

Constitutive Expression of Calreticulin in Osteoblasts Inhibits Mineralization

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Abstract. Recent studies have shown that the multifunctional protein calreticulin can localize to the cell nucleus and regulate gene transcription via its ability to bind a protein motif in the DNA-binding domain of nuclear hormone receptors. A number of known modulators of bone cell function, including vitamin D, act through this receptor family, suggesting that calreticulin may regulate their action in bone cells. We have used a gain-of-function strategy to examine this putative role of calreticulin in MC3T3-E1 osteoblastic cells. Purified calreticulin inhibited the binding of the vitamin D receptor to characterized vitamin D response elements in gel retardation assays. This inhibition was due to direct protein-protein interactions between the vitamin D receptor and calreticulin. Expression of calreticulin transcripts declined during MC3T3-E1 osteoblastic differentiation. MC3T3-E1 cells were transfected with calreticulin expression vectors; stably transfected cell lines overexpressing recombinant cal-

reticulin were established and assayed for vitamin D-induced gene expression and the capacity to mineralize. Constitutive calreticulin expression inhibited basal and vitamin D-induced expression of the osteocalcin gene, whereas osteopontin gene expression was unaffected. This pattern mimicked the gene expression pattern observed in parental cells before down-regulation of endogenous calreticulin expression. In long-term cultures of parental or vector-transfected cells, $1\alpha,25$ -dihydroxyvitamin D₃ ($1,25[\text{OH}]_2\text{D}_3$) induced a two- to threefold stimulation of ⁴⁵Ca accumulation into the matrix layer. Constitutive expression of calreticulin inhibited the $1,25(\text{OH})_2\text{D}_3$ -induced ⁴⁵Ca accumulation. This result correlated with the complete absence of mineralization nodules in long-term cultures of calreticulin-transfected cells. These data suggest that calreticulin can regulate bone cell function by interacting with specific nuclear hormone receptor-mediated pathways.

CALRETICULIN was initially identified as the major Ca²⁺ storage protein in the sarcoplasmic reticulum of skeletal muscle (Ostwald and MacLennan, 1974). Subsequent work has revealed that the protein can also be detected in the endoplasmic reticulum of nonmuscle tissues (Fliegel et al., 1989; Opas et al., 1991). Calreticulin possesses many diverse functional domains such as high affinity, low capacity- and low affinity, high capacity-Ca²⁺-binding sites, a COOH-terminal KDEL endoplasmic reticulum retention signal, and a nuclear localization signal (for review see Michalak et al., 1992).

Recent studies have shown that calreticulin can indeed be detected in the nucleus (Opas et al., 1991; Dedhar, 1994; Dedhar et al., 1994). Although the precise molecular mechanisms allowing the escape of calreticulin from the endoplasmic reticulum and regulating its translocation to

the nucleus are currently unknown, two elegant series of experiments have elucidated the function of nuclear calreticulin (Burns et al., 1994; Dedhar et al., 1994). The protein recognizes a conserved amino acid motif, KXFFK/RR (where X is either G, A, or V), common to the DNA-binding domain of all known members of the nuclear hormone receptor family (Burns et al., 1994; Dedhar et al., 1994). These receptors act as ligand-inducible transcriptional regulators (Fuller, 1991). By interacting with the conserved motif located between the DNA-binding zinc fingers (for review see Glass, 1994) of the nuclear receptors, calreticulin inhibits their capacity to bind DNA in vitro and to activate gene expression in vivo (Burns et al., 1994; Dedhar et al., 1994). The ability of calreticulin to inhibit nuclear hormone receptor activity has been directly demonstrated for the glucocorticoid (Burns et al., 1994), the androgen (Dedhar et al., 1994), and the retinoic acid receptors (Dedhar et al., 1994). The functional importance of this inhibition has been demonstrated by the observation that calreticulin overexpression can influence the retinoic acid-dependent dif-

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ferentiation of multipotential embryonal carcinoma cells (Dedhar et al., 1994). Interestingly, a similar KLGFFKR amino acid motif is also found in the cytoplasmic domains of all integrin α subunits (Rojiani et al., 1991), and calreticulin has also been shown to interact with α -integrins through this sequence (Leung-Hagesteijn et al., 1994). These results suggest the existence of a calreticulin-dependent signal transduction pathway linking the binding of extracellular matrix components by the integrin family of cell-surface receptors to the coordinate regulation of gene expression in the nucleus (Dedhar, 1994).

The active form of vitamin D, $1\alpha,25$ -dihydroxyvitamin D_3 ($1,25$ -[OH] $_2D_3$,¹ also named calcitriol), is a known modulator of bone cell growth and function (for review see Narbaiz, 1992). Vitamin D mainly exerts its pleiotropic effects after binding to its specific receptor, which is itself a member of the steroid hormone receptor superfamily (Fuller, 1991). The ligand-bound vitamin D receptor (VDR) then interacts with its cognate-binding site, termed vitamin D response element (VDRE), to affect the transcription of target genes (for review see Ozono et al., 1991). In osteoblastic cells, vitamin D can stimulate the activity of alkaline phosphatase (Kurihara et al., 1986), and the expression of type I collagen (Kurihara et al., 1986), osteocalcin (Yoon et al., 1988; Demay et al., 1989), and osteopontin (Noda et al., 1990). Functional VDREs have been identified and characterized for the promoters of the osteocalcin (Kerner et al., 1989; Demay et al., 1990) and osteopontin (Noda et al., 1990) genes.

In addition to the binding of the VDR to certain VDREs as a homodimer (Carlberg et al., 1993), the VDR can form heterodimers with retinoid X receptors (RXRs) (Yu et al., 1991; Kliewer et al., 1992). The RXRs are a family of nuclear receptors binding the retinoid *9-cis* retinoic acid (Heyman et al., 1992; Levin et al., 1992; Mangelsdorf et al., 1992). Both the VDR and RXRs possess the conserved KGFFK/RR amino acid motif within their respective zinc finger DNA-binding domains (Dedhar et al., 1994; Burns et al., 1994), suggesting that VDR-mediated transcriptional activation in bone cells may be inhibited by calreticulin and thus that calreticulin may be involved in the regulation of osteoblastic differentiation and function.

We have used the osteoblastic MC3T3-E1 cell line (Sudo et al., 1983) to investigate this putative regulatory function of calreticulin. We report that purified calreticulin inhibited the binding of VDR homodimers and VDR-RXR heterodimers to the previously characterized osteopontin and osteocalcin VDREs. Direct protein-protein interactions between the VDR and calreticulin were demonstrated. The expression of the endogenous calreticulin gene was shown to be down-regulated during osteoblastic differentiation of MC3T3-E1 cells. Constitutive expression of calreticulin achieved by transfection of MC3T3-E1 cells with calreticulin expression vectors inhibited basal and vitamin D-induced stimulation of osteocalcin expression. However, the stimulation of the expression of osteopontin by vitamin D was unaffected. Mineralization was also inhibited in the calreticulin-expressing clones, as assessed by

the reduction in calcium incorporation into the extracellular matrix and the complete absence of mineralization nodules. These results support a role for calreticulin in the regulation of osteogenesis through interaction with specific nuclear hormone receptor-mediated pathways.

Materials and Methods

Gel Retardation Assays

The VDR and RXR- β cDNAs were kind gifts from Dr. J. W. Pike (Ligand Pharmaceuticals, San Diego, CA) and Dr. V. Giguère (McGill University, Montréal, Québec), and were in vitro transcribed and translated using the TNT-coupled wheat germ lysate system following the instructions of the manufacturer (Promega Corp., Madison, WI). Purified recombinant VDR and RXR- α expressed in insect cells using baculovirus expression vectors (MacDonald et al., 1991) were generously provided by Dr. M. Haussler (University of Arizona, Tucson, AZ). For gel-retardation assays, 5 μ l from the TNT reactions or 40 ng of the baculovirus-expressed receptors were incubated with or without purified calreticulin (Rojiani et al., 1991) together with 4×10^3 cpm of probe in gel retention mix (10 mM Tris-HCl, pH 7.3, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 2 mM MgCl₂, 0.2% NP-40, 12% glycerol, 1.3 mg/ml BSA, 10^{-7} M $1,25$ -[OH] $_2D_3$) in a final vol of 25 μ l. Nonspecific competitor DNA consisted of 8 μ g poly (dI-dC) and 0.2 μ g salmon sperm DNA or 500 ng of poly (dI-dC) for the in vitro-translated and baculovirus-expressed receptors, respectively. The receptor and calreticulin proteins were preincubated for 30 min on ice before addition of the probe DNA. The probes used were synthetic oligonucleotides corresponding to the murine osteopontin VDRE (Noda et al., 1990) or the murine osteocalcin VDRE (Rahman et al., 1993). Probes were labeled by Klenow fill-in or kinasing (St-Arnaud and Moir, 1993). After incubation for 20 min at room temperature, bound probe was separated from free oligonucleotide on a 7% 30:1 acrylamide/bisacrylamide nondenaturing gel in $1 \times$ TBE (89 mM Tris-borate [pH 8.3], 2 mM Na₂EDTA). Samples were migrated at 150 V for 3 h with recirculating $1 \times$ TBE. Gels were then dried and autoradiographed.

Immunoprecipitation

The VDR was labeled with [³⁵S]methionine using the in vitro express translation kit as instructed by the manufacturer (Stratagene Corp., La Jolla, CA). The labeled receptor was incubated for 60 min on ice with or without purified calreticulin (Rojiani et al., 1991) in RIPA buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate) before addition of 10–30 μ l of anti-calreticulin antibody (Lieu et al., 1988). To ascertain that VDR and calreticulin interacted through the KXFFK/RR motif, an additional immunoprecipitation reaction included 100 μ g of the synthetic peptide KLGFFKR (Dedhar et al., 1994). Immunoprecipitation reactions were incubated for 4 d at 4°C and antigen-antibody complexes were collected by the addition of protein A-Sepharose (Pharmacia Canada, Baie d'Urfé, Québec). The immunocomplexes were washed four times in RIPA buffer before analysis by SDS-PAGE on a 7.5% gel. The fixed, dried gel was subsequently autoradiographed.

Cells and Tissue Culture Conditions

The MC3T3-E1 cells were maintained as previously described (Candelieri et al., 1991). Mineralization was accelerated by supplementing the medium with 10% FBS, 50 μ g/ml ascorbic acid, and 5 mM β -glycerophosphate (Ecarot-Charrier et al., 1983). Cultures were stained for mineralization nodules using the von Kossa method (Mallory, 1942).

RNA Extraction and Analysis

Cells were cultured for 14 d in medium supplemented for mineralization and then treated with 10^{-8} M $1,25$ -[OH] $_2D_3$ in ethanol or vehicle alone for 24 h. Total RNA was isolated by the lithium chloride-urea technique of Auffray and Rougeon (1980). Northern blot hybridization was performed as described elsewhere (St-Arnaud et al., 1988). The probes used included the 1.9-kb SacI fragment from the human calreticulin cDNA (McCauliffe et al., 1992); a 470-bp EcoRI-PstI fragment from the murine osteocalcin cDNA (Celeste et al., 1986); the full-length murine osteopontin cDNA (Smith and Denhardt, 1987); and the MAT1.1 probe for mouse α -tubulin (Lemishka et al., 1981).

1. Abbreviations used in this paper: $1,25$ (OH) $_2D_3$, $1\alpha,25$ -dihydroxyvitamin D_3 ; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D response element.

Expression Vectors and Transfections

The SV-40 promoter-driven pECE-Cal expression vector was constructed by subcloning the 1.9-kb HindIII-calreticulin cDNA fragment from Cal-2 (Dedhar et al., 1994) into the corresponding site of the pECE vector (Ellis et al., 1986) in the sense orientation. 20 µg of pECE-Cal together with 0.3 µg of the pGEM7(KJ1)R selection plasmid (Rudnicki et al., 1992) were transfected into MC3T3-E1 cells by electroporation (2×10^6 cells; 300 V; 1000 µF; 0.2 cm electrode gap). To establish control clones, MC3T3-E1 cells were transfected with 2 µg of pGEM7(KJ1)R selection plasmid alone. After electroporation, the cells were plated in 100-mm-diam dishes. Selection was initiated the next day by adding 1.2 mg/ml G-418 (Canadian Life Technologies, Burlington, Ontario) to the culture medium. Three control clones and four clones transfected with pECE-Cal were picked and established. The clones were maintained as the parental MC3T3-E1 cells except that the culture medium included 200 µg/ml of G-418. Expression of the recombinant calreticulin protein in transfected clones was assessed by Western blotting of whole-cell extracts with an anti-calreticulin antibody (Rokeach et al., 1991) as previously described (Dedhar et al., 1994).

Assay of ^{45}Ca Accumulation

Accumulation of ^{45}Ca into the matrix layer was measured as described by Matsumoto et al. (1991). Cells were cultured for 14 d before treatment with 10^{-8} M 1,25-(OH) $_2\text{D}_3$ or vehicle for 2 d.

Results

Calreticulin Inhibition of DNA Binding by VDR

We used gel retardation assays with in vitro-translated or baculovirus-expressed VDR and RXR proteins to determine the effect of purified calreticulin on the binding of VDR homodimers and VDR/RXR heterodimers to characterized VDREs. Fig. 1 A shows that calreticulin inhibited the binding of the heterodimer to the murine osteopontin VDRE (Noda et al., 1990) in a dose-dependent manner (lanes 8–11). A similar result was obtained using the VDRE from the promoter region of the murine osteocalcin gene (Rahman et al., 1993) (Fig. 1 B, lanes 3–6). The amount of calreticulin required for the inhibition of VDR binding to each VDRE was not significantly different. The inhibition by calreticulin was also observed with VDR homodimers (Fig. 1 A, lanes 4–7), demonstrating that calreticulin can interact with the VDR protein, and that the inhibition of DNA binding was not mediated solely through interaction of calreticulin with RXR. Unprogrammed wheat germ lysate (Fig. 1 A, lane 2) or purified calreticulin alone (Fig. 1 A, lane 3 and B, lane 2) did not bind to the VDRE probes.

Recent results have suggested that calreticulin interacts directly with nuclear hormone receptors through a conserved KXFFK/RR amino acid motif nested within the DNA-binding domain of the receptor proteins (Dedhar et al., 1994; Burns et al., 1994). We addressed the VDR-calreticulin interaction using immunoprecipitation reactions with labeled VDR, purified calreticulin, and anti-calreticulin antibodies. Antibodies directed against calreticulin did not immunoprecipitate the in vitro-labeled VDR (Fig. 1 C, lane 1). However, when purified unlabeled calreticulin was added to the reaction, the labeled VDR protein was coimmunoprecipitated by the anti-calreticulin antibodies (Fig. 1 C, lane 2). This result demonstrates a direct interaction between VDR and calreticulin. This reaction was mediated through the KGFFRR motif of the VDR, as a synthetic KLGFFKR peptide added simultaneously with

calreticulin competed for the interaction of the VDR with calreticulin and substantially decreased the amount of coimmunoprecipitated labeled receptor (Fig. 1 C, lane 3).

Calreticulin Expression during Osteoblastic Differentiation

MC3T3-E1 cells exhibit a preosteoblastic phenotype during exponential growth. On reaching confluency, the cells begin to express osteoblastic phenotype markers such as alkaline phosphatase and type I collagen (Sudo et al., 1983). Long-term cultures form multilayers of cells that express late osteoblastic differentiation markers and form calcified bone tissue (nodules of mineralization) in vitro (Sudo et al., 1983). This pattern closely recapitulates the osteoblastic differentiation sequence (Stein et al., 1990; Zhou et al., 1994).

We extracted RNA from subconfluent, confluent, and mineralizing cultures of MC3T3-E1 cells to analyze the expression of the endogenous calreticulin gene. Fig. 2 B shows that the 1.9-kb calreticulin mRNA could be detected in early and confluent cultures (lanes 1 and 2, respectively). No calreticulin expression could be detected in mineralizing cultures (Fig. 2 B, lane 3). Fig. 2 A shows that comparable amounts of total RNA were loaded in each lane. This pattern of expression of the endogenous calreticulin gene was observed in two independent experiments (data not shown).

We also probed the RNA blot for expression of osteoblastic phenotype markers. Fig. 2 C shows that the osteopontin gene was expressed at all stages of culture. Thus osteopontin expression could be detected in cultures that did or did not express the endogenous calreticulin gene (compare Fig. 2, B and C). On the contrary, the osteocalcin mRNA was only detected in mineralizing cultures (Fig. 2 D). Thus osteocalcin gene expression appeared to coincide with the inhibition of endogenous calreticulin gene expression in the cultured cells (Fig. 2, B and D, lane 3).

Expression of Recombinant Calreticulin

We isolated four clones stably transfected with the pECE-Cal vector (clones SV-Cal 4, SV-Cal 5, SV-Cal 6, and SV-Cal 12). Three clones transfected with the selection vector alone served as controls.

Fig. 3 shows the relative expression levels for the calreticulin protein in parental MC3T3-E1 cells and the control and transfected clones. Elevated calreticulin protein expression was achieved in all four clones transfected with the calreticulin expression vector. The increase in calreticulin expression was estimated to vary between two- and fivefold over parental levels. The expression of the recombinant calreticulin protein was constitutive and maintained in long-term cultures of the transfected clones (data not shown). In both parental MC3T3-E1 cells and transfected clones, calreticulin was detected in the nucleus and the perinuclear region using immunocytochemistry (not shown), as demonstrated for other cell types (Dedhar, 1994); the relative abundance of nuclear calreticulin was increased in the clones transfected with the calreticulin expression vector (data not shown).

Enhanced calreticulin expression affected the morphology of the cells. This change was more evident in early cultures

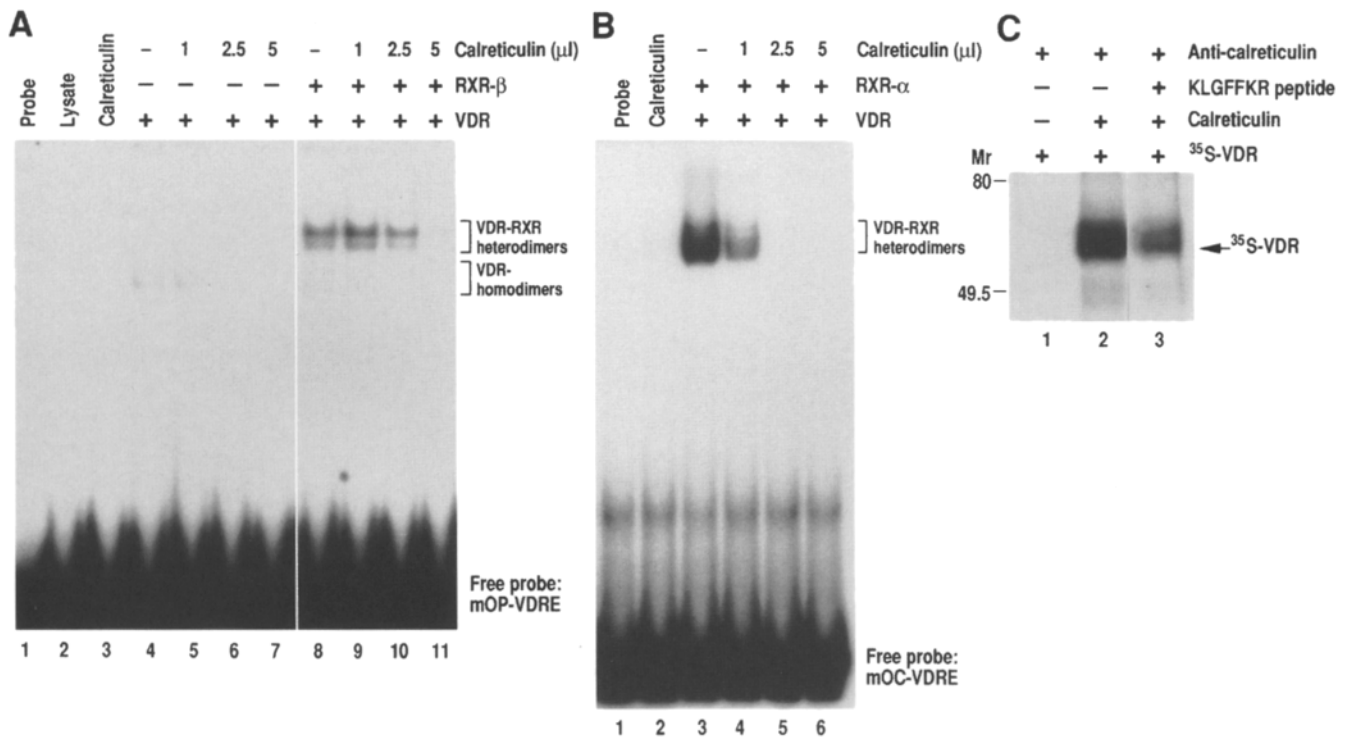


Figure 1. Calreticulin interacts with the VDR protein to inhibit the binding of VDR homodimers and VDR-RXR heterodimers to characterized VDREs. (A) VDR and RXR-β were in vitro translated and incubated with a labeled oligonucleotide corresponding to the murine osteopontin VDRE in the presence or absence of purified calreticulin (10 ng/μl). Probe, unprogrammed lysate, and calreticulin alone were migrated as controls in lanes 1, 2, and 3, respectively. (B) Baculovirus-expressed VDR and RXR-α were incubated with a murine osteocalcin VDRE oligonucleotide probe. Increasing amounts of purified calreticulin (10 ng/μl) were added as indicated above lanes 4–6. Probe and calreticulin were migrated in lanes 1 and 2. The binding reactions were analyzed using the gel retardation assay on a 7% nondenaturing gel. (C) SDS-PAGE analysis of immunoprecipitation reactions. ³⁵S-labeled VDR was incubated with or without purified calreticulin and an anti-calreticulin antibody. In the presence of calreticulin, the labeled receptor was associated with the immunocomplexes (lanes 2 and 3). Addition of a synthetic peptide (KLGFFKR; 100 μg) substantially reduced the amount of immunoprecipitated receptor (lane 3).

that had just reached confluency. The clones overexpressing calreticulin had a spindle-shaped appearance compared with the characteristic cuboidal osteoblastic morphology of the parental cells and the control clones (Fig. 4). This morphological change became less apparent in long-term, multilayer cultures (data not shown).

Calreticulin Overexpression Selectively Inhibits Gene Expression

Long-term (14 d) cultures of control and calreticulin-expressing clones were treated with 1,25-(OH)₂D₃ for 24 h and total RNA was extracted to analyze the influence of calreticulin overexpression on vitamin D-stimulated gene expression in bone cells. Fig. 5 shows that constitutive calreticulin expression inhibited the induction of osteocalcin expression by vitamin D in all clones studied. Basal levels of osteocalcin mRNA were also drastically down-regulated in calreticulin-expressing clones (Fig. 5). These observations are consistent with the pattern of osteocalcin gene expression observed in parental cells, where osteocalcin mRNA could not be detected in subconfluent and confluent cultures of MC3T3-E1 cells that expressed endogenous calreticulin mRNA (Fig. 2). Similarly, vitamin D treatment of subconfluent and confluent MC3T3-E1 cul-

tures did not induce osteocalcin gene expression (data not shown). Interestingly, the vitamin D-induced stimulation of the expression of the osteopontin gene was unaffected by calreticulin (Fig. 5). This result is in accord with the data obtained in parental cell cultures, where osteopontin expression could readily be detected in early cultures of MC3T3-E1 cells that expressed significant levels of endogenous calreticulin mRNA (Fig. 2). Thus our observations suggest that calreticulin overexpression had specific effects on the modulation of gene expression by nuclear hormone receptors and did not inhibit gene expression in general. Indeed, the expression of α-tubulin, which was analyzed to monitor for equal loading of RNA for each sample, was unaffected by calreticulin overexpression (Fig. 5).

Constitutive Calreticulin Expression Inhibits Mineralization

Multilayer cultures of MC3T3-E1 cells deposit mineral and form calcified bone tissue after 14–21 d in culture (Sudo et al., 1983). This osteogenic process can be documented by counting the number of mineralization nodules and by measuring the incorporation of labeled calcium into the extracellular matrix. The calcium accumulation

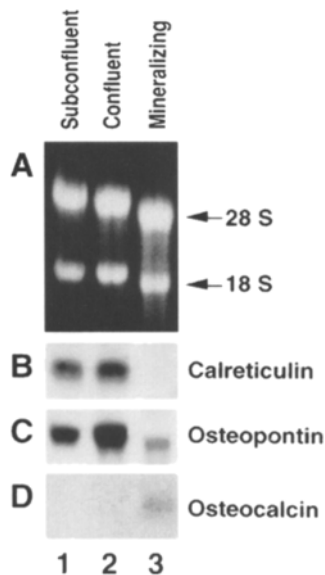


Figure 2. Expression pattern of calreticulin and osteoblast phenotype markers during MC3T3-E1 osteoblastic differentiation. Total RNA was isolated from subconfluent (day 2), confluent (day 4), and mineralizing (day 21) MC3T3-E1 cells and analyzed by Northern blot assay. (A) Ethidium bromide-stained ribosomal RNA showing equivalent loading for all samples. The RNA was transferred onto a nylon membrane, cross-linked, and hybridized to specific probes for calreticulin (B), osteopontin (C), and osteocalcin (D). The major 1.5-kb osteopontin transcript is shown in C.

into the matrix has been shown to be stimulated by treatment of the cultures with $1,25\text{-(OH)}_2\text{D}_3$ (Matsumoto et al., 1991). We have used both assays to analyze the influence of constitutive calreticulin expression on mineralization in osteoblastic cells.

Fig. 6 A shows a 14-d-old culture of the control clone G7KJ1-2 that was stained for mineralization nodules using the von Kossa method (Mallory, 1942). Long-term cultures of all three control clones yielded similar results that were representative of the mineralization observed in parental MC3T3-E1 cultures (not shown). Fig. 6 B illustrates the complete absence of mineralization nodules observed in calreticulin-overexpressing clones. The data from a typical mineralization experiment is summarized in Table I. The cultures of the control clone G7KJ1-2 contained 53 ± 4 nodules (mean \pm SEM, $n = 4$), whereas the cultures from all the clones expressing calreticulin constitutively contained no nodules (0.6 ± 0.3 ; mean \pm SEM, $n = 2$ for each of the four clones).

Vitamin D-induced stimulation of ^{45}Ca incorporation

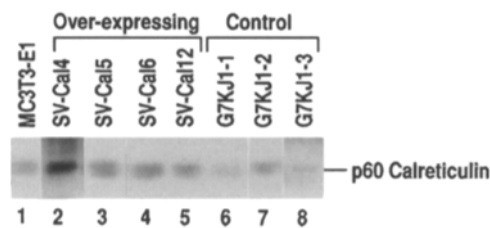


Figure 3. Expression of the calreticulin protein in parental cells, overexpressing clones, and control clones. MC3T3-E1 cells were electroporated with the pGEM7(KJ1)R selection plasmid alone or in combination with the pECE-Cal expression vector for calreticulin as described in Materials and Methods. G-418-resistant clones were picked and expanded, and total-cell extracts from four clones transfected with pECE-Cal and three control clones transfected with pGEM7(KJ1)R alone were analyzed for calreticulin protein expression by Western blot assay. The levels of calreticulin expressed in untransfected MC3T3-E1 cells is shown for comparison purposes in lane 1.

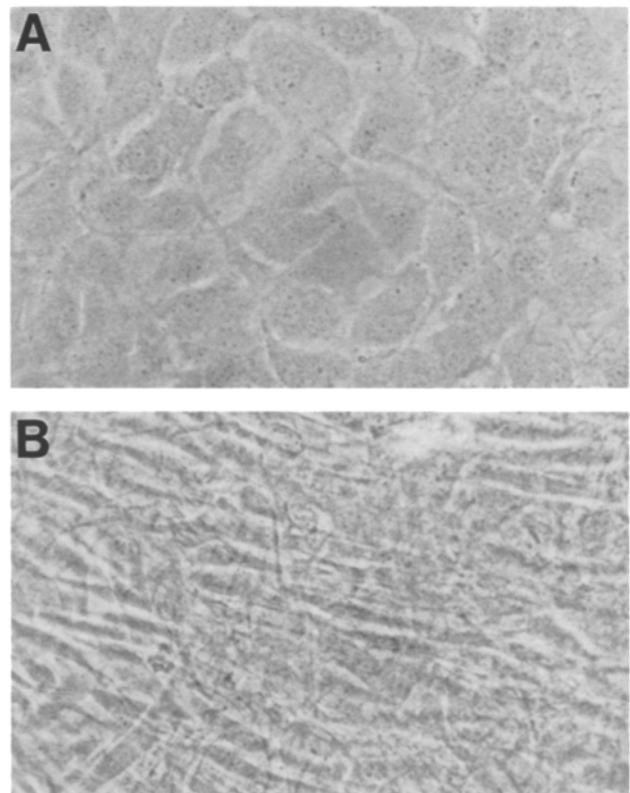


Figure 4. Calreticulin overexpression affects the morphology of osteoblastic cells at confluence. Phase-contrast photomicrographs of the control clone G7KJ-2 (A) and the calreticulin overexpressing clone SV-Cal 4 (B) grown to confluence (day 4) in medium supplemented for mineralization. Notice the characteristic osteoblastic cuboidal morphology of the control cells and the spindle-shaped, fibroblastic appearance of the cells expressing increased levels of recombinant calreticulin.

into the extracellular matrix was also inhibited in calreticulin-transfected clones. We observed the previously reported twofold stimulation of calcium incorporation into the matrix by vitamin D (Matsumoto et al., 1991) in cultures of MC3T3-E1 cells and in cultures of all three control clones (Fig. 7). Constitutive expression of calreticulin inhibited calcium incorporation in response to vitamin D treatment in all four clones (Fig. 7).

Discussion

We have shown that constitutive expression of calreticulin in osteoblastic cells inhibited mineralization. This inhibition was most likely mediated through specific modulation of gene expression by calreticulin, as we have demonstrated that calreticulin could interact with the vitamin D receptor to inhibit its binding to cognate response elements *in vitro*, and selectively inhibit certain vitamin D-mediated transcriptional responses *in vivo*. Our results support a role for calreticulin in the regulation of osteoblastic differentiation and function.

The skeleton is the body's major repository of calcium in the form of hydroxyapatite crystals. Mineralization, i.e., the deposition of mineral along the fibrils of the collagen

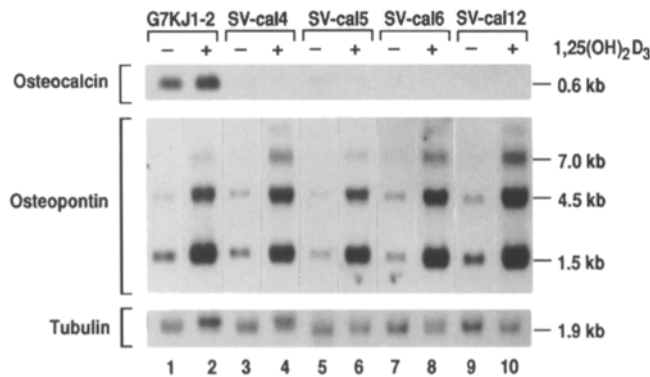


Figure 5. Calreticulin overexpression selectively inhibits the vitamin D-induced stimulation of osteocalcin expression without affecting vitamin D-stimulated osteopontin expression. Total RNA was isolated from mineralizing (day 14) cultures of one control clone (lanes 1 and 2) and the four calreticulin overexpressing clones (lanes 3–10) treated with (even-numbered lanes) or without (odd-numbered lanes) 10^{-8} M $1,25\text{-(OH)}_2\text{D}_3$ for 24 h. The RNA was analyzed by Northern blot assay with specific probes for osteocalcin, osteopontin, and α -tubulin (to monitor for variations in sample loading). Observed transcript sizes are indicated on the right. Multiple transcripts for murine osteopontin have been described previously (Miyazaki et al., 1990).

matrix of bone, is under the control of the osteoblast (Termin, 1993). But the role of the osteoblast is not to act as a calcium-storage compartment since a proportion of the skeletal content of calcium is freely exchangeable with the extracellular fluids and serves as the major storage pool of calcium (Broadus, 1993). Moreover, the function of the osteoblast is not dependent on the availability of a large intracellular store of calcium, in contrast, for example, to the function of skeletal myotubes which require rapid calcium release to initiate muscle contraction. Thus it is likely that the role of calreticulin in osteoblasts is not limited to a calcium storage function.

Indeed, we have shown that calreticulin can influence gene expression and mineralization. The inhibition of mineral deposition cannot be simply explained by the sequestration of calcium inside the cells by the recombinant calreticulin protein. First, the levels of expression that we achieved in the transfected clones were not disproportionately high. Second, under the conditions used, the extracellular fluid was supersaturated with regard to calcium and phosphate. In experiments measuring the stimulation of labeled calcium into the extracellular matrix by vitamin D (Fig. 7), the calreticulin-expressing clones had similar baseline amounts of ^{45}Ca incorporated in the matrix (not shown). The mechanisms responsible for the actual inhibition of calcium incorporation when the calreticulin-transfected clones were treated with vitamin D are unclear. Vitamin D treatment had no effect on the expression of the endogenous calreticulin gene in MC3T3-E1 cells (Prud'homme, J., and R. St-Arnaud, unpublished observations).

In addition to vitamin D, a number of steroid hormones or morphogens binding to members of the nuclear hormone receptor superfamily can modulate gene expression in osteoblasts. In MC3T3-E1 cells, glucocorticoids have been shown to inhibit prostaglandin E_2 synthesis (Hughes-Fulford et al., 1992) and increase the expression of betaglycan

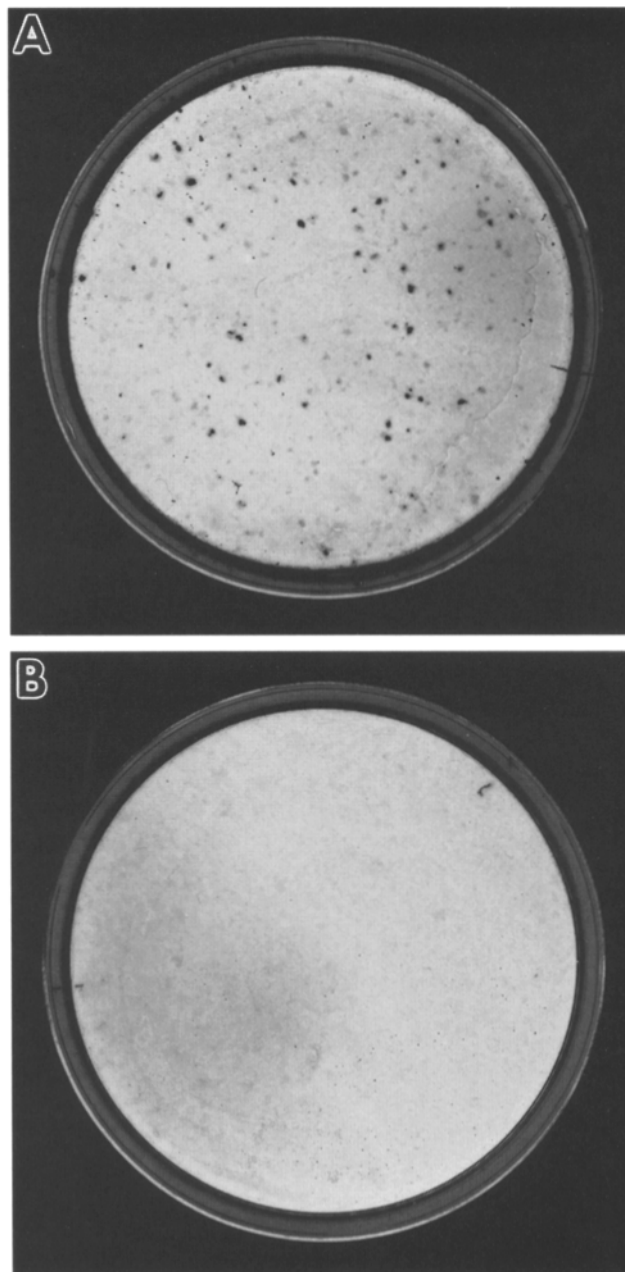


Figure 6. Constitutive calreticulin expression inhibits mineralization. Long-term (day 14) cultures, supplemented for mineralization, of control clone G7KJ1-2 (A) and calreticulin-expressing clone SV-Cal 12 (B) were stained for mineralization nodules using the von Kossa method. The nodules appear as dark spots in the bright-field photograph.

(Nakayama et al., 1994). Estrogen receptors are expressed in osteoblasts (Eriksen et al., 1988) and $17\text{-}\beta$ estradiol down-regulates the transcriptional activity of the interleukin-6 gene promoter and inhibits the expression of the glycoprotein 130, a subunit of cytokine receptors (Manolagas, S. C. 1995. *Bone*. 16 [Suppl. 1]:91S.). Retinoic acid stimulates the expression of alkaline phosphatase and induces differentiation of preosteoblastic cell lines (Ng et al., 1988; Heath et al., 1989). All of these receptor-mediated responses represent putative targets for modulation by calreticulin. Our results, showing that calreticulin inhibited some but

Table 1. Number of Mineralization Nodules in Long-Term Cultures of Control and Calreticulin-transfected Clones

Clone	Number of nodules	Mean \pm SEM
G7KJ1-2 (control)	47; 52; 50; 63	53 \pm 4
SV-Cal 4	2; 0	
SV-Cal 5	0; 0	
SV-Cal 6	0; 1	
SV-Cal 12	0; 2	0.6 \pm 0.3

Cells were grown for 14 d in media supplemented for mineralization, then stained using the von Kossa method. Mineralization nodules were counted manually. The control clone G7KJ1-2 was grown in quadruplicate, whereas the calreticulin-transfected clones were cultured in duplicate dishes.

not all of the transcriptional responses mediated by the VDR, suggest that certain of these nuclear hormone receptor-dependent pathways will be left intact while others may be affected. It would be interesting to investigate transcriptional responses to various steroids in bone cells expressing calreticulin constitutively.

The response to vitamin D was not completely abrogated in clones expressing the recombinant calreticulin protein (Fig. 5). This could suggest that the VDR was in stoichiometric excess over calreticulin in the transfected clones, thus allowing the excess of ligand-bound VDR to escape inhibition by calreticulin and activate transcription. Indeed, vitamin D treatment has been shown to induce the expression of VDR mRNA and protein in bone cells (Mahonen et al., 1990). Alternatively, the residual vitamin D response observed in calreticulin-transfected clones could be due to the documented nongenomic effects of vitamin D (for review see Norman et al., 1992), which may not be affected by constitutive calreticulin expression.

Our results have demonstrated that calreticulin inhibits VDR binding to both the osteopontin and the osteocalcin VDRE (Fig. 1). However, the two genes responded dissimilarly when the calreticulin-expressing clones were challenged with vitamin D (Fig. 5). Despite differences between

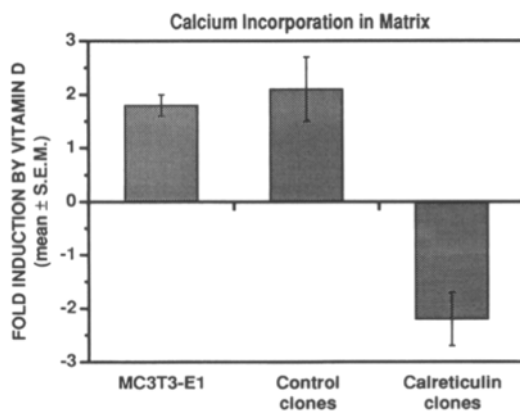


Figure 7. Calreticulin overexpression inhibits the vitamin D-induced stimulation of calcium incorporation into the extracellular matrix. ^{45}Ca incorporation in long-term (day 14) cultures of parental MC3T3-E1 cells, three control clones and four calreticulin-expressing clones treated with or without vitamin D was measured as described by Matsumoto et al. (1991). Results are expressed as mean fold induction \pm SEM of three independent experiments performed in duplicate.

the sequences of the two VDREs (Kerner et al., 1989; Demay et al., 1990; Rahman et al., 1993) that could affect VDR binding affinity to each response element (Nishikawa et al., 1993), we were unable to detect any difference in the capacity of calreticulin to inhibit VDR binding to each VDRE. Thus the structure of the response element cannot seem to account for the variation in transcription. It is more likely that the expression of each gene is dictated by the structure of the entire promoter region, and not just a single element. The pattern observed in the parental cells supports this view. Indeed, the osteopontin mRNA was readily detected in early cultures of MC3T3-E1 cells that also expressed calreticulin (Fig. 2). On the contrary, osteocalcin transcripts were only observed in long-term cultures that had down-regulated calreticulin expression (Fig. 2). Thus it appears that calreticulin expression inhibits both steady-state and stimulated osteocalcin expression, whereas it does not affect the transcription of the osteopontin gene. In this respect, the pattern of expression of these two osteoblastic phenotype markers that we measured in the calreticulin-expressing clones closely mimicked what was observed in the parental MC3T3-E1 cells.

There was no correlation between the level of calreticulin overexpression achieved in independent clones and the inhibition of basal and stimulated osteocalcin transcription, as all clones exhibited an identical blunted osteocalcin response (see Figs. 3 and 5). Our results suggest that it was not the magnitude of the expression of the recombinant protein per se that affected the osteocalcin response, but the fact that calreticulin expression was constitutive and therefore not down-regulated in long-term cultures of the transfected clones. Again, this observation parallels the pattern of osteocalcin gene expression that we documented in nontransfected MC3T3-E1 cultures (Fig. 2).

Attachment to extracellular matrix components modulates gene expression and differentiation of bone cells. For example, differentiation of canalicular cell processes was observed after contact of MC3T3-E1 cells with laminin (Vukicevic et al., 1990), and gene expression is affected when preosteoblastic cells are plated on various extracellular matrix components (Traianedes et al., 1993). Osteoblasts express various forms of the integrin family of cell surface receptors for matrix proteins (Hughes et al., 1993; Saito et al., 1994), and inhibition of integrin function using subunit-specific antibodies has been shown to prevent cytokine-induced osteoblastic differentiation of osteosarcoma cells (Dedhar, 1989). Calreticulin binds to α -integrin subunits (Leung-Hagesteijn et al., 1994) and recent results suggest that this interaction can modulate the affinity state of integrins (Dedhar, 1994). Moreover, calreticulin can modulate nuclear hormone receptor-dependent gene expression (Dedhar et al., 1994; Burns et al., 1994). Taken together, these observations support the existence of a calreticulin-modulated signal transduction pathway linking substratum attachment via integrin receptors to the control of gene expression. Our results further support an important role for this pathway in the regulation of osteoblastic differentiation and function.

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Note Added in Proof. After completion of this work, a report was published confirming the inhibition of vitamin D signal transduction by calreticulin (Wheeler, D. G., J. Horsford, M. Michalak, J. H. White, and G. N. Hendy. 1995. *Nucleic Acids Res.* 23:3269–3274).

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