reduced rates of chondrocyte differentiation (48). These genetic experiments support our findings in that cyclic AMP/PKA signal transduction regulates the activation of the two important transcription factors, CREB and AP-1, that mediate PTHrP effects in chondrocytes. In contrast, PKC signaling appears less relevant for these events both in vivo and in vitro.

To establish whether either CREB or AP-1 transcriptional regulation is important for the effect of PTHrP on chondrocyte phenotype, experiments were performed using ACREB and AFOS. In the absence of PTHrP, the effects of these signaling inhibitors was minimal, suggesting relatively low basal activation of these signaling pathways. However, large effects were observed in the presence of PTHrP, consistent with the activation of CREB and induction of c-Fos by PTHrP. Although the magnitude of the individual signaling effects was greater in retroviral infected cultures, the transient transfection experiments demonstrated interactive effects between CREB and AP-1 signaling in chondrocyte maturation.

Chondrocyte maturation was determined by the expression...
of type X collagen and alkaline phosphatase, both of which are markedly elevated during chondrocyte differentiation and are important for the normal process of endochondral ossification (49, 50). As a suppressor of chondrocyte differentiation, PTHrP inhibited both type X collagen and alkaline phosphatase activity. Here we used two strategies to examine the role of CREB and AP-1 in this process: use of transient transfection and long-term retroviral expression vectors. Although transient transfection allows for modest expression for a shorter period of time, it also permits use of both dominant-negative constructs in combination. Thus, the effects we observed in these experiments were smaller but we were able to define synergistic effects and show interdependence between AP-1 and CREB mediated effects. In contrast, the effects observed in the retrovirally infected cultures were greater and further confirmed the importance of these transcription factors in PTHrP mediated signaling events. The phenotype that we obtained by inhibiting CREB and AP-1 signaling is similar to the phenotype of the PTHrP knockout mouse; accelerated chondrocyte differentiation of chondrocytes, resulting in premature endochondral ossification at many sites (7, 51).

Although, PTHrP has an accepted role as an inhibitor of maturation, its role in proliferation is controversial. While Lee et al. (52), observed similar expression of H4-histone mRNA (marker for S-phase of the cell cycle) in wild-type and PTHrP-deficient animals, others have shown diminished [3H]thymidine incorporation and a marked reduction in the number of proliferating chondrocytes in the knockout animals (51, 53). In vitro studies have supported an important role for PTHrP in chondrocyte proliferation (54–57). In the current experiments, PTHrP doubled the number of cells entering mitosis, and stimulated [3H]thymidine incorporation and cyclin D1 promoter activation (54–57). Consistent with reports in the literature (58), our results point out a role of CREB/ATF and AP-1 family members in the activation of cyclin D1 following PTHrP stimulation.

Chondrocytes integrate a complex array of signals during the process of differentiation. PTHrP is one of the major regulators but other factors such as Indian hedgehog, bone morphogenetic proteins, and transforming growth factor-β also affect the differentiation through overlapping or complementary pathways (54, 59, 60). Our experiments show that both CREB and AP-1 transcription factors are important regulators of the rate of chondrocyte differentiation. Additional studies will be required to define how other growth factors interact with CREB and AP-1 signaling and thereby modulate PTHrP effects during chondrocyte differentiation.

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REFERENCES

1. Wright, E., Hargrave, M. R., Christiansen, J., Cooper, L., Kun, J., Evans, T., Gangadharan, U., Greenfield, A., and Koopman, P. (1995) Nat. Genet. 9, 15–20.
2. Buckwalter, J. A., Mower, D., Ungar, R., Schaeffer, J., and Ginsberg, B. (1986) J. Bone Joint Surg. Am. 68, 243–55.
3. Linsenmayer, T. F., Chen, Q. A., Gibney, E., Gordon, M. K., Marchant, J. K., Lumsden, A., and Mercola, D. (1994) Cell Calcium 16, 123–132.
4. Schipani, E., Lanske, B., Hunzelman, J., Luz, A., Kovesz, C. S., Lee, K., Pirro, A., Kronenberg, H. M., and Juppner, H. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 13689–13694.
5. Zuscik, M. J., Puzas, J. E., Rosier, R. N., Gunter, K. K., and Gunter, T. E. (1994) Arch. Biochem. Biophys. 315, 352–361.
6. Zuscik, M. J., Gunter, T. E., Rosier, R. N., Gunter, K. K., and Puzas, J. E. (1994) Cell Calcium 16, 123–132.
7. Segre, G. V., Abou-Samra, A. B., Juppner, H., Schipani, E., Force, T., Urena, P., Freeman, M., Koonce, F. X., Kolakowski, L. F., Jr., and Hock, J. (1992) J. Endocrinol. Invest. 15, 367–373.
8. Montminy, M. (1997) Annu. Rev. Biochem. 66, 807–822.
9. Gonzalez, G. A., and Montminy, M. R. (1989) Cell 59, 675–680.
10. Barton, K., Muthusamy, N., Chanyangam, M., Fischer, C., Clemenlin, and, Puzas, J. E. (1996) Mol. Endocrinol. 10, 2641–2652.
11. Reimold, A. M., Grusby, M. J., Kosaras, B., Fries, J. W., Mori, R., Maniwa, S., Silverman, D., and Silbermann, M. (1992) J. Bone Joint Surg. Am. 74, 1149–1160.
12. Zuscik, M. J., Gunter, T. E., Rosier, R. N., Gunter, K. K., and Puzas, J. E. (1994) Cell Calcium 16, 123–132.
13. Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L., Kronenberg, H. M., and Mulligan, R. C. (1994) Genes Dev. 8, 277–289.
14. Lanske, B., Karaplis, A. C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Reimold, A. M., Grusby, M. J., and Mulligan, R. C. (1994) Endocrinology 134, 1119–1130.
15. Gonzalez, G. A., Yamamoto, K. K., Fischer, W. H., Karr, D., Menzel, P., Biggs, R. G., and Greenberg, M. E. (1998) Mol. Cell. Biol. 18, 5609–5619.
16. Anouar, Y., Lee, H. W., and Eiden, L. E. (1999) Mol. Pharmacol. 56, 162–169.
17. Brown, J. R., Nigh, E., Lee, R. J., Ye, H., Thompson, M. A., Sandou, F., Pestell, R. G., and Greenberg, M. E. (1998) Mol. Cell. Biol. 18, 5609–5619.
18. Asins, G., Hegardt, F. G., and Serra, D. (2000) Cell Calcium 27, 749–752.
19. Zuscik, M. J., Puzas, J. E., Rosier, R. N., Gunter, K. K., and Gunter, T. E. (1994) Arch. Biochem. Biophys. 315, 352–361.
20. Reimold, A. M., Grusby, M. J., Kosaras, B., Fries, J. W., Mori, R., Maniwa, S., Silverman, D., and Silbermann, M. (1992) J. Bone Joint Surg. Am. 74, 1149–1160.
21. Reimold, A. M., Grusby, M. J., Kosaras, B., Fries, J. W., Mori, R., Maniwa, S., Silverman, D., and Silbermann, M. (1992) J. Bone Joint Surg. Am. 74, 1149–1160.
53. Suda, N., Shibata, S., Yamazaki, K., Kuroda, T., Senior, P. V., Beck, F., and Hammond, V. E. (1999) *J. Bone Miner Res.* **14**, 1838–1847
54. Grimsrud, C. D., Romano, P. R., D’Souza, M., Puzas, J. E., Reynolds, P. R., Rosier, R. N., and O’Keefe, R. J. (1999) *J. Bone Miner Res.* **14**, 475–482
55. Ishikawa, Y., Wu, L. N., Genge, B. R., Mwale, F., and Wuthier, R. E. (1997) *J. Bone Miner Res.* **12**, 356–366
56. Klaus, G., May, T., Hugel, U., von Eichel, B., Rodriguez, J., Fernandez, P., Reichrath, J., Ritz, E., and Mehlis, O. (1997) *Kidney Int.* **52**, 45–51
57. Zerega, B., Cermelli, S., Bianco, P., Cancredda, R., and Cancredda, F. D. (1999) *J. Bone Miner Res.* **14**, 1281–1289
58. Beier, F., Lee, R. J., Taylor, A. C., Pestell, R. G., and LuValle, P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 1433–1438
59. Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M., and Tabin, C. J. (1996) *Science* **273**, 613–622
60. Pateder, D. B., Rosier, R. N., Schwarz, E. M., Reynolds, P. R., Puzas, J. E., D’Souza, M., and O’Keefe, R. J. (2000) *Exp. Cell Res.* **256**, 555–562