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NEW MECHANISMS OF REGULATION OF THE GENOMIC ACTIONS OF VITAMIN D IN BONE CELLS: INTERACTION OF THE VITAMIN D RECEPTOR WITH NON-CLASSICAL RESPONSE ELEMENTS AND WITH THE MULTIFUNCTIONAL PROTEIN, CALRETICULIN

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1. ABSTRACT

Vitamin D exerts its genomic effects following binding to a specific receptor which is a member of the steroid hormone receptor superfamily. The vitamin D receptor (VDR) forms heterodimers with retinoid X receptors (RXRs) and the dimer then interacts with its cognate binding site, termed vitamin D response element (VDRE), to affect the transcription of target genes.

Recent studies have identified novel sequence motifs for VDREs as well as novel protein-protein interactions involving the VDR. These will be reviewed with particular emphasis on the complex VDRE from the c-fos proto-oncogene promoter region and the inhibition of the vitamin D signal transduction pathway by the multifunctional protein, calreticulin.

Thus research on the control of gene transcription by vitamin D reveals examples of molecular interplay between transcriptional regulatory pathways and provides new insight into the molecular mechanism of action of vitamin D.

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2. INTRODUCTION

2.1. Vitamin D and bone cells

The hormonal form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25 $(OH)_2D_3$, also named calcitriol), is a known modulator of osteoblastic growth and function. A number of effects have been described when primary cultures of bone cells or established osteoblastic cell lines are treated with 1,25 $(OH)_2D_3$ *in vitro*. Vitamin D treatment inhibits type I collagen expression by osteoblasts (1-5) but enhances the expression of genes that are specific markers of osteoblastic differentiation. These include alkaline phosphatase (6-9), osteopontin (10, 11), osteocalcin (12, 13), and matrix-Gla protein (14-16). Overall, the direct effects of 1,25 $(OH)_2D_3$ on bone cells are consistent with the induction of differentiation of osteoblasts *in vitro*. Accordingly, the metabolite has been shown to stimulate mineralization in cultures of clonal osteoblast-like cells (17).

2.2. VDREs

Vitamin D mainly exerts its pleiotropic effects following binding to a specific receptor which is a member of the steroid hormone receptor superfamily

Table 1: DR3-type VDREs

Gene	VDRE sequence	Refs
m Osteopontin	GGTTCAcgaGGTTCA	21
r Osteocalcin	GGGTGAatgAGGACA	22
h Osteocalcin	GGGTCAacgGGGGCA	39, 40
r Calbindin D_{9k}	GGGTGTcggAAGCCC	23
c Integrin β_3	GAGGCAgaaGGGAGA	24
r 24-hydroxylase	CGCACCcgcTGAACC	25

[a] abbreviations used: m, mouse; h, human; r, rat; c, chicken.

(reviewed in ref. 18). 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 (19).

A number of natural and synthetic VDREs have been identified. These response elements consist of direct repeats (DR) of two hexameric core binding sites spaced by varying numbers of nucleotide residues. Binding studies using the VDR DNA binding domain have revealed the order of relative binding affinities for various hexameric core binding sites, with GGTTCA > AGGTCA > AGGACA >GGGTGA (20). The DR3-type element, exampled by the mouse osteopontin VDRE (21), consists of two GGTTCA hexameric core sites spaced by 3 residues. DR3-type elements have been described in the promoters of the rat osteocalcin gene (22), the rat calbindin D-9k gene (23), the avian integrin β3 subunit gene (24) and the rat calcidiol 24-hydroxylase gene (25) (Table 1).

Several DNA sequences that diverge from the canonical DR3-type elements have also been shown to bind the VDR and to mediate vitamin D-dependent transcriptional activation. These non-classical VDREs will be described in section 3 below.

2.3. VDR dimerization

In addition to the binding of the VDR to certain VDREs as a homodimer (26), it has been known for some time that the *in vitro* binding affinity of the VDR is enhanced by the addition of nuclear extracts, suggesting the existence of nuclear accessory factors (27). Some of these accessory factors have now been identified. The VDR forms heterodimers with retinoid X receptors (RXRs) (28-30). The RXRs are a family of nuclear receptors binding the retinoid 9-*cis* retinoic acid (31-33). The molecular cross-talk involving the VDR is further evidenced by results showing that it can heterodimerize with retinoic acid receptors (RARs) (34) and thyroid hormone receptors (T_3Rs) (35). The VDR dimerizing partner seems to affect the binding affinity to and the transcriptional response of particular VDREs (35, 36).

3. NON-CLASSICAL VDREs

3.1. In various promoters

Rhodes *et al.* (37) have recently described a composite retinoic acid (RA)- and vitamin D-response element (RDE). This element has a DR4-type structure with two AGTTCA hexameric sites (on the non-coding strand) spaced by 4 residues. The transcriptional response to 1,25 $(OH)_2D_3$ mediated through the RDE was exceptionally high and the element was shown to bind VDR-RXR heterodimers, but not VDR homodimers (37). A DR4-like element has also been described in the promoter of the calbindin D-28k gene (38). The VDR was shown to bind that element but the putative dimerizing partners involved were not characterized (38).

In contrast, the DR6-type element of the human osteocalcin VDRE consists of two GGGTGA hexameric consensus sites spaced by 6 residues (39, 40). It has been shown to bind preferentially to VDR-RAR and VDR-T_3R heterodimers (34, 35), as well as to VDR homodimers, although with lower affinity (26; 35).

Other structural motifs that have been shown to mediate transcriptional induction by the VDR alone, and thus inferred to bind VDR homodimers, include a palindrome of the GGGTGA core site with no intervening residue and an inverted palindrome of the same hexameric site spaced by 12 residues (26). Following this initial observation that inverted palindromic elements can mediate the transcriptional response to vitamin D, the laboratory of Carsten Carlberg in Geneva initiated an exhaustive search for inverted palindromes that would respond to each of the vitamin D transcriptional pathways (VDR homodimers and VDR-RXR, VDR-RAR, and VDR-T_3R heterodimers). First utilizing synthetic elements, they have shown that VDR-RAR dimers exhibit preferential binding affinity for motifs in which the core hexameric sites are spaced by 11 residues (inverted palindrome 11 or IP11) (41), whereas VDR-RXR and VDR-T_3R heterodimers show maximal affinity for IP9 and IP7 motifs, respectively (35; 41).

Until recently, the physiological significance of these unusual response element motifs remained unclear. Evidence for a functional role of these non-classical VDREs in mediating natural vitamin D-dependent transcriptional activation was accumulated through the identification and characterization of two natural inverted palindrome VDREs (Table 2) (42). These elements, identified in the promoter regions of the human calbindin D_{9k} gene and the rat osteocalcin gene both consist of inverted palindromes spaced by nine residues (IP9s) (42). The functional response through the IP9 elements was demonstrated both in the context of the native promoter regions and with chimeric promoters in which one copy of the IP9 binding site was subcloned upstream of an heterologous promoter (42).

Table 2: Non-classical VDREs from natural promoters

Gene[a]	VDRE Seq	VDRE type[b]	Refs
m Calbindin D28k	GGGGGAtgtgAGGAGA	DR4	38
m Pit-1	TGAACTctcaTGAACT	DR4	37
h Osteocalcin	GGGTGActcaccGGGTGA	DR6	39, 40
h Calbindin D9k	TGCCCTtccttatggGGTTCA	IP9	42
r Osteocalcin	TGCACTgggtgaatgAGGACA	IP9	42
m c-fos	AGGTGAAAGATGTATGCCAAGACGGGGGTTGAAAG	n/a	48

[a] abbreviations used: m, mouse; h, human; r, rat; n/a, not applicable. [b] see text

As previously observed with the synthetic elements, the natural IP9s preferentially bound VDR-RXR heterodimers with high affinity (42). Binding of the receptors to the sites allowed transcriptional activation by vitamin D and the transcriptional response was further enhanced by addition of 9-*cis* retinoic acid (9-*cis* RA) (42), the RXR ligand (31-33). The authors utilized the response of the IP9 element to both vitamin D and 9-*cis* RA to reconcile their data with previously published observations: indeed, two groups had previously shown that binding of 9-*cis* RA to the RXR would destabilize the VDR-RXR heterodimerization and thus blunt the transcriptional response mediated by vitamin D through classical VDREs (30; 43). Schräder *et al.* (42) found that the response to 9-cis RA is dependent on the VDR to RXR ratio. When the VDR is in excess of the RXR (16:1), concomitant treatment with 9-*cis* RA inhibited the transcriptional response to vitamin D. On the other hand, excess of RXR molecules relative to VDR (4:1) led to additive effects of treatment with both ligands on the activation of transcription mediated through the IP9 (42). This observation suggests that both the 9-*cis* RA ligand and the levels of expression of its specific receptor, RXR, play important roles in vitamin D-dependent gene transcription.

It should be mentioned that the core binding motifs of characterized VDREs diverge from the consensus motif GGTTCA (Table 1). Similarly, the IP9 elements are imperfect inverted palindromes (42). This variation from the consensus sequence in each 'half-site' of the response elements seems to allow for specific binding polarities of the nuclear receptors; VDR-RXR heterodimers bind their response elements with RXR occupying the 5'-'half-site' while the VDR occupies the 3'-motif (44). The same binding polarity was observed for the IP9 binding elements (42). The polarity-directed binding of the nuclear receptors to their cognate binding site may have implications for the specific cellular response to particular ligands (36).

3.2. In the promoter of the c-fos proto-oncogene

We have been interested in studying the effects of vitamin D treatment on the expression of the members of the fos and jun families of proto-oncogenes in bone cells. c-fos encodes a nuclear phosphoprotein (c-Fos) that heterodimerizes with the members of the jun family of proto-oncogenes to form the transcription factor AP-1, which binds specific sites in the promoter region of target genes to regulate their transcription (reviewed in ref. 45). There is a growing body of experimental evidence demonstrating that the c-fos proto-oncogene plays a key role in the regulation of bone tissue metabolism (46). We have shown that 1,25 $(OH)_2D_3$ can transiently stimulate the expression of c-fos in osteoblasts and that this stimulation is mediated at the level of gene transcription (47).

We have pursued this work by identifying and characterizing the VDRE in the promoter of c-fos (48). The 1,25 $(OH)_2D_3$ -responsive region was delineated between residues -178 to -144 upstream of the c-fos transcription start site. Transient transfection assays using wild-type or mutated versions of the c-fos VDRE in the context of natural or chimeric promoters demonstrated that the c-fos VDRE is a functional response element. The structure of the c-fos VDRE was found to be unusual with the following sequence:

5' AGGTGAAAGATGTATGCCAAGACGGGGGTTGAAAG 3'.

When analyzed in the context of the DR-type structures, three putative hexameric core sites can be identified (underlined). These would give the c-fos VDRE either a DR7-like conformation or a DR20-type configuration. However, mutational analysis has revealed that these alignments are not valid (48).

We have not detected homodimeric binding of the VDR to the c-fos VDRE (48). Our data also revealed that VDR-RAR and VDR-T_3R heterodimers do not bind the c-fos VDRE (not shown). Moreover, while both the VDR protein and the RXR molecule were detected in the complex from bone cells that bound the c-fos VDRE, the VDR-RXR heterodimer bound to the element *in vitro* yielded a complex of a different size than the complex observed in nuclear extracts from bone cells (48). We interpreted these observations to mean that the vitamin D-responsive complex binding the c-fos VDRE in nuclear extracts from bone cells was composed of the VDR and RXR proteins interacting with a third

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Murine:     -178 AG GTGAAAGATG TATGCCAAGA CGGGGGTTGA AAG -144
                    |   | ||     ||||||  ||||||  |   ||
Human       -185 GA GATTAGGACA CGCGCCAAGG CGGGGGCAGG GAG -151
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Fig. 1: Sequence comparison between the murine c-fos VDRE and the human c-fos promoter sequence. Alignment of the putative human c-fos VDRE with the murine sequence was performed by computer analysis using Tetra software (SoftGene Gmbh, Berlin, Germany).

component. Indeed, our data also showed that a putative osteoblast specific NF-1 family member bound the response element in conjunction with the nuclear hormone receptors (48). These results will be discussed in more detail in section 4.2 below.

A region showing sequence similarity to the murine c-fos VDRE can be identified in the human c-fos promoter (Fig.1). This sequence also binds the VDR and mutations affecting binding to the murine VDRE also inhibit binding to the human sequence (Fig.2). Although, the functionality of the human element remains to be established, these results support the physiological importance of the c-fos VDRE.

4. PROTEIN-PROTEIN INTERACTIONS OF THE VDR

Association with multiple protein targets appears like a common mechanism through which activators can fully stimulate transcription. The dimerization of the VDR with itself or with RXR, RAR, and T_3R has been described above. This section will review the interaction of the VDR with other partners. These protein-protein interactions are hypothesized to play key regulatory roles in the control of vitamin D-dependent gene transcription.

4.1. Interactions with the basic transcriptional machinery

Recent genetic and biochemical evidence has revealed that the activation of gene expression involves sequence-specific DNA binding transcriptional activators such as the VDR, the basic or general initiation factors TFIIA, B, D, E, F and H, and a third class of molecules that mediate interaction between the activators and the basal factors via direct protein-protein contacts (reviewed in ref. 49). At least one of the protein targets of the VDR within the basic transcriptional machinery has been identified: using the powerful yeast two-hybrid screening methodology (50), MacDonald *et al.* (51) have shown that the VDR interacts directly with TFIIB. The domains of the two proteins involved in the interaction were identified using deletion mutants both *in vitro* and *in vivo* (51, 52). Interestingly, cooperative interactions between VDR and TFIIB were demonstrated using transient transfection assays in embryonic cells; cooperativity was modulated by cell-type-specific accessory factor(s) (52). These accessory factors could be functionally equivalent to the transcriptional coactivators that have been shown

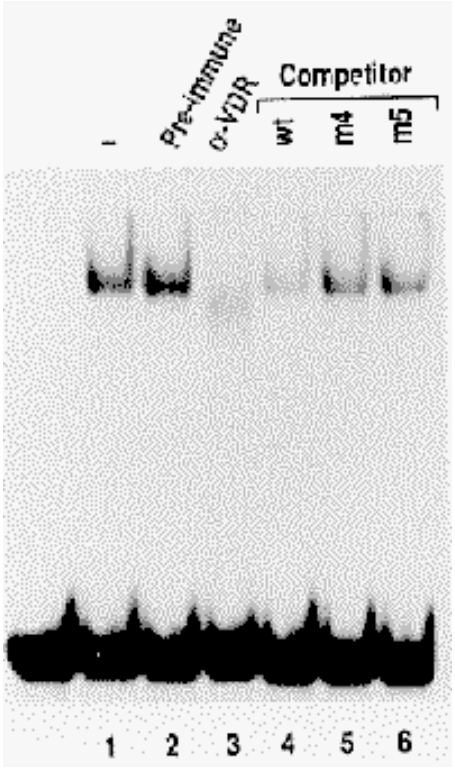

Fig. 2: The VDR complex from bone cells interacts with the putative human c-fos VDRE. Gel retardation assay using as probe an oligonucleotide corresponding to the putative human c-fos VDRE. The probe was incubated with nuclear extracts from bone cells (lane 1) in the presence of either pre-immune serum (lane 2), anti-VDR antibody (lane 3) or cold, unlabeled oligonucleotides corresponding to the wild-type murine c-fos VDRE (wt, lane 4) or mutant sequences that were shown to be defective for the binding of the vitamin D-responsive complex from bone cells (m4 and m5, lanes 5 and 6, respectively) (see ref. 48). Sequence of the m4 and m5 mutants were: 5'-AGATGAAAGATGTAT ACCAAGACGGGGATTGAAAT-3' and 5'-AGGTGA AAGATGTATACCAAGACGGGGGTTGAAAG-3', respectively. Mutated residues are underlined.

to be essential for activated gene transcription (49; 53). Cell-specific coactivators have recently been characterized (54, 55). The identification of coactivator molecules interacting with the VDR will undoubtedly increase our comprehension of the molecular

mechanismsimplicated in vitamin D-stimulated transcriptional responses.

4.2. Interactions with NF1

Recent work suggests that interactions between different families of transcription factors and steroid hormone receptors may provide another level of regulation of receptor function. One such class of factors is the NF1 gene family.

The nuclear factor 1 (NF-1) family of proteins have been shown to be implicated in the transcriptional activation of many cellular and viral genes (56-58) as well as in the stimulation of adenovirus DNA replication (59). NF-1 family members bind the CCAAT box (hence their original designation as CCAAT-binding transcription factors or CTF), making contact with TGG residues on the opposite strand to activate transcription (59). The name 'CCAAT-binding transcription factors' was deemed confusing considering the fact that a variety of transcription factors, some of which definitely unrelated to NF-1, had been shown to bind the CCAAT motif (examples include C/EBP and CBF/NF-Y). Thus NF-1 was selected as the 'family name' and the various gene family members received letter designations based either on their sequence of publication or some connection with previous names. Thus CTF/NF-1 has been replaced by the name NF1-C. The NF-1 proteins consist of a family of related polypeptides generated by the alternative splicing of RNA (60). To date, four family members encoded by multiple genes have been cloned (61).

Mutation of the NF-1 binding site in the mouse mammary tumor virus (MMTV) long terminal repeat abolishes the glucocorticoid responsiveness of this promoter (62). Similarly, mutating the NF-1 site within the vitellogenin promoter inhibits estrogen inducibility (63). Thus functional interactions between nuclear hormone receptors and NF-1 family members appear to influence transcriptional activation by steroid hormones. Such synergism has also been described between the VDR and NF1-C, as the VDR transactivates 10 times more strongly when a NF-1 binding site is added to a reporter plasmid containing a DR-3 type VDRE (64).

As previously mentioned, we identified VDR and RXR as components of the complex that bound the c-fos VDRE (48). However, our results also showed that a putative NF-1 family member bound the response element in conjunction with the nuclear hormone receptors. Interestingly, the study of the promoter elements implicated in the control of the expression of the human c-fos gene has revealed the importance of an element centered at a position corresponding to the murine c-fos VDRE and hypothesized to bind members of the NF-1 family (65).

Three lines of evidence support the involvement of a NF-1 family member as a component of the 1,25 $(OH)_2D_3$ -responsive complex binding to the c-fos VDRE. First, the core sequence of the response element (5'-GCCAAG-3') responsible for binding specificity (48) is a high affinity NF-1 binding site (59). Second, a canonical NF-1 binding site (from the adenovirus E5 promoter) efficiently competed for the binding of the vitamin D-responsive complex to the c-fos VDRE (not shown). Finally, antibodies directed against NF-1 recognized the VDRE-bound complex in gel retardation assays (48). However, our results suggest that previously identified members of the NF-1 family (NF1-C1, NF1-C2, and NF1-C3) cannot interact with VDR and RXRα to bind the c-fos VDRE: mixing of recombinant VDR and RXRα proteins with nuclear extracts from HeLa cells, which express high levels of functional NF1-C1, 2, and 3 binding activity (60), failed to reconstitute the binding activity to the c-fos VDRE (48). Moreover, we have expressed the cDNAs for NF1-C1, NF1-C2, and NF1-C3 using *in vitro* transcription and translation systems and have failed to demonstrate interactions with the recombinant receptors (not shown). Moreover, the vitamin D-responsive complex binding the element could only be detected in nuclear extracts from bone cells (48). This supports the notion of a bone-specific NF-1 family member involved in the regulation of target genes in osteoblasts.

Our results suggest a model of trimeric binding where the NF-1 family member anchors the complex to the response element, and the VDR and RXR proteins are then tethered to the site by a combination of protein-protein and protein-DNA interactions. This model raises the intriguing possibility that interactions with the nuclear hormone receptors would endow a ligand-independent transcriptional activator, the NF-1 family member, with dual ligand-switching capabilities, *i.e.* the capacity to respond both to 1,25 $(OH)_2D_3$ and 9-*cis* retinoic acid.

The characterization and molecular cloning of the osteoblast-specific NF-1 factor interacting with the VDR, an ongoing effort in our laboratory, should further our understanding of osteoblastic differentiation and provide new insight into the control of bone-specific gene expression and the molecular mechanism of action of vitamin D.

4.3. Interactions with calreticulin

A number of local and systemic factors have been shown to influence gene expression in osteoblasts. Of particular importance are the effect of attachment of preosteoblasts to various components of the extracellular matrix as well as the modulation of gene expression mediated by members of the nuclear hormone receptor family.

Binding to extracellular matrix components mediated through the integrin family of cell surface receptors modulates gene expression and differentiation

of bone cells. For example, differentiation of canalicular cell processes was observed following contact of osteoblastic cells with laminin (66). At the molecular level, both constitutive and retinoic acid (RA)-induced gene expression is affected when preosteoblastic cells are plated on various substrates (67). These observations suggest that factors that can influence substratum attachment via integrin receptors as well as factors that modulate the function of nuclear hormone receptors may have dramatic effects on gene expression, differentiation and function of osteoblasts. We have shown that the multifunctional protein calreticulin can regulate the affinity state of integrins (68, 69) and inhibit DNA binding by nuclear hormone receptors (70). Interestingly, our most recent results also show that calreticulin plays an important role in osteoblast function and bone formation (71).

Calreticulin has been considered to be a major calcium binding protein of the endoplasmic reticulum of non-muscle cells (72-74). The functions of calreticulin, however, still remain obscure, and as described below, the protein has all the hallmarks of a multifunctional protein. The primary sequence of calreticulin contains putative recognition sequences for phosphorylation by protein kinase C (in the N terminal domain), casein kinase II, and tyrosine kinase (74). In addition calreticulin also appears to have a sequence with marked similarity to the active site of protein kinase C. In fact, calreticulin has recently been described as a phospho-protein with possible autokinase activity (75). Other functional motifs in calreticulin include a nuclear targeting signal, a proline-rich region, and a concentration of acidic residues in the C-terminal of the protein, ending with the ER retention signal, KDEL. Consistent with these putative signals, calreticulin has been localized in the ER and recently also in the nucleus (76). Thus, these interesting features of calreticulin suggest that it may have multiple functions resulting from covalent modifications, *e.g.* by phosphorylation or calcium binding, and depending on its intracellular localization.

Calreticulin has previously been demonstrated by us to function as an integrin binding protein. Specifically, calreticulin binds to the highly conserved KXGFFKR motif present in the cytoplasmic domains of all integrin α-subunits (77). A computer analysis of protein data banks for the presence of the KXGFFKR sequence motif in other proteins revealed that a highly homologous sequence, KGFFKR, was present in the DNA binding domain of all known members of the steroid receptor family (78). In some of the receptors there is a conservative substitution of the G (glycine) to a V (valine) or an A (alanine). Also of interest is the observation that the second amino acid in the integrin motif KXGFFKR which is the most variant in this family, is not present in the steroid receptors. This motif appears in the DNA binding domain of steroid receptors between the two Zn^{2+} fingers, but adjacent to the first Zn^{2+} finger. Site-directed mutagenesis of amino acids in this motif results in either the alteration of DNA sequence specificity, or the complete abrogation of the binding of the receptor to its DNA element (79). Thus, this sequence motif is a DNA binding motif involved in the binding of steroid receptors to DNA during the transcriptional regulation of target genes by these receptors. By analogy with the interaction of calreticulin with the KXGFFKR sequence in the cytoplasmic domain of integrin α-subunits, it was felt that calreticulin may also bind to steroid receptors via this motif and thus modulate gene expression. Thus some aspects of cellular differentiation and function may be regulated by 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 (Fig.3) (80).

Indeed, we and others have demonstrated that calreticulin can interact with members of the steroid hormone and nuclear receptor family and inhibit their binding to DNA response elements (70, 71; 81-83). This inhibition results in an inhibition of transcription via these receptors. Calreticulin has now been demonstrated to interact in a functional manner with the androgen receptor (70), the GR (81), the RAR (70), the peroxisome proliferator-activated receptor (83) and the VDR (71; 82). Purified calreticulin inhibited the binding of the VDR to characterized VDREs in gel retardation assays (71; 82). This inhibition was due to direct protein/protein interactions between the vitamin D receptor and calreticulin (71) and resulted in the abrogation of the vitamin D-dependent transcriptional activation through the VDRE (71; 82). Hence calreticulin may be a major regulator of gene expression via nuclear hormone receptors and as such may have a profound effect on many aspects of cellular physiology, including the response of bone cells to vitamin D.

We have used a gain-of-function strategy to examine this putative role of calreticulin in the regulation of vitamin D-dependent transcriptional activation and mineralization in osteoblastic cells. Northern blot assays revealed that expression of calreticulin transcripts declined during the differentiation of MC3T3-E1 osteoblastic cells (71). Interestingly, preliminary data from our laboratory suggests expression of calreticulin transcripts in preosteoblastic cells from embryonic bone (not shown), supporting the results obtained with the *in vitro* model of osteoblastic differentiation.

Osteoblastic cells were transfected with calreticulin expression vectors; stable transfected cell lines overexpressing recombinant calreticulin 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

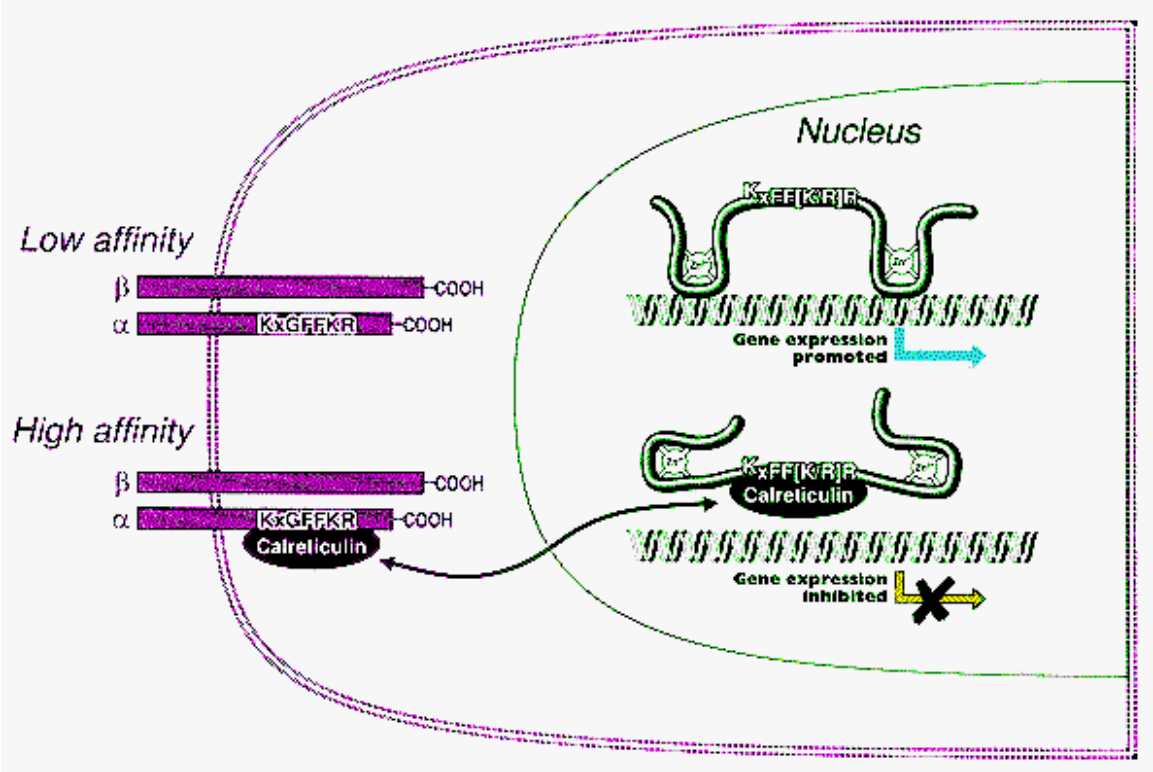

Fig. 3: Schematic representation of the calreticulin-binding motif (KXGFFKR) in the cytoplasmic domain of integrin alpha-subunits and the DNA-binding domain of nuclear receptors (see text). The diagram also illustrates that occupation of integrin KXGFFKR by calreticulin locks the integrins into a 'high-affinity' state that allows ligand binding. Interaction of calreticulin with the KXFF[K/R]R motif of nuclear hormone receptors inhibits receptor activity and receptor-dependent gene transcription.

osteopontin gene expression was unaffected. This pattern mimicked the gene expression pattern observed in parental cells prior to down-regulation of endogenous calreticulin expression. Our results have demonstrated that calreticulin inhibits VDR binding to both the osteopontin and the osteocalcin VDRE (71). However, the two genes responded dissimilarly when the calreticulin-expressing clones were challenged with vitamin D (71). Despite differences between the sequences of the two VDREs (22; 39; 84) that could affect VDR binding affinity to each response element (20), 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. Indeed, developmental and physiologic responsiveness of the osteocalcin gene to 1,25 $(OH)_2D_3$ has been shown to involve, in addition to specific ligand-receptor interactions and binding to DNA response elements, a complex set of event including enhanced receptor gene expression, critical receptor phosphorylation, formation of multiple receptor-protein complexes, as well as overlapping DNA elements at the VDRE locus (85 and references therein). It is likely that calreticulin influences only one aspect of this complex regulatory pathway, namely the interaction of the ligand-bound receptor to its response element, as we have demonstrated *in vitro*.

Aberrant calreticulin expression in bone cells perturbs the differentiation and function of these cells. In long-term cultures of parental or vector-transfected cells, 1,25 $(OH)_2D_3$ induced a 2- to 3-fold stimulation of ^{45}Ca accumulation into the matrix layer (17; 71). Constitutive expression of calreticulin inhibited the 1,25 $(OH)_2D_3$ - induced ^{45}Ca accumulation (71). This result correlated with the complete absence of mineralization nodules in long-term cultures of calreticulin-transfected cells (71).

As previously mentioned, calreticulin binds to alpha-integrin subunits (68) and recent results suggest that this interaction can modulate the affinity state of integrins (69). Moreover, calreticulin can modulate nuclear hormone receptor-dependent gene expression (70, 71; 81-83). 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.

5. PERSPECTIVES

Research on the vitamin D endocrine system is at a crossroad. Non-genomic responses to 1,25 $(OH)_2D_3$ have been described (reviewed in ref. 86) and mutant mice strains deficient in key vitamin D metabolic pathways have been engineered and begin to reveal new aspects of vitamin D function that were previously unrecognized (87). Concerning the vitamin D-dependent transcriptional responses, new VDRE structures have been characterized and novel VDR dimerization partners identified. Sequence similarity searches based on the non-classical response elements may identify previously unrecognized vitamin D-responsive genes. The characterization of additional factors interacting with the VDR, such as the osteoblast-specific NF-1 family member or transcriptional coactivators, is bound to reveal new information on the molecular mechanism of action of vitamin D. Finally, the interactions of the VDR with the multifunctional protein calreticulin have to be tested at the physiological level *in vivo*. The targeted inactivation of calreticulin in embryonic stem cells and the subsequently derived calreticulin 'knock-out' mice, already being studied in our laboratory, will unravel the role of calreticulin in embryonic development and vitamin D-mediated signal transduction.

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