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Prevention of Glucocorticoid-Induced Apoptosis in Osteocytes and Osteoblasts by Calbindin-D_{28k}

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ABSTRACT: This study show for the first time that calbindin-D_{28k} can prevent glucocorticoid-induced bone cell death. The anti-apoptotic effect of calbindin-D_{28k} involves inhibition of glucocorticoid induced caspase 3 activation as well as ERK activation.

Introduction: Recent studies have indicated that deleterious effects of glucocorticoids on bone involve increased apoptosis of osteocytes and osteoblasts. Because the calcium-binding protein calbindin-D_{28k} has been reported to be anti-apoptotic in different cell types and in response to a variety of insults, we investigated whether calbindin-D_{28k} could protect against glucocorticoid-induced cell death in bone cells.
Materials and Methods: Apoptosis was induced by addition of dexamethasone (dex; 10^{-6} M) for 6 h to MLO-Y4 osteocytic cells as well as to osteoblastic cells. Apoptosis percentage was determined by examining the nuclear morphology of transfected cells. Caspase 3 activity was evaluated in bone cells and in vitro. SELDI mass spectrometry (MS) was used to examine calbindin-D_{28k}-caspase 3 interaction. Phosphorylation of calbindin-D_{28k} was examined by ^{32}P incorporation as well as by MALDI-TOF MS. ERK activation was determined by Western blot.
Results: The pro-apoptotic effect of dex in MLO-Y4 cells was completely inhibited in cells transfected with calbindin-D_{28k} cDNA (5.6% apoptosis in calbindin-D_{28k} transfected cells compared with 16.2% apoptosis in vector-transfected cells, $p < 0.05$). Similar results were observed in osteoblastic cells. We found that dex-induced apoptosis in bone cells was accompanied by an increase in caspase 3 activity. This increase in caspase 3 activity was inhibited in the presence of calbindin-D_{28k}. In vitro assays indicated a concentration-dependent inhibition of caspase 3 by calbindin-D_{28k} ($K_i = 0.22$ μM). Calbindin-D_{28k} was found to inhibit caspase 3 specifically because the activity of other caspases was unaffected by calbindin-D_{28k}. The anti-apoptotic effect of calbindin-D_{28k} in response to dex was also reproducibly associated with an increase in the phosphorylation of ERK 1 and 2, suggesting that calbindin-D_{28k} affects more than one signal in the glucocorticoid-induced apoptotic pathway.
Conclusion: Calbindin-$D_{28k,}$ a natural non-oncogenic protein, could be an important target in the therapeutic intervention of glucocorticoid-induced osteoporosis.
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Key words: calbindin-D_{28k}, glucocorticoid-induced osteoporosis, apoptosis, caspase 3, extracellular signal regulated kinase 1 and 2

INTRODUCTION

GLUCOCORTICOIDS ADMINISTERED therapeutically have both anti-inflammatory and immunosuppressive effects. Glucocorticoids are used in the treatment of autoimmune, pulmonary, and gastrointestinal disorders, as well as in transplantation. A frequent side effect of long-term glucocorticoid therapy is reduction in bone density involving cortical and cancellous bone of the axial skeleton.[1–3] Adverse effects of excessive cortisol have been known for over 60 years.[4] Bone loss resulting from glucocorticoid therapy is a relatively common disorder. It is the third most prevalent form of osteoporosis after postmenopausal and senile osteoporosis.[2] Glucocorticoid-induced reduction in bone density has been proposed to be caused by diminished intestinal calcium absorption, increased renal clearance of calcium, and sex steroid deficiency.[1,3,5,6] Studies in mice and humans, as well as in vitro experiments, strongly suggest that the deleterious effects of glucocorticoids on the skeleton are also caused by direct effects on bone cells.[7–10] Glucocorticoid excess can promote osteoclasts survival, inhibit recruitment and activity of osteoblasts, and cause apoptosis of osteoblasts and osteocytes, resulting in a signif-

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icant reduction in bone formation.[7–10] Increased osteocyte apoptosis has been suggested to lead to accumulation of bone microdamage and to increase bone fragility, resulting in the collapse of the femoral head.[7,11] Thus, the decrease in bone formation resulting from glucocorticoid treatment is associated with suppression of osteoblastogenesis and induction of apoptosis in osteoblasts and osteocytes.

Apoptosis, a term that defines the biological process of programmed cell death, is characterized by morphological alterations including condensation and fragmentation of nuclear chromatin, cytoplasmic contraction, and plasma membrane blebs.[12] A cascade of cysteine proteases known as caspases is important in effecting the apoptotic process. The caspases are synthesized as proenzymes and are activated through autocatalysis or a caspase cascade. Once caspases are activated, they contribute to apoptosis by cleaving an ever-increasing list of cellular target proteins. Caspase 3 is a key mediator of apoptosis and is a common downstream effector of multiple apoptotic signaling pathways.[13] The involvement of caspase 3 activation in glucocorticoid-induced apoptosis of thymocytes is known and has been suggested to be involved in glucocorticoid-induced bone cell apoptosis.[14,15]

The calcium-binding protein, calbindin-D_{28k}, originally thought to function primarily as a facilitator of calcium diffusion in intestine and kidney, has been reported to be present in many other tissues including bone, pancreas, and brain and to play an important role in protecting against apoptotic cell death.[16,17] In earlier studies, the anti-apoptotic property of calbindin-D_{28k} was suggested to be because of its ability to buffer calcium and therefore to inhibit calcium-dependent cytotoxic events.[18–22] In more recent studies, calbindin-D_{28k} was found to protect against TNF-induced apoptosis of osteoblasts.[23] The mechanism involved inhibition of caspase 3 activity.[23] The importance of calbindin-D_{28k} is that, besides the inhibitor of apoptotic proteins (IAPs),[24] calbindin-D_{28k} is the only other known natural, non-oncogenic inhibitor of caspase 3.

In this study, we present evidence for the first time that calbindin-D_{28k} can prevent glucocorticoid-induced osteoblastic and osteocytic cell apoptosis. The mechanism of that protection is at least partially because of calbindin-D_{28k}'s ability to inhibit endogenous caspase 3 activity. Calbindin-D_{28k} was found to inhibit caspase 3, but not other caspases. We also found that the anti-apoptotic effect of calbindin-D_{28k} involves activation of extracellular signal regulated kinase (ERK) 1 and 2. These findings have important implications for the therapeutic intervention of glucocorticoid-induced osteoporosis.

MATERIALS AND METHODS

Calbindin-D_{28k} was purified from rat kidney as previously described.[25] Purified bovine calbindin-D_{9k}, calmodulin, dexamethasone (dex), collagenase, type 1 collagen solution (C8919), total ERK1/2 antibody (M7927), and phosphorylated ERK1/2 antibody (M8159) were purchased from Sigma (St Louis, MO, USA). The secondary anti-mouse and anti-rabbit antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All caspases, caspase inhibitors, caspase substrates, and caspase assay buffers were obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Purified protein kinase C (PKC), phosphotidylserine, and PKC phosphorylation assay dilution buffer II were purchased from Upstate Biotechnology (Lake Placid, NY, USA). [γ-^{32}P]ATP (3,000 Ci/mM) was obtained from DuPont-NEN (Boston, MA, USA). Purified calf intestinal alkaline phosphatase was purchased from New England BioLabs (Beverly, MA, USA). Lipofectamine, MIRUS TransIT-LT1, and ECL reagents were purchased from Life Technologies (Carlsbad, CA, USA), Mirus Corp. (Madison, WI, USA), and Perkin Elmer Life Science (Boston, MA, USA), respectively. The enhanced green fluorescent protein (Clontech Laboratories, Palo Alto, CA, USA), containing the SV40 large T antigen nuclear localization sequence[26] attached to the carboxyl terminus, was provided by Dr Charles O'Brien (University of Arkansas for Medical Sciences, Little Rock, AR, USA). PKC inhibitor Gö6850 and mitogen-activated protein kinase kinase (MEK)1/2 inhibitor UO126 were purchased from Cell Signaling (Beverly, MA, USA) and Calbiochem (San Diego, CA, USA), respectively. Phenol red-free α-MEM, DMEM/F12, phenol red-free DMEM, FBS, bovine calf serum, and PSN antibiotic mixture were purchased from GIBCO Invitrogen Corp. (Carlsbad, CA, USA). Trypsin, sequencing grade, was from Roche Diagnostics (Mannheim, Germany). Phosphothreonine and phosphoserine antibodies were obtained from Sigma.

Cell culture

The murine long bone–derived osteocytic cell line MLO-Y4 was provided by Dr L Bonewald (University of Texas Health Center at San Antonio, San Antonio, TX, USA). Cells were cultured in phenol red-free α-MEM supplemented with 5% FBS, 5% bovine calf serum, and 100 U/ml penicillin. Cells were plated on type 1 collagen–coated plates as previously described.[27] UMR-106 osteoblastic cells were cultured in DMEM/F12 supplemented with 10% FBS and 100 U/ml penicillin. Primary bone cells isolated from neonatal murine calvaria (2- to 5-day-old C57BL/6 mice), totally digested with collagenase as previously described,[28] were cultured in phenol red-free DMEM supplemented with 10% FBS and 100 U/ml penicillin.

Transient transfection of cells

Transient transfection of MLO-Y4 cells and UMR cells was carried out in 12-well culture plates using lipofectamine. Primary bone cells were transfected in 12-well plates using Mirus TransIT-LT1. Cells (0.1×10^6/well) were transfected with 2 μg of the expression vector pREP4 (Invitrogen) or pREP4-calbindin-D_{28k}, together with the expression vector for nuclear green fluorescent protein. The calbindin-D_{28k} expression plasmids were prepared as described before.[23] In the experiments evaluating the requirement of BAD phosphorylation for calbindin-D_{28k} action, 0.2 μg of the wildtype BAD or AAA mutant BAD (dominant negative BAD in which serine 112, 136, and 155 were all mutated to alanine) cloned into a pcDNA3-HA vector was

cotransfected with pREP4 or pREP4-calbindin-D_{28k} and the expression vector for nuclear green fluorescent protein. Wildtype BAD and dominant negative BAD expression vectors were provided by X-M Zhou (Apoptosis Technology, Cambridge, MA, USA).[29] After 48 h, apoptosis was induced by addition of 10^{-6} M dex and quantified as indicated below.

Quantification of apoptotic cells

Apoptotic cells were quantified by nuclear fragmentation assay and trypan blue staining. The percentage of bone cells exhibiting trypan blue staining has previously been shown to correlate with the percentage of apoptotic cells.[14,23,30] MLO-Y4 cells or UMR-106 cells were plated on chamber slides and transfected with the expression vectors pREP4 alone or pREP4-calbindin-D_{28k} together with the expression vector for nuclear green fluorescent protein. Forty-eight hours after transfection, cells were exposed to 10^{-6} M dex for 6 h. Subsequently, cells were fixed, mounted, and examined under Olympus confocal laser scanning microscope. Confocal fluorescent images were obtained using an argon laser of 488 nm wavelength and a 530-nm-long pass barrier filter. The percentage of apoptosis was determined by examining the nuclear morphology of >100 transfected (fluorescent) cells. Primary osteoblasts transfected with the expression vectors pREP4 alone or pREP4-calbindin-D_{28k} were treated with 10^{-6} M dex for 6 h, and cell death was quantified by trypan blue staining. The effect of Gö6850 (1 μM) or UO126 (1 μM) was evaluated by pretreating the cells with the inhibitors for 3 h before a 6-h dex treatment.

Western blot analysis

Total cell lysates were prepared using 4% SDS lysis buffer with protease and phosphatase inhibitors (4% SDS in 100 mM Tris buffer with 0.1 mM sodium vanadate, 1 mM sodium fluoride, and 1 mM sodium molybdate). For Western blot analysis, 50 μg of protein was loaded onto a 12% SDS polyacrylamide gel and separated by electrophoresis. Protein was transferred onto Immun-Blot polyvinylidenedifluoride membranes (Bio-Rad) with semi-dry transfer cell (Bio-Rad). Membranes were incubated overnight at 4°C with appropriate primary antibody (1:1000 dilution in 5% nonfat milk for calbindin-D_{28k} antibody and 1:5000 dilution in 5% nonfat milk for total and phosphorylated ERK1/2 antibodies), followed by incubation for 1 h with the corresponding secondary antibody conjugated with horseradish peroxidase. Blots were developed by enhanced chemiluminescence (ECL).

Endogenous caspase 3 activity

Changes in caspase 3 activity in transfected cells were quantified by analyzing the subcellular localization of a caspase 3 sensor (YFP-caspase 3; Clontech, Palo Alto, CA, USA). For this experiment, cells were transfected with either the pREP4 vector or with the pREP4 containing calbindin-D_{28k}, along with cyan fluorescent protein targeted to the nucleus (nCFP; Clontech), to allow the visualization of the cell nuclei, and an expression vector containing a construct that codifies for a caspase 3 sensor protein, which allows the evaluation of the caspase 3 activity in transfected cells only. The sensor protein contains a dominant N-terminal nuclear export signal, a caspase 3 cleavage site (DEVD), a yellow fluorescent protein (YFP), and a C-terminal nuclear localization signal. When caspase 3 is inactive, the nuclear export sequence prevails and the fluorescent protein is located in the cytosol. When caspase 3 is activated, it cleaves off the protein in the DEVD sequence, removing the nuclear export, and as a consequence, the fluorescent protein is located in the nucleus. Eighteen hours after transfection, cells were treated for 6 h with vehicle or 10^{-6} M dex and fixed in neutral buffer formalin for 8 minutes. The percentage of cells exhibiting co-localization of YFP with nCFP, an index of active caspase 3, was determined using fluorescence microscopy. At least 250 cells from fields selected by systematic random sampling were examined for each experimental condition.

Caspase activity assays

Caspase activities were measured in a cell-free assay by determining the degradation of different colorimetric substrates that contain the amino acid sequences of the cleavage site of different caspases (caspases 1–10).[31] Thirty units of human recombinant active caspase were combined with 200 μM of the appropriate substrates (Ac-YVAD-pNA for caspase 1, Ac-LEHD-pNA for caspases 2, 4, 5, and 9, Ac-DEVD-pNA for caspases 3 and 7, Ac-VEID-pNA for caspase 6, and Ac-IETD-pNA for caspases 8 and 10) in the absence or presence of purified renal calbindin-D_{28k} in 100 μl reaction volume. The assay for caspase 3 activity was also done in the presence or absence of 0.1 μM caspase 3 inhibitor, Asp-Glu-Val-Asp-aldehyde (DEVD-CHO). The activity of all caspases except caspase 9 was examined in assay buffer with 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM EDTA, 10% glycerol, and 10 mM dithiothreitol (DTT). Caspase 9 activity was examined in assay buffer with 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6.5), 10% polyethylene glycol, 0.1% CHAPS, and 10 mM DTT. Enzyme activity was calculated based on absorption values at 405 nm at different time points (0–120 minutes) with a Bio-Rad microplate reader. The inhibition constant (K_i) for caspase 3 by calbindin-D_{28k} was determined by plotting $V_0/V_i - 1$ against inhibitor concentration (I).

Protein–protein interaction studies using surface-enhanced laser desorption/ionization time of-flight mass spectrometry

A surface-enhanced laser desorption/ionization (SELDI) Protein Chip Biology System (Ciphergen Biosystems, Palo Alto, CA, USA), which combines chromatography and mass spectrometry (MS), was used to examine calbindin-D_{28k}-caspase 3 interaction.[32] Five microliters of a 100-μg/ml solution of purified rat renal calbindin-D_{28k} or calmodulin in PBS pH 7.4 was applied to an individual spot of the SELDI chip (Ciphergen PS-1 chip; Ciphergen Biosystems) coated with a preactivated surface and incubated in a humidified chamber for 2 h at room temperature (which allows the peptide to covalently bind to the carbonyl diimi-

dazole moiety on the chip). Residual active sites were blocked by adding 3 μl of 1 M ethanolamine (in PBS, pH 8.0; incubation in a humidified chamber for 20 minutes at room temperature). After washing (PBS, pH 7.4; 0.5% Triton X 100), for analysis of the interaction of calbindin-D_{28k} or calmodulin with caspase 3, 1 μl of a 100-μg/ml solution of activated caspase 3 (from Biomol) was added to each spot and incubated overnight at 4°C in a humidified chamber. After washing (three times with PBS, pH 7.4; 0.2% Triton X 100 to achieve specificity of the protein–protein interaction), sinapinic acid (energy absorbing molecule; 1 μl) was added, and the chip was analyzed on a SELDI mass analyzer MRS-1 with a linear time-of-flight (TOF) mass spectrometer (Ciphergen Biosystems). An accurate mass was determined based on TOF analysis. MS analysis of purified caspase 3 (input) was used as a control for comparison to the mass of the protein bound to calbindin-D_{28k}. Purified caspase 3 was placed on a normal phase protein chip, 1 μl of sinapinic acid in 50% acetonitrile and 0.1% trifluroacetic acid was added, and the protein was analyzed by MS. All experimental conditions described were determined to be optimal. Real-time signal averages of 100 laser shots were used to generate each spectrum.

Phosphorylation of calbindin-D_{28k} by PKC

Purified rat renal calbindin-D_{28k} (1 μg) was mixed with 20 ng purified PKC from rat brain diluted in was diluted 20 mM MOPS (pH 7.2) containing 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM $CaCl_2$, and 200 μM phosphatidylserine (which was sonicated on ice for 1 minute before use) in the presence of 0.25 mM ATP with or without [γ^{32}P]ATP (5 μCi) in a reaction volume of 60 μl and incubated at 30°C. The phosphorylation reaction was stopped by adding 2% SDS. The ^{32}P signal was detected by autoradiography after 3.5–17% SDS-PAGE. Reaction conditions were found to be optimal, and phosphorylation was observed in a substrate concentration-dependent manner. EGTA or staurosporin was added to the reaction as inhibitors of the PKC phosphorylation reaction. Dephosphorylation of calbindin-D_{28k} (1 μg) was carried out using 2–3 U of calf intestinal alkaline phosphatase (CIP) and incubation at 37°C for 5 h.

Tryptic digestion

The 28,000 mw calbindin-D_{28k} band was excised from the SDS gel, cut into pieces, placed in a 1.5-ml microfuge tube, washed with 500 μl of 25 mM ammonium bicarbonate and 50% acetonitrile (ACN), dehydrated in ACN (100%), and dried in a SpeedVac Concentrator (Savant, Farmingdale, NY, USA). The gel pieces were re-swollen in 20 μl ammonium bicarbonate containing 200 ng trypsin at 4°C (sequencing grade; Roche Diagnostics). After 15 minutes, 20 μl of 25 mM ammonium bicarbonate was added to keep the gel pieces moist during trypsin digestion (37°C overnight). To extract the peptides, 50 μl 60% ACN/5% formic acid (FA) was added, and the samples were sonicated for 5 minutes. The separated liquid was dried under vacuum. In some experiments, tryptic samples were separated and purified using a C-18 Vydac RP-HPLC column using a linear 0–45% acetonitrile gradient in 0.1% trifluoroacetic acid. PKC phosphorylation without [γ^{32}P]ATP (2 h at 30°C) and CIP dephosphorylation reactions were conducted before tryptic digestion of calbindin-D_{28k}.

Matrix-Assisted Laser-Desorption Ionization-MS analysis of phosphoproteins

For matrix-assisted laser-desorption ionization (MALDI)-MS, the peptides were purified over a C18 reversed-phase minicolumn filled in micropipette tip (ZipTip C18; Millipore, Bedford, MA, USA). For C18 ZipTip desalting, after extraction and evaporation (see Tryptic digestion), the tryptic peptides were resuspended in 0.1% FA and adsorbed onto the tip by repeated aspiration and dispensing cycles. Peptides were eluted with 60% ACN/0.1% FA. Additionally, before MALDI-MS, to enrich for phosphopeptides, the C18 Zip Tip desalted peptides were further purified by immobilized metal ion affinity chromatography (IMAC) using ZipTip pipette tips according to Millipore's (Bedford, MA, USA) instructions. The peptides were bound using 10% ACN in 0.1% acetic acid and eluted from the IMAC zip tip with 0.3N ammonium hydroxide. After evaporation of the eluant to dryness and resuspension in 50% ACN in water the samples were analyzed by MS. α-Cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI, USA) was used as the matrix. Peptide analysis was performed on a MALDI-TOF MS using a AB1 DE Pro MALDI-TOF instrument (Applied Biosystems, Framingham, MA, USA) according to published protocols.[33] All samples were analyzed in positive linear and reflectron mode using a standard 337-nm nitrogen laser. Up to 250 laser flashes per sample were averaged into a single spectrum, and each spectrum was calibrated using mass standards purchased from Sigma. A difference of less than 0.1% between the observed and calculated mass values was the criteria used to identify the peptides. Mass spectra of PKC- or CIP-treated calbindin-D_{28k} tryptic fragments were analyzed and compared.

Statistical analysis

Data are expressed as means ± SD. The statistical significance of difference between mean values was determined by one-way ANOVA and Student's *t*-test.

RESULTS

Calbindin-D_{28k} prevents glucocorticoid-induced apoptosis of osteocytic and osteoblastic cells

Dex treatment (10^{-6} M for 6 h) induced nuclear fragmentation of MLO-Y4 osteocytic cells transfected with empty pREP4 vector (5.8% versus 16.2% apoptotic cells in −dex and +dex, respectively). In contrast, the pro-apoptotic effect of dex was inhibited in MLO-Y4 cells transfected with pREP4-calbindin-D_{28k} (4.8% versus 5.6% apoptotic cells in −dex and +dex, respectively; Fig. 1A). Dex significantly induced and calbindin-D_{28k} significantly decreased the number of apoptotic cells ($p < 0.05$). To test whether calbindin-D_{28k} can also prevent the dex-induced apoptosis in osteoblastic cells, UMR-106 osteoblastic cells

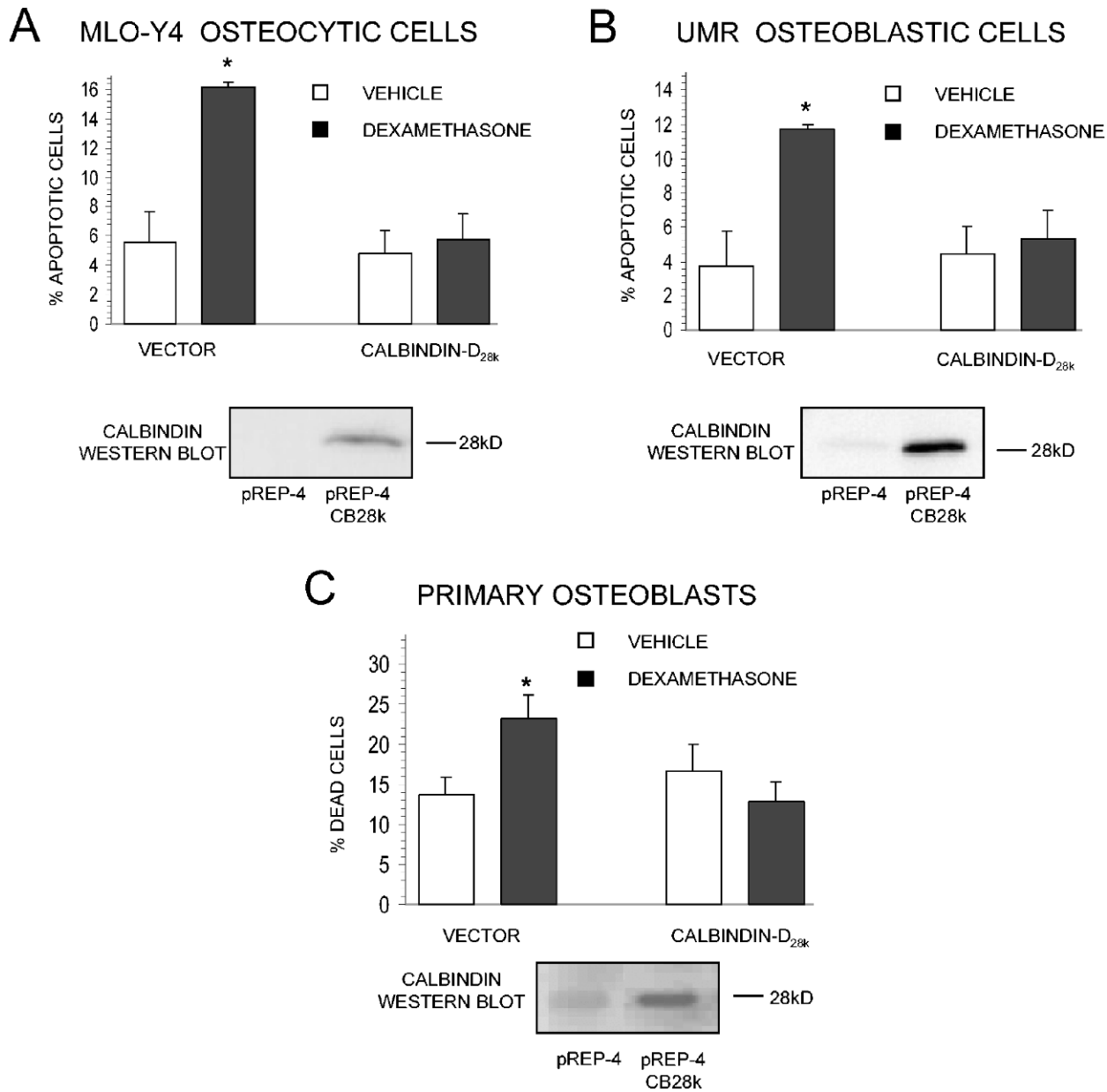

FIG. 1. Calbindin-D_{28k} inhibits glucocorticoid-induced apoptosis of osteocytic and osteoblastic cells. (A) MLO-Y4 osteocytic cells and (B) UMR osteoblastic cells were transiently transfected for 48 h with pREP4 alone (*vector*) or containing the cDNA for calbindin-D_{28k} (*calbindin-D_{28k}*) together with an expression vector for nuclear green fluorescent protein, and apoptosis was induced by addition of dex (10^{-6} M) to cells. After 6 h, cells were examined under an Olympus confocal laser-scanning microscope. Confocal fluorescent images were obtained using an argon laser of 488 nm wavelength and a 530-nm-long pass barrier filter. Apoptosis of (A) MLO-Y4 osteocytic cells and (B) UMR osteoblastic cells after dex treatment were examined by evaluation the nuclear morphology of >100 transfected (fluorescent) cells selected by systematic random. Apoptotic percentage results were obtained in three different experiments. (C) Primary osteoblasts were transiently transfected with pREP4 vector or pREP4-calbindin-D_{28k} for 48 h. Cell death of primary osteoblasts was induced by addition of dex (10^{-6} M) for 6 h, and death percentage was quantified by trypan blue uptake. The pro-apoptotic effect of dex was significantly attenuated ($p < 0.05$) in primary osteoblasts transfected with pREP4-calbindin-D_{28k}. (A–C) Total cell lysates were collected 48 h after the transient transfection for analysis of calbindin-D_{28k} by Western blotting. Although basal levels of calbindin-D_{28k}, above the levels observed in MLO-Y4 cells and UMR cells, were detected in vector transfected primary osteoblasts (Western blot, C), 13.8% dead cells were noted in the vector transfected cells, suggesting that basal calbindin-D_{28k} levels, although detectable, were below the range needed for protection.[22,23]

were transfected with pREP4-calbindin-D_{28k} or pREP4 empty vector (control). Consistent with the results obtained using MLO-Y4 cells, calbindin-D_{28k} significantly ($p < 0.05$) decreased the dex-induced UMR cell apoptosis (3.7% −dex versus 11.7% +dex in vector transfected cells and 4.4% −dex versus 5.3% +dex in calbindin-D_{28k} transfected cells; Fig. 1B). Similar results were obtained in studies of dex-induced cell death of primary osteoblastic cells (Fig.

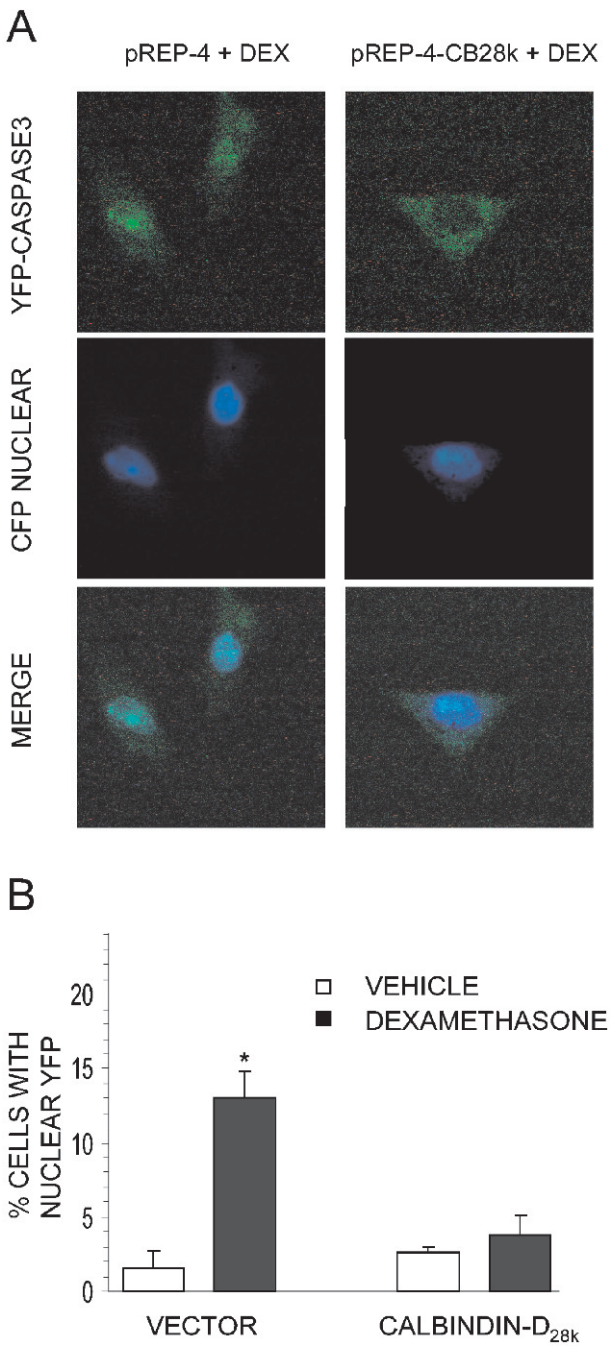

FIG. 2. Inhibition by calbindin-D_{28k} of dex-induced caspase 3 activation in MLO-Y4 osteocytic cells. MLO-Y4 cells were transiently transfected with empty vector or with the expression vector for calbindin-D_{28k}, along with YFP-caspase 3 and nCFP. Eighteen hours after transfection, cells were treated with 10^{-6} M dex for 6 h. The percentage of cells presenting nuclear accumulation of YFP was evaluated under a fluorescence microscope. (A) Fluorescence images show nuclear or cytoplasmic localization of YFP-DEVD caspase 3 sensor. The sensor protein contains a dominant N-terminal nuclear export signal, the caspase 3 cleavage site, DEVD, a yellow fluorescent protein (YFP), as well as a C-terminal nuclear localization signal. When caspase 3 is not activated, the nuclear export signal prevails and the protein is localized in the cytosol (pREP-4-CB28k + DEX). When caspase 3 is activated (pREP-4 + DEX), it cleaves the protein in the DEVD sequence, removing nuclear export, and as a consequence, the fluorescent protein is located in the nucleus. Representative cells from dex-treated cultures transfected with vector alone (pREP-4) or calbindin-D_{28k} (pREP-4-CB28k) are shown. Top panels show the nuclear or cytosolic localization of YFP, respectively; middle panels show the nuclei of the same cells labeled with CFP (to allow visualization of the cell nuclei); and bottom panels correspond to the merged images. (B) Apoptosis was quantified as indicated in the Materials and Methods section. Bars indicate means ± SD of three independent measurements. *$p < 0.05$ vs. vehicle-treated cultures by one-way ANOVA.

1C). Increased expression of calbindin-D_{28k}, as indicated by Western blot (Figs. 1A–1C), correlated with the protection of the transfected cells from glucocorticoid-induced cell death.

Calbindin-D_{28k} protects against dex-induced MLO-Y4 cell apoptosis by inhibiting endogenous caspase-3 activity

For this experiment, cells were transfected with either the pREP4 vector or with the pREP4 containing calbindin-D_{28k} along with an expression vector containing a caspase 3 sensor construct (DEVD-YFP). YFP is localized in the cytoplasm of cells exhibiting inactive caspase 3, whereas activation of caspase 3 induces nuclear translocation of YFP. We found that dex significantly increased the percentage of cells exhibiting nuclear YFP, an index of active caspase 3, in vector transfected cultures, whereas it did not in cultures transfected with calbindin-D_{28k} (Fig. 2).

Calbindin-D_{28k} inhibits caspase 3 activity but not the activity of other caspases

Whether calbindin-D_{28k} can inhibit caspases other than caspase 3 is not known. The IAPs were found to inhibit both caspases 3 and 7, which are downstream caspases in the apoptotic pathway cascade, but not other upstream caspases.[24,34–36] We tested the ability of calbindin-D_{28k} to inhibit the activity of purified active caspases 1–10. Purified caspases were incubated with or without calbindin-D_{28k} (0.09–0.72 μM) in the presence of the appropriate substrates (200 μM). Only caspase 3 activity was markedly reduced by calbindin-D_{28k} (Fig. 3). In contrast, calbindin-D_{28k} did not alter the activity of the other caspases more than 10% even at 1000-fold molar excess. Other calcium-binding proteins, calbindin-D_{9k} and calmodulin, used as control proteins, were not able to inhibit caspase 3 activity. Calbindin-D_{28k} inhibited caspase 3 with an estimated K_i of 200 nM, which is higher than the K_i reported for cIAP-1 (108 nM). However, individual calbindin-D_{28k} peptides (~50) isolated by tryptic digestion fragment followed by reversed-phase HPLC were unable to inhibit caspase 3 when used at concentrations of up to 1000-fold molar excess (data not shown). Calbindin-D_{28k} was found not only to inhibit but also to bind to caspase 3. Using the Ciphergen protein chip system, calbindin-D_{28k} or control protein (calmodulin) was immobilized on the protein chip, and active caspase 3 (a tetramer of two large subunits [17 kDa] and two small subunits [12 kDa][37]) was added on the chip to determine whether there is a specific protein–protein inter-

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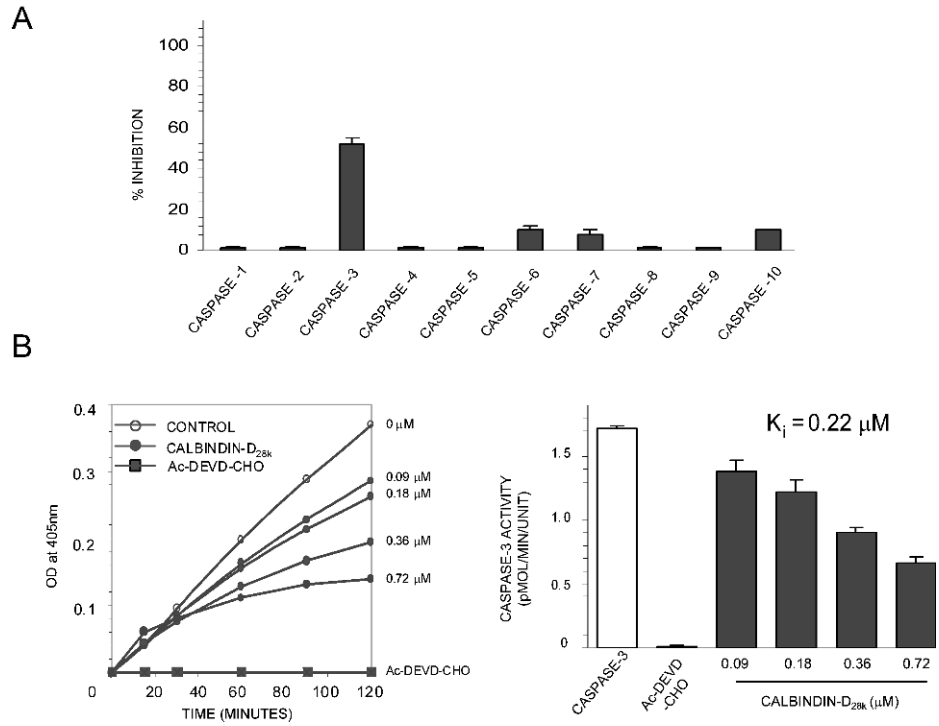

FIG. 3. Calbindin-D_{28k} inhibits caspase 3 activity but not other caspases. (A) Caspase activity was calculated based on absorption values of cleaved substrates at 405 nm. Percentage of caspase activity inhibition was calculated by comparison of caspase activity in the presence and absence of calbindin-D_{28k}. (B) Thirty units of human recombinant active caspase 3 were combined with 200 μM DEVD-pNA in the absence or presence of 0.1 μM Ac-DEVD-CHO inhibitor or purified renal calbindin-D_{28k} (0.09–0.72 μM). Cleaved substrate absorption values were accessed at 405 nm at different time points. Caspase 3 activity was examined in the absence or presence of 0.1 μM Ac-DEVD-CHO inhibitor or purified renal calbindin-D_{28k} (0.09–0.72 μM). The inhibition constant (K_i) for caspase 3 activity by calbindin-D_{28k} was determined by plotting V_0/V_i-1 against inhibitor calbindin-D_{28k} concentration.

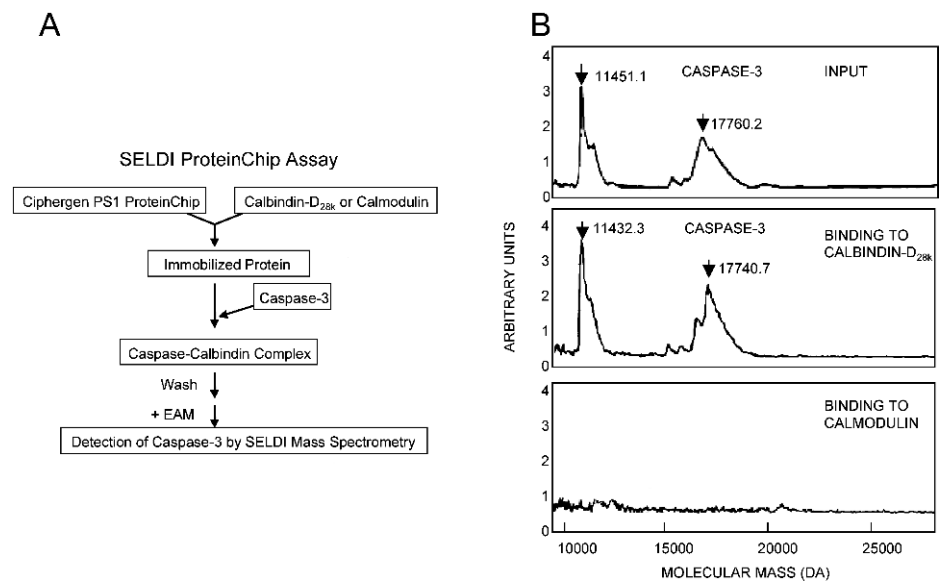

FIG. 4. Direct interaction between calbindin-D_{28k} and caspase 3. (A) Caspase 3 binding to calbindin-D_{28k} was analyzed using the SELDI protein chip. (B) Calbindin-D_{28k} or control protein calmodulin (500 ng of each protein) was immobilized on the PS1 protein chip. Mass spectrometry analysis of input caspase 3 (top panel) was used as a control to compare with proteins bound to calbindin-D_{28k}. Direct interaction between calbindin-D_{28k} and caspase 3 (middle panel) was detected, but not between calmodulin and caspase 3 (bottom panel). The mass of caspase 3 bound to calbindin-D_{28k} was indistinguishable from the mass of purified caspase 3 (input, top panel). Results are representative of at least 8–10 separate experiments. Direct interaction between calbindin-D_{28k} and caspase 3 was also observed when caspase 3 was immobilized on the protein chip (data not shown).

action by SELDI-TOF MS analysis (Fig. 4A). Calbindin-D_{28k} was found to interact directly with active caspase 3 (Fig. 4B, middle panel). The mass of caspase 3 bound to calbindin-D_{28k} was indistinguishable from the mass of purified caspase 3 (Fig. 4, input, top panel). Similar findings were observed with a glutathione-*S*-transferase (GST) pull-down assay using GST-calbindin-D_{28k} and purified activated caspase 3 (data not shown). Calmodulin was used as

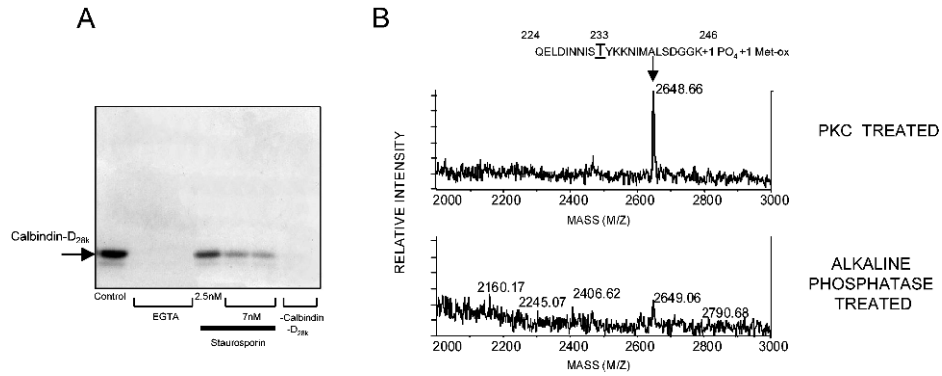

FIG. 5. Rat calbindin-D_{28k} is phosphorylated by PKC. (A) Purified rat renal calbindin-D_{28k} was incubated with PKC in a buffer containing [γ^{32}P] ATP at 30°C for 10 minutes. After SDS-PAGE, phosphorylated proteins were examined by autoradiography. EGTA (2.5 mM) or the PKC inhibitor staurosporin (2.5 nM or 7 nM) blocked phosphorylation. The last lane represents a control incubation (no calbindin-D_{28k} was added). (B) MALDI mass spectra of trypsinized calbindin-D_{28k} after phosphorylation with PKC in vitro (top) or after alkaline phosphatase treatment (bottom). IMAC was used before MALDI-MS to enrich for phosphopeptides. The peak with m/z 2648.66 corresponds to the sequence QELDINNISTKKNIMALSDGGK of rat calbindin-D_{28k} (peptides 224–246). The monophosphorylated peptide 224–246 encompasses the putative phosphorylation site for PKC, T-233.

a negative control in the protein chip assay and was unable to bind to caspase 3 (Fig. 4B, bottom panel).

Rat calbindin-D_{28k} is phosphorylated by PKC

Phosphorylation has been known to play an important role in regulation of the function of proteins involved in the apoptotic pathway.[38–40] Consensus phosphorylation sites for PKC are present in calbindin-D_{28k} at Thr(106) and Thr (233).[41] Thus, we investigated whether phosphorylation has a role in regulating the anti-apoptotic function of calbindin-D_{28k}. We found that calbindin-D_{28k} can act as a substrate of PKC phosphorylation and that EGTA or the PKC inhibitor staurosporin blocked that phosphorylation (Fig. 5A). Analysis by MALDI MS of tryptic peptides revealed an ion fragment that is present in the in vitro PKC-phosphorylated calbindin-D_{28k} spectrum but is missing in the spectrum of alkaline phosphatase–treated calbindin-D_{28k}. The relevant phospho-peptide deduced from the tryptic map of calbindin-D_{28k} and the MS profile corresponded to amino acids 224–246 (QELDINNIS***T***YKKNIMALSDGGK) of rat calbindin-D_{28k}. Within this peptide is a PKC consensus motif (STYKK), suggesting that the PKC phosphorylation site is at Thr(233) (Fig. 5B, underlined). In addition, using Western blot analysis, in vitro PKC-phosphorylated calbindin-D_{28k} was detected with a phosphothreonine but not a phosphoserine antibody (data not shown). To test whether PKC phosphorylation affects calbindin-D_{28k}'s ability to inhibit caspase 3, caspase 3 activity was assayed in the presence of calbindin-D_{28k} or alkaline phosphatase–treated calbindin-D_{28k}. Alkaline phosphatase treatment did not affect calbindin-D_{28k}'s ability to inhibit caspase 3 activity (Fig. 6A). Alkaline phosphatase itself has no effect on caspase 3 activity (data not shown). Using the Ciphergen protein chip system, we also found that alkaline phosphatase–treated calbindin-D_{28k} was still able to interact with active caspase 3 (data not shown). Additionally, in the presence of the specific PKC inhibitor Gö6850 (1 μM), calbindin-D_{28k} was still able to protect against dex-induced MLO-Y4 cell death (Fig. 6B). This result indicates that inhibition of PKC does not affect calbindin-D_{28k}'s ability to protect against dex-induced bone cell death.

Anti-apoptotic effect of calbindin-D_{28k} involves ERK activation

Because calbindin-D_{28k} has been reported to be involved in the regulation of signaling pathways,[42,43] we asked whether the anti-apoptotic effect of calbindin-D_{28k} may also be associated with activation of ERK 1 and 2, which has been shown to promote bone cell survival.[14] MLO-Y4 cells were transfected with pREP4-calbindin-D_{28k} or pREP4 empty vector (control). Forty-eight hours after transfection, phosphorylated ERK and total ERK protein levels were examined by Western blot analysis. In calbindin-D_{28k} transfected cells, there was an increase in the phosphorylated fraction of ERK1/2 compared with vector transfected cells. In both calbindin-D_{28k} and vector transfected cells, total ERK1/2 levels were similar (Fig. 7A). Pretreatment of MLO-Y4 cells with the specific MEK1/2 inhibitor UO126 before dex treatment blocked the activation of ERK1/2 in calbindin-D_{28k} transfected cells and decreased the anti-apoptotic effect of calbindin-D_{28k} (Fig. 7B). These results suggest an association between the calbindin-D_{28k}'s anti-apoptotic effect and ERK1/2 activation.

Anti-apoptotic effect of calbindin-D_{28k} involves BAD phosphorylation

To determine whether BAD phosphorylation is downstream of ERK1/2 activation and is involved in the protective effect of calbindin-D_{28k}, the effect of calbindin-D_{28k} was evaluated in MLO-Y4 cells cotransfected with wildtype BAD or AAA mutant BAD, which cannot be phosphorylated. A significant decrease in calbindin-D_{28k}'s ability to prevent dex-induced MLO-Y4 cells apoptosis was observed when cells were transfected with AAA mutant BAD (Fig. 8). Because the AAA mutant BAD cannot be phosphory-

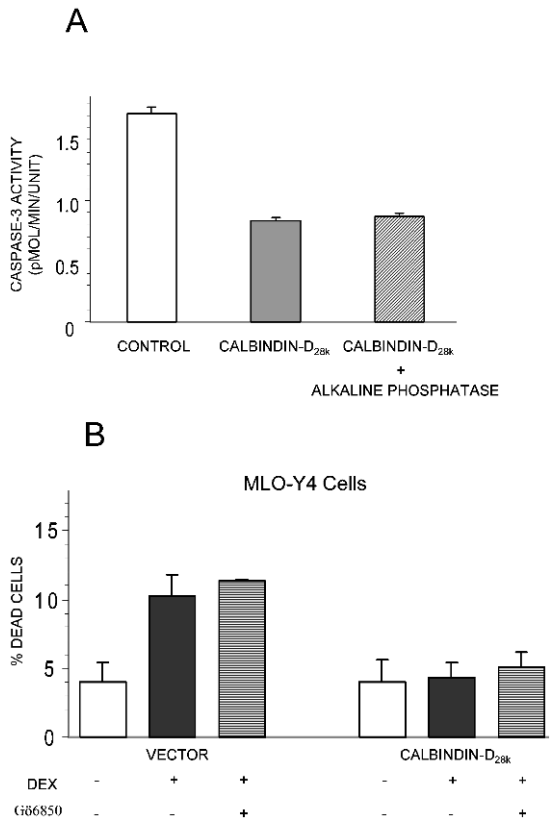

FIG. 6. PKC phosphorylation does not affect calbindin-D_{28k}'s ability to inhibit caspase 3. (A) Thirty units of human recombinant active caspase 3 were combined with 200 μM DEVD-pNA in the absence or presence of purified renal calbindin-D_{28k} (0.36 μM), or presence of purified renal calbindin-D_{28k} (0.36 μM) pretreated with alkaline phosphatase. Caspase 3 activity was calculated based on absorption value of cleaved substrates at 405 nm. (B) MLO-Y4 osteocytic cells were transiently transfected with pREP4 vector or pREP4-calbindin-D_{28k} for 48 h. Cell death was induced by addition of dex (10^{-6} M) for 6 h, and death percentage was quantified by trypan blue uptake. Effect of PKC inhibitor Gö6850 (1 μM) was evaluated by pretreating the cells with the inhibitor for 3 h before dex treatment.

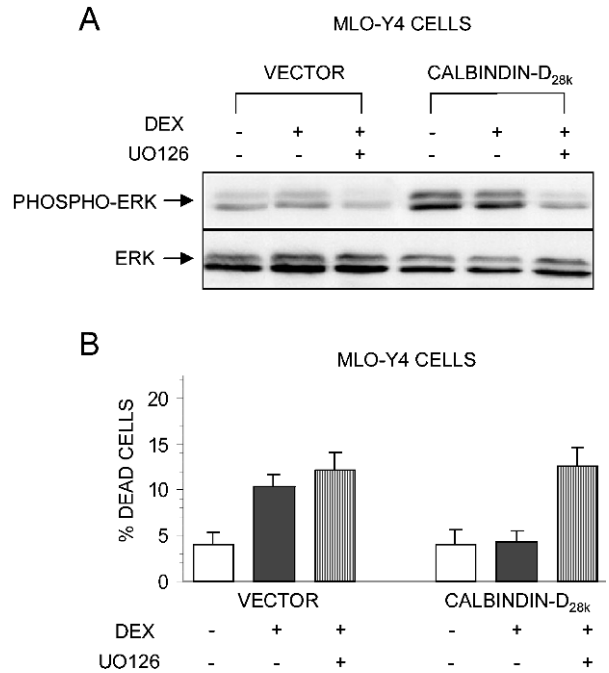

FIG. 7. Anti-apoptotic effect of calbindin-D_{28k} involves ERK activation. (A) MLO-Y4 osteocytic cells were transiently transfected with pREP4 vector or pREP4-calbindin-D_{28k}. Forty-eight hours after transfection, cells were treated with vehicle or MEK1/2 inhibitor UO126 (1 μM) for 3 h, followed by a 6-h treatment of dex (10^{-6} M). Cell lysates were collected for analysis of phospho-ERK1/2 and total ERK1/2 by Western blotting. Similar results were observed in four separate experiments. (B) Death percentage of MLO-Y4 cells was quantified by trypan blue uptake.

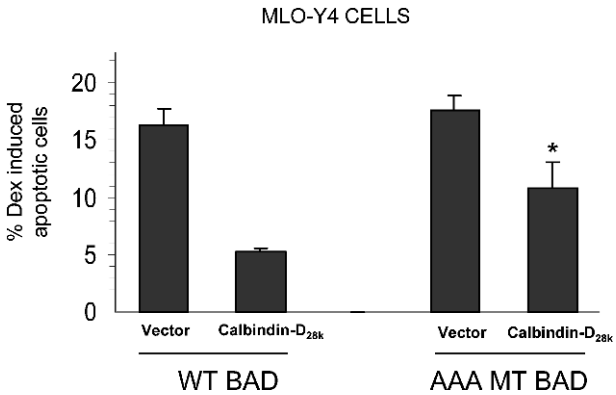

FIG. 8. Anti-apoptotic effect of calbindin-D_{28k} involves BAD phosphorylation. MLO-Y4 cells were transiently cotransfected with pREP4-calbindin-D_{28k} or pREP4 vector and wildtype BAD or AAA mutant BAD, together with an expression vector for nuclear green fluorescent protein. Forty-eight hours after transfection, cells were treated with dex (10^{-6} M) for 6 h. Apoptotic percentage of dex-induced MLO-Y4 cells was quantified. Bars indicate the mean $\pm$ SD (*$p < 0.05$ vs. cells transfected with calbindin-D_{28k} and WT BAD).

lated, this result suggests that the protective effect of calbindin-D_{28k} involves, at least in part, BAD phosphorylation.

DISCUSSION

These studies show for the first time that calbindin-D_{28k} can inhibit glucocorticoid-induced osteoblastic and osteocytic cell apoptosis. Prevention of glucocorticoid-induced osteoblast/osteocyte cell death by calbindin-D_{28k} is correlated with calbindin-D_{28k}'s ability to inhibit caspase 3. The anti-apoptotic effect of calbindin-D_{28k} also involves activation of ERK, and BAD is at least one of the downstream factors phosphorylated by activated ERK.

Our study indicates that, similar to findings in thymocytes,[13] glucocorticoid-induced apoptosis of bone cells involves caspase 3 activation. Calbindin-D_{28k} interfered with the glucocorticoid-induced apoptotic pathway, in part, by direct inhibition of caspase 3. In previous studies, we showed that calbindin-D_{28k} protects against TNF-α induced osteoblastic cell apoptosis also, at least in part, by inhibiting caspase 3 activity.[23] Because chelation of calcium by EGTA as well as other calcium binding proteins did not

inhibit caspase 3, these results suggest that calbindin-D_{28k}'s ability to inhibit caspase 3 is unrelated to calbindin-D_{28k}'s ability to buffer calcium.[23] Thus, calbindin-D_{28k} has a major role in protecting against cellular degeneration in different cell types including bone cells, and calbindin-D_{28k}'s anti-apoptotic properties result not only from buffering calcium as previously reported for neuronal cells, pancreatic β cells, and lymphocytes,[18–20,22] but also from its ability to inhibit caspase 3. Besides calbindin-D_{28k}, Bcl-2 can also inhibit apoptosis. Bcl-2 inhibits apoptosis by inhibiting the release of cytochrome c, which can trigger apoptosis.[44] In addition, Bcl-2 has been reported to bind to pro-apoptotic proteins such as Bax.[45] Recent studies indicate that estrogen can prevent glucocorticoid-induced apoptosis in osteoblasts by increasing the Bcl-2/Bax ratio.[46] Calbindin-D_{28k}'s ability to bind to the mature form of caspase 3 and inhibit its activity does not resemble the mechanism of action of Bcl-2, but rather, it resembles the mechanism by which the IAP family of proteins acts to inhibit apoptosis. The IAPs block the activity of specific caspases including caspase 3.[24,34] Calbindin-D_{28k} inhibited caspase 3 with a K_i of 220 nM, which is high compared with the K_i reported for the inhibition of the cIAP-1 and cIAP-2 proteins (30–120 nM),[34] but lower than the K_i reported for the viral protein CrmA, which also inhibits caspase 3 ($\sim$500 nM).[47] These findings suggest that structural differences exist between these proteins that affect how well they bind to and inhibit caspase 3. Structural differences are also suggested by the lack of a baculoviral IAP repeat (BIR) domain in calbindin-D_{28k}. This domain of $\sim$70 amino acids in the N-terminal region of the IAP family of proteins is thought to play a role together with the linker region in caspase interaction and inhibition.[48,49] In addition, individual calbindin-D_{28k} peptides isolated by tryptic digestion followed by reversed-phase HPLC were unable to inhibit caspase 3, suggesting the importance of secondary structure for the inhibition of caspase 3 by calbindin-D_{28k}. In contrast to the viral proteins p35 and CrmA, which are potent inhibitors of several caspases, the IAP and XIAP proteins are more selective (they inhibit caspases 3 and 7), and calbindin-D_{28k} seems to be selective for only caspase 3.[34,47,50] Although caspases 3 and 7 share 53% homology (the highest overall identity among the members of the caspase family),[34] calbindin-D_{28k}, unlike the IAPs, did not affect caspase 7 activity, further suggesting differences in the contact regions of the inhibitory proteins to caspase. Recent studies have indicated an interaction between an IAP family member, NAIP, and a brain calcium-binding protein (hippocalcin), and the coexpression of these two proteins enhanced both the protective effect of each protein and caspase 3/7 inhibitory activity.[51] It will be of interest in future studies to determine if there is a similar interrelationship between calbindin-D_{28k} and the IAP family members. Because our study has shown that glucocorticoid-induced bone cell death involves an increase in caspase 3 activity, insights into the mechanisms by which calbindin-D_{28k} inhibits caspase 3 may prove important for the therapeutic intervention of glucocorticoid-induced osteoporosis.

The anti-apoptotic effects of calbindin-D_{28k} were also associated with activation of ERKs. Previous studies have shown that the protective effects of bisphosphonates and calcitonin against glucocorticoid-induced osteocyte and osteoblast apoptosis are also associated with activation of ERK.[14] In addition, activated ERK was reported to mediate the anti-apoptotic effect of 17β-estradiol on osteoblasts and osteocytes.[52] Activated ERK phosphorylates the pro-apoptotic protein Bad. Phosphorylation of Bad inhibits binding of Bad to Bcl-X_L and frees Bcl-X_L to promote survival.[53] Because a Bad mutant lacking the ability to undergo phosphorylation by activated ERK has been reported to abrogate the anti-apoptotic action of estrogen on osteoblasts and osteocytes, it was suggested that Bad phosphorylation by activated ERK is required for the anti-apoptotic effect of estrogen.[54] Our results indicate that protection against glucocorticoid-induced osteoblast and osteocyte cell death by calbindin-D_{28k}, which is associated with ERK activation, involves at least in part phosphorylation of Bad. Thus, we have identified a dual role of calbindin-D_{28k} in inhibiting glucocorticoid-induced apoptosis of bone cells: caspase 3 inhibition and activation of ERK. XIAP, a member of the IAP family of proteins, has recently been reported to block apoptosis by both inhibiting caspase activity and by activating c-Jun N-terminal kinase (JNK)1.[55] Activation of JNK1 did not necessarily correlate with the ability of IAPs to inhibit caspases. XIAP and c-IAP-2 are both capable of inhibiting caspases, but transient transfection of XIAP and not c-IAP-2 in COS or 293 cells was able to activate JNK1, suggesting that XIAP anti-apoptotic properties are achieved by two separate mechanisms.[55,56] Similar to XIAP, cell survival by calbindin-D_{28k} seems to be mediated by more than one pathway. Thus, calbindin-D_{28k} is a multifunctional protein that employs more than one pathway, including caspase 3 inhibition and ERK activation, as mechanisms to inhibit glucocorticoid-induced apoptosis of osteoblasts and osteocytes. Increasing evidence suggests that calbindin-D_{28k} is a key factor in the anti-apoptotic process. Understanding the mechanisms involved in calbindin-D_{28k}'s anti-apoptotic function will be important to use these mechanisms for modulation of glucocorticoid-induced bone cell apoptosis.

Cell survival is known to involve post-transcriptional regulation. In addition to Bad phosphorylation, the activity of pro-survival members of the Bcl-2 protein family is also regulated by phosphorylation. Phosphorylation has been reported to negatively regulate Bcl-X_L activity and to both activate and repress Bcl-2 activity.[38,39] Phosphorylation of caspase 9 results in its inactivation and suppression of apoptosis.[40] Although calbindin-D_{28k} can be phosphorylated by PKC, the phosphorylation site and the significance of the phosphorylation of calbindin-D_{28k} were not known.[41] Our study suggests by MALDI-MS that the calbindin-D_{28k} PKC phosphorylation site is at Thr(233). However, further studies are needed to identify definitively the phosphorylated peptide. Additionally, we provide evidence that calbindin-D_{28k} is a phosphoprotein and show that phosphorylation of calbindin-D_{28k}, unlike the phosphorylation of the Bcl-2 protein family, does not affect its regulation of apoptosis. The significance of calbindin-D_{28k} phosphorylation may not be to regulate its anti-apoptotic activity but rather to enhance its expression by altering its stability.[41]

Thus, activation of PKC by growth factors may result in enhanced levels of calbindin-D_{28k} and greater protection. Because of the protective effect of calbindin-D_{28k}, it will be important in future studies to understand more about calbindin-D_{28k}'s regulation in bone. It is known that glucocorticoids decrease insulin-like growth factor (IGF)-1, which can inhibit osteoblast apoptosis, resulting in a decreased bone formation rate.[8,57,58] It is of interest that IGF-1 induces the expression of neuronal calbindin-D_{28k}.[59] It will be of interest in future studies to determine whether IGF-1 can enhance the expression of calbindin-D_{28k} in bone cells and whether the protective effect of IGF-1 may be mediated in part through an increase in calbindin-D_{28k}.

In conclusion, glucocorticoid-induced osteoporosis is a common, clinically relevant problem. Effects of glucocorticoids include inhibitory effects on bone forming cells. Our study suggests that calbindin-D_{28k} employs at least two mechanisms, caspase 3 inhibition and ERK activation, to inhibit glucocorticoid-induced apoptosis of bone cells. This study is the first to show the prevention of glucocorticoid-induced bone cell death by calbindin-$D_{28k.}$ Calbindin-D_{28k}, a natural non-oncogenic protein, could be an important target in the therapeutic intervention of glucocorticoid-induced osteoporosis.

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REFERENCES

1. Patschan D, Loddenkemper K, Buttgereit F 2001 Molecular mechanisms of glucocorticoid-induced osteoporosis. Bone **29:**498–505.
2. Luckert B 1996 Glucocorticoid-induced osteoporosis. In: Marcus R, Feldman D, Kelsey J (eds.) Osteoporosis. Academic Press, San Diego, CA, USA, pp. 801–820.
3. Reid IR 1997 Glucocorticoid osteoporosis–mechanisms and management. Eur J Endocrinol **137:**209–217.
4. Cushing H 1932 The basophil adenomas of the pituitary body and their clinical manifestations(pituitary basophilism). Bull Johns Hopkins Hosp **50:**137–195.
5. Lukert BP, Raisz LG 1990 Glucocorticoid-induced osteoporosis: Pathogenesis and management. Ann Intern Med **112:**352–364.
6. Feher JJ, Wasserman RH 1979 Intestinal calcium-binding protein and calcium absorption in cortisol-treated chicks: Effects of vitamin D3 and 1, 25-dihydroxyvitamin D3. Endocrinology **104:**547–551.
7. Manolagas SC 2000 Birth and death of bone cells: Basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocr Rev **21:**115–137.
8. Canalis E, Delany AM 2002 Mechanisms of glucocorticoid action in bone. Ann NY Acad Sci **966:**73–81.
9. Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC 1998 Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest **102:**274–282.
10. Weinstein RS, Chen JR, Powers CC, Stewart SA, Landes RD, Bellido T, Jilka RL, Parfitt AM, Manolagas SC 2002 Promotion of osteoclast survival and antagonism of bisphosphonate-induced osteoclast apoptosis by glucocorticoids. J Clin Invest **109:**1041–1048.
11. Weinstein RS, Nicholas RW, Manolagas SC 2000 Apoptosis of osteocytes in glucocorticoid-induced osteonecrosis of the hip. J Clin Endocrinol Metab **85:**2907–2912.
12. Green DR 1998 Apoptotic pathways: The roads to ruin. Cell **94:**695–698.
13. Thornberry NA, Lazebnik Y 1998 Caspases: Enemies within. Science **281:**1312–1316.
14. Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T 1999 Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. J Clin Invest **104:**1363–1374.
15. Mann CL, Hughes FM Jr, Cidlowski JA 2000 Delineation of the signaling pathways involved in glucocorticoid-induced and spontaneous apoptosis of rat thymocytes. Endocrinology **141:**528–538.
16. Christakos S, Gabrielides C, Rhoten WB 1989 Vitamin D-dependent calcium binding proteins: Chemistry, distribution, functional considerations, and molecular biology. Endocr Rev **10:**3–26.
17. Christakos S, Beck J, Hyllner SJ 1997 Calbindin-D28k. In: Feldman D, Glorieux F, Pike JW (eds.) Vitamin D. Academic Press, San Diego, CA, USA, pp. 209–221.
18. Mattson MP, Rychlik B, Chu C, Christakos S 1991 Evidence for calcium-reducing and excito-protective roles for the calcium-binding protein calbindin-D28k in cultured hippocampal neurons. Neuron **6:**41–51.
19. Guo Q, Christakos S, Robinson N, Mattson MP 1998 Calbindin D28k blocks the proapoptotic actions of mutant presenilin 1: Reduced oxidative stress and preserved mitochondrial function. Proc Natl Acad Sci USA **95:**3227–3232.
20. Dowd DR, MacDonald PN, Komm BS, Haussler MR, Miesfeld RL 1992 Stable expression of the calbindin-D28K complementary DNA interferes with the apoptotic pathway in lymphocytes. Mol Endocrinol **6:**1843–1848.
21. Turner PR, Mefford S, Christakos S, Nissenson RA 2000 Apoptosis mediated by activation of the G protein-coupled receptor for parathyroid hormone (PTH)/PTH-related protein (PTHrP). Mol Endocrinol **14:**241–254.
22. Rabinovitch A, Suarez-Pinzon WL, Sooy K, Strynadka K, Christakos S 2001 Expression of calbindin-D(28k) in a pancreatic islet beta-cell line protects against cytokine-induced apoptosis and necrosis. Endocrinology **142:**3649–3655.
23. Bellido T, Huening M, Raval-Pandya M, Manolagas SC, Christakos S 2000 Calbindin-D28k is expressed in osteoblastic cells and suppresses their apoptosis by inhibiting caspase-3 activity. J Biol Chem **275:**26328–26332.
24. Deveraux QL, Reed JC 1999 IAP family proteins–suppressors of apoptosis. Genes Dev **13:**239–252.
25. Christakos S, Rhoten WB, Feldman SC 1987 Rat calbindin D28K: Purification, quantitation, immunocytochemical localization, and comparative aspects. Methods Enzymol **139:**534–551.
26. Kalderon D, Roberts BL, Richardson WD, Smith AE 1984 A short amino acid sequence able to specify nuclear location. Cell **39:**499–509.
27. Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF 1997 Establishment of an osteocyte-like cell line, MLO-Y4. J Bone Miner Res **12:**2014–2023.
28. Bellido T, Borba VZ, Roberson P, Manolagas SC 1997 Activation of the Janus kinase/STAT (signal transducer and activator of transcription) signal transduction pathway by interleukin-6-type cytokines promotes osteoblast differentiation. Endocrinology **138:**3666–3676.
29. Zhou XM, Liu Y, Payne G, Lutz RJ, Chittenden T 2000 Growth factors inactivate the cell death promoter BAD by phosphorylation of its BH3 domain on Ser155. J Biol Chem **275:**25046–25051.
30. Jilka RL, Weinstein RS, Bellido T, Parfitt AM, Manolagas SC 1998 Osteoblast programmed cell death (apoptosis): Modulation by growth factors and cytokines. J Bone Miner Res **13:**793–802.
31. Schlegel J, Peters I, Orrenius S, Miller DK, Thornberry NA, Yamin TT, Nicholson DW 1996 CPP32/apopain is a key interleu-

kin 1 beta converting enzyme-like protease involved in Fas-mediated apoptosis. J Biol Chem **271:**1841–1844.
32. Issaq HJ, Veenstra TD, Conrads TP, Felschow D 2002 The SELDI-TOF MS approach to proteomics: Protein profiling and biomarker identification. Biochem Biophys Res Commun **292:** 587–592.
33. Shou W, Verma R, Annan RS, Huddleston MJ, Chen SL, Carr SA, Deshaies RJ 2002 Mapping phosphorylation sites in proteins by mass spectrometry. Methods Enzymol **351:**279–296.
34. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC 1997 The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J **16:**6914–6925.
35. Chai J, Shiozaki E, Srinivasula SM, Wu Q, Datta P, Alnemri ES, Shi Y, Dataa P 2001 Structural basis of caspase-7 inhibition by XIAP. Cell **104:**769–780.
36. Fernandes-Alnemri T, Litwack G, Alnemri ES 1995 Mch2, a new member of the apoptotic Ced-3/Ice cysteine protease gene family. Cancer Res **55:**2737–2742.
37. Mittl PR, Di Marco S, Krebs JF, Bai X, Karanewsky DS, Priestle JP, Tomaselli KJ, Grutter MG 1997 Structure of recombinant human CPP32 in complex with the tetrapeptide acetyl-Asp-Val-Ala-Asp fluoromethyl ketone. J Biol Chem **272:**6539–6547.
38. Basu A, Haldar S 2003 Identification of a novel Bcl-xL phosphorylation site regulating the sensitivity of taxol- or 2-methoxyestradiol-induced apoptosis. FEBS Lett **538:**41–47.
39. Ruvolo PP, Deng X, May WS 2001 Phosphorylation of Bcl2 and regulation of apoptosis. Leukemia **15:**515–522.
40. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC 1998 Regulation of cell death protease caspase-9 by phosphorylation. Science **282:**1318–1321.
41. Gagnon A, Welsh J 1997 Modulation and phosphorylation of calbindin-D28K correlates with protein kinase C activation. Biochem Cell Biol **75:**33–40.
42. Choi WS, Chun SY, Markelonis GJ, Oh TH, Oh YJ 2001 Overexpression of calbindin-D28K induces neurite outgrowth in dopaminergic neuronal cells via activation of p38 MAPK. Biochem Biophys Res Commun **287:**656–661.
43. Berggard T, Szczepankiewicz O, Thulin E, Linse S 2002 Myo-inositol monophosphatase is an activated target of calbindin D28k. J Biol Chem **277:**41954–41959.
44. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X 1997 Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked. Science **275:**1129–1132.
45. Korsmeyer SJ 1995 Regulators of cell death. Trends Genet **11:** 101–105.
46. Gohel A, McCarthy MB, Gronowicz G 1999 Estrogen prevents glucocorticoid-induced apoptosis in osteoblasts in vivo and in vitro. Endocrinology **140:**5339–5347.
47. Zhou Q, Snipas S, Orth K, Muzio M, Dixit VM, Salvesen GS 1997 Target protease specificity of the viral serpin CrmA. Analysis of five caspases. J Biol Chem **272:**7797–7800.
48. Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS, Reed JC 1998 A single BIR domain of XIAP sufficient for inhibiting caspases. J Biol Chem **273:**7787–7790.
49. Huang Y, Park YC, Rich RL, Segal D, Myszka DG, Wu H 2001 Structural basis of caspase inhibition by XIAP: Differential roles of the linker versus the BIR domain. Cell **104:**781–790.
50. Bump NJ, Hackett M, Hugunin M, Seshagiri S, Brady K, Chen P, Ferenz C, Franklin S, Ghayur T, Li P, Licari P, Mankovich J, Shi L, Greenberg AH, Miller LK, Wong WW 1995 Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. Science **269:**1885–1888.
51. Mercer EA, Korhonen L, Skoglosa Y, Olsson PA, Kukkonen JP, Lindholm D 2000 NAIP interacts with hippocalcin and protects neurons against calcium-induced cell death through caspase-3-dependent and -independent pathways. EMBO J **19:**3597–3607.
52. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC 2001 Nongenotropic, sex-nonspecific signaling through the estrogen and androgen receptors: Dissociation from transcriptional activity. Cell **104:**719–730.
53. Scheid MP, Schubert KM, Duronio V 1999 Regulation of bad phosphorylation and association with Bcl-x(L) by the MAPK/Erk kinase. J Biol Chem **274:**31108–31113.
54. Kousteni S, Han L, Chen JR, Almeida M, Plotkin LI, Bellido T, Manolagas SC 2003 Kinase-mediated regulation of common transcription factors accounts for the bone-protective effects of sex steroids. J Clin Invest **111:**1651–1664.
55. Sanna MG, da Silva Correia J, Ducrey O, Lee J, Nomoto K, Schrantz N, Deveraux QL, Ulevitch RJ 2002 IAP suppression of apoptosis involves distinct mechanisms: The TAK1/JNK1 signaling cascade and caspase inhibition. Mol Cell Biol **22:**1754–1766.
56. Sanna MG, Duckett CS, Richter BW, Thompson CB, Ulevitch RJ 1998 Selective activation of JNK1 is necessary for the anti-apoptotic activity of hILP. Proc Natl Acad Sci USA **95:**6015–6020.
57. Delany AM, Durant D, Canalis E 2001 Glucocorticoid suppression of IGF I transcription in osteoblasts. Mol Endocrinol **15:**1781–1789.
58. Hill PA, Tumber A, Meikle MC 1997 Multiple extracellular signals promote osteoblast survival and apoptosis. Endocrinology **138:**3849–3858.
59. Nieto-Bona MP, Busiguina S, Torres-Aleman I 1995 Insulin-like growth factor I is an afferent trophic signal that modulates calbindin-28kD in adult Purkinje cells. J Neurosci Res **42:**371–376.

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