Parathyroid Hormone Induces c-fos Promoter Activity in Osteoblastic Cells through Phosphorylated cAMP Response Element (CRE)-binding protein Binding to the Major CRE*

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Many parathyroid hormone (PTH)-mediated events in osteoblasts are thought to require immediate early gene expression. PTH induces the immediate early gene, c-fos, in this cell type through a cAMP-dependent pathway. The present work investigated the nuclear mechanisms involved in PTH regulation of c-fos in the osteoblastic cell line, UMR 106-01. By transiently transfecting c-fos promoter 5′ deletion constructs into UMR cells, we demonstrated that PTH induction of the c-fos promoter requires the major cAMP response element (CRE). Point mutations created in the major CRE within the largest construct inhibited both PTH-stimulated and basal expression. This element, therefore, performs concerted basal and PTH-responsive cis-acting functions. Gel retardation and Western blotting techniques revealed that CRE-binding protein (CREB) constitutively binds the major CRE but becomes phosphorylated at its cAMP-dependent protein kinase consensus recognition site following PTH treatment. CREB was functionally implicated in c-fos regulation by coexpressing a dominant CREB repressor, KCREB (killer CREB), with the c-fos promoter constructs. KCREB suppressed both basal and PTH-mediated c-fos induction. We conclude that PTH activates c-fos in osteoblasts through cAMP-dependent protein kinase-phosphorylated CREB interaction with the major CRE in the promoter region of the c-fos gene.

We have shown previously that parathyroid hormone (PTH)1 stimulates c-fos transcription in the osteoblastic UMR 106-01 cell line through a cAMP-mediated pathway (1). However, the events that follow cAMP induction are less clear. The present work was undertaken to describe the nuclear mechanisms involved in PTH-mediated c-fos induction in osteoblasts. Many PTH-responsive genes in osteoblasts are thought to be secondary responses due to their delayed nature and requirement for ongoing protein synthesis (2, 3). By definition, these genes require the expression of primary response genes, such as c-fos, for their induction.

Several in vivo models have identified Fos as a player in bone biology. This factor was first linked to bone when it was discovered in a mouse osteosarcoma as the product of v-fos, the viral homolog of c-fos (4). Similarly, several groups have engineered mice that overexpress c-fos and display bone abnormalities including non-malignant bone neoplasms and collagenase-producing bone tumors (5, 6). Conversely, Fos null mice exhibit osteopetrosis and disorganized bone growth (7). Transgenic mice, which express a fos-lacZ fusion gene, also identified bone as one of the major sites for c-fos expression (8). In agreement with the rodent models, evidence for Fos involvement in human bone disease has been provided by high c-fos expression in Pagetic bone (9) and human osteosarcomas (10).

c-fos is regulated in a cell-specific manner through a variety of mechanisms. These signaling pathways most likely act through different combinations of highly conserved sites within the promoter region (11). The mechanism for c-fos induction in osteoblasts by the bone resorptive agent, PTH, has not yet been determined. Observations described in this and previous studies conducted in our laboratory (1) demonstrate that PTH activates c-fos transcription independent of de novo protein synthesis and mainly through increased intracellular cAMP. We, therefore, hypothesized that cAMP response element-binding protein (CREB) becomes phosphorylated by PKA allowing transcriptional activation through a cAMP response element (CRE). Current information supports this model because CREB is a constitutively expressed protein, which becomes activated upon phosphorylation within its P-box domain. CREB is then able to trans-activate genes that possess the CRE sequence. The c-fos 5′ regulatory region has several imperfect CRE sequences. However, only one CRE markedly causes cAMP-dependent c-fos promoter activation in Balb/c3T3 cells (12). This site has therefore been referred to as the major CRE and was the focus of our experimental approach.

We present a dissection of the PTH-responsive segment within the c-fos 5′ regulatory region as well as describe the transcription factor activation mechanism. Specifically, the major CRE is required for PTH-mediated c-fos induction. This site confers basal expression through unphosphorylated CREB. However, PTH causes CREB to become phosphorylated within its PKA recognition site resulting in enhanced promoter function. These observations led to the following model. In unstimulated cells, CREB participates in basal c-fos expression through the major CRE. Upon PTH exposure, PKA phosphorylates, and thereby activates, CREB trans-activation functions. Increased Fos protein then participates in gene regulation in bone.

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1 The abbreviations used are: PTH, parathyroid hormone; ATF, activating transcription factor; AP-1, activator protein-1; CRE, cyclic AMP response element; CREB, CRE-binding protein; CAT, chloramphenicol acetyltransferase; PMA, phorbol 12-myristate 13-acetate; CTF/NF1, CAAT transcription factor/nuclear factor-1; PKA, cAMP-dependent protein kinase; PBS, phosphate-buffered saline; 8-Br-cAMP, 8-bromo-cAMP.
**EXPERIMENTAL PROCEDURES**

**Materials**—Radiolabeled [3H]chloramphenicol and enhanced chemiluminescence (ECL) reagents were obtained from Amersham Corp. DuPont NEN supplied [35S]pATP. Polyvinylidene difluoride membrane was synthesized by Millipore (Bedford, MA). Oligonucleotides were synthesized by Midland Certified Reagent Co. (Midland, TX). Poly(dI-dC) was a product of Boehringer Mannheim. Tissue culture media and reagents were obtained from the Washington University Tissue Culture Center (St. Louis, MO). All other chemicals were obtained from Sigma or Fisher Scientific.

**Antibodies**—Anti-Fos antibodies were either purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) or a gift from Dr. Natalie Teich (Imperial Cancer Research Fund, London, United Kingdom). Anti-CREB antibody, raised against the P-box region of CREB (13), was a gift of Dr. Joel Habener (Massachusetts General Hospital and Howard Hughes Medical Institute, Harvard Medical School, Boston, MA). Antibodies to ATF-1 (hybridoma) and ATF-2 (mouse ascites) were gifts from Dr. James Hoeffer (University of Colorado Health Sciences Center, Denver, CO). Anti-phosphoCREB was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from both Bioprobe and Santa Cruz Biotechnology, Inc.

**Plasmids**—Mouse fos-CAT 5′ deletion constructs—56 fos-CAT, −71 fos-CAT, −151 fos-CAT, and −356 fos-CAT (14–18)—were provided by Dr. Michael Gilman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). KCREB expression plasmid (17) was a gift from Dr. John Chrivia (Saint Louis University School of Medicine, St. Louis, MO). pSV-β-galactosidase control vector, pSV2CAT, and pSV0CAT plasmids were purchased from Promega Corp. (Madison, WI).

**Cell Culture**—UMR 106-01 cells were cultured as described previously (18). The treatment medium contained either 2% fetal bovine serum or 0.1% bovine serum albumin.

**Western Blotting**—For Fos detection, cell monolayers were washed with PBS (pH 7.6), scraped into PBS, and pelleted by centrifugation (200 × g, 10 min, room temperature). All further steps were conducted at 4°C or on ice as rapidly as possible and in the presence of 0.2 mM phenylmethylsulfonyl fluoride because Fos protein is extremely labile at 4°C or on ice. Samples to be assayed for β-galactosidase expression were quickly frozen to −70°C. Fos protein was detected by the Western blotting techniques. The membranes were then washed with 0.1% TTBS (Tris-buffered saline (TBS) supplemented with 0.1% Tween 20) three times for 5 min. Secondary antibody was applied for 30 min in all cases. For autoradiography, the gel was dried and complexes were visualized by autoradiography. The sequences of the oligonucleotide probes were shown in many figures as mean ± S.E.

**RESULTS**

**PTH Increases Fos Protein Levels through a Mechanism That Is Dependent on Time, PTH Concentration, and Intracellular cAMP**—We have previously demonstrated that the c-fos gene transcription rate and steady state mRNA levels increase in UMR 106-01 cells after PTH treatment in a time- and dose-dependent manner (1). However, Fos protein is the final determinant in AP-1-mediated gene activation. To assess the time course of PTH-induced Fos accumulation, UMR 106-01 cells were treated for the indicated time periods with 10−8 M PTH (Fig. 1A). Fos protein levels were noticeably elevated after 30 min, becoming maximal by 60 min. The effect was transient with Fos returning to basal levels approximately 2 h after PTH treatment. Previously, we in our laboratory revealed that c-fos mRNA accumulation was dependent on PTH concentration (1). In order to determine whether Fos protein accumulated in the same PTH dose-dependent manner, UMR cells were treated with media containing the indicated PTH concentrations (Fig. 1B) and Fos protein levels measured. Fos protein accumulation was found to be PTH dose-dependent, with an increase first observed following 10−10 M PTH treatment for 1 h. Maximal stimulation was achieved with 10−8 M PTH treatment. This relationship is in agreement with that of c-fos mRNA accumulation.
PTH increases intracellular cAMP (23) and calcium (24) as well as phosphatidylinositol hydrolysis (25) in osteoblastic cells. In order to dissect the second messenger responsible for PTH-induced Fos in this cell type, UMR cells were treated with PTH, 8-Br-cAMP, or PMA, either individually or combined, as indicated in Fig. 1C. All treatments caused an increase in Fos protein. Alone or combined, 8-Br-cAMP and PTH-induced Fos to the same extent, while PMA exceeded the response pattern was similar for both enhancer regions. Like the Fos protein measurements, the PTH effect was mimicked by 8-Br-cAMP with –71 fos-CAT producing the highest fold stimulation, although lowest basal expression. PTH in combination with 8-Br-cAMP activated both constructs to nearly the same extent as either agent alone, suggesting a common signaling pathway. While –356 fos-CAT was slightly responsive to PMA, this agent alone had no effect on –71 fos-CAT. However, analogous to Fos protein measurements, PMA, when combined with either PTH or 8-Br-cAMP, increased CAT activity synergistically. This synergism was not abolished by pretreating with either PMA or myristoylated protein kinase C inhibitor peptide, possibly due to the extended length of time that transfected cells must be treated with the agents (data not shown). However, as stated above, we have previously ruled out diacylglycerol-inducible protein kinase C as a participant in PTH-induced c-fos expression (1).

**Fig. 1. Characterization of PTH-induced Fos protein accumulation.** UMR 106-01 cells were treated as indicated and subjected to Western blot analysis. All blots are representative of three independent experiments. A, UMR 106-01 cells were treated for the indicated time points with control- or PTH-containing (10−8 m) media. The left panel was detected with anti-Fos antibody (from Dr. Natalie Teich) and the right with preimmune rabbit serum. B, UMR 106-01 cells were treated with the indicated PTH concentration for 60 min. The left panel was detected with anti-Fos antibody (Santa Cruz) and the center panel with rabbit nonimmune IgG. The right panel had been treated with anti-Fos antibody, which was preadsorbed with the peptide against which it was raised. C, UMR 106-01 cells were treated with control medium (C), PTH (10−8 m), 8-Br-cAMP (5 × 10−3 m), or PMA (2.6 × 10−7 m) either individually or in the indicated combinations. The left panel was detected with anti-Fos antibody (Santa Cruz), the center panel with anti-Fos antibody that had been preadsorbed with the peptide against which it was raised, and the right panel with nonimmune rabbit IgG.

**Fig. 2.** Characterization of PTH-induced Fos protein accumulation—(A) UMR 106-01 cells were treated with control medium (C), PTH (10−8 m), 8-Br-cAMP (5 × 10−3 m), or PMA (2.6 × 10−7 m) either individually or combined, as indicated in Fig. 1C. All treatments caused an increase in Fos protein. Alone or combined, 8-Br-cAMP and PTH-induced Fos to the same extent, while PMA exceeded the maximal PTH response. We also noted that either PTH or 8-Br-cAMP combined with PMA resulted in a synergistic effect on Fos protein accumulation. However, the response is in excess of the maximal PTH-induced Fos level, indicating an alternate signaling mechanism. It should be noted that we have shown previously that activation of the protein kinase C pathway is not necessary for PTH stimulation of c-fos mRNA (1). We postulate that the synergism results from the combined effect of PKA- and protein kinase C-mediated phosphate addition to a transcription factor or factors.

**Mouse c-fos Promoter Constructs Require the Major CRE-containing Region for PTH and 8-Br-cAMP-mediated Gene Expression**—We have thus far made it clear that PTH increases Fos levels in UMR cells through a cAMP-dependent pathway. However, the mechanistic description is incomplete without mapping the minimal PTH-responsive region within the c-fos promoter region and identifying the cis- and trans-acting elements responsible for transcriptional activation. To this end, mouse c-fos promoter 5′ deletion constructs −356, −151, −71, and −56 fos-CAT (Fig. 2A) (14–16) were transiently transfected into UMR cells. These constructs include several highly conserved regions, which respond to different agonists in other cell types. Upon PTH treatment, −356, −151, and −71 were stimulated 2.6-, 2.8-, and 4.6-fold, respectively (Fig. 2B). Conversely, −56 fos-CAT, which is deleted 3′ to the main CRE was unaffected by PTH treatment. It is worth emphasizing that the shortest of the three CRE-containing constructs (−71 fos-CAT) gave the highest fold stimulation although basal expression sharply decreased (Fig. 2B, inset). However, there was no difference between PTH induction of −151 and −356 fos-CAT. Human c-fos promoter CAT constructs responded similarly to PTH when transfected into UMR cells (data not shown). PTH dose-response experiments showed that −71 fos-CAT displayed a PTH concentration dependence similar to the endogenous gene (Fig. 2C). Increased promoter activity was first detected following 10−10 m PTH treatment. Maximal stimulation was detected after 10−8 m PTH, and half-maximal response was calculated to occur with 5.5 × 10−10 m PTH.

Elegant experiments utilizing mice that express a fos-lacZ fusion gene established that different combinations of regulatory elements interact to control c-fos in different systems (8). In order to determine if the same element and second messenger pathway activate –71 as do –356 fos-CAT, these constructs were transiently transfected into UMR 106-01 cells followed by treatment with PTH, 8-Br-cAMP, and PMA either alone or in combination (Fig. 2D). An identical pattern would signify that both constructs activate transcription through the same region. The response pattern was similar for both enhancer regions. Like the Fos protein measurements, the PTH effect was mimicked by 8-Br-cAMP with –71 fos-CAT producing the highest fold stimulation, although lowest basal expression. PTH in combination with 8-Br-cAMP activated both constructs to nearly the same extent as either agent alone, suggesting a common signaling pathway. While –356 fos-CAT was slightly responsive to PMA, this agent alone had no effect on –71 fos-CAT. However, analogous to Fos protein measurements, PMA, when combined with either PTH or 8-Br-cAMP, increased CAT activity synergistically. This synergism was not abolished by pretreating with either PMA or myristoylated protein kinase C inhibitor peptide, possibly due to the extended length of time that transfected cells must be treated with the agents (data not shown). However, as stated above, we have previously ruled out diacylglycerol-inducible protein kinase C as a participant in PTH-induced c-fos expression (1).
106-01 cells at this site. Proteins in cell lysates from both control and PTH-treated cells bound the mouse major CRE (TGACGTAG), creating an identical shift pattern (Fig. 4). Titration with cold probe demonstrated that a 5-fold molar excess efficiently competed for all protein-DNA complexes, while 25-fold excess oligonucleotide containing a CTF/NF1 element did not. This titration also revealed that the lower shifted band is less efficiently competed for by cold probe, indicating a lower relative affinity than the upper bands. Other experiments revealed that other non-CRE elements (AP-1, GRE, SP-1) were unable to compete (data not shown) for binding.

The unaltered shift pattern following PTH treatment is in agreement with current thinking that CREB binds to the consensus CRE with equal affinity regardless of its phosphorylation state but activates transcription only when phosphorylated (27, 28). To determine if our system fits this model, we...
included an antibody that interacts with CREB only if the latter is phosphorylated at Ser133 within the PKA consensus phosphorylation site. This antibody supershifted the upper bands only when protein was derived from PTH-treated cells (Fig. 5A). A nondiscriminating anti-CREB antibody super-shifted these bands independent of PTH treatment (data not shown). Likewise, phosphorylation without change in CREB protein abundance was confirmed by Western blot. In this experiment, PTH did not alter the total CREB signal as visualized with CREB antibody while phosphoCREB was detected only in PTH-treated cells (Fig. 5B). The band running below the CREB-containing complex in gel shift experiments appears to be ATF-1 since it was abolished by antibody to ATF-1. c-Jun antibody had no effect on any protein-DNA complex.

During the course of this study, the rat c-fos promoter region was published (11) and shown to be very similar to the human and mouse promoters. Rat c-fos bears the same nonconsensus major CRE as mouse but with different flanking sequences. Because UMR 106-01 cells are rat-derived, we assayed UMR 106-01 proteins binding to rat fos CRE in its native context. Similar to the mouse sequence, this element bound protein from both control- and PTH-treated UMR 106-01 cells equally, creating a shifted complex that could be supershifted by CREB antibody (data not shown).

The Dominant CREB Inhibitor KCREB Suppresses PTH Induction of Mouse c-fos-CAT Constructs—KCREB (killer CREB) is a dominant inhibitor of CREB function (17). The protein product has an amino acid substitution within the leucine zipper, which results in KCREB-CREB heterodimers with no DNA binding function. We cotransfected a KCREB expression

FIG. 4. PTH effect on UMR 106-01 cell protein binding to the mouse major CRE. UMR 106-01 cells were treated with control medium (C) or PTH (10^{-8} M) for 20 min and a cell lysate prepared. Protein binding to a double-stranded 27-mer oligonucleotide, which included the c-fos major CRE, was assessed by gel mobility shift analysis. Three separate experiments were performed with similar results. The fold excess of unlabeled oligonucleotides included in the binding reaction is indicated.

FIG. 5. PTH effect on CREB phosphorylation. A, protein from control- and PTH (10^{-8} M)-treated UMR cells was subjected to gel mobility shift analysis using mouse c-fos major CRE-containing oligonucleotide probe. Antibodies against phosphorylated CREB (P-CREB), ATF-1, and c-Jun were included in the indicated reactions. B, UMR 106-01 cells were treated with control medium (C) or PTH (10^{-8} M) for 20 min, a cell lysate prepared, and subjected to Western blot analysis. The panel on the left was detected with anti-phosphoCREB antibody, the middle panel with anti-CREB antibody, and the right panel with nonimmune rabbit IgG. Results are representative of three separate experiments.

Gel mobility shift experiments, which included a mutant CREB-containing oligonucleotide probe, confirmed the loss of CREB-containing bands (Fig. 6). The mutations were those incorporated into Mut 1 and Mut 3. This experiment confirmed our speculation that a single point mutation within the major CRE allows residual protein binding as determined by overexposing the film (data not shown). Residual CREB interaction with the mutant CREs was inversely proportional to basal expression of Mut 1 and Mut 3 constructs. Conversely, ATF-1 binding was not altered.
plasmid along with the mouse fos-CAT constructs into UMR 106-01 cells and treated the cells with control- or PTH-containing media. KCREB inhibited PTH-mediated activation of both −71 and −356 fos-CAT promoter constructs by 60% (Fig. 7). Conversely, expression of −56 fos-CAT, which lacks the major CRE as well as PTH responsiveness, was unaffected by KCREB (data not shown).

**DISCUSSION**

The data presented in this work identify three steps in the mechanism through which PTH induces c-fos gene expression in osteoblastic cells. (i) PTH induces c-fos expression using cAMP as a second messenger. (ii) Activated PKA catalytic subunit phosphorylates CREB at Ser133 within the P-box. (iii) Phosphorylated CREB bound to the major CRE in the c-fos promoter results in transcriptional activation.

Despite causing increased intracellular calcium and phosphoinositide turnover, PTH induces many osteoblastic responses through cAMP-dependent mechanisms (3). Accordingly, our observation that a cAMP analog mimics the effect of PTH on both c-fos promoter activation and Fos protein accumulation is in agreement with this pattern. In addition, failure of maximal concentrations of 8-Br-cAMP and PTH in combination to significantly increase their effect on c-fos further supports this interpretation. This is because each agent maximally activates the same pathway making additional c-fos expression through this mechanism impossible. Although PMA induced Fos protein both alone and combined with PTH or 8-Br-cAMP, the response was always in excess of the maximal PTH induction. This point, coupled with convincing experiments performed by Clohisy et al. (1), refutes any protein kinase-C involvement in the PTH-induced transduction of c-fos. Therefore, all available evidence identifies cAMP as the major functional second messenger in this and many other PTH-mediated responses in osteoblasts.

We have previously defined PTH-induced c-fos expression as a primary response (1). De novo synthesized transcriptional activators are, by definition, not participants in this mechanism. Therefore, post-translational modification must be involved. Partridge et al. (29) have demonstrated PKA activation in response to PTH. These findings combined with the current data are in agreement with the classical CREB-mediated pathway. Specifically, constitutively expressed CREB is phosphorylated at Ser133 by PKA. CREB is then activated, and as a dimer bound to a CRE, can activate gene transcription (for review, see Ref. 30). Our observations that CREB is necessary for the full PTH response coupled with PTH-induced PKA activity and CREB phosphorylation at the PKA consensus site are in complete support of this mechanism. It should be noted that Ca2+/calmodulin-dependent protein kinases types II and IV have also been shown to phosphorylate CREB at Ser133. However, we have clearly eliminated calcium involvement in induction of c-fos by PTH (1).

While Ser133 phosphorylation is required for CREB activity, additional kinases may also participate in CREB activation (28). Accordingly, PKA-mediated phosphorylation is required to create a casein kinase II consensus site, which, upon phosphorylation, activates CREB in vitro (31). The latter and similar observations involving other kinases have resulted in a hierarchical phosphorylation model (32). This states that, although CREB remains inactive without phosphorylation at the PKA site, the differential action of kinases on additional residues is required for its full activation and allows for cell-specific regulation. In this mechanism, Ser133 phosphorylation may cause a conformational change in the CREB protein, making other phosphorylated residues available for protein-protein interaction or exposing other residues to kinases. Work is currently in progress to identify any additional phosphorylation events that may be part of the PTH-mediated signal transduction pathway in osteoblasts. In fact, we postulate that this mechanism causes the synergism that we observed between PMA and 8-Br-cAMP.

Proteins other than CREB are able to interact with the CRE
sequence. However, this report clearly establishes CREB as one, if not the only, of these factors active and required at the c-fos major CRE. Gel shift assays combined with transfected fos-CAT constructs containing mutant CREs provide the most direct proof. Although ATF-1 interaction with the major CRE was visualized by gel mobility shift, we reject this factor as a candidate for four reasons. (i) ATF-1 binding was not altered by CRE mutations that inhibited PTH induction of −356 c-fos-CAT. (ii) Although the P-CREB antibody cross-reacts with a homologous site on ATF-1, we did not see ATF-1 phosphorylation. (iii) ATF-1 bound the major CRE with a lower affinity than CREB. (iv) No CREB-ATF-1 dimers bound to the major CRE. In summary, we demonstrate that PTH induces c-fos transcription primarily through the cAMP/PKA pathway. This event requires that CREB becomes phosphorylated by PKA at Ser133 and acts at the major CRE within the c-fos promoter region. This is the first demonstration that PTH induces gene transcription through phosphorylation of a constitutively expressed transcription factor and completes the signaling pathway from the cell membrane to the nucleus.

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