Vitamin D Receptor Zinc Finger Region Binds to a Direct Repeat as a Dimer and Discriminates the Spacing Number between Each Half-site*

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1α,25-Dihydroxyvitamin D₃, the most active metabolite of vitamin D₃, is a multifunctional agent. The actions of 1α,25-dihydroxyvitamin D₃ are mediated through its receptor that activates the specific genes in a ligand-dependent manner. In order to investigate the details of DNA binding properties of vitamin D receptor, we have developed the overexpression and purification system of vitamin D receptor DNA binding domain. The purified peptide could specifically bind to the osteopontin-derived vitamin D responsible element (VDRE) but not to the osteocalcin and the calbindin D-9k-derived VDREs, as determined by bandshift analysis. The osteopontin VDRE contains a direct repeat of GGTTCA motif separated by 3 nucleotides, whereas the osteocalcin and calbindin D-9k VDREs have inadequate direct repeat. Further analyses using synthetic oligonucleotides revealed that vitamin D receptor DNA binding domain could discriminate the spacing number between the consensus steroid-responsed element motif and had different affinities to direct repeats that consisted of various related sequences. These studies give insight into ways in which vitamin D receptor mediates the signal of 1α,25-dihydroxyvitamin D₃.

A vitamin D receptor (VDR) is a ligand-dependent transcriptional factor that belongs to the steroid and thyroid hormone (3, 5, 3′-triiodothyronine) receptor superfamily as well as glucocorticoid receptor, thyroid hormone receptor, estrogen receptor, and so on (1, 2). These receptors exist in the nucleus and can bind to cognate ligands. The conformational change by ligand binding confers on them the ability to act as transcriptional activators through the interaction with other factors and/or phosphorylation. By these processes the signal of ligands is mediated to cellular function through transcriptional activation of the target genes.

Sequence comparison among steroid hormone receptors and mutational analysis of VDR in mammalian cells have identified functional domains for transcriptional activation, DNA binding, and ligand binding (3). The DNA binding domain exists in the amino terminus and includes a cysteine-rich cluster that comprises two zinc finger-like structures (4-6).

Steroid hormones exert their effect by the binding of receptor-ligand complexes to the short DNA sequences called hormone response elements (HREs) in the regulatory regions of the genes (7, 8). Although vitamin D responsible elements (VDREs) have been identified in human osteocalcin (OST) (9, 10), rat OST (11, 12), mouse osteopontin (mSPP-1) (13), and rat calbindin D-9k (CaBP) (14) gene, the DNA binding of an exclusive VDR to VDRE has not been achieved. In order to bind the OST VDRE, VDR requires the additional nuclear accessory factor (15-19). However, information on the sequence that a sole VDR can recognize is crucial for understanding how VDR discriminates between DNA binding sites and, in particular, how VDR might bind cooperatively or competitively to DNA in situations where a number of different transcription factors are present within a cell. Such information is also essential as a basis for interpreting three-dimensional structure data concerning the molecular mechanism of DNA sequence recognition.

Detailed biochemical and structural characterization of VDR has been prevented from the analysis by the limited availability of the pure protein because of its scarcity and lability. Although VDR has been expressed in Saccharomyces cerevisiae (15) and insect cells using a baculovirus system (20, 21), the protein production in Escherichia coli can be superior to the above mentioned system in consumed time and quantity. Therefore, we have focused on the DNA binding of VDR and developed the expression system of VDR DNA binding domain. In this paper, we describe the overproduction of VDR zinc finger region in E. coli and its DNA binding properties.

MATERIALS AND METHODS

Construction of Plasmids—The expression plasmid pMAL-c2, carrying the P勋 promoter positioned to transcribe a malE gene, was purchased from New England Biolabs. The VDR zinc finger DNA fragment was synthesized by the RNA/polymerase chain reaction technique (22). The mRNA was purified from rat kidney and used as template. After gel purification, the polymerase chain reaction-amplified fragment was inserted into pMAL-c2 so that it was in the same translational reading frame as the vector’s malE gene. The recombinant plasmid was transformed into E. coli JM109 cells. The entire sequence inserted was confirmed by the digest method (23).

Production of Fusion Protein in Bacterial Cells—The VDR zinc finger DNA fragment was inserted downstream from the malE gene, which encoded maltose-binding protein (MBP), the VDR zinc finger peptide (VDRDBD) was expressed in the form of fusion protein with MBP (Fig. 1B). For production of MBP-VDRDBD fusion protein in E. coli, the overnight culture was inoculated into Luria-Bertani medium supplemented with 0.2% glucose and incubated at 37 °C to an optical density at 600 nm of 0.4; then IPTG was added at a final concentration of 0.3 mM. Cells were harvested after 2-h incubation, resuspended in TEGD200 (50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA,
10% glycerol, 2.5 mM dithiothreitol, and 200 mM KCl), and disrupted by sonication. The production of fusion protein was estimated by staining the gel with Coomassie Brilliant Blue after SDS-PAGE of the cell lysate.

Purification of VDRDBD—The purification was started from the cytosolic fraction of IPTG-induced bacteria, which was obtained as supernatant by centrifugation of cell disruption at 10,000 x g for 10 min. The crude extract was applied to an amylose resin column that had high affinity for MBP and washed with 10 column volumes of TEGDZm. The bound MBP-VDRDBD fusion protein was eluted with 50 mM maltose in TEGDZm. The protein-containing fractions were pooled and subjected to factor Xa cleavage. Factor Xa is a sequence-specific protease, and the factor Xa cleavage site (Ile-Glu-Arg) exists between MBP and VDRDBD. Factor Xa was added to the amylose resin eluate at the ratio of 0.5% to the amount of fusion protein. The reaction mixture was incubated for 2 days at 4°C. After the factor Xa cleavage, the reaction mixture was applied to a hydroxylapatite column equilibrated with 10 mM phosphate buffer, pH 7.0. Under this condition, VDRDBD appeared in a flow-through fraction to be homogeneous.

DNA Binding Analysis—Double-stranded oligonucleotides used for the binding analysis were synthesized by a DNA synthesizer (Milligen). All oligonucleotides synthesized here were purified by urea-polyacrylamide gel electrophoresis. To use the oligonucleotides as probes, 5'-overhangs were filled in with [cy-32P]dCTP (Du Pont-New England Nuclear) using Klenow fragment. The purified fusion protein or VDRDBD (6.25 μl) was mixed with the same volume of 20 mM Tris-HCl (pH 7.5), 10% glycerol, 2 mM dithiothreitol, 10 mM EDTA, 0.32 mg/ml poly(dI-dC), and radiolabeled probe (10,000 cpm/10 fmol). The binding reaction was performed at room temperature for 30 min. Each reaction mixture was loaded on a 4% nondenaturing polyacrylamide gel, electrophoresed at 150 V for 1 h, fixed with 10% methanol and 10% acetic acid, and autoradiographed overnight at −80°C.

RESULTS

Overproduction and Purification of VDRDBD—Because the expression vector pMAL-c2 contained a strong inducible tac promoter and the malE translational initiation signals, the vector gave high level expression of the cloned gene, followed by one-step affinity purification for MBP to facilitate isolation of the fusion protein (25). The induction was started by addition of IPTG to the culture medium in which transformed E. coli JM109 cells were growing. During the IPTG induction, cells were sampled at given times and analyzed by 7.5% SDS-PAGE for total cytoplasmic protein (Fig. 1C, lanes 2–5). A predominant band at M, 56,000 (solid arrow) was evident in the IPTG-induced cells (Fig. 1C, lanes 2–5), whereas it was undetectable in the extract from uninduced cells (Fig. 1C, lane 1). It was confirmed by Western blot analysis using anti-MBP that this band corresponded to the MBP-VDRDBD fusion protein (data not shown). The production of the fusion protein was increasing in a time-dependent manner and saturated at 2 h, so that for the purification, cells were induced for 2 h.

The MBP-VDRDBD fusion protein was partially purified by amylose resin affinity chromatography, and the protein was analyzed for the purity by a 7.5–15% gradient gel (Fig. 2, lane 4). The purified MBP-VDRDBD fusion protein was digested with factor Xa (0.5% of the fusion protein) for 2 days at 4°C. Under these conditions, about 50% of fusion protein was cleaved to 42 kDa of MBP and 14 kDa of VDRDBD (Fig. 2, lane 4). This size is consistent with the calculated M, 13,638 corresponding to 119 amino acids (101 + 18 amino acids derived from the vector). Increasing the concentration of factor Xa to 1% of the fusion protein resulted in about 90% cleavage (data not shown). When the reaction mixture was applied to the hydroxylapatite column, MBP and the uncleaved fusion protein were adsorbed to hydroxylapatite, and VDRDBD appeared in the flow-through fraction (Fig. 2, lane 6). After completion of the final chromatographic step, VDRDBD was concentrated with a microconcentrator (Amicon), and the purity was checked by SDS-PAGE (Fig. 2, lane 8). Amino acid sequence of the purified peptide was analyzed, and a single N-terminal region of 27 amino acids was found corresponding to rat VDR (26). This result also confirmed the homogeneity of VDRDBD. We could obtain the 10-mg peptide of the pure VDRDBD from a 5-liter culture.

Interaction of Purified VDRDBD with Various VDREs—The ability of recombinant VDRDBD to bind DNA was investigated. The interaction of VDRDBD with specific DNA
sequences can be assessed through bandshift analysis as a VDR-DNA complex retarded in migration from the free DNA probe. Because VDRDBD was unstable at a low protein concentration, we used the relatively high amount of protein.

As shown in Fig. 3A, VDREs derived from human osteocalcin (hOST), rat osteocalcin (rOST), and CaBP (but not mSPP-1) genes were not bound to VDRDBD (lanes 1–4). Therefore, we concluded that the mSPP-1 VDRE was the only sequence recognized by the VDR zinc finger region itself among the VDREs reported up to now, although all VDREs used in this experiment had been shown to be vitamin D inducible by chloramphenicol acetyltransferase assay in mammalian cells (9, 11, 13, 14).

As a further test for the specificity of the detected complex, competition experiments using unlabeled oligonucleotides were performed (Fig. 3B). The complex formation of VDR-DNA was inhibited significantly by addition of a 100-fold excess of unlabeled mSPP-1 VDRE (Fig. 3B, lane 2). In contrast, the rOST VDRE did not interrupt the binding of VDRDBD to the mSPP-1 even though a 500-fold excess of unlabeled rOST was added (Fig. 3B, lane 6). This result also suggested that the VDR DNA binding region had much higher affinity for the mSPP-1 VDRE than the rOST VDRE. The mSPP-1 VDRE consisted of a complete direct repeat of the HRE half-sites in contrast to the rOST VDRE (Fig. 4). It has been reported that the spacing of core binding motifs (AGGTCA (N), AGGTCA) is important for dictating selective transcriptional effects by each of the steroid hormone receptors (24). Spacers of 4 and 5 bp confer relatively specific transcriptional responses for 3,5,3′-triiodothyronine and retinoic acid, respectively, while a spacer of 3 bp confers transcriptional response for 1α,25-dihydroxyvitamin D₃.

VDRDBD Discriminates the Spacing Number between Half-sites—The good agreement of our results with this 3–4–5 rule led us to examine the binding of VDRDBD to DR-1, DR-3, DR-4, and DR-5, which were typical direct repeat HREs separated by 1, 3, 4, and 5 nucleotides used in the study of Umesono et al. (24). The VDRDBD allowed us to detect the specific binding to DR3 but not to DR-1, DR-4, and DR-5 in both lower (Fig. 5A) and higher concentrations of VDRDBD (Fig. 5B). It was surprising that VDRDBD could discriminate the spacing number even though it did not have the major dimerization domain at the C terminus because the discrimination of spacer number was thought to be conducted through dimerization. This result suggested that another dimerization domain existed in the VDR zinc finger region and that its domain was important to discriminate the spacing number.

VDRDBD Binds to the Direct Repeated VDRE as a Dimer—It has been known that the other steroid hormone receptors such as glucocorticoid receptor, estrogen receptor, and thyroid hormone receptor bind to the palindromic HREs as a dimer. Since these receptor-DNA complexes are symmetric, it is assumed that the dimerization domain of each receptor on a single half-site is directed to be nearby, where it is easy to achieve the protein-protein interaction. Thus, the homodimerization of receptor on DNA was thought to be accomplished by binding to the palindromic HRE. However, purified VDRDBD can bind to the direct repeated VDRE (Figs. 3 and 5). Therefore, we next examined whether VDRDBD binds to DNA as a dimer or not, by using purified VDRDBD and MBP-VDRDBD fusion protein.

As shown in Fig. 6B (using the mSPP-1 VDRE as a probe), the fusion protein-DNA complex showed further retarded migration than the VDRDBD-DNA complex (lane 1 or lane 2) due to the molecular mass difference between the fusion protein and VDRDBD, 56 and 13 kDa, respectively. Using a mixture of the fusion protein and VDRDBD, three bands corresponding to protein-DNA complexes could be seen; the slowest migrating band corresponded to the homodimer of fusion protein bound to DNA (compare to lane 2), and the most rapid corresponded to the homodimer of VDRDBD.
Table 1. Alignment of VDRE sequences on hOST, rOST, mSPP-1, CaBP gene, and DR-3.

| Gene    | Sequence      | VDR binding |
|---------|---------------|-------------|
| hOST    | GGGTGA AGG GGGGCA |            |
| rOST    | GGGTGA AGT AGGACA |            |
| CaBP    | GGGTGT CGG AGGCCC |            |
| mSPP-1  | GGTCA CGA GGTTCGA | +          |
| DR-3    | AGGTCA AGG AGGTCA | +          |

**Fig. 4.** Alignment of VDRE sequences on hOST, rOST, mSPP-1, CaBP gene, and DR-3.

**Fig. 5.** Effect of the spacing number between half-sites on VDRDBD binding. Lower (200 ng, panel A) and higher (1000 ng, panel B) amounts of VDRDBD were incubated with oligonucleotides containing the core motif AGGTCA as a direct repeat with a half-site spacing of 1 bp (DR-1, lanes 1 and 5); 3 bp (DR-3, lanes 2 and 6); 4 bp (DR-4, lanes 3 and 7), or 5 bp (DR-5, lanes 4 and 8). The oligonucleotide sequences used in this study are as follows: DR-1, 5'-gatcTTCCAGGTTCACAGGTCAGAT-3'; DR-3, 5'-gatcTTCCAGGTTCACAGGTCAGAT-3'; DR-4, 5'-gatcTTCCAGGTTCACAGGTCAGAT-3'; DR-5, 5'-gatcTTCCAGGTTCACAGGTCAGAT-3'.

Bound to DNA (compare with lane 1). The presence of an intermediate band is indicative of a DNA-bound heterodimer between MBP-VDRDBD fusion protein and VDRDBD (lane 3). While we examined the dimerization experiments by using homobifunctional cross-linking agents, disuccinimidyl suberate and 3,3'-dithiobis(succinimidyl propionate), we could not find the homodimer of VDRDBD in the absence of DNA (data not shown).

To test the dimerization of VDRDBD on direct repeat, we synthesized the oligonucleotide consisting of one mSPP-1 half-site and compared it with native mSPP-1 VDRE as a function of the protein concentration (Fig. 6A). In the case of the mSPP-1 half-site, the lower band thought to be a monomeric complex was observed (lane 12), although it required the 8-fold excess of protein compared with native mSPP-1 VDRE (lanes 1–6). Furthermore, when the native mSPP-1 was used as a probe, the monomeric complex was not observed even at a relatively low concentration of VDRDBD (lane 3). This result might be explained because the dimer of VDRDBD had higher affinity for the mSPP-1 VDRE than the monomer, and therefore the dimer preferentially bound to DNA. From these results, we concluded that VDRDBD was monomeric in solution and the binding of one VDRDBD monomer to the mSPP-1 half-site facilitated the binding of the second monomer to the other half-site and dimerization on DNA.

**Effect of Orientation and Half-Site Sequence on VDRDBD Binding**—We have shown in this paper that the spacing number between the half-sites was critical for the binding of VDRDBD. Therefore, we next examined the effect of orientation and sequence of the half-site, which should be important factors for the DNA binding. As shown in Fig. 7B, VDRDBD could bind to palindromes similar to direct repeats in the case of either the mSPP-1 (lanes 1 and 2) or DR-3 (lanes 3 and 4) half-site. Thus, the orientation of half-site was not effective for the binding of VDRDBD to DNA or at most less effective. However, the sequence of the half-site was strongly effective (compare lanes 1, 3, 5, and 6). The direct repeat of the mSPP-1 half-site sequence GGTTCA had higher affinity than the estrogen receptor (ER)/thyroid hormone receptor (TR) subfamily consensus motif AGGTCA. The direct repeat of glucocorticoid response element half-site AGGACA, which is identical to the second half-site of rOST.
VDRE, showed a weak affinity for VDRDBD. VDRDBD could not bind to a direct repeat of the rOST first half-site GGTTCA. VDRDBD had the following order of relative binding affinity for the direct repeat series: GGTTCA > AGGTCA > AGGACA > GGTTGA. From these results, it was concluded that the half-site sequence and spacing number between half-sites were important for DNA recognition of VDRDBD and that the orientation of the half-site was much less effective.

**DISCUSSION**

In this report, we describe the sequence that the DNA binding domain of VDR can recognize independently. Although the purified peptide fragment of VDR zinc finger region did not bind to the hOST, rOST, and CaBP VDREs, it was able to bind to the VDREs of mSPP-1 (Fig. 3). The VDRE of mSPP-1 contained a complete direct repeat of half-site; in contrast, the osteocalcin and CaBP VDRE contained an inadequate direct repeat (Fig. 4). Furthermore, the DNA binding of VDRDBD was affected by the spacing number between half-sites (Fig. 5). These data demonstrated that VDR might bind to DNA as a dimer. The VDR expressed in yeast and insect cells also did not have the ability of binding to the osteocalcin VDRE independently (15, 20). However, these receptors could bind to the VDRE only in the presence of other nuclear accessory factors derived from mammalian cells (15, 19, and 20) or retinoid X receptor (17). These facts may be explained by dimerization of VDR on DNA as a homodimer or a heterodimer provides sufficient free energy to stabilize the protein-DNA complex. That is to say, one VDR binds to each half-site of the direct repeat, and the protein-DNA complex is stabilized through the dimer formation of VDR on DNA. If the VDRE consists of an imperfect direct repeat such as hOST, rOST, and CaBP, the VDR molecule cannot interact on DNA by itself, and VDR forms a heterodimer with another factor in order to bind to DNA. In the case of VDRDBD, the dimerization may be accomplished by use of the second zinc finger that is thought to have a weak dimerization domain in the case of the other steroid hormone receptors (27-29). These differences in protein-DNA complex formation between the VDRE of vitamin D-inducible genes might possibly produce the diversity of vitamin D action.

The P box in the first zinc finger is important for discriminating the half-site sequences (30, 31). According to the P box amino acid sequence, steroid hormone receptors are classified into glucocorticoid receptor subfamily and ER/TR subfamily. The P box sequence of VDR is Glu-Gly, and it belongs to the ER/TR subfamily. The half-site recognized by VDR is predicted to be A/TGGTCA. However, VDRDBD showed higher affinity for a direct repeat of the mSPP-1 half-site GGTTCA than AGGTCA. This demonstrates that there is diversity in concerning half-site recognition among subfamilies.

Finally, VDRDBD could bind to both the direct repeat and palindrome with 3-nucleotide spacing (Fig. 7). In the case of palindrome, it is easy to imagine that the dimerization domain of each receptor on a single half-site is directed to be nearby, where it is easy to achieve the protein-protein interaction. In fact, this hypothesis was confirmed by three-dimensional structural analysis using the crystal of the GRDBD-glucocorticoid response element complex (29). However, the precise mechanism by which protein binds to the direct repeat remains to be elucidated until the three-dimensional structure of the VDRDBD-DNA complex will be dissolved. In this paper, we have described the overexpression system of the VDR zinc finger region in E. coli with the simple protocol, leading to the isolation of a large quantity of the peptide. Having a rich source of the functional VDRDBD should provide the starting material for structural studies by NMR, x-ray crystallography, and others.

**Acknowledgment**—We thank the colleagues in Dr. Muramatsu’s laboratory at Saitama Medical School for oligonucleotide synthesis.

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