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Calcium hydroxide regulates bone sialoprotein gene transcription in human osteoblast-like Saos2 cells

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Abstract: Bone sialoprotein (BSP) is a mineralized tissue-specific protein expressed in differentiated osteoblasts that appears to function in the initial mineralization of bone. Calcium hydroxide ($Ca(OH)_2$) is a basic salt that has been widely used for a variety of applications in dentistry, due to its antimicrobial effects and its capability of inducing hard tissue formation. However, details of the mechanism involved in the mineralization induced by $Ca(OH)_2$ are still unclear. In the present study, $Ca(OH)_2$ (0.4 mM) was found to increase the levels of BSP and Runx2 mRNA at 3 h in human osteoblast-like Saos2 cells. Transient transfection assays were performed using chimeric constructs of the human BSP gene promoter linked to a luciferase reporter gene. Treatment of Saos2 cells with $Ca(OH)_2$ (0.4 mM) increased the luciferase activities of the constructs between -60LUC and -927LUC at 12 h. Gel shift analysis showed that $Ca(OH)_2$ (0.4 mM) increased the binding of nuclear protein to CRE1, CRE2 and FRE. Antibodies against CREB1, c-Fos, c-Jun, JunD, Fra2 and P300 disrupted the formation of the CRE1- and CRE2-protein complexes, and antibodies against Dlx5, Msx2, Runx2 and Smad1 disrupted the formation of the FRE-protein complex. These findings demonstrate that $Ca(OH)_2$ stimulates BSP transcription by targeting the CRE1, CRE2 and FRE elements in the human BSP gene promoter. (J Oral Sci 53, 77-86, 2011)

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Introduction

Calcium hydroxide ($Ca(OH)_2$) is a basic salt that has been shown to induce the formation of fibrous tissue and immature bone when placed in direct contact with host tissue (1). Since the 1920s, $Ca(OH)_2$ has been broadly used for endodontic treatments, such as inter-appointment medication or root canal sealing, or as temporary medication for apexification. In an aqueous environment, $Ca(OH)_2$ dissociates into the calcium ion (Ca^{2+}) and the hydroxyl ion (OH^-). Various biological properties have been attributed to $Ca(OH)_2$, such as antimicrobial activity (2-4), tissue-dissolving ability (5), and induction of repair through formation of hard tissue (6).

Bone sialoprotein (BSP) is a highly sulfated, phosphorylated, and glycosylated protein that is expressed almost exclusively in mineralizing tissues (7,8). It is characterized by its ability to bind to hydroxyapatite through polyglutamic acid sequences, and to mediate cell attachment through an RGD sequence (9,10). High BSP expression coincides with *de novo* bone formation (11). BSP is primarily expressed by mature osteoblasts, osteoclasts and hypertrophic chondrocytes (12), but is also expressed in breast, lung, thyroid and prostate cancers (13-15). The human BSP gene has been cloned and partially characterized (16-20). Its promoters have an inverted TATA box (nt -28 to -23) (16-20), an inverted CCAAT box (ATTGG; -54 to -50) that is required for basal transcription (18-21), and two cAMP response elements (CRE1; -79 to 72 and CRE2; -674 to -667) (18-20,22). In addition, a fibroblast growth factor 2 (FGF2) response element (FRE;

-96 to -89) (18,19,23-25), three activator protein 1 (AP1) response elements (AP1(1); -148 to -142, AP1(2); -483 to -477 and AP1(3); -797 to -791) (18,19), and a homeodomain protein-binding site (HOX; -200 to -191) (16,20,24) have been characterized.

To elucidate the molecular mechanism of BSP gene regulation by $Ca(OH)_2$, we have analyzed the effects of $Ca(OH)_2$ on the expression of the BSP gene in human osteoblast-like Saos2 cells. We found that the transcription induced by $Ca(OH)_2$ was mediated through the CRE1, CRE2 and FRE elements in the proximal promoter of the human BSP gene.

Materials and Methods

Materials

Alpha minimum essential medium (α-MEM), fetal calf serum (FCS), lipofectamine, penicillin, streptomycin, and TrypLE^{TM} Express were obtained from Invitrogen (Carlsbad, CA, USA). PGL3-basic vector, pSV-β-galactosidase (β-Gal) control vector and U0126 (ERK 1/2 inhibitor) were purchased from Promega Co. (Madison, WI, USA). The protein kinase C (PKC) inhibitor H7 and the cAMP-dependent protein kinase (PKA) inhibitor KT5720 were purchased from Sigma-Aldrich Japan (Tokyo, Japan). The tyrosine kinase inhibitor herbimycin A (HA) was purchased from Wako Pure Chemical Industries (Tokyo, Japan). A EXScript RT reagent kit and SYBR Premix Ex Taq were purchased from Takara (Tokyo, Japan). All chemicals used were of analytical grade.

Preparation of the $Ca(OH)_2$ test solution

The $Ca(OH)_2$ solution was prepared by dissolving 0.0121 g of $Ca(OH)_2$ in 10.0 ml of distilled water at 23°C. This solution was stirred and centrifuged, filtered, and titrated to ensure that the pH of the culture medium (pH 7.76) would not be significantly altered (26).

Cell culture

The human osteosarcoma cell line, Saos2, was cultured in α-MEM containing 10% FCS. The cells were grown to confluence in 60-mm tissue culture dishes and then cultured in α-MEM without serum and incubated with or without $Ca(OH)_2$ (0.4 mM) for time periods extending over 3-24 h. Nuclear proteins were extracted by addition of extra proteinase inhibitors (the extraction buffer was 0.42 M NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 25% (v/v) glycerol, 0.5 mM phenylmethyl-sulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 1 μg/ml aprotinin, pH 7.9) (24,25).

Real-time PCR

Total RNA (1 μg) was used as a template for cDNA, which was prepared using the EXScript RT Reagent Kit. Quantitative real-time PCR was performed using the following primer sets: BSP forward, 5'-CTGGCACAGGGTATACAGGGTTAG-3'; BSP reverse, 5'-ACTGGTGCCGTTTATGCCTTG-3'; Runx2 forward, 5'-ATGTGTGTTTGTTTCAGCAGCA-3'; Runx2 reverse, 5'-TCCCTAAAGTCACTCGGTATGTGTA-3'; Osterix forward, 5'-GCCATTCTGGGCTTGGGTATC-3'; Osterix reverse, 5'-GAAGCCGGAGTGCAGGTATCA-3'; GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-3'; GAPDH reverse, 5'-ATGGTGGTGAAGACGCCAGT-3' using the SYBR Premix Ex Taq in a TP800 thermal cycler dice real time system (Takara, Tokyo, Japan). The amplification reactions were performed in a final volume of 25 μl containing 2×SYBR Premix EX Taq (12.5 μl), 0.2 μM forward and reverse primers (0.1 μl) and 100 ng cDNA (10 μl). To reduce variability between replicates, PCR premixes, which contained all reagents except for cDNA, were prepared and aliquoted into 0.2-ml Hi-8 tubes (Takara). The thermal cycling conditions were 1 cycle at 95°C for 10 s, 40 cycles at 95°C for 5 s, and 60°C for 30 s. Post-PCR melting curves confirmed the specificity of the single-target amplification, and the fold expression of human BSP and Runx2 relative to GAPDH was determined in quadruplicate; that of Osterix relative to GAPDH was determined in triplicate.

Transient transfection assays

Exponentially growing Saos2 cells were used for transfection assays. At 24 h after plating, the cells at 40-60% confluence were transfected using Lipofectamine reagent. The transfection mixture included 1 μg of a luciferase (LUC) construct (17-20) and 2 μg of β-Gal vector as an internal transfection control. The human BSP promoter sequences were cloned into the Bgl II site of the multiple cloning site of the pGL3-promoter luciferase plasmid. Two days after transfection, the cells were deprived of serum for 12 h, and $Ca(OH)_2$ (0.4 mM) was added for 12 h prior to harvest. The luciferase assays were performed in accordance with the supplier's protocol (PicaGene, ToyoInki, Japan) using a Luminescence reader (Acuu FLEX Lumi 400; Aloka) to measure the luciferase activities. The protein kinase inhibitors KT5720 (100 nM) and H7 (5 μM) were used to inhibit protein kinases A and C. HA (1 μM) and U0126 (5 μM) were used to inhibit tyrosine kinase and ERK1/2. Oligonucleotide-directed mutagenesis by PCR was utilized to introduce dinucleotide substitutions using the Quikchange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). All constructs were sequenced

as described previously to verify the fidelity of the mutagenesis.

Gel mobility shift assays

Confluent Saos2 cells in T-75 flasks incubated for 3, 6, and 12 h with Ca(OH)$_2$ (0.4 mM) in α-MEM without serum were used to prepare nuclear extracts. Double-stranded oligonucleotides encompassing the inverted CCAAT (nts, -64 to -41, 5'-CGTGACAGTGATTGGCT GTTGGAA-3'), CRE1 (nts, -89 to -63, 5'-ATCCACGT TCTGACATCACCTTGGTCG-3'), FRE (nts, -102 to -83, 5'-TTTTCTGGTGAGAATCCACG-3'), AP1(1) (nts, - 158 to -129, 5'-CGTTTCTTGTTTATTCAACTG AGCCTGTGT-3'), HOX (nts, -208 to -180, 5'-CTA AACCTTCAATTAAATTCCACAATGCA-3') and CRE2 (nts, -680 to -658, 5'-ATCAGCTGACCTCACA TGCACGA-3') in the human BSP promoter were employed. For gel shift analysis, the double stranded-oligonucleotides were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Nuclear protein extracts (3 μg) were incubated for 20 min at room temperature (21°C) with 0.1 pM radiolabeled double-stranded oligonucleotide in buffer containing 50 mM KCl, 0.5 mM EDTA, 10 mM Tris-HCl, pH 7.9, 1 mM dithiothreitol, 0.04% Nonidet P-40, 5% glycerol, and 1 μg of poly (dI-dC). After incubation, the protein-DNA complexes were resolved by electro-phoresis on 5% nondenaturing acrylamide gels (38:2 acrylamide/ bis acrylamide) run at 200 V at room temperature (27). The gels were then dried, and autoradiograms were prepared and analyzed using an image analyzer. Supershift experiments were performed using antibodies against c-Jun (sc-44), c-Fos (sc-253), JunD (sc-74), Fra2 (sc-604), P300 (sc-585), Msx2 (sc-15396), Smad1 (sc-7965) (Santa Cruz Biotechnology, Inc.), CREB1 (p43, Rockland), Dlx5 (AB5728, Chemicon) and Runx2 (PC287L, Calbiochem). The antibodies were added to each reaction mixture and incubated for 4 h at 4°C before electrophoresis under the same conditions as those described above.

Statistical analysis

Quadruplicate or triplicate samples were analyzed for each experiment, and experiments were replicated to ensure the consistency of the responses to drugs. Significance of differences between controls and treatments were determined using unpaired Student's *t* test.

Results
Effects of Ca(OH)$_2$ on expression of BSP, Runx2 and Osterix mRNAs

To study the regulation of BSP transcription by Ca(OH)$_2$, we performed real-time PCR analysis of total RNA extracted from human osteoblast-like Saos2 cells. First, the dose-response relationship of BSP induction by Ca(OH)$_2$ was established by treating Saos2 cells with different concentrations of Ca(OH)$_2$ for 12 h. Ca(OH)$_2$ increased the level of BSP mRNA at 0.12, 0.4 and 1.2 mM, and its effect was maximal at 0.4 and 1.2 mM (Fig. 1A). Therefore, 0.4 mM Ca(OH)$_2$ was used to determine the time courses of BSP, Runx2 and Osterix mRNA expression. It was found that treatment of Saos2 cells with 0.4 mM Ca(OH)$_2$ significantly increased the levels of BSP and Runx2 mRNA at 3 h, becoming maximal at 12 h (Fig. 1B). Ca(OH)$_2$ (0.4 mM) induced the expression of Osterix mRNA at 12 h, and its effect became maximal at 24 h (Fig. 1B).

Transient transcription analysis of human BSP promoter constructs

As the effects of Ca(OH)$_2$ likely involved the interaction of transcription factors in the promoter region of the human BSP gene, studies were performed to investigate this possibility. The human BSP promoter regions ligated to luciferase reporter genes, comprising -43 to +60 (-43LUC), -60 to +60 (-60 LUC), -84 to +60 (-84 LUC), -108 to +60 (-108 LUC), -116 to +60 (-116 LUC), -184 to +60 (-184LUC), -311 to +60 (-311LUC), -428 to +60 (-428LUC), -868 to +60 (-868LUC) and -927 to +60 (-927LUC), were transiently transfected into Saos2 cells, and the subsequent transcriptional activities were determined in the presence or absence of Ca(OH)$_2$. The results of luciferase assays indicated that Ca(OH)$_2$ (0.4 mM) increased the transcription of BSP after 12 h of treatment for constructs between -60LUC and -927LUC (Fig. 2). Included within the DNA sequence unique to the human BSP promoter region is an inverted CCAAT box (ATTGG; -54 to -50), together with CRE1 (-79 to -72), FRE (-96 to -89), AP1(1) (-148 to -142), HOX (-200 to -191) and CRE2 (-674 to -667) elements (Fig. 3). We introduced 2-bp mutations in the CCAAT, CRE1, FRE and CRE2 elements within the -184LUC and -868LUC constructs (Fig. 4). When mutations were made in CCAAT, CRE1, FRE and CRE2 in -184LUC or -868LUC, Ca(OH)$_2$-induced activities of -184LUCmCCAAT, -184LUCmCRE1, -184LUCmFRE, -868LUCmCRE1, -868LUCmFRE and -868LUCmCRE2 were partially inhibited. When mutations were made in pairs of CRE1 and CRE2 in -868LUC (-868mCRE1/mCRE2), the effects of Ca(OH)$_2$ on luciferase activity were almost totally abrogated (Fig. 4). These results suggested that CRE1 and CRE2 act as functional response elements for Ca(OH)$_2$ regulation of human BSP gene transcription.

Since protein kinases mediate Ca(OH)$_2$ signaling
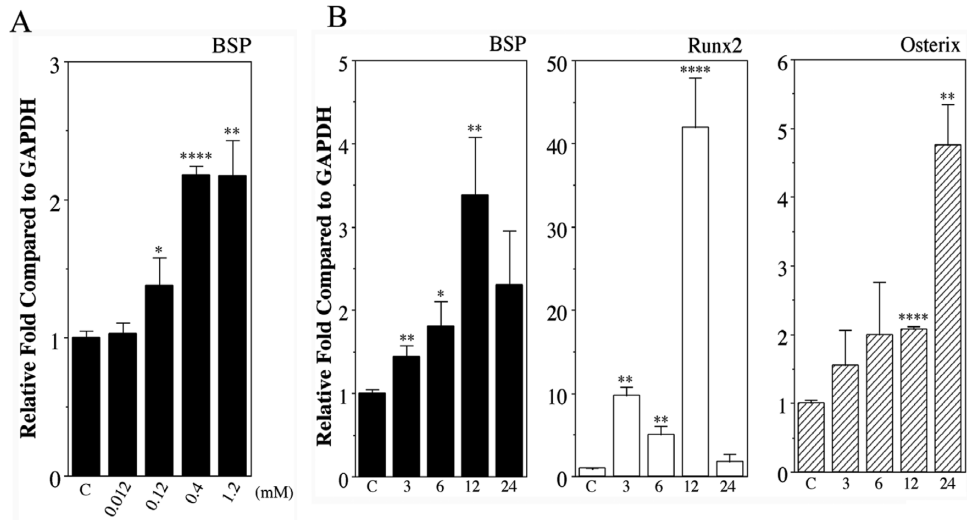
Fig. 1 Effects of Ca(OH)$_2$ on expression of mRNA for BSP, Runx2 and Osterix in Saos2 cells. The expression of mRNA for BSP, Runx2, Osterix and GAPDH in Saos2 cells was measured by real-time PCR. A: Dose-response effect of Ca(OH)$_2$ on BSP mRNA levels in Saos2 cells treated for 12 h. B: Saos2 cells were untreated or treated with Ca(OH)$_2$ (0.4 mM) for 3, 6, 12, and 24 h. The relative amounts of mRNA for BSP, Runx2 and Osterix to GAPDH were calculated. The experiments were performed in quadruplicate (BSP and Runx2) or triplicate (Osterix) for each data point. Quantitative analyses of the data sets are shown with standard errors. Significant differences from control: *($P < 0.1$), **($P < 0.05$), ****($P < 0.01$).
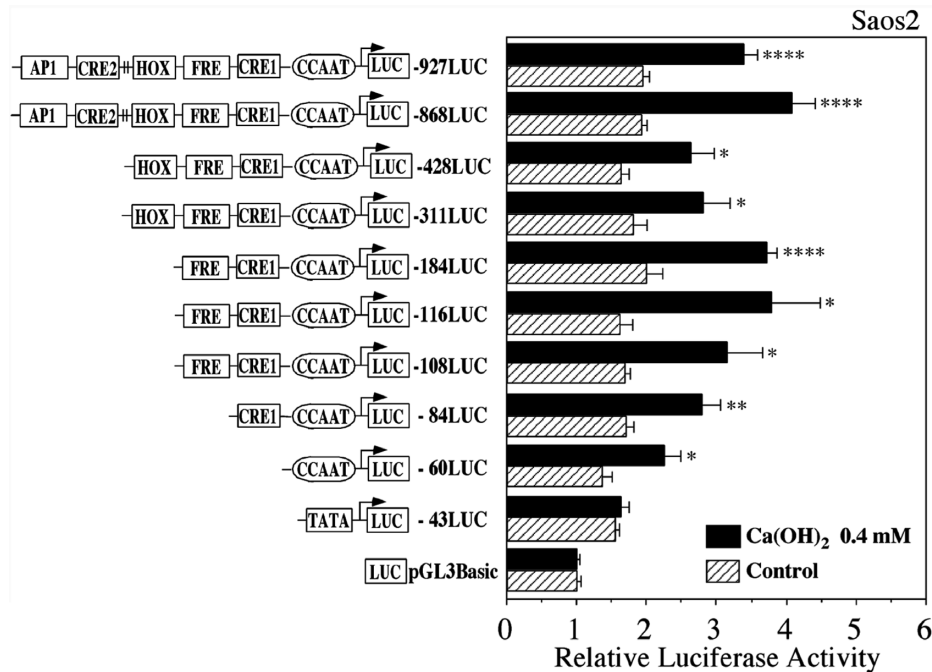

Fig. 2 Ca(OH)$_2$ up-regulates human BSP gene promoter activities. Transient transfection of Saos2 cells in the presence or absence of Ca(OH)$_2$ (0.4 mM) for 12 h was performed to determine the transcriptional activities of chimeric constructs that included various regions of the human BSP gene promoter ligated to a luciferase gene. The transcriptional activities obtained from four separate transfections with constructs, pGL3 basic and -43LUC to -927LUC, have been combined, and the values are expressed with standard errors. *($P < 0.1$), **($P < 0.05$), ****($P < 0.01$).
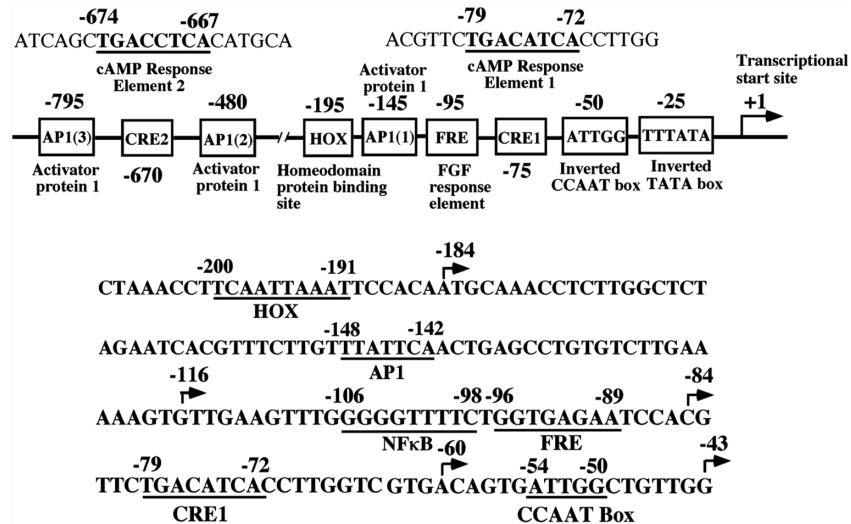
Fig. 3 Regulatory elements in the human BSP gene promoter. The positions of the inverted TATA and CCAAT boxes, CRE1, FRE, HOX, CRE2 and three AP1 sites are shown in the proximal promoter region of the human BSP gene. The nucleotides are numbered relative to the transcription start site (+1). The nucleotide sequences of two cAMP response elements (CRE1 and CRE2) in the human BSP gene promoter are shown from -79 to -72 (TGACATCA) and from -674 to -667 (TGACCTCA) (upper panel). The nucleotide sequences of the human BSP gene promoter encompassing an inverted CCAAT box, CRE1, FRE, NFκB, AP1 and HOX, are shown from -208 to -43 (lower panel).
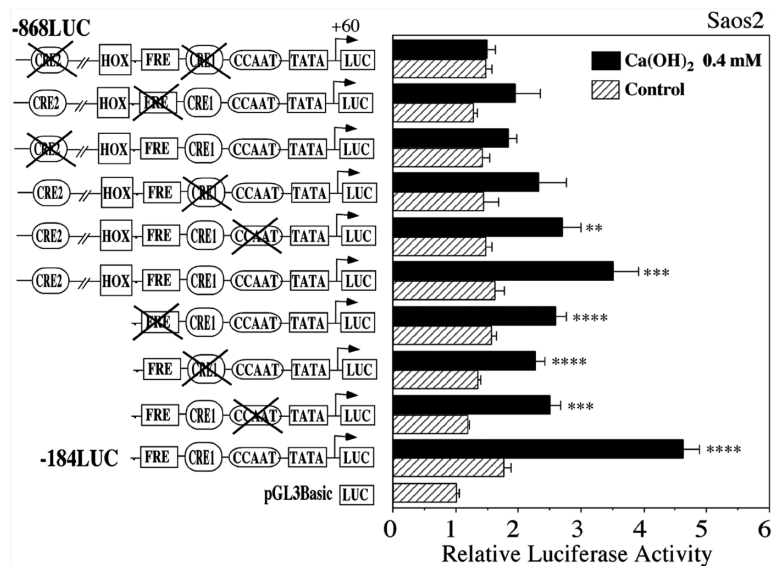

Fig. 4 Site mutation analysis of luciferase activities. Dinucleotide substitutions were made within the context of the homologous -184 to +60 (-184LUC) and -868 to +60 (-868LUC) BSP promoter fragments. The constructs -184mCCAAT and -868mCCAAT (ATTtt), -184mCRE1 and -868mCRE1 (TGACAgaA), -184mFRE and -868mFRE (GGcaAGAA), -868mCRE2 (TGACCgaA), and -868mCRE1/mCRE2 were analyzed for relative promoter activity after transfection into Saos2 cells and examined for induction after treatment with Ca(OH)$_2$ (0.4 mM) for 12 h. The results of transcriptional activity obtained from four separate transfections with the constructs were combined, and the values are expressed with standard errors. Significant differences from control: **($P < 0.05$); ***($P < 0.02$); ****($P < 0.01$).

activities, we investigated the effects of the PKC inhibitor H7, the PKA inhibitor KT5720, the tyrosine kinase inhibitor HA, and the ERK1/2 inhibitor U0126 on Ca(OH)$_2$-mediated transcription. Whereas Ca(OH)$_2$-induced BSP transcription (-184LUC and -868LUC) was inhibited by H7, KT5720 and U0126 were not inhibited by HA, indicating the involvement of PKC, PKA and ERK1/2 in the signaling pathways (Fig. 5).

Gel mobility shift assays

To identify which nuclear proteins are able to bind to the promoter region, double-stranded oligonucleotides of inverted CCAAT, CRE1, FRE, AP1(1), HOX and CRE2 elements were end-labeled and incubated with equal amounts (3 μg) of nuclear proteins extracted from confluent Saos2 cells that were either untreated (control) or treated with Ca(OH)$_2$ (0.4 mM) for 3, 6 and 12 h. When we used the inverted CCAAT as a probe, the DNA-protein complex did not change after stimulation with Ca(OH)$_2$ (Fig. 6, lanes 1-4). With nuclear extracts from confluent control cultures

of Saos2 cells, a shift of FRE-protein complexes was evident (Fig. 6, lane 5). After stimulation with Ca(OH)$_2$ (0.4 mM) (3, 6 and 12 h), FRE-protein complexes were increased at 6 and 12 h (Fig. 6, lanes 7 and 8). With nuclear extracts from confluent control cultures of Saos2 cells, shifts of the CRE1- and CRE2-protein complexes were evident (Fig. 6, lanes 9 and 13). After stimulation with Ca(OH)$_2$ (0.4 mM) (3, 6 and 12 h), the CRE1- and CRE2-protein complexes were increased at 3 and 12 h (Fig. 6, lanes 10-12 and lanes 14-16). Ca(OH)$_2$ did not induce formation of AP1(1)- and HOX-protein complexes (data not shown). To further characterize the proteins in the complexes formed with FRE, CRE1 and CRE2, we used antibodies against several transcription factors. Addition of antibodies against CREB1, c-Fos, c-Jun, JunD, Fra2 and P300 disrupted the formation of CRE1- and CRE2-protein complexes (Fig. 7A), whereas addition of antibodies against Dlx5, Msx2, Runx2 and Smad1 disrupted the formation of the FRE-protein complex (Fig. 7B).
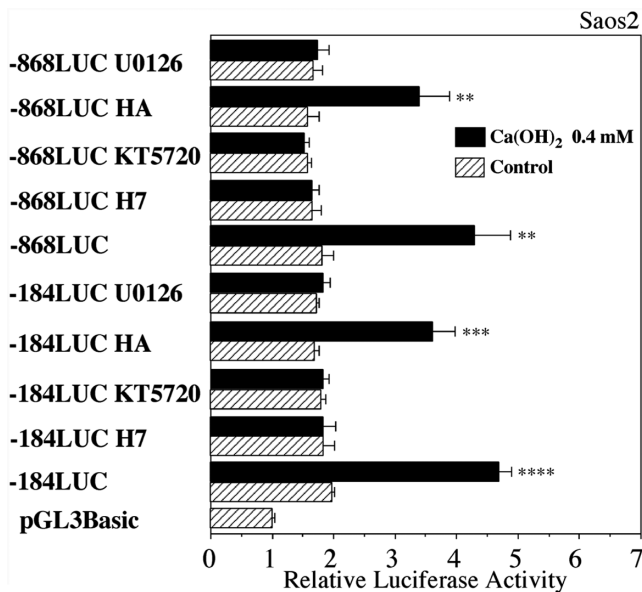

Fig. 5 Effect of kinase inhibitors on transcriptional activation by Ca(OH)$_2$. Transient transfection analysis of -184LUC and -868LUC in the presence or absence of Ca(OH)$_2$ (0.4 mM) in Saos2 cells is shown together with the effects of PKC inhibitor (H7, 5 μM), PKA inhibitor (KT5720, 100 nM), tyrosine kinase inhibitor (HA, 1 μM) and ERK1/2 inhibitor (U0126, 5 μM). The results of transcriptional activity obtained from four separate transfections with constructs were combined, and the values are expressed with standard errors. Significant differences from control: **($P < 0.05$); ***($P < 0.02$); ****($P < 0.01$).
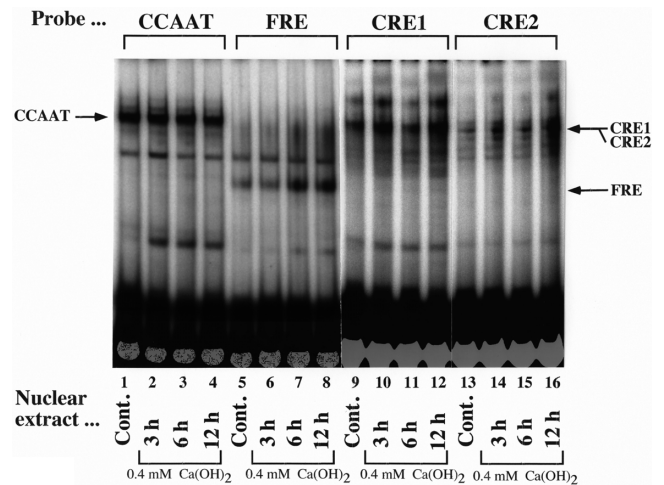

Fig. 6 Ca(OH)$_2$ up-regulates nuclear proteins that recognize FRE, CRE1 and CRE2. Radiolabeled double-stranded CCAAT (-64 CGTGACAGTG**ATTGG**CTGTTGG AA -41), FRE (-111 GTTTGGGGGTTTTCT**GGT GAGAA**TCCACGTTCTG -78), CRE1 (-89 ATCC ACGTTC**TGACATCA**CCTTGGTCG -63) and CRE2 (-680 ATCAGC**TGACCTCA**CATGCACGA -658) oligonucleotides were incubated with nuclear protein extracts (3 μg) obtained from Saos2 cells without (lanes 1, 5, 9, and 13) or with Ca(OH)2 (0.4 mM) stimulation for 3 h (lanes 2, 6, 10, and 14), 6 h (lanes 3, 7, 11, and 15) and 12 h (lanes 4, 8, 12, and 16). DNA-protein complexes were separated on 5% poly-acrylamide gel in low-ionic-strength Tris-borate buffer, dried under a vacuum, and exposed to an imaging plate for quantitation using an image analyzer.
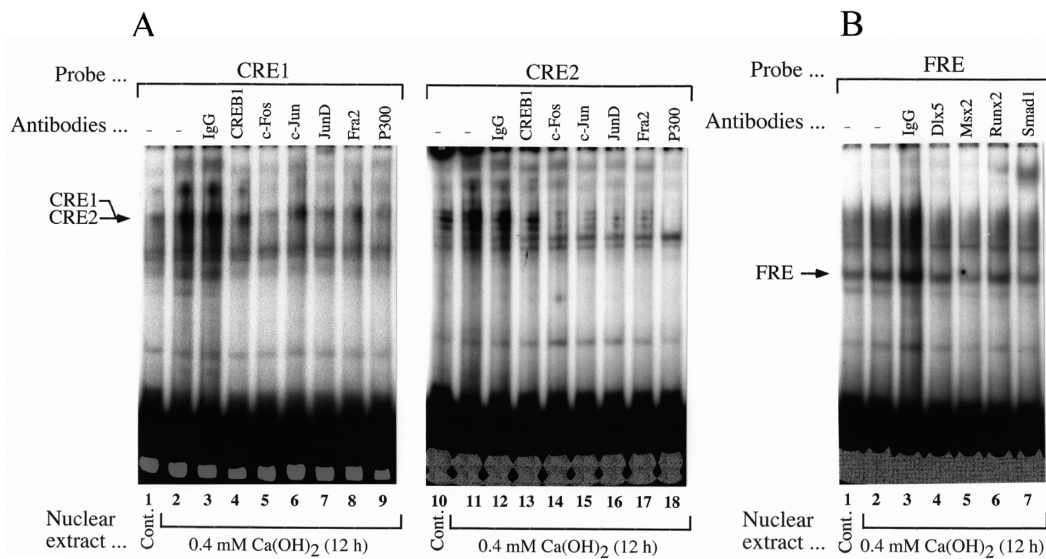
Fig. 7 Specific binding of nuclear proteins to CRE1, CRE2 and FRE. CRE1 and CRE2 (A), and FRE (B) were incubated with nuclear protein extracts (3 μg) obtained from Saos2 cells without or with Ca(OH)$_2$ (0.4 mM) stimulation for 12 h. Supershift experiments were performed with 0.4 μg antibodies that were added separately to each gel shift reaction.

Discussion

Ca(OH)$_2$ is one of the most effective pulp capping materials for inducing repair of the dentine-pulp complex (dentine bridge formation) following injury (28). As Ca(OH)$_2$ dissociates into Ca^{2+} and OH$^-$ in aqueous solution, the effects of OH$^-$ must be considered. It has been suggested that a rise in pH combined with the availability of Ca^{2+} and OH$^-$ ions affects both enzymatic pathways and mineralization (29). We have found that when Ca(OH)$_2$ (0.4 mM) solution is added to α-MEM, the pH of the medium does not change. It has also been shown that incubation with OH$^-$ does not have any effect on the proliferation and mineralization of osteoblasts (30), and other strong alkaline substances do not induce mineralization *in vivo* (31). Therefore, the mineralization of osteoblasts induced by Ca(OH)$_2$ may be dependent on the presence of dissociated Ca^{2+}. Furthermore, it has been demonstrated that when 38-50% Ca(OH)$_2$ paste is added to the root canal, Ca^{2+} is released for at least 100 days after application (32). In the present study, we demonstrated that Ca(OH)$_2$ increased the expression of the gene for BSP, a major non-collagenous protein of mineralized connective tissues that is involved in osteogenic differentiation and bone formation. In Saos2 osteoblast-like cells, Ca(OH)$_2$ up-regulated the levels of BSP and Runx2 mRNA simultaneously at 3 h, the effect becoming maximal at 12 h (Fig. 1). Runx2 is a crucial transcription factor involved

in osteoblast differentiation and bone formation. Runx2-deficient mice display an absence of bone due to arrested osteoblast differentiation (33). By transient transfection analyses, we identified the Ca(OH)$_2$ response elements FRE, CRE1 and CRE2 in the human BSP gene promoter (Figs. 2 and 4). The results of gel shift assays using specific antibodies (Fig. 7) suggested that Ca(OH)$_2$ induced BSP transcription through Dlx5, Msx2, Runx2 and Smad1 targeting the FRE (Fig. 7B), and through CREB1, c-Fos, c-Jun, JunD, Fra2 and P300 targeting CRE1 and CRE2, in the human BSP gene promoter (Fig. 7A). CREB/ATF and AP1 family transcription factors can bind to CRE as homodimers or heterodimers (34), and we found that AP1 family transcription factors (c-Fos, c-Jun, JunD and Fra2) and P300 interacted with CRE1 and CRE2 in the human BSP gene promoter. P300 is a multifunctional transcription factor that serves as an adapter for several transcription factors, and possesses intrinsic histone acetyltransferase activity. P300 plays an architectural role in the osteocalcin gene promoter by interacting with Runx2 and the vitamin D3 receptor, and by connecting factors bound to the distal and proximal promoters (35). Smad1, 5 and 8 are activated by bone morphogenetic protein (BMP) receptors and transduce BMP signaling (36). Runx2 induction by retinoic acid is potentiated by BMP2 signaling through interaction with Smad1 on the type X collagen promoter in chondrocytes (37). Runx2 and Msx2 mediate the expression of BSP in human breast cancer cells (38). We reported

previously that the FRE binding proteins in Saos2 cells are Runx2, Dlx5 and Smad1, and that an anti-Smad1 antibody co-precipitates Smad1 and Runx2 (24). Dlx5 is a transcription factor expressed by osteoblasts and chondrocytes (39), and binds to the HOX site in the rat and mouse BSP gene promoter (24,40). BMP2-induced Runx2 expression is mediated by Dlx5 (41). Therefore, it is conceivable that Dlx5, Msx2, Runx2 and Smad1 may interact with CRE1 and CRE2, because CRE1 is located adjacent to FRE in the proximal promoter of the human BSP gene. The CREB1 and AP1 transcription factors JunD and Fra2 regulate BSP gene expression in human breast cancer cells (23). The effect of $Ca(OH)_2$ on BSP transcription was almost completely inhibited by PKC, PKA and ERK1/2 inhibitors. It has recently been reported that increased intracellular cAMP levels may not only activate PKA but also directly stimulate Ras/Raf signaling, which is linked to the MAP kinase pathway in a PKA-independent manner (42). Furthermore, cAMP is able to stimulate MAP kinase activity (43). In this study using Saos2 osteoblast-like cells, we demonstrated that Dlx5, Msx2, Runx2 and Smad1 interacted with FRE. Furthermore, CREB1, c-Fos, c-Jun, JunD and Fra2 were able to bind the two CRE sites in the human BSP gene promoter together with p300, and regulate BSP gene expression.

In conclusion, our present results suggest that $Ca(OH)_2$ induces expression of the BSP gene in human osteoblast-like cells via FRE, CRE1 and CRE2 in the gene promoter, being mediated by the CREB1 and AP1 family, and that Dlx5, Msx2, Runx2 and Smad1, and P300, may connect the distal and proximal human BSP gene promoters.

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