A Negative Vitamin D Response DNA Element in the Human Parathyroid Hormone-related Peptide Gene Binds to Vitamin D Receptor Along with Ku Antigen to Mediate Negative Gene Regulation by Vitamin D*

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We found that the human parathyroid hormone-related peptide (hPTHrP) gene contained a DNA element (nVDREhPTHrP) homologous to a negative vitamin D response element in the human parathyroid hormone gene. It bound to vitamin D receptor (VDR) but not retinoic acid receptor (RXRa) in the human T cell line MT2 cells. VDR binding to this element was confirmed by the Southwestern assay combined with immunodepletion using anti-VDR monoclonal antibody, and this binding activity was repressed by 1,25-dihydroxyvitamin D3. Such a repression was reversed by acid phosphatase treatment, suggesting that 1,25-dihydroxyvitamin D3 phosphorylates VDR to weaken its binding activity to nVDREhPTHrP. In electrophoretic mobility shift assay, we found anti-Ku antigen antibody specifically supershifted the MT2 nuclear protein-nVDREhPTHrP complex. The nVDREhPTHrP-bearing reporter plasmid produced vitamin D-dependent inhibition of the reporter activity in MT2 cells, which was markedly masked by the introduction of the Ku antigen expression vector in the antisense orientation. On the other hand, such a procedure did not perturb the vitamin D response element-mediated gene stimulation by vitamin D. These results indicate that nVDREhPTHrP interacts with Ku antigen in addition to VDR to mediate gene suppression by vitamin D.

The mechanism by which steroid/thyroid nuclear hormone receptors activate gene transcription has been extensively studied (1–8). Among them there have been so many lines of solid evidence showing that vitamin D receptor (VDR),1 thyroid hormone receptor, and retinoic acid receptor employ a common machinery to exert their ligand-dependent specific effects; all of them utilize a retinoic acid X receptor (RXR) in common as a partner of a heterodimer (2, 4). However, this mechanism seems to be confined only to gene stimulation but not to gene repression. This situation led us to address one of the key points in hormonal biology, negative feedback mechanism. The levels of almost all the hormones synthesized at a specific organ are under rigid control to keep their levels within a very narrow range. One of the representative mechanisms is the so-called end product inhibition. In the cases of the nuclear hormone receptors, negative transcriptional regulation of several pituitary trophic peptide hormone genes by glucocorticoid or sex steroid hormones is a good example. Although individual nuclear hormone receptors might well be involved in such regulation, detailed unified molecular mechanisms such as the manner of dimerization are largely unknown, with a few exceptions (9–14). Likewise, the mechanism of negative gene regulation by VDR and vitamin D is only partially understood (15–21). In this case, an active form of vitamin D, 1,25-dihydroxyvitamin D3, can be considered an end product of parathyroid hormone (PTH) action, and this metabolite, in turn, inhibits the synthesis of PTH mRNA to keep the blood calcium level constant. Demay et al. (16) first reported that a negative vitamin D response element (nVDRE) exists in the upstream region of the human PTH gene to mediate such gene repression. This element contains a homologous sequence to only one of the two hexameric DNA sequences that form the core sequence of the consensus DNA sequence (VDRE) for positive gene regulation by vitamin D (2, 4, 22–25). In this process, it was shown that VDR, but not RXR, was involved, and the presence of another unknown partner protein(s) of VDR was proposed (17). However, there have been no reports confirming that the nVDRE is conserved among the genes whose expression is negatively regulated by vitamin D. We have found that the human PTH-related peptide (PTHrP) gene, which is assumed to be derived from an ancestral gene in common with the PTH gene (26), contains a DNA sequence very homologous to the nVDRE of hPTH. Inoue et al. (27) reported that expression of the hPTHrP gene was inhibited by 1,25-dihydroxyvitamin D3 at the transcriptional level in human adult T cell lymphoma/leukemia virus-infected T cells, MT2 cells. With these cells, the therapeutic potential of vitamin D to decrease the blood calcium level due to the inhibition of PTHrP synthesis was discussed. Unlike parathyroid cells, from which it is hard to establish cultured cell lines, MT2 cells are transfectable cultured cell lines. Here, we examined the molecular mechanism of negative regulation of the PTHrP gene by vitamin D in MT2 cells.

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1 The abbreviations used are: VDR, vitamin D receptor; PTH, parathyroid hormone; PTHrP, PTH-related peptide; hPTHrP, human PTHrP; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; Tk, thymidine kinase; VDRE, vitamin D response element; VDREapo, VDRE in the mouse osteopontin gene; RXRa, retinoic acid X receptor α; nVDRE, negative vitamin D response element; nVDREhPTHrP, nVDRE in the human PTHrP gene; nVDREhPTH, nVDRE in the human PTH gene; bp, base pair.
MATERIALS AND METHODS

Homology Search by Computer Analysis—The DNA sequences homologous to the nVDRE of human PTH (16) were searched in the EMBL gene bank, including the human PTHrP gene, and some of them are shown in Fig. 1. We also compared the nVDRE with other DNA elements reported to be responsible for negative as well as positive gene regulation by vitamin D (16–24).

Synthetic Oligonucleotides and Plasmid Constructions—All the oligonucleotides used in this report were made by a DNA synthesizer (Biogen Inc.). They were synthesized as follows: nVDRE\textsubscript{hPTHrP}, 5'-GTGCTCAGCTCCGTTCTGCTA(T)-3'; 3'-GGATCCATCGGTAGTTTTGATGCGAT(AGATC)-5'. VDRE\textsubscript{mp}, 5'-GATCCAGGAGTAATACAGTACAAGCTGAA(T)-3'; 3'-GTTATAGGAAACGCGGTATA(TAGATC)-5'. Bases in the parentheses are BamHI and XbaI cohesive ends to facilitate subsequent ligations to a BamHI-XbaI larger fragment of PUTKAT1 (26).

Transfection and CAT Assay—MT2 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum unless otherwise mentioned. The CAT plasmids or the expression vector encoding p70 subunit of Ku antigen in the antisense orientation were described (29).

Preparation of Nuclear Extracts and Gel Shift Assay—Nuclear extracts were prepared by the method of Schreiber et al. (31) from MT2 cells 40 h after maintaining the cells in the media as indicated. The synthetic oligonucleotides were end-labeled with 32P-ATP by T4 polynucleotide kinase. 104 cpm of the probe (109 cpm/g of the nuclear protein along with 1 g of poly[dI-dC] in each reaction for 30 min at room temperature. Final KCl concentration was adjusted to 80 mM by the binding buffer containing no KCl (10, 31). The probe was incubated with 105 cells for 15 min before the addition of the nuclear extracts. The bound fractions were collected and analyzed by autoradiography as described (12–14). Where indicated, 0–50-fold molar excess of nonradiolabeled oligonucleotides was used as competitors. When anti-human VDR rat monoclonal antibody, 9A7 (BIOMOL Research Laboratory Inc.), or anti-human RXR a rabbit polyclonal antibody, D-20 (Santa Cruz Biotech. Inc.) was used, each of 1/20 and/or 1/4 dilution and the respective control IgGs (1 g/ml) supplied by manufacturers were included in the reaction for 15 min before the addition of the radiolabeled probes. We also used 1 g of anti-Ku antigen human antiserum, which specifically recognizes the p70 subunit of Ku antigen (29) and control serum in the same manner as electrophoretic mobility shift assay (EMSA). To explore the effects of vitamin D metabolites on the protein-DNA interaction, cells were treated in the same manner as described in transfection method. Where indicated, 0.1 unit of potato acid phosphatase (Sigma) was directly included in the reaction mixture at 37°C for the indicated period. Protein concentrations were determined by the Bio-Rad assay kit.

Southwestern Analyses, Immunodepletion, and Immunoblotting—Thirty µg of the nuclear proteins obtained as described above was mixed with an equal volume of 2× denaturing buffer (5% N-lauroylsarcosine, 10% glycerol, 0.1% Trit-Cl (pH 6.8), 2% SDS, 50 mM Tris, 50 mM dithiothreitol). After a 15-min incubation at room temperature, samples were loaded onto an 8% (or 10%) SDS-polyacrylamide gel. Further details were described elsewhere (28), except that we used a radiolabeled oligon(nVDRE\textsubscript{hPTHrP} or VDRE\textsubscript{mp}) in this study. Where indicated, we used MT2 nuclear proteins in which VDR was immunodepleted with the 9A7 shown above, according to the method reported previously (28).

Immunoblotting with 1 µg/ml of the 9A7 or 1/1000 diluted anti-Ku antigen human antiserum also shown above was performed by the enhanced chemiluminescence method as reported (29).

RESULTS

Putative nVDRE Sequences in PTH and PTHrP Genes from Several Species—In the upstream region of the human PTHrP gene, we found a DNA sequence homologous to a nVDRE in the human PTHrP gene (nVDRE\textsubscript{hPTHrP}). The numbers shown above each sequence represent the locations from the respective transcription start sites. The bases similar to the consensus direct repeat, AG(G/A)TTCA (2), are underlined. The identical bases between nVDRE\textsubscript{hPTHrP} and a given sequence are shown as bold letters. The sources of each putative nVDRE are as follows: nVDRE\textsubscript{rPTHrP} (18), nVDRE\textsubscript{ePTHrP} and nVDRE\textsubscript{ePTHrP} (18), nVDRE\textsubscript{ePTHrP} (19), nVDRE\textsubscript{ePTHrP} (21), and the nVDRE-like sequence of chicken TGFβ2 (20). One of the positive VDREs, VDRE\textsubscript{mp}, was also included.

Putative nVDRE in the hPTHrP Gene Binds to VDR and Ku Antigen

Fig. 1. DNA sequences homologous to a putative negative vitamin D response element (nVDRE) in the human PTHrP gene (nVDRE\textsubscript{hPTHrP}). The numbers shown above each sequence represent the locations from the respective transcription start sites. The bases similar to the consensus direct repeat, AG(G/A)TTCA (2), are underlined. The identical bases between nVDRE\textsubscript{hPTHrP} and a given sequence are shown as bold letters. The sources of each putative nVDRE are as follows: nVDRE\textsubscript{rPTHrP} (18), nVDRE\textsubscript{ePTHrP} and nVDRE\textsubscript{ePTHrP} (18), nVDRE\textsubscript{ePTHrP} (19), nVDRE\textsubscript{ePTHrP} (21), and the nVDRE-like sequence of chicken TGFβ2 (20). One of the positive VDREs, VDRE\textsubscript{mp}, was also included.
TGFβ2 gene very homologous to nVDREs of both hPTH and hPTHrP. Intriguingly, expression of the chicken TGFβ2 gene was shown to be inhibited by vitamin D in mesangial cells (20). On the other hand, as shown in Fig. 1, the sequences of other recently proposed nVDREs such as those in the chicken PTH (21) gene and the rat PTHrP gene (19) or positive VDRE in the recently proposed nVDREs such as those in the chicken PTH and TGFβ2 genes are considerably different from these three nVDREs.

**Interaction between Nuclear Protein(s) in MT2 Cells and nVDRE**

We synthesized oligonucleotides corresponding to nVDRE_{hPTHrP} and VDRE_{mop} and examined the interactions between each of these oligonucleotides and nuclear protein(s) from MT2 cells by EMSA. As shown in Fig. 2, both of the oligonucleotides formed two protein-DNA complexes. Since each of the lower bands did not consistently appear, we focused on the upper bands in this study. Although the migrating positions of both complexes seemed similar in EMSA, the MT2 protein-nVDRE_{hPTHrP} complex was competed out only by a 10–50-fold molar excess of the nonradiolabeled VDRE_{mop} (lanes 2 and 3) or nVDRE_{hPTHrP} (lanes 4 and 5) were included. The arrows indicate the specific protein DNA complex.

**nVDRE in the hPTHrP Gene Binds to VDR and Ku Antigen**

Our next question was what is the effect of vitamin D on the binding between nVDRE_{hPTHrP} and MT2 proteins. As shown in EMSA (Fig. 5A), 1,25-dihydroxyvitamin D₃ weakened the binding between nVDRE_{hPTHrP} and MT2 nuclear proteins, although it strengthened the binding between VDRE_{mop} and these proteins in a dose-dependent manner. In the Southwestern assay, using a radiolabeled nVDRE_{hPTHrP} as a probe (Fig. 5B), similar dose-dependent effects of 1,25-dihydroxyvitamin D₃ were observed on the band corresponding to about 50 kDa in size but not on the other bands. We then employed MT2 nuclear proteins in which VDR was immunodepleted with the 9A7 shown above in the Southwestern assay. As shown in Fig. 5C, immunodepletion treatment clearly and selectively abolished the binding of this 50-kDa band but not higher molecular mass bands, strongly suggesting this 50-kDa band corresponded to the VDR itself. On the other hand, the radiolabeled VDRE_{mop} yielded only one band around 96 kDa in size, which was unaffected by the immunodepletion. We assume these higher molecular weight proteins are nonspecific DNA binding proteins. Immunoblotting with 9A7 revealed that treatments with 1,25-dihydroxyvitamin D₃ did not affect the amounts of VDR protein (Fig. 5D). Together, these results suggest that 1,25-dihydroxyvitamin D₃ treatment weakens the binding between nVDRE_{hPTHrP} and VDR in MT2 cells, thereby exerting its inhibitory effect on the expression of nVDRE_{hPTHrP}-bearing gene(s) in these cells.

**Ku Antigen, Along with VDR, Mediates Negative Regulation of the Gene(s) Containing nVDRE_{hPTHrP} by Vitamin D**

In order to find a clue for a probable partner of VDR in the nVDRE_{hPTHrP}-MT2 protein complex, we reexamined the sequence of nVDRE_{hPTHrP} and noticed that it contained an octamer ATTTCGAT-like sequence following the core motif. This sequence, ATTTCGTT, is reminiscent of one of the negative calcium-responsive elements, oligo(A). We previously showed that the footprint in the oligo(A) HeLa nuclear protein was localized exactly in this position (15) and demonstrated that the nuclear protein specifically binding to oligo(A) contained Ku antigen (29). Various roles of Ku antigen such as double strand break repair or DNA recombination by virtue of its ability to bind DNA ends nonspecifically have been reported (32–34). However, a recent report demonstrating that Ku antigen recognizes a specific internal DNA element in the mouse mammary tumor virus long terminal repeat to inhibit glucocorticoid receptor-mediated transcriptional stimulation (35) prompted us to examine whether Ku antigen is involved in nVDRE_{hPTHrP}-mediated gene inhibition by vitamin D. As shown in Fig. 6, inclusion of anti-Ku antigen antibody (29) in EMSA significantly reduced the nVDRE_{hPTHrP}-MT2 protein complex but not the VDRE_{mop}-MT2 protein complex. Of note, this treatment seemed to strengthen the binding activity of the
faster migrating band when we used nVDRE<sub>hPTHrP</sub> as a probe. However, as noted earlier, because the migrating position of the faster migrating band overlaps that of the occasionally observed lower band (Fig. 2), we did not pursue this issue in this manuscript.

To show the effect of Ku antigen on nVDRE<sub>hPTHrP</sub>-mediated gene repression by vitamin D<sub>3</sub> in vivo, we transiently introduced an expression vector encoding the p70 subunit of Ku antigen in its antisense orientation (29) along with the TkCAT-based reporter plasmids bearing either nVDRE<sub>hPTHrP</sub> or VDRE<sub>mop</sub>. Immunoblotting with anti-Ku antigen antiserum, which specifically recognizes the p70 subunit of Ku antigen (Fig. 7A), revealed that this treatment inhibited the synthesis of the p70 subunit of Ku antigen by about 50%. As shown in Fig. 7B, although 1,25-dihydroxyvitamin D<sub>3</sub> had similar repressive effects on nVDRE<sub>hPTHrP</sub>-CAT activity in MT2 cells transfected with an empty vector, it lost the inhibitory action in MT2 cells when the p70 subunit of Ku antigen was expressed in the antisense orientation. On the other hand, 1,25-dihydroxyvitamin D<sub>3</sub> exerted similar stimulatory effects on the CAT activity by VDRE<sub>mop</sub>-TkCAT with or without expression of Ku antigen.

Phosphatase Treatment of VDR Restores Its Binding to nVDRE<sub>hPTHrP</sub> Once Attenuated by Vitamin D—Recently, Ku antigen was shown to have an inhibitory effect on the glucocorticoid receptor-mediated transcriptional stimulation of the mouse mammary tumor virus long terminal repeat gene (35). It was subsequently shown that the catalytic subunit of Ku antigen (DNA-dependent protein kinase), along with both regulatory subunits of Ku antigen (p70 and p86), phosphorylates glucocorticoid receptor after binding to its specific binding DNA element called negative response element (36). Although the authors did not refer to the direct role of this type of phosphorylation on gene repression (36), their report prompted us to speculate that presumable phosphorylation of VDR by the Ku antigen, which would have been triggered by the treatment with 1,25-dihydroxyvitamin D<sub>3</sub>, might weaken its activity to bind to nVDRE<sub>hPTHrP</sub>, leading to vitamin D-mediated gene repression. To examine this possibility, we treated nuclear proteins obtained from vehicle- or 1,25-dihydroxyvitamin D<sub>3</sub>-administered MT2 cells with potato acid phosphatase for different times and examined their activity to bind nVDRE<sub>hPTHrP</sub> by the Southwestern assay as shown in Fig. 8. As expected, the binding of the VDR to nVDRE<sub>hPTHrP</sub>, once attenuated by the treatment with 10<sup>−7</sup> M 1,25-dihydroxyvitamin D<sub>3</sub> (see Fig. 5), but not with vehicle alone, was up-regulated by the phosphatase treatment. On the other hand, the binding of the nonspecific bands above the VDR was not altered by the treatment. These results suggest, albeit indirectly, that phosphorylation of VDR by a certain kinase (e.g. DNA-dependent protein kinase) might decrease its activity to bind to nVDRE<sub>hPTHrP</sub>.
We have shown that the human PTHrP gene contains a DNA element (nVDRE_hPTHrP) very homologous to the negative vitamin D response element found in the human PTH gene (16). Although it is in reverse orientation compared with the nVDRE_hPTH, we demonstrated that nVDRE_hPTHrP functioned similarly to nVDRE_hPTH in terms of transcriptional repression by vitamin D. Sequence comparison between nVDRE_hPTHrP and nVDRE_hPTH revealed 11 identical bases out of 13 nucleotides. Furthermore, it contains a heptameric AG(G/A)TTCA, mimicking a hexameric AG(G/A)TCA motif proposed to be a core element (2) for the binding of the nuclear receptors for thyroid hormone, vitamin D (VDR), or retinoic acid (retinoic acid receptor and RXR). However, unlike VDRE, consisting of two such motifs separated by a three-base spacer (Refs. 2 and 22; see VDRE_hop), nVDRE_hPTHrP and nVDRE_hPTH possess only one motif. This observation led us to speculate that the content of nuclear proteins binding to the nVDREs was different from the usual VDR-RXR heterodimer, as is found in the authentic VDRE (see below). On the other hand, by computer search, we found the nVDRE motif in the 21100-bp upstream region of the mouse TGFb2 gene (Fig. 1). 12 out of 13 bases were identical between the two. The only difference lay in the heptamer region. In the chick TGFb2 gene, AGATTCA in the hPTHrP gene was changed to AGGTTCA, which was, in turn, identical to the heptamer in the hPTH gene. Very interestingly, expression of this gene in the mesangial cells was reported to be inhibited by 1,25-dihydroxyvitamin D3 (20). This finding further supports that nVDRE might play a general role in vitamin D-mediated gene inhibition.

A couple of recent reports demonstrated that the rat PTHrP gene contains several DNA sequences responsible for negative gene regulation by vitamin D. Falzon (18) reported two nVDREs in the upstream region of the rat PTHrP gene. He showed that both of them were necessary for vitamin D-mediated gene inhibition. One of them located at the −726-bp upstream region possesses one heptameric sequence, AGGTTCCT.
GTGCT, lying -7 to -3 bases preceding the core heptamer were identical between the two nVDREs found in the human and rat PTHrP genes; such identity was not observed in nVDRE hPTHrP. Interestingly, this AGGTCCA was repeated with a 3 bases preceding the core heptamer in the nVDRE in the human PTH gene was found in the binding to the positive regulatory element. Furthermore, of particular interest, the DNA sequence of VDR and RXR

that another unknown partner (16, 17) might be involved. Although we cannot rule out the possibility that RXRs other than RXRα are involved here, the unique association of VDR and RXRα, which is found in the binding to the positive VDRE mop as shown in Fig. 3, is not predominant in the binding between nVDREhPTHrP and MT2 nuclear proteins. This notion was supported by the Western assay, in which MT2 VDR was immunodepleted by the anti-VDR antibody (Fig. 5C).

In this assay, we demonstrated that nVDREhPTHrP could not bind to 50-kDa nuclear proteins after immunodepletion, whereas VDRE mop did not bind to this band irrespective of immunodepletion. This result suggests that VDR mop, unlike nVDREhPTHrP, can bind to MT2 VDR only when the latter is associated with RXRα.

On the other hand, our experiments using anti-Ku antigen antibody, which recognizes one of its subunits, p70, demonstrated that it supershifted the complex between MT2 nuclear proteins and nVDREhPTHrP but not VDRE mop (Fig. 3). Furthermore, our experiments using the antisense-oriented Ku antigen expression vector (Fig. 6) demonstrated that negative regulation by vitamin D via the nVDREhPTHrP was abrogated by the introduction of such a vector. Because such an abrogation was not observed in the case of the VDRE mop-TkCAT, we concluded that Ku antigen was crucially involved in negative gene regulation by vitamin D in MT2 cells. Although the mode of action of Ku antigen has been under extensive study (32-34), only recently has its role as a transcription factor been established (29, 33). Ku antigen consists of two regulatory subunits, p70 and p86, as well as the catalytic unit of another large protein of DNA-dependent protein kinase, DNA-dependent protein kinase (32). Its ability to bind DNA nonspecifically could explain its function such as double strand break repair or DNA recombination. We (29) recently demonstrated that p70 and p86 subunits of Ku antigen could interact in association with another nuclear protein, redox factor 1 (1), with one of the negative calcium-responsive elements in a sequence-specific manner. We showed that such an interaction led to extracellular calcium-mediated transcriptional inhibition of the genes bearing negative calcium-responsive elements. Furthermore, of particular interest, the DNA sequence
following the heptamer in the nVDRE is directed to the detailed analyses of the interaction between nVDRE and MT2 VDR as shown in Fig. 5, is currently unclear. Although there have been several reports describing up-regulatory effects of vitamin D on the amount of VDR protein or on its binding activity to VDRE (37), the mechanism by which vitamin D inhibits the binding of VDR to nVDRE has not been addressed. One attractive hypothesis is that Ku antigen might play some role in such negative regulation as shown in this manuscript. Particularly, the recent report suggesting that protein kinase activity of DNA-dependent protein kinase, the catalytic subunit of Ku antigen, would modulate glucocorticoid receptor activity after the specific binding to DNA (35, 36) prompted us to carry out the Southwestern assay in which the VDR from 1,25-dihydroxyvitamin D3-administered MT2 cells was dephosphorylated by potato acid phosphatase (Fig. 8). Although dephosphorylation of VDR did not affect its binding to nVDRE when VDR was obtained from MT2 cells treated with vehicle alone, it reversed VDR binding to nVDRE pre-attenuated by the treatment with 1,25-dihydroxyvitamin D3. Therefore, we raise the possibility that phosphorylation of VDR by the Ku antigen, which would have been triggered by the treatment with 1,25-dihydroxyvitamin D3, might weaken its activity to bind to nVDRE followed by vitamin D-mediated gene repression, although direct evidence to support this contention is still lacking.

If vitamin D mediates conditional inhibition of PTHrP gene as suggested here, what is the significance of the constitutively active binding between nVDRE and MT2 nuclear protein(s) in the unliganded state? We speculate that such a binding might be somewhat related to constitutively active production of PTHrP in such cell lines as MT2 cells, leading to humoral hypercalcemia of malignancy, the most common cause of human hypercalcemia (26). We suggest here that a therapeutical approach to targeting to the transcriptional apparatus would be of potential benefit. In this paper, we focused on the difference between positive and negative VDREs as described above. Future investigation is directed to the detailed analyses of the interaction between nVDRE and VDR including mutagenesis of nVDRE as well as characterization of VDR domain involved here, all of which are now in progress in our laboratory.

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