Parathyroid Hormone Induction of the Osteocalcin Gene
REQUIREMENT FOR AN OSTEOBLAST-SPECIFIC ELEMENT 1 SEQUENCE IN THE PROMOTER AND INVOLVEMENT OF MULTIPLE SIGNALING PATHWAYS*

Received for publication, October 21, 2003, and in revised form, November 18, 2003
Published, JBC Papers in Press, November 21, 2003, DOI 10.1074/jbc.M311547200

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Parathyroid hormone (PTH) is an important peptide hormone regulator of bone formation and osteoblastic activity. However, its mechanism of action in bone cells is largely unknown. This study examined the effect of PTH on mouse osteocalcin gene expression in MC3T3-E1 preosteoblastic cells and primary cultures of bone marrow stromal cells. PTH increased the levels of osteocalcin mRNA 4–5-fold in both cell types. PTH also stimulated transcriptional activity of a 1.3-kb fragment of the mouse osteocalcin gene 2 (mOG2) promoter. Inhibitor studies revealed a requirement for protein kinase A, protein kinase C, and mitogen-activated protein kinase pathways in the PTH response. Deletion of the mOG2 promoter sequence from −1316 to −116 caused no loss in PTH responsiveness whereas deletion from −116 to −34 completely prevented PTH stimulation. Interestingly, this promoter region does not contain the RUNX2 binding site shown to be necessary for PTH responsiveness in other systems. Nuclear extracts from PTH-treated MC3T3-E1 cells exhibited increased binding to OSE1, a previously described osteoblast-specific enhancer in the mOG2 promoter. Furthermore, mutation of OSE1 in DNA transfection assays established the requirement for this element in the PTH response. Collectively, these studies establish that actions of PTH on the osteocalcin gene are mediated by multiple signaling pathways and require OSE1 and associated nuclear proteins.

Parathyroid hormone (PTH) is a major regulator of calcium homeostasis. PTH has catabolic and anabolic effects on osteoblasts and bone in vitro and in vivo, which depend on the temporal pattern of administration; continuous administration decreases bone mass whereas intermittent administration increases bone mass (1–4). PTH functions through the PTH-1 receptor, a G protein-coupled receptor that is expressed in osteoblasts (5–7). Binding of PTH to its receptor activates multiple intracellular signaling pathways that involve cyclic cAMP, inositol phosphates, intracellular Ca²⁺, protein kinases A and C (2), and the extracellular signal-regulated kinase/mitogen-activated protein kinase (MAPK) pathway (8, 9).

Osteocalcin (OCN), a 5700-dalton γ-carboxyglutamic acid-containing noncollagenous protein, is a major component of the bone extracellular matrix. Although the functions of OCN are poorly understood, gene deletion studies suggest possible functions in bone remodeling (10). The expression of OCN is regulated by a number of calcitropic hormones and growth factors including 1,25-dihydroxy vitamin D₃ (11–13), glucocorticoids (14, 15), PTH (16), bone morphogenetic proteins (17), basic fibroblast growth factor 2 (18), tumor necrosis factor-α (19), and transforming growth factor β (20). A number of transcription factors that bind to specific regions of the osteocalcin gene promoter have also been identified. These factors include AP-1 family members (21, 22), MSX-2/HOX 8.1 (23, 24), DLX-5 (25), CCAAT/enhancer binding proteins (26), and RUNX2 (27), the osteoblast-specific product of the Chst1 gene. These factors play important roles in osteoblast and bone development and mediate the response of osteoblasts to the differentiation signals mentioned above. Among these factors, only RUNX2 has unique roles in bone formation, which are essential for the differentiation of hypertrophic chondrocytes and osteoblasts (27–30). RUNX2 expression and functional activity are regulated by a number of factors including bone morphogenetic proteins, fibroblast growth factor 2, PTH, tumor necrosis factor-α, and extracellular matrix signals (17–19, 31, 32).

Two regions of the mouse osteocalcin gene 2 (mOG2) promoter are required for its bone-specific expression, OSE2 (osteoblast-specific element 2) (33), the binding site for RUNX2, and OSE1, which binds a partially characterized factor designated as Osf1 (34). OSE1 has a core sequence of TTACATCA, located at −55 to −48 relative to the transcription start site. Schinke and Karsenty (34) showed that OSE1 and OSE2 are equally important in terms of their contribution to mOG2 promoter activity in osteoblasts both in vitro and in vivo. Osf1 is present in nuclear extracts of osteoblasts at the early stage of cell differentiation but is absent in differentiated osteoblasts, suggesting that it may play a role in osteoblastic proliferation and early differentiation (33, 34). A preliminary report indicated that Osf1 may play a role in the regulation of bone mass (35); however, the mechanism of action is not well understood.

The ability of PTH to regulate gene expression is dependent on the activation of specific transcription factors such as cAMP-response element-binding protein (CREB) (36, 37), AP-1 family members (38, 39), pituitary-specific transcription factor 1 (40), and RUNX2 (38). Although it is well known that PTH induces OCN gene expression, the mechanism mediating this regulation is not known. In the present study, we show for the first time that PTH induction of the OCN gene requires an intact OSE1 sequence in the mOG2 promoter and increased Osf1 in osteoblast nuclear extracts.
EXPERIMENTAL PROCEDURES

Reagents—Tissue culture medium and fetal bovine serum were obtained from HyClone (Logan, UT). Other reagents including forskolin, GF109203X, H89, and cycloheximide were obtained from Sigma; U0126 was obtained from Promega (Madison, WI), U0124 from Calbiochem (La Jolla, CA), and PTH-(1–34) from Bachem (Torrance, CA). All other chemicals were of analytical grade.

Cell Cultures—Two previously described MC3T3-E1 subclonal cell lines with high osteoblast differentiation potential were used in this study (32). Both subclones (MC-4 and MC-42 cells) express osteoblast phenotypic marker genes and mineralize only after growth in a medium containing ascorbic acid. MC3T3-E1 subclone 42 (MC-42) cells have the same phenotype as MC-4 cells and also contain stably integrated copies of a 1.3-kb mOG2 promoter driving a firefly luciferase reporter gene. Luciferase expression closely follows levels of endogenous OCN mRNA (32). Both cell lines were maintained in ascorbic acid free α-minimum

Fig. 1. PTH increases OCN mRNA levels in MC3T3-E1 preosteoblastic cells and in BMSCs. A and B, effect of various concentrations of PTH on OCN mRNA expression. MC-4 cells or BMSCs were seeded at a density of 50,000 cells/cm² in 35-mm dishes and cultured in 10% FBS medium overnight. Cells were then switched to 0.1% FBS in the absence or presence of various concentrations of PTH for 6 h. For each group, total RNA (20 μg/lane) was loaded for Northern hybridization using cDNA probes for mouse OCN mRNA and 18 S rRNA (for normalization). Northern blots were scanned, and OCN mRNA signals (top) were normalized to 18 S rRNA (bottom) (A, MC-4 cells; B, BMSCs). C, time course of PTH-induced OCN mRNA expression. MC-4 cells were treated as in A for indicated times in the absence (●) or presence (○) of 10⁻⁷ M PTH. D, effect of cycloheximide (CHX) treatment on PTH induction of OCN mRNA. MC-4 cells were treated with vehicle or 10 μg/ml cycloheximide in the absence or presence of PTH for 6 h. OCN mRNA and 18 S rRNA were determined by Northern blot analysis. Experiments were repeated a minimum of two times, and qualitatively identical results were obtained.
Eagle’s medium (Invitrogen), 10% fetal bovine serum, and 1% penicillin/streptomycin and were not used beyond passage 15.

**Mouse Bone Marrow Stromal Cell Cultures (BMSCs)**—Isolation of mouse BMSCs was described previously (41). Briefly, 6-week-old male C57BL/6 mice were sacrificed by cervical dislocation. Tibiae and femurs were isolated, and the epiphyses were cut. Marrow was flushed with Dulbecco’s modified Eagle’s medium containing 20% FBS, 1% penicillin/streptomycin, and 10⁻⁶ M dexamethasone into a 60-mm dish, and the cell suspension was aspirated up and down with a 20-gauge needle to break up clumps of marrow. The cell suspension (marrow from two mice/flask) was then cultured in a T75 flask in the same medium. After 10 days, cells reached confluency and were ready for experiments.

**DNA Constructs**—All luciferase reporter plasmids (except wild-type and mutant 4OSE2-luc, which were constructed in −34mOG2pGL3B-luc vector) were constructed by cloning mOG2 promoter inserts and multimeric oligonucleotides into the p4-luc promoterless luciferase expression vector as previously described (33). p657OSE2mut-luc, which contains a 2-bp substitution mutation in OSE2 at positions −134 and −133 (CCAGAACCA), was described previously (33). p116OSE1mut3-luc and p657OSE1mut3-luc, which both contain a 3-bp substitution mutation in OSE1 at positions −52, −51, and −50 (TTAGTACA), were generated from p116-luc and p657-luc by PCR amplification using the QuickChange XL site-directed mutagenese...
sis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The two primers used for making these mutant constructs were GCACTCCCCGTCTCTGTCTTTAGTACAGAGGAGCGAGAGTAGGCGG (forward) and CCGGTACTTGGTCCTGTACTAAAGCCAGGGAGGCAGGGTCG (reverse). –34 mOG2 or 13 mOG2 minimal promoter region into the BgII/HindIII site in pGL3-basic vector (Promega). Multimers (four copies) of wild-type or mutated (containing OSE1mut3) oligonucleotide were cloned into the BgII site of the –34 mOG2 or 13 mOG2 promoter vector. All sequences were verified by automatic DNA sequencing.

Transfections—Cells were plated on 35-mm dishes at a density of 5 × 10⁴ cells/cm². After 24 h, cells were transfected with LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Each transfection contained 0.5 µg of the indicated plasmid plus 0.05 µg of pRL-SV40 containing a cDNA for Renilla reniformis luciferase to control for transfection efficiency. Cells were harvested and assayed using the dual luciferase assay kit (Promega) on a Monolight 2010 luminometer (BD Biosciences, San Diego, CA).

Preparation of Nuclear Extracts and Gel Mobility Shift Assays—Nuclear extracts were prepared, and gel mobility shift assays were conducted as described previously (32). Each reaction contained 1 µg of nuclear extracts. The OSE2 wild-type and mutant oligonucleotides used in gel mobility shift assays in this study were described elsewhere (32). Additional oligonucleotides used in this study are described under “Results.”

RNA Analysis—RNA was isolated using TRIzol (Invitrogen) reagent according to the manufacturer’s protocol. Aliquots were fractionated on 1% agarose-formaldehyde gels and blotted onto nitrocellulose paper as described by Thomas (42). Mouse OCN cDNA probe used for hybridization was obtained from Dr. John Wozney (Genetics Institute, Boston, MA) (43). The cDNA inserts were excised from plasmid DNA with the appropriate restriction enzymes and purified by agarose gel electrophoresis before labeling with α-³²PdCTP using a random primer kit (Roche Applied Science). Hybridizations were performed as previously described using a Bellco Autoblot hybridization oven (44) and quantitatively scanned using a Packard A2024 InstantImager. All values were normalized for RNA loading by probing blots with cDNA to 18S rRNA (45).

Statistical Analysis—All experiments were repeated two to five times, and qualitatively identical results were obtained. Statistical analyses were performed using Instat 3.0 software (GraphPad, Inc., San Diego, CA). Unless indicated otherwise, each value reported is the mean and standard deviation of triplicate independent samples.

RESULTS

PTH Stimulates Osteocalcin Gene Expression and Promoter Activity—The effect of PTH on mouse OCN gene expression was evaluated in MC-4 cells and primary mouse bone marrow stromal cells. Confluent cells were treated with increasing concentrations of PTH for 6 h. As illustrated in Fig. 1A, PTH stimulated OCN mRNA expression in a dose-dependent manner with a significant stimulatory effect first detected at 10⁻¹⁰ µM. PTH also stimulated OCN message levels in primary mouse bone marrow stromal cells (Fig. 1B). Time course experiments showed that the levels of OCN mRNA began to rise within 1 h of PTH administration and remained elevated for at least 6 h (Fig. 1C). PTH stimulation was not blocked by the protein synthesis inhibitor, cycloheximide, suggesting that de novo protein synthesis is not required for the PTH response (Fig. 1D).

To establish whether the mOG2 promoter can be activated by PTH, MC-42 cells, which contain stably integrated copies of a 1.3-kb mOG2 promoter driving a firefly luciferase reporter gene, were treated with various concentrations of PTH (from 10⁻¹³ to 10⁻⁷ M) for 6 h. Cells were then harvested and assayed for luciferase activity. As shown in Fig. 2A, PTH stimulated promoter activity in a dose-dependent manner with a detectable response seen at a PTH concentration of 10⁻⁹ M (significance at p < 0.001). Measurable activation of the mOG2 promoter was observed 1 h after PTH addition with maximal induction occurring between 3 and 6 h and lasting for more than 6 h (Fig. 2B).

Multiple Signaling Pathways Mediate PTH Stimulation—PTH signals mainly through the PKA pathway, although involvement of PKC and MAPK pathways has also been described. To identify signaling pathways mediating PTH induction of the mOG2 gene, we examined the effects of various inhibitors or activators. As shown in Fig. 3A, H89, a selective inhibitor of the PKA pathway, completely abolished the PTH-stimulated OCN gene expression. Forskolin, a well known activator of PKA, increased OCN expression. In combination with PTH, the effect of forskolin was not additive, indicating that the PKA pathway is maximally stimulated. GP190203X, a specific inhibitor of the PKC pathway, also dramatically decreased PTH stimulation. Interestingly, U0126 (a specific inhibitor of MAPK) but not its inactive analog, U0124, also partially suppressed PTH stimulation. Similar results were obtained when PTH-induced mOG2 promoter activity was examined in MC-42 cells (all three inhibitors significantly blocked PTH induction, p < 0.001; Fig. 3B). The concentrations of the inhibitors or activators used in this study are in the ranges reported by us and others to selectively affect the relevant pathways (18, 47–49). We found no evidence of toxicity. Compounds did not reduce cell DNA or protein under the current conditions (data not shown). Collectively, these results indicate that PTH stimulation of OCN gene expression requires PKA and PKC as well as MAPK signaling pathways.

Minimal PTH-response Region Is within 116 bp of the Transcriptional Start Site—Several deletion mutants of the mOG2 promoter driving firefly luciferase gene expression have been described and shown to be active in osteoblast cell lines (32, 33). To determine in which region PTH is necessary to activate transcription, these constructs were transiently transfected into MC-4 cells in the presence or absence of PTH. Results showed that luciferase activity of both control and PTH-treated groups decreased with progressively larger 5’ deletions. However, a major drop in PTH stimulation was observed when the promoter was deleted from the –116 to –34 bp region (Fig. 4, inset). Thus, it is clear from these results that a major PTH-responsive element is located in the –116 to –34 mOG2 promoter region.

PTH Treatment Increases Binding of Nuclear Proteins to OSE1—As an initial step to identify the DNA sequence responsible for the PTH response, an 82-bp DNA oligonucleotide from –116 to –34 of the mOG2 promoter was synthesized and used as a probe for gel mobility shift assays using nuclear extracts from MC-4 cells with or without PTH treatment. Results revealed that PTH treatment increased nuclear protein binding
to this region (data not shown). To further identify the DNA sequence responsible for PTH responsiveness, four smaller overlapping DNA oligonucleotides (designated oligonucleotide 1, 2, 3, and 4, located at −116 to −89, −98 to −69, −80 to −50, and −61 to −34, respectively) covering the entire 82-bp region from −116 to −34 of the mOG2 promoter were synthesized (Fig. 5A). These oligonucleotides were then used as probes for gel mobility shift assays using nuclear extracts from MC-4 cells with or without PTH treatment. As shown in Fig. 5B, PTH had virtually no effect on the binding of nuclear proteins to oligonucleotides 1 and 2 (containing an E-box motif, CACATG, and a CCAAT/enhancer binding protein motif, CCAATT, respectively). In contrast, PTH treatment dramatically increased binding to oligonucleotides 3 and 4 (Fig. 5B, bands 1 and 2). The lower shifted band (band 3) bound to and was not competed by the four oligonucleotides and thus was clearly nonspecific.
Because the region shared by oligonucleotides 3 and 4 contains the OSE1 core sequence, TTACATCA, we predicted that a PTH-induced nuclear factor binds to this region. To confirm this, a set of double-stranded DNA oligonucleotides containing 3-bp mutations that span the entire OSE1 region was synthesized and evaluated in competitive gel mobility shift assays using wild-type oligonucleotide 4 as the probe and nuclear extracts from PTH-treated MC-4 cells (Fig. 6A). As shown in Fig. 6B, PTH-stimulated band 2 was completely abolished by the addition of 100-fold molar excess of unlabeled wild-type or mutant oligonucleotides 1, 5, and 6 but not by unlabeled mutant oligonucleotides 2, 3, and 4. These latter three oligonucleotides have 3-bp substitution mutations that exactly cover the OSE1 core sequence (TTACATCA). The band 2 increased by PTH treatment was further demonstrated to be the result of sequence-specific interactions in that it was disrupted by a 25–100-fold molar excess of OSE1 oligonucleotide but was not affected by the mutant oligonucleotide 3 (from CAT to GTA, mutant 3) (Fig. 6C). Band 1 was also competed by both unlabeled wild-type and mutant oligonucleotides 1, 5, and 6. However, mutant oligonucleotides 2, 3, and 4 could still partially compete its binding to the DNA oligo. It remains to be determined whether the band 1 and 2 protein-DNA complexes share common nuclear factors. These results clearly show that PTH treatment of cells increases the binding of a nuclear factor to OSE1.

Antibodies recognizing conserved domains of CREB, c-Fos, c-Jun, Fra-1, Fra-2, ATF-1, and RUNX2 did not supershift or disrupt the complex binding to OSE1 (data not shown).

**Mutation of the OSE1 Site Dramatically Reduces PTH Stimulation**—If OSE1 is indeed required for the activation of mOG2 by PTH, mutation of this element should abolish the PTH response. Furthermore, OSE1 fused to a minimal promoter should also respond to PTH. The results shown in Fig. 7 confirm these predictions. The introduction of a mutation of the core OSE1 sequence TTACATCA to TTAGTACA (mutant 3 in Fig. 6A) in the context of either 116mOG2-luc (Fig. 7A) or 657mOG2-luc (Fig. 7B) essentially abolished PTH responsiveness. The result with 657mOG2-luc is of particular significance in that this promoter fragment contains sufficient information to direct osteoblast-specific expression in vivo (46). Finally, an artificial promoter containing four copies of OSE1 fused to a /H11002 to /H11001 minimal mOG2 promoter was induced by PTH treatment whereas the same construct containing mutated OSE1 was not (Fig. 7C).

RUNX2 has been reported to mediate PTH responses in other systems (38). To address the possible involvement of this factor in the PTH response, we used two different approaches. In the first, we focused on the OSE2 located at /H11002 to /H11001 in the mOG2 promoter using a 147-bp mOG2-luc reporter gene. Although the introduction of a 2-bp mutation that renders this sequence nonfunctional as a RUNX2 binding site decreased basal promoter activity, it did not affect PTH induction (10.7-fold for the wild-type versus 10.3-fold for the OSE2 mutant) (Fig. 8A). Similar results were obtained when this site was
mutated either alone or together with a second OSE2 at -608 to -602 in the context of a 647-bp mOG2 promoter (result not shown). Consistent with these results, PTH treatment did not alter binding of RUNX2 to OSE2 DNA in gel mobility shift assays (Fig. 8B). Because RUNX2 may also be able to form complexes with other tissue-specific nuclear factors bound to non-OSE2 sites, we examined whether anti-RUNX2 antibodies could alter the mobility of the complex binding to OSE1 oligonucleotide, but no changes in mobility were observed (data not shown). In summary, we were unable to obtain any evidence for the involvement of RUNX2 or its DNA binding site in the PTH response.

DISCUSSION

This study examined the molecular mechanism and signaling pathways mediating PTH induction of the mouse OCN gene. Our studies show that: 1) PTH rapidly stimulates OCN
gene expression and mOG2 promoter activity in mouse MC3T3-E1 preosteoblastic cells and primary bone marrow stromal cells; 2) the PTH response does not require induction of a protein intermediate and involves PKA, PKC, and MAPK pathways; 3) the actions of PTH on the endogenous OCN gene can be reproduced with a short 116-bp mOG2 promoter and that this promoter lacks the RUNX2 transcription factor binding site that has been implicated in PTH action in other systems; and 4) PTH treatment increases binding of nuclear proteins to an OSE1 sequence in the proximal mOG2 promoter, and this sequence is essential for PTH responsiveness.

A number of signaling transduction pathways have been associated with PTH (8, 9, 47–50). For example, Boguslawski et al. (16) showed that PKA and PKC pathways cooperatively interact in PTH-mediated OCN gene expression in rat and human osteoblast cell lines. Consistent with these reports, the present study shows that these two pathways are also required for PTH-induced mouse OCN gene expression in MC3T3-E1 preosteoblast cells. Furthermore, our results show that the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/MAPK) pathway is also required for PTH responsiveness.

PTH can activate pre-existing Osf1 through post-translational modifications such as phosphorylation. PTH is known to phosphorylate and activate CREB (36, 37) and may also phosphorylate RUNX2 (47). Alternatively, PTH could increase the stability of Osf1 protein. Regulation of ubiquitin/proteasome activity has been linked to osteoblast activity and bone formation (59). Accumulating evidence has shown that PTH can regulate osteoblast and bone metabolism through the ubiquitin/proteasome pathway (60, 61). Many targets of PTH such as p27 Kip, c-Fos, c-Jun, UBP41, and RUNX2 are regulated in both osteoblasts (49, 56). In contrast, PTH rapidly induces osteoblast differentiation in immature cells while inhibiting it in more mature cells. The basis for this difference remains to be elucidated. Osf1 was reported to be present only in proliferating osteoblasts (33), which may play a role in this regard. In the future, it will be important to address whether Osf1 contributes to the anabolic effect of PTH on osteoblasts and bone under physiological and pathological conditions. A preliminary report describes Osf1 as a leucine zipper-containing transcription factor having a role in the regulation of osteoblastic proliferation (35). The precise mechanism through which PTH regulates Osf1 is not known. Because PTH stimulation of OCN gene expression occurs rapidly (within 1 h) and does not require protein synthesis, it is highly possible that PTH can activate pre-existing Osf1 through post-translational modifications such as phosphorylation. PTH is known to phosphorylate and activate CREB (36, 37) and may also phosphorylate RUNX2 (47). Alternatively, PTH could increase the stability of Osf1 protein. Regulation of ubiquitin/proteasome activity has been linked to osteoblast activity and bone formation (59). Accumulating evidence has shown that PTH can regulate osteoblast and bone metabolism through the ubiquitin/proteasome pathway (60, 61). Many targets of PTH such as p27 Kip, c-Fos, c-Jun, UBP41, and RUNX2 are regulated in this way (2, 62–65). Whether Osf1 is regulated by this mechanism needs to be determined. Finally, it remains to be elucidated whether the actions between Osf1 and other proteins such as it modifies interactions between RUNX2 and AP-1 factors on the collagenase gene (39, 53). Determining the precise mechanisms through which PTH regulates Osf1 transcriptional activity must await the availability of molecular reagents for Osf1.

Acknowledgments—We thank Dr. Laurie K. McCauley for critical reading of this manuscript, Dr. Abraham Schneider for assistance with preparation of mouse bone marrow stromal cells, and Drs. Chunxi Ge and Zhuoran Zhao for technical support.

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