Calmodulin and Calmodulin-dependent Kinase IIα Regulate Osteoblast Differentiation by Controlling c-fos Expression*

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Majd Zayzafoon‡, Keertik Fulzele¶, and Jay M. McDonald§§

From the ‡Department of Pathology, University of Alabama at Birmingham and the ¶Veterans Administration Medical Center, Birmingham, Alabama 35233

Ca2+/calmodulin-dependent protein kinase IIα (α-CaMKII) was once thought to be exclusively expressed in neuronal tissue, but it is becoming increasingly evident that CaMKII is also expressed in various extraneural cells. CaMKII plays a critical role in regulating various signaling pathways leading to modulation of several aspects of cellular functions, including proliferation, differentiation, cytoskeletal structure, and gene expression. The purpose of this study was to examine the expression of CaMKII in osteoblast-like cells (MC4) and to elucidate its role in osteoblast differentiation. We demonstrated that CaMKII, specifically the α isoform, is expressed in osteoblasts both in vitro and in vivo. Inhibition of CaMKII by the calmodulin antagonist trifluoperazine (KN93) or the CaMKII antagonist KN93 reduces alkaline phosphatase activity and mineralization, as well as causes 85% and 56% decreases in alkaline phosphatase and osteocalcin gene expression, respectively. CaM and CaMKII antagonists, using the newborn mouse calvaria in vivo model, cause a 50% decrease in osteoblast number (N.Ob-BS) and a 32% decrease in mineralization (BV/TV). Pharmacologic and genetic inhibition of α-CaMKII by using trifluoperazine, KN93, and α-CaMKII small interfering RNA decreases the phosphorylation of ERK and of cAMP-response element-binding protein, leading to a significant decrease in the transactivation of serum response element and cAMP-response element. Inhibition of α-CaMKII decreases the expression of c-fos, AP-1 transactivation, and AP-1 DNA binding activity. Our findings demonstrated that α-CaMKII is expressed in osteoblasts and is involved in c-fos expression via regulation of serum response element and cAMP-response element. Inhibition of α-CaMKII results in a decrease in c-fos expression and AP-1 activation, leading to inhibition of osteoblast differentiation.

Cellular responses to several hormones, cytokines, mechanical stimuli, and many other signaling molecules involve the activation of the Ca2+/calmodulin pathway in numerous cell types, including osteoblasts (1, 2). Upon activation, calmodulin (CaM) is known to bind to and activate numerous targets, including calcium/calmodulin-dependent protein kinase II (CaMKII). It has also been shown from transgenic mice models that the elevation of CaM expression leads to an increase in the autonomous activity of CaMKII (3). The ubiquitously expressed CaMKII is involved in several aspects of cellular function (4). There are now over 30 identified isoforms of CaMKII, with molecular masses ranging from 52 to 83 kDa, resulting from alternative messenger RNA splicing of four genes (α, β, γ, and δ) (5). The CaMKII isoforms α and β are highly homologous, with the exception of an ~30-amino acid sequence in the β isoform that serves as an actin-targeting domain (5). These two isoforms were once thought to be expressed only in the brain (6), but it is becoming increasingly evident that the α isoform is expressed in other cells such as thyroid, hepatic, and smooth muscle cells (7, 8). In contrast, the γ and δ isoforms are known to be expressed in various extraneural cells but predominantly in embryonic cells and lymphocytes, respectively (9). All CaMKII isoforms are capable of homo- and hetero-multimerization via their C-terminal domain to form 300–700-kDa holoenzymes, with the subunit composition being dictated in a stochastic manner by relative isoform expression levels (10–12).

At the cellular level, the activation of CaMKII ultimately promotes its autophosphorylation on threonine 286. This process increases the affinity of CaMKII for Ca2+/CaM by ~1000-fold and helps maintain the enzyme in a partially activated or autonomous state even after dissociation of CaM (13). The phosphorylated CaMKII then modulates the activity of several transcription factors, including CREB (14), ATF (15), CAAT-enhancer-binding protein β (16), and serum response factor (17). Ultimately, the activation of CaMKII and its downstream signaling cascade is involved in regulating a wide variety of cellular events, including proliferation, differentiation, and apoptosis (4).

Osteoblasts originate from mesenchymal progenitors that, with the appropriate stimulation, undergo proliferation and differentiate into preosteoblasts and then into mature, functional osteoblasts (18). In culture, as in vivo, osteoblasts form bone-like mineralized nodules by undergoing three stages of development: proliferation, extracellular matrix maturation, and mineralization (19, 20). During each stage of development, specific subsets of genes are sequentially expressed or repressed. For example, collagen I is known to be a marker for proliferation, alkaline phosphatase for the extracellular matrix maturation, and osteocalcin for mineralization (20). The regulation of gene expression in osteoblasts during development

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‡ To whom correspondence should be addressed: The University of Alabama at Birmingham, 220 West Pavilion, 619 S. 19th St., Birmingham, AL 35233-7331. Tel.: 205-934-4303; Fax: 205-934-5499; E-mail: mcdonald@path.uab.edu.

¶ The abbreviations used are: CaM, calmodulin; CaMKII, calcium/calmodulin-dependent protein kinase II; CRE, CAMP-response element; CREB, CRE-binding protein; ERK, extracellular signal-regulated kinase; ATF, activating transcription factor; ALP, alkaline phosphatase; TFP, trifluoperazine; PBS, phosphate-buffered saline; CMV, cytomegalovirus; PTH, parathyroid hormone; siRNA, small interfering RNA; SRE, serum-response element; RT-PCR, reverse transcription-PCR.

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and differentiation occurs predominantly at the transcriptional level. Several transcription factors, such as AP-1, CREB/ATF2, and Runx2 have been shown to play a major role in the regulation of osteoblast gene expression, phenotype, and ultimately bone formation (21–23).

AP-1 is a dimeric transcription factor composed of either homodimers of the Jun family (c-Jun, JunB, and JunD) or heterodimers of the Jun and Fos family (c-Fos, Fra-1, Fra-2, and FosB) (24) or the Jun and ATF family (ATF-2, ATF-3, and ATF-4) (22, 25). Dimerization of AP-1 family members is a prerequisite for DNA binding to their G-12-O-tetradecanooylphorbol 13-acetate (TPA) response element (TRE) and CRE consensus elements (25, 26). The role of AP-1 in skeletal development has been elucidated mainly by genetic studies in mice (27–29). The importance of the Fos family in bone formation has been demonstrated by the c-Fos knock-out model that leads to a complete inhibition of osteoclast formation, the development of osteopetrosis (30), and a decrease in osteoblast differentiation (31). In contrast, the overexpression of c-Fos leads to osteosarcoma caused by osteoblast transformation (32). The overexpression of FosB leads to osteosclerosis caused by an increase in osteostasticogenesis of mesenchymal stem cells (28, 33).

CREB/ATF is another family of transcription factors that plays an important role in osteoblast differentiation. It has been reported that during osteoblast differentiation there is an increase in the phosphorylation and activation of CREB. In addition, during mineralization, most of the CREB bound to the CRE consensus sequence has been shown to be phosphorylated. Furthermore, the increase in CRE activity in osteoblasts has been reported to alter the composition of AP-1 family members and ultimately to decrease osteoblast differentiation (22). The c-fos promoter has been shown to be regulated by two important response elements, the SRE and CRE, located within the 400-bp region directly upstream of the transcriptional start site. The activation of several upstream kinases, such as ERK and CREB, can ultimately lead to increasing the transactivation of SRE and CRE, respectively (34, 35). Interestingly, CaMKII has been shown to regulate the activation of both ERK and CREB (8, 36).

The aims of this study were to examine the expression of CaMKII isoforms, specifically the α isoform in osteoblasts, and to elucidate the role of this kinase in osteoblast differentiation. Here we have demonstrated that the α and γ CaMKII isoforms are expressed in osteoblasts and that α-CaMKII regulates CRE and SRE transactivation and c-fos expression. We have also shown that pharmacologic and specific genetic inhibition of α-CaMKII inhibits the activity of the CRE and SRE response elements, leading to a decrease in AP-1 transactivation and DNA binding activity. Ultimately, this leads to a decrease in osteoblast differentiation, both in vitro and in vivo.

MATERIALS AND METHODS

Cell Culture and Differentiation—The murine osteoblastic cell line MC3T3-E1, Subclone 4 (MC4) (37) was purchased from the American Type Culture Collection (Manassas, VA). These cells have been shown to possess high osteoblastic potential (37). Cells were maintained in minimum essential Eagle's medium, α modification (α-MEM) (Sigma) containing 10% fetal bovine serum (Atlanta Biologicals), 100 units/ml of penicillin G, and 100 μg/ml streptomycin (Invitrogen) at 37 °C with 5% CO2. Cells were passaged every 2–3 days and were not used beyond passage 25. Osteoblastic induction was performed by supplementing the maintenance medium with 5 mM β-glycerophosphate and 50 μM ascorbic acid-2-phosphate (22).

Animal Studies—Balb/c mice, 5 days old, were injected daily in the calvarial region with 75 μl of phosphate-buffered saline (PBS) solution containing either Me2SO (control) or 10 μM calmodulin antagonist (TFP or KN93) as described previously (22). Briefly, a needle (26 gauge) was inserted at the base of the calvaria and pushed until it reached the central region of the skull. Solution was then injected subcutaneously over the parietal region of the skull. After 7 days, mice were sacrificed, and calvariae were harvested. Tissues were washed with PBS, fixed with 10% formalin, and embedded in methyl methacrylate. Undemineralized sections (5-μm thick) were cut and stained with Goldner's Trichrome and von Kossa stains. A region of interest was selected and remained constant for all animals regardless of the shape of the section. Standard bone histomorphometry as described by Parfitt et al. (38) was performed using the BioQuant image analysis software (R & M Biometrics, Nashville, TN) in the Histomorphometry and Molecular Analysis Core Laboratory of the University of Alabama at Birmingham Bone Center. In addition, the brains of some of the animals were collected and flash-frozen in liquid nitrogen. RNA was extracted, and RT-PCR was performed as described below and used as a positive control for the confirmation of the expression of CaMKII isoforms. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

RNA Extraction and RT-PCR—Total RNA was extracted by the TRIzol method as recommended by the manufacturer (Invitrogen). One μg of RNA was reverse-transcribed using M-MLV reverse transcriptase, and the equivalent of 10 ng was used for TaqMan real-time quantitative RT-PCRs as described previously (23). The expression of 18 S rRNA was used as a control. The sequences and conditions for the specific primers used in this study were as described previously. ALP (23), osteocalcin (23), c-fos (39), α-CaMKII (40), β-CaMKII (40), γ-CaMKII (40). Regular PCR was performed using recombinant TaqDNA polymerase and a Bio-Rad i-Cycler (Hercules, CA). Amplified products were then electrophoresed on a 1.5% agarose gel. Digital images were taken and analyzed using the Kodak Digital Science one-dimensional image analysis software (Eastman Kodak Co., Rochester, NY).

Whole Cell Protein Extraction—At the end of the study, MC4 cells were washed with chilled PBS, centrifuged at 800 × g for 5 min at 4 °C, and resuspended in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 10% glycerol). Protease and phosphatase inhibitors were added to the lysis buffer (41). Samples were then centrifuged at 14,000 × g for 30 min at 4 °C, and the supernatant protein concentration was measured by the Bio-Rad DC protein assay (41).

Western Blot Analysis—Protein extracts were loaded (30 μg/lane) onto a mini-SDS-PAGE system. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, Immunoblot-P (Millipore Co.), using a Bio-Rad wet transfer system. Protein transfer efficiency and size determination were verified using specific membrane markers were blocked with Tris-buffered saline-Blotto B (Santa Cruz Biotechnology) for 1 h at room temperature and subsequently incubated overnight with antibodies directed against α-CaMKII, ERK, CREB, phosphorylated ERK, phosphorylated CREB (Ser-133), and actin (Santa Cruz Biotechnology). Signals were detected using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection kit (ECL; Amersham Biosciences) (23).

Alkaline Phosphatase Staining—Cells were washed with cold PBS, fixed in 2% paraformaldehyde/PBS for 10 min, and stored at 4 °C in 100 mM cacodylic acid buffer (pH 7.4). Cells were then incubated at 37 °C with freshly prepared alkaline phosphatase substrate solution (100 mM Tris-maleate buffer (pH 8.4), 2.8% N,N-dimethyl formamide (v/v), 1 mg/ml Fast Red TR, and 0.5 mg/ml naphthyl AS-MX phosphate). The reaction was terminated after 30 min by removal of the substrate solution and washing with 100 mM cacodylic acid buffer.

Mineral Deposition—Mineralization was assessed by either von Kossa staining of the cultures (5 min in 3% AgNO3) (42) or by calcium measurement in the culture as described previously (41). Briefly, cells were collected by scraping into 10 mM Tris-buffered saline (pH 7.2) containing 0.2% Triton X-100. An aliquot was removed for determination of protein concentration, and the remaining material was incubated in 0.5 M HCl (final concentration) at 70 °C for 15 min, followed by spectrophotometric calcium detection (Arsenazo III, Sigma).

Nuclear Protein Extraction—MC4 cells were washed with chilled PBS and centrifuged at 800 × g for 5 min at 4 °C. Nuclei were then prepared by scraping cells with an Nucleic acid buffer containing 10 mM Tris, 10 mM NaCl, 3 mM MgCl2, 0.5% Nonidet P-40, and 0.5 μl sucrose. Nuclei were then treated with a hypotonic solution containing 10 mM HEPES, 1.5 mM MgCl2, and 10 mM KCl followed by a 30-min incubation at 4 °C in an extraction buffer containing 20 mM HEPES, 20% glycerol, 600 mM KCl, 1.5 mM MgCl2, and 0.2 mM EDTA. Nuclei were finally centrifuged at 14,000 × g for 30 min at 4 °C, and the supernatant protein concentration was measured by the Bio-Rad DC
protein assay. All solutions in this procedure contained a mixture of protease and phosphatase inhibitors (41).

**Electrophoretic Mobility Shift Assay**—The AP-1 oligonucleotide was labeled as previously described (41). The oligonucleotide sequence used as a probe was as follows: 5′-GCGTGGATGAGTCAGCCGGGAA-3′ (43). Four μg of nuclear extracts were then incubated for 20 min at room temperature with a 32P-labeled oligonucleotide containing the AP-1 consensus sequence in a loading buffer, pH 7.5 (50 mM NaCl, 5 mM dithiothreitol, 10 mM Tris-HCl, 4% glycerol, 0.5 mM EDTA, 1 mM MgCl2, and 50 μg/ml poly(dI-dC)). DNA-protein complexes were resolved by 5% native PAGE. Gels were dried and exposed to x-ray film at −80 °C with an intensifying screen.

**Immunofluorescence**—MC4 osteoblasts were cultured on coverslips for 24 h. Cells were then washed with cold PBS and fixed in methanol. Tibias were collected from 5–6-week-old mice, fixed with 4% paraformaldehyde, dehydrated in 25% and, 50% and 95% ethanol, and 95% ethanol and 4% paraffin. Tibias were collected from 5–6-week-old mice, fixed with 4% paraformaldehyde, decalcified in 0.25M EDTA, and 5-μm bone sections were obtained. Samples were then blocked for 1 h in Fc receptor blocker (Innovex Biosciences, Richmond, CA). α-CaMKII antibody (Santa Cruz Biotechnology) was diluted in Fc blocker solution and applied to the samples for overnight incubation at 4 °C. Fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz Biotechnology) was then used for 30 min. A nonspecific IgG was used for negative controls. At least 10 randomly selected microscopic fields were examined using ×10 and ×40 objectives. Photos were taken with a SPOT digital camera (41).

**Western Blot Analysis**—The proteins were solubilized and subjected to gel electrophoresis as previously described (41). Western blots were exposed to x-ray film at −80 °C with an intensifying screen.

**RESULTS**

To characterize the expression of CaMKII in osteoblasts, MC4 cells were cultured for 10 days in the presence of β-glycerophosphate and ascorbic acid, and the expression of CaMKII isoforms was examined. Differentiated osteoblasts expressed the genes for α and γ isoforms of CaMKII, as shown by RT-PCR (Fig. 1A). Expression of the β isoform was not detectable in osteoblasts. Positive controls on RNA extracted from mouse brain show expression of all three isoforms (Fig. 1A). Protein expression of the α-CaMKII isoforms was seen in osteoblasts both in vitro and in vivo as demonstrated by Western blotting and immunofluorescence, respectively (Fig. 1, B and C). To examine the phosphorylation states and localization of α-CaMKII in osteoblasts, immunofluorescence was performed using an antibody directed against phosphorylated α-CaMKII (Thr-286), which displays distinct speckled perinuclear localization (Fig. 1D). We also overexpressed α-CaMKII tagged with green fluorescent protein, generously provided by Dr. Tobias Meyer, Stanford University (44), to monitor the localization of α-CaMKII in osteoblasts. Similar to immunofluorescence, overexpression of α-CaMKII-green fluorescent protein produced a distinct pattern of perinuclear localization (data not shown).

The role of CaMKII during osteoblast differentiation was examined using the known CaM and CaMKII antagonists, TFP and KN93, respectively. MC4 cells were cultured, with or without 10 μM inhibitors, for 10 and 18 days in the presence of β-glycerophosphate and ascorbic acid. At the end of the study, cells were fixed and stained for ALP activity and for mineralization. Fig. 2A demonstrates that inhibiting both calmodulin and CaMKII decreases both ALP activity (red color in control) and mineralization (black color in control), thus demonstrating the importance of CaM/CaMKII in osteoblast differentiation.

**Gene Silencing by siRNA**—MC4 osteoblasts were plated at a density of 2 × 104 cells/cm2 in 6-well plates. 24 h after plating, cells were transfected with 1 μg of luciferase reporter plasmids driven by the AP-1, CRE, SRE sequences (Clontech), CMV-CaMKII-green fluorescent protein (provided by Dr. Tobias Meyer), or a CMV-β-galactosidase reporter construct (as a control). Transfections were performed using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. 24 h post-transfection, the medium was changed and cells were treated with CaM or CaMKII inhibitors as indicated. Cells were lysed and reporter activity was measured using a luciferase (Promega) or β-galactosidase (Clontech) assay system and a luminometer (22). Transfection efficiency was evaluated by transfecting CMV-green fluorescent protein, and an efficiency of 70–80% was observed.

**Silencing by siRNA**—MC4 osteoblasts were plated at a density of 2 × 104 cells/cm2 in 6-well plates. α-CaMKII siRNA (Dharmacon Research Inc., Lafayette, CO.) or control non-functional siRNA (Santa Cruz Biotechnology) was transfected into the cells using TransIT-TKO transfection reagent as recommended by the manufacturer (Mirus, Madison, WI). The medium was changed 24 h post-transfection, and the cells were cultured with fresh medium. Cells were harvested 72 h later. The success of the siRNA transfection was confirmed by demonstrating the inhibition of CaMKII protein expression (Fig. 6). The α-CaMKII 1 siRNA sequence is as follows: 5′-CACACCCAUUGGACGAAATdTdT-3′, 3′-dTdTUGGGUGGUAAACUCGCUCU-5′.

**Statistical Analysis**—All statistical analyses were performed using the Microsoft Excel data analysis program for Student’s t test analysis. Experiments were repeated at least three times unless otherwise stated. Values were expressed as a mean ± S.E.

**RESULTS**

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To examine the in vivo effects of CaM/CaMKII antagonists on osteoblast differentiation, we used the mouse calvaria in vivo model. CaM/CaMKII antagonists were injected subcutaneously for 7 days into the calvaria region of mouse pups (5 days old). Mice were then sacrificed, calvariae were harvested, and the tissue was processed for staining and bone histomorphometry. Fig. 4 shows the effects of in vivo treatment with TFP and KN93 on bone. TriChrome staining (Fig. 4A) demonstrates that both TFP and KN93 decrease osteoblast number (purple) and bone volume (green). In addition von Kossa staining (Fig. 4B) demonstrates a decrease in mineralization (black). These parameters were quantitated using the BioQuant image analysis system (Fig. 4, C and D) and show that in vivo treatment with TFP and KN93 results in a 50% decrease in osteoblast number (N.Ob-BS) and a 32% decrease in mineralized bone (BV/TV). These results clearly demonstrate that CaM/CaMKII play a critical role in osteoblast formation and differentiation.

To elucidate the mechanisms by which CaM/CaMKII inhibit osteoblast differentiation, we examined the effects of TFP and KN93 on two signaling pathways (ERK/mitogen-activated protein kinase and CREB/ATF) that are known to be regulated by CaM/CaMKII and are also important for osteoblast differentiation (8, 36). In addition, we examined the specific role of the α isoform of CaMKII on these signaling pathways by using siRNA designed to specifically target α-CaMKII (Fig. 5). The effectiveness α-CaMKII siRNA in silencing α-CaMKII was confirmed by demonstrating a decrease in α-CaMKII protein expression 72 h after transfection (Fig. 5). MC4 cells were cultured for 10 days and treated with Me2SO, 10 μM TFP, or 10 μM KN93 for 15 min before harvesting. The pharmacological inhibition of both CaM and CaMKII decreases the phosphorylation of ERK 42/44 in MC4 cells (Fig. 6A). Furthermore, the genetic silencing of α-CaMKII by siRNA also resulted in a
decrease in the phosphorylation of ERK 42/44 (Fig. 6B). We also examined the effects of inhibition of CaM and CaMKII and α-CaMKII silencing on signaling, downstream of ERK. MC4 cells were transfected with SRE-luciferase; 24 h later, cells were treated with 10 μM TFP or 10 μM KN93 for 24 h. In addition, cells were either transfected with scrambled non-functional siRNA (control) or α-CaMKII siRNA prior to SRE-Luc transfection. At the end of the study, MC4 cells were lysed and luciferase activity was measured. Both TFP and KN93 decreased the transactivation of SRE by 25% (Fig. 6C). The specific role of α-CaMKII in this response was confirmed by silencing α-CaMKII using siRNA. Silencing α-CaMKII results in a 75% decrease in the transactivation of SRE (Fig. 6D). These data demonstrate the critical role of α-CaMKII in the activation of ERK/SRE signaling pathway, which undoubtedly will affect osteoblast gene expression and phenotype.

Furthermore, we examined the effects of TFP, KN93, and α-CaMKII siRNA on the activation of CREB/ATF family members. Treatment with TFP and KN93 as well as the silencing of α-CaMKII decrease the phosphorylation of CREB (Ser-133) (Fig. 7A and B). Treatment with CaM/CaMKII antagonists or α-CaMKII silencing did not affect the phosphorylation of ATF-1 and -2 (data not shown). We also examined the effects of the pharmacologic inhibition of CaM and CaMKII by TFP and KN93, respectively, on CRE transactivation, which is known to be a downstream target of CREB. Here we have shown that both TFP and KN93 reduce CRE transactivation by 46% (Fig. 7C). The response to specific inhibition of α-CaMKII by siRNA was more severe. CRE transactivation was dramatically reduced, 86%, as result of silencing α-CaMKII (Fig. 7D). These data clearly demonstrate that CaMKII, and more specifically the α isoform, is a critical kinase in osteoblast.

The expression of c-fos has been shown by others to be regulated by two important regulatory response elements, CRE...
**FIG. 2.** TFP and KN93 inhibit osteoblast differentiation. **A**, MC4 osteoblasts were cultured for 14 (left) and 28 days (right, and panel **B**). Cells were treated beginning at day 4 with 10 μm TFP or 10 μm KN93, freshly added every 2 days, for the duration of the culture. Me2SO was added to the control group. Following fixation, samples were stained for alkaline phosphatase activity (red) and mineralization by von Kossa staining (brown). Similar results were seen in three other experiments. **B**, at the end of the study, cells were lysed and calcium content was measured. Values were obtained from three separate experiments, each repeated in triplicate, and represent the mean ± S.E. of calcium content relative to total protein. *, *p* ≤ 0.01.

**FIG. 3.** TFP and KN93 decrease ALP and osteocalcin gene expression in osteoblasts. Osteoblasts cultured for 14 days were treated with 10 μm TFP or 10 μm KN93 for 24 h. Control (C) was treated with Me2SO. At the end of the study, cells were harvested and RNA extracted. Regular and semiquantitative RT-PCR reactions were performed using primers for ALP (**A**), osteocalcin (**B**), and 18 S. Representative pictures from three different experiments of regular RT-PCR are also shown. Values were obtained by semiquantitative RT-PCR from the same number of experiments as above and represent the mean ± S.E. of ALP and osteocalcin messenger RNA expression relative to 18 S rRNA expression. *, *p* ≤ 0.01.
and SRE (34, 35). Therefore, we examined the effects of TFP and KN93 on c-fos expression in MC4 cells. Cells were cultured for 10 days and then treated with CaM or CaMKII antagonists for times ranging from 5 min to 24 h. We were only able to detect expression of c-fos at 1 h after feeding, because c-fos is an immediate response gene. Not surprisingly, treatment with both TFP and KN93 decreased c-fos expression in MC4 cells (Fig. 8). To demonstrate that the decrease in c-fos in response to treatment with CaM or CaMKII antagonists, indeed, affects AP-1 activity, we transfected MC4 osteoblasts with AP-1 luciferase followed by treatment with TFP or KN93. The transactivation of AP-1 was decreased ~68% in response to a 24-h treatment with either TFP or KN93 (Fig. 9A). The specific role of α-CaMKII in the transactivation of AP-1 was confirmed by using α-CaMKII siRNA. Similar to TFP and KN93, silencing α-CaMKII decreased AP-1 transactivation by ~85% (Fig. 9B). In addition, AP-1 DNA binding activity was decreased in response to CaM/CaMKII antagonists (Fig. 9C). MC4 cells were also treated with phorbol 12-myristate 13-acetate (50 ng/ml) for 3 h as positive control for AP-1 transactivation and DNA binding (data not shown).

Taken together, our results have provided a novel mechanism in which α-CaMKII, in response to the activation of CaM, regulates the activity of both ERK and CREB, which in turn affects the transactivation of SRE and CRE. This leads to regulation of c-fos gene expression, AP-1 transactivation, and AP-1 DNA binding activity and ultimately influences osteoblastic differentiation (Fig. 10).

DISCUSSION

Calcium (Ca^{2+}) is a well established regulator of transcriptional changes in gene expression that affects the transcription of numerous phenotypic genes and transcription factors, important for cellular function, proliferation, and differentiation.
At rest, the cytoplasmic Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_i\) is \(\approx 100\) nM, but this level rises to 500–1000 nM upon activation. The elevation of [Ca\(^{2+}\)]\(_i\) regulates cellular processes depending on the speed, amplitude, and spatiotemporal diversity of the increased [Ca\(^{2+}\)]\(_i\) (45). Many cellular responses to Ca\(^{2+}\) signals are modulated by Ca\(^{2+}\)/calmodulin-dependent enzymes, such as CaMKII, which has been shown to regulate the activation of many transcription factors, including AP-1. During differentiation, osteoblasts express different stage-specific gene markers. Transcription factors, including AP-1, have been found to play a critical role in osteoblast differentiation and the expression of some of these markers. The role of AP-1 in skeletal development has been elucidated mainly by genetic studies in mice (27–29). The importance of the Fos family in bone formation has been demonstrated using the c-Fos knock-out model, which leads to a complete inhibition of osteoclast formation (30) and a decrease in osteoblast differentiation and responsiveness to PTH (31). Interestingly, c-fos expression has been shown to be regulated by Ca\(^{2+}\) and CaMKII in glomerular mesangial cells (46). Therefore, the present study investigated the role of CaM/CaMKII in osteoblast differentiation and c-fos expression.

We first characterized the pattern of CaMKII expression in osteoblasts. For our in vitro studies we used the well characterized, murine pre-osteoblast cell line MC4, a subclone of MC3T3-E1 murine pre-osteoblasts (37). RT-PCR analysis showed that MC4 osteoblasts express the \(\alpha\) and \(\gamma\) isoforms of CaMKII, whereas the \(\beta\) isoform was not detectable. Our data are consistent with previously published data stating that the \(\gamma\) and \(\delta\) isoforms are expressed in tissues other than the brain (9, 40). In addition, we have shown here that both \(\alpha\)-CaMKII

**Fig. 6.** TFP, KN93, and \(\alpha\)-CaMKII silencing decrease the phosphorylation of ERK and the transactivation of SRE in osteoblasts. 
A, MC4 cells were cultured for 14 days. Cells were either treated with Me\(_2\)SO (control, C) or 10 \(\mu\)M TFP or 10 \(\mu\)M KN93. B, cells were transfected with \(\alpha\)-CaMKII siRNA 24 h after plating and were harvested 72 h after transfection. Cells were lysed for whole cell protein extraction. Extracts (30 \(\mu\)g/lane) were separated by 10% SDS-PAGE. Immunoblots were developed using antibodies directed against total ERK or phosphorylated ERK. The blots are representative of three separate experiments. C, MC4 osteoblasts were transfected (24 h after plating) with an SRE-luciferase reporter construct. 24 h after transfection, medium was changed and cells were treated with Me\(_2\)SO as a control, 10 \(\mu\)M TFP, or 10 \(\mu\)M KN93 for 24 h. D, cells were initially transfected for 24 h with \(\alpha\)-CaMKII siRNA. Reporter activity was then measured. Data are expressed relative to an internal control (CMV-\(\beta\)-galactosidase) and are the means \(\pm\) S.E. of three separate experiments, each performed in triplicate. *, \(p \leq 0.01\).
RNA and protein are expressed in MC4 osteoblasts. Furthermore, we are the first to have shown that CaMKII is expressed in vivo in osteoblasts lining trabecular bone of mice. Our results are complementary to a previously published report showing that the expression of collagenase, in response to PTH, in osteoblasts is mediated by activation of CaMKII (47). Here we have shown that this response could be mediated by any of the two isoforms detected in osteoblasts. The lack of CaMKII expression could be explained by the known nature and function of this kinase in neuronal cells. It has been shown that CaMKII, but not CaMKII, binds to F-actin in the absence of Ca\(^2\+)/CaM, enhancing filopodia extension and dendritic motility (48). Interestingly, the expression of CaMKII peaks early in development (48). Therefore, it is possible that in differentiating osteoblasts, CaMKII is not expressed due to the lack of need for motility and filopodia extension or it is expressed briefly during early development and subsequently turned off. Finally, this study has shown that in osteoblasts, total and phosphorylated CaMKII display a distinct speckled perinuclear pattern. It is known that when cells express different isoforms of CaMKII, cellular localization is determined by the relative ratio of the isoforms. When an excess of the cytoplasmic isoform of CaMKII is co-expressed along with nuclear isoform, all isoforms will be localized to the cytoplasm (49).

The rules of calmodulin and CaMKII in osteoblast differentiation were examined using the known antagonists, TFP and KN93, respectively. Treatment with both TFP and KN93 de-
creased ALP staining and mineralization, suggesting that CaM and CaMKII play a critical role in osteoblast differentiation. Not surprisingly, treatment with the antagonists decreased the expression of the osteoblastic gene markers ALP and osteocalcin. It is known that a decrease in osteoblast proliferation would ultimately lead to changes in the ability of osteoblasts to differentiate. Our results have shown that TFP and KN93 do not change [3H]thymidine incorporation (data not shown), thus treatment in our system did not affect proliferation. Although TFP is known to inhibit CaM activity, which in turn affects the activation of CaMKII, it is unlikely that the effects of TFP in inhibiting osteoblast differentiation are due solely to inhibition of CaMKII. Our data, using both TFP and KN93, suggested that CaMKII plays a critical role in regulating osteoblast differentiation, but this does not rule out involvement of other signaling molecules downstream of CaM, other than CaMKII, including CaMI and CaMKIV.

To examine the effects of CaM and CaMKII inhibition, we used a mouse pup in vivo calvaria model (22) showing that TFP and KN93 decrease osteoblast formation and mineralization in vivo. This method of examining the effects of treatment on osteoblast growth and differentiation, by injecting subcutaneously into the calvaria region, has the advantage of obtaining direct results with minimal risks of nonspecific data resulting from indirect effects of the treatment on other systems in the body. This is very important in this case as all cells in the body depend on CaM for various critical functions.

CaMKII is known to regulate signaling pathways in many systems, most importantly mitogen-activated protein kinase and CREB/ATF. In osteoblasts, these pathways are not only important for normal growth and differentiation but also for response to hormones such as PTH, PTH-related protein, bone morphogenetic protein, and TGF-β (50, 51). In addition, it has been suggested that the CaMK (II and IV) could play important roles in the osteoblast response to PTH and vitamin D (47, 52). Here we have shown that pharmacologic inhibition of both CaM and CaMKII and the gene-specific silencing of CaMKII by siRNA results in a significant decrease in activation of both ERK and CREB. These results are consistent with studies in other systems where CaMKII inhibition leads to a decrease in the activation of ERK, such as in vascular smooth muscle cells (53, 54), and CREB in differentiating neurons (55). In contrast, Shimomura et al. (15) demonstrate by in vitro studies that CREB is not involved in the CaMKII transcriptional regulation of gene expression. Furthermore, they demonstrate that ATF-1 is the mediator between the CaMKII and CREB signaling pathways. In osteoblasts, we found that ATF-1 was not involved, as its phosphorylation was unaffected by TFP or KN93 (data not shown). This difference could be because Shimomura’s study was an in vitro study using recombinant proteins. Interestingly, it has been shown by several investigators that both ERK/mitogen-activated protein kinase and CREB/CREB pathways play critical roles in osteoblast differentiation and response to hormones, such as PTH and PTH-related protein (56, 57). Our results implicated a novel upstream regulator of both ERK and CREB that could not only be responsible for osteoblast differentiation but also could be a controller of cellular responses to other hormones utilizing the Ca2+ signaling pathway.
α-CaMKII Regulates Osteoblast Differentiation

Transcription factors, namely AP-1, are known to play critical roles in the differentiation of many cells, including osteoblasts (58). Interestingly, c-Fos and its related antigen, Fra-1, have been shown to be critical in the osteoblast response to hormones (31, 59) and bone matrix formation (60). The expression of c-fos is regulated by two response elements, SRE and CRE (34, 35). Here we have shown that the inhibition of CaM or CaMKII leads to decreases in the transactivation of both SRE and CRE. This is most likely a result of the decrease in activation of ERK and CREB (61, 62). Our results were consistent with those of others who show that the decrease in activation of CRE in response to CaM and CaMKII inhibition leads to a decrease in c-fos gene expression. In support of our results, it has been shown c-fos is decreased in smooth muscle cells after the inhibition of CaMKII by KN93 (64).

Dimmerization of AP-1 family members is a prerequisite for DNA binding and the regulation of gene expression (25). It has been shown that a decrease in the availability of one AP-1 member can be substituted by another. Although this could still happen in osteoblasts after the decrease in c-fos expression, we have shown that AP-1 transcriptional activity is decreased because of the lack of c-Fos. Similarly, it has been shown in mesangial cells that CaMKII regulates c-fos promoter activation and ultimately modulates AP-1 activity (65). These results are significant not only because of the role of AP-1 in osteoblast differentiation but also because other transcription factors are known to regulate gene expression by interacting with AP-1. Therefore, the decrease in AP-1 activity in response to CaM and CaMKII inhibition could potentially decrease AP-1 interaction with other transcription factors such as Runx2, Smad, and estrogen receptor, ultimately leading to a decrease in osteoblast differentiation.

Our results indicate that CaMKII, and more specifically α-CaMKII, is a critical regulator of osteoblast differentiation. It acts by controlling different signaling pathways (CREB/ATF and mitogen-activated protein kinase) and AP-1 transcription factor activity by regulating c-fos expression (Fig. 10). This presents a potential target for development of anabolic drugs and therapeutic interventions to combat the decrease in osteoblast differentiation seen in osteoporosis.

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