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Structural analysis of RXR–VDR interactions on DR3 DNA☆

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Abstract

The Vitamin D receptor (VDR) is a ligand-responsive transcription factor that forms homo- or heterodimers on response elements composed of two hexameric half-sites separated by three base pairs of spacer DNA. Binding of 1α,25-dihydroxyvitamin D_3 to the full-length VDR causes destabilization of the VDR homodimer and formation of a heterodimeric complex with the 9-*cis* retinoic acid receptor (RXR). VDR and RXR DNA-binding domains (DBDs) do not mimic this behavior, however: VDR DBD homodimers are formed exclusively, even in the presence of excess RXR DBD. Exploiting the asymmetry of the heterodimer and our knowledge of the homodimeric DBD interface, we have engineered VDR mutants that disfavor the homodimeric complex and allow for the formation of heterodimeric DBD complexes with RXR on DR3 elements. One of these complexes has been crystallized and its structure determined. However, the polarity of the proteins relative to the DNA is non-physiological due to crystal packing between symmetry-related VDR DBD protomers. This reveals a flattened energy landscape that appears to rely on elements outside of the core DBD for response element discrimination in the heterodimer.
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1. Introduction

The Vitamin D receptor (VDR) is a ligand-activated transcription factor that plays a central role in calcium homeostasis and has been implicated in regulating diverse biological functions, including cellular proliferation and differentiation [1–4]. VDR belongs to the steroid and nuclear hormone receptor superfamily whose members include receptors for thyroid hormone (TR), all-*trans* retinoic acid (RAR), estrogen (ER), glucocorticoids (GR), 9-*cis* retinoic acid (RXR) and >150 others [5,6].

Receptors bind to hormone response elements (HREs) via their DNA-binding domains (DBDs). These consist of a highly conserved 66 residue core [7,8] and an adjacent C-terminal extension (CTE) of the DBD that imparts additional sequence or dimerization specificity. Hormone receptors also contain a C-terminal domain (LBD) that binds the hormone. The canonical view of hormone receptors asserts that the LBD controls ligand binding, dimerization partner selection, and transcriptional activation. Likewise, the DBDs are necessary and sufficient for DNA binding and target discrimination [9–13]. Evidence for this separation of function is seen in experiments with chimeric proteins: when the DBD and hinge regions of VDR were linked to the hinge and LBD of TR, the resulting molecule bound to Vitamin D response elements (VDREs), but activated transcription in response to thyroid hormone, not Vitamin D [14].

Vitamin D response elements typically consist of two hexameric half-sites whose consensus sequence is 5′-AGGTCA-3′. Additionally, the half-sites are arranged as a direct repeat with three neutral base pairs separating the half-sites (DR3) [15]. Unliganded VDR can occupy its response elements as a homodimer [16]. Upon binding of ligand, VDR forms a heterodimer with RXR through their LBDs and this species binds to VDREs with the RXR occupying the 5′ DNA half-site. This complex mediates the transcriptional effects of Vitamin D.

Here we show the structure of an RXR DBD–VDR DBD–DR3 DNA complex in which the polarity of the proteins relative to the DNA target is reversed from that of the physiological heterodimer. The reversed polarity complex is stabilized by crystal packing contacts made by symmetry-related VDR molecules when bound to the upstream half-site, at the expense of the expected RXR–VDR interface. This and other evidence suggests that

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the heterodimeric interface between correctly placed RXR and VDR DBDs is likely to be very weak or non-existent compared to that of other heterodimeric nuclear receptors.

2. Materials and methods

2.1. Protein and DNA purification

The human VDR DBD (residues 16–125) P61A/F62A/H75A mutant was expressed in *Escherichia coli* as inclusion bodies and purified as described previously [17]. The human RXRα DBD (residues 130–228) was expressed in *E. coli* as a GST fusion and purified as described previously [18]. Synthetic oligonucleotides were purchased from the Keck Oligonucleotide Synthesis Facility at Yale University and were detritylated and purified on a reversed-phase column (Rainin Dynamax-300 Å PureDNA). Concentrated, purified strands were annealed by heating to 95 °C and slowly cooling to room temperature.

2.2. Crystallization and data collection

Samples for co-crystallization contained DNA, VDR DBD, and RXR DBD at concentrations of 0.33 mM each in 5 mM Tris, pH 7.6, 50 mM NaCl and 2 mM DTT. Crystals were grown by hanging drop vapor diffusion method at 18 °C using polyethylene glycol precipitants. Detailed methods for crystallization and data collection will be published elsewhere.

Table 1
Summary of data collection and refinement statistics for RXR–VDR–DNA complex

Diffraction data	
Source	APS-14 IDB
Space group	C2
a, b, c (Å), β (°)	123.10, 57.05, 73.44, 110.31
Resolution[a] (Å)	50–3.00 (3.11–3.00)
Completeness (%)	96.3 (97.5)
Average I/σ_I	31.2 (2.6)
R_{merge}[b] (%)	4.5 (34)
Phasing power[c]	1.44
FOM (after DM)[d]	0.21 (0.95)
Crystallographic refinement	
Resolution range (Å)	50–3.00
Reflections ($F > 2\sigma_F$)	16398 (14420)
Non-solvent atoms	2070
Protein model (RXR–VDR)	134–206/18–113
RMS deviation from ideality	
Bond lengths (Å), bond angles (°)	0.0140, 1.67
R-value ($F > 2\sigma_F$)[e] (%)	24.6 (23.5)
R_{free} ($F > 2\sigma_F$)	28.4 (27.3)

[a] The resolution limit was defined as $I/\sigma_I \geq 2.0$.
[b] $R_{merge} = \Sigma_{hkl}\Sigma_I|I_{i(hkl)} - \langle I_{(hkl)}\rangle| / \Sigma_{hkl}\Sigma_i I_{(hkl)}$.
[c] Phasing power = $<[|F_H|/E]>$, where E is the residual lack of closure.
[d] Figure of merit = $<|\Sigma P(\alpha)e^{i\alpha}/\Sigma P(\alpha)|>$, where α is the phase and $P(\alpha)$ is the phase probability distribution.
[e] $R = \Sigma|F_o - F_c|/\Sigma F_o$: 10% of the reflections were used to calculate R_{free}.

2.3. Structure determination and refinement

The structure presented here was solved and refined using CNS [19]. Initial phases were obtained by molecular replacement. The extended model was built using O, and manual rebuilding was followed by simulated annealing refinement and restrained individual B-factors [20]. The extent of the model and the refinement statistics are given in Table 1.

2.4. Gel filtration assay

The gel filtration assay for determining the composition of proteins present in the DBD–DNA complex was performed as described previously [17].

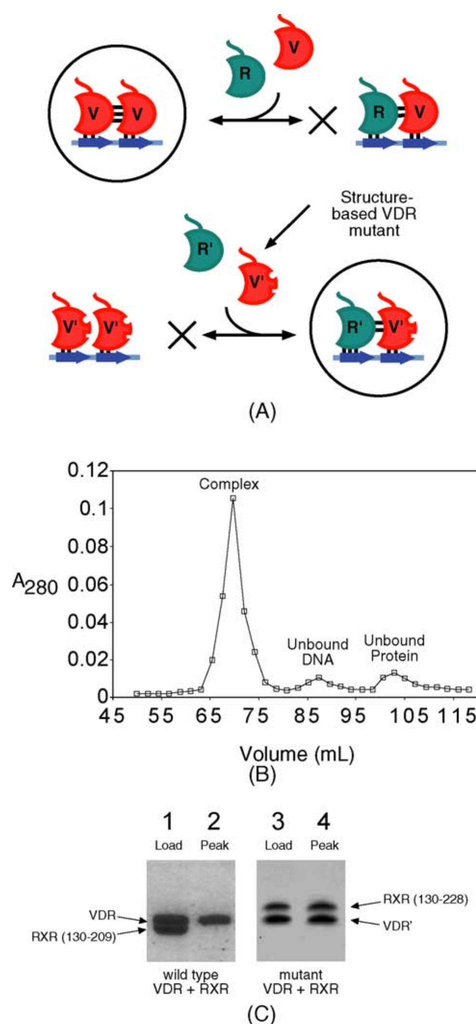

Fig. 1. RXR–VDR DBD heterodimer formation. (A) Experimental plan for structure-based mutations of the VDR DBD to disfavor homodimerization. (B) Typical chromatogram of elution of DBD–DNA complexes on Superdex 75. (C) SDS-PAGE analysis of proteins found in the complex peak. Lanes titled 'load' show the mixture of proteins applied to the column, and those labeled 'peak' show the composition of the isolated peak fraction. Wild type VDR and RXR DBDs were used in lanes 1 and 2. Mutant VDR and wild type RXR DBDs were used in lanes 3 and 4.

3. Results

3.1. Mutant VDR DBD and WT "extended" RXR DBD form heterodimeric complexes

As shown previously, in the presence of a DR3 DNA target VDR and RXR DBDs do not form heterodimeric complexes (Fig. 1C, lanes 1 and 2), as assayed by gel filtration (Fig. 1B) [17]. This assay was employed in these experiments because it allows the determination of both the protein composition and protein–DNA stoichiometry of the complex. Also, as shown previously, exploiting the consistent and distinct polarity of the RXR–VDR–DNA heterodimer (Fig. 1A), structurally-based mutations of the VDR and RXR DBDs were made that allowed formation of heterodimeric complexes on DR3 targets.

When stoichometric amounts of the triple mutant of VDR DBD (Pro61Ala, Phe62Ala, His75Ala) and RXR DBD (residues 130–228) are mixed with DR3 DNA and analyzed, both proteins are observed in the peak complex fraction (Fig. 1C, lanes 3 and 4). Excess amounts of either DBD did not prevent heterodimer formation (not shown). To show that the triple mutant of VDR used in these experiment does not impair heterodimer formation and DNA binding in the context of the full-length receptors, in vivo studies of WT and mutant VDR were performed. No difference in transcriptional activation from DR3 response elements was observed, indicating that the mutant is functional (Freedman, personal communication).

3.2. Structure of a VDR DBD–RXR DBD–DNA complex

The structure of a VDR DBD–RXR DBD–DNA ternary complex was solved at 3.0 Å resolution by molecular replacement, refined, and independently verified with maps calculated from single wavelength anomalous dispersion (SAD) data collected near the zinc edge.

The asymmetric unit consists of one VDR DBD–RXR DBD–DNA complex, shown in Fig. 2, complex 1. Surprisingly, while each protein subunit fully engages its hexameric half-site, the RXR DBD occupies the 3′, or downstream, half-site, instead of the upstream half-site. All biochemical data to date has indicated that the full-length receptors form a heterodimeric complex on DNA in which the RXR molecule is bound to the 5′, or upstream half-site [21]. Alignment of the VDR and RXR DBD cores with those

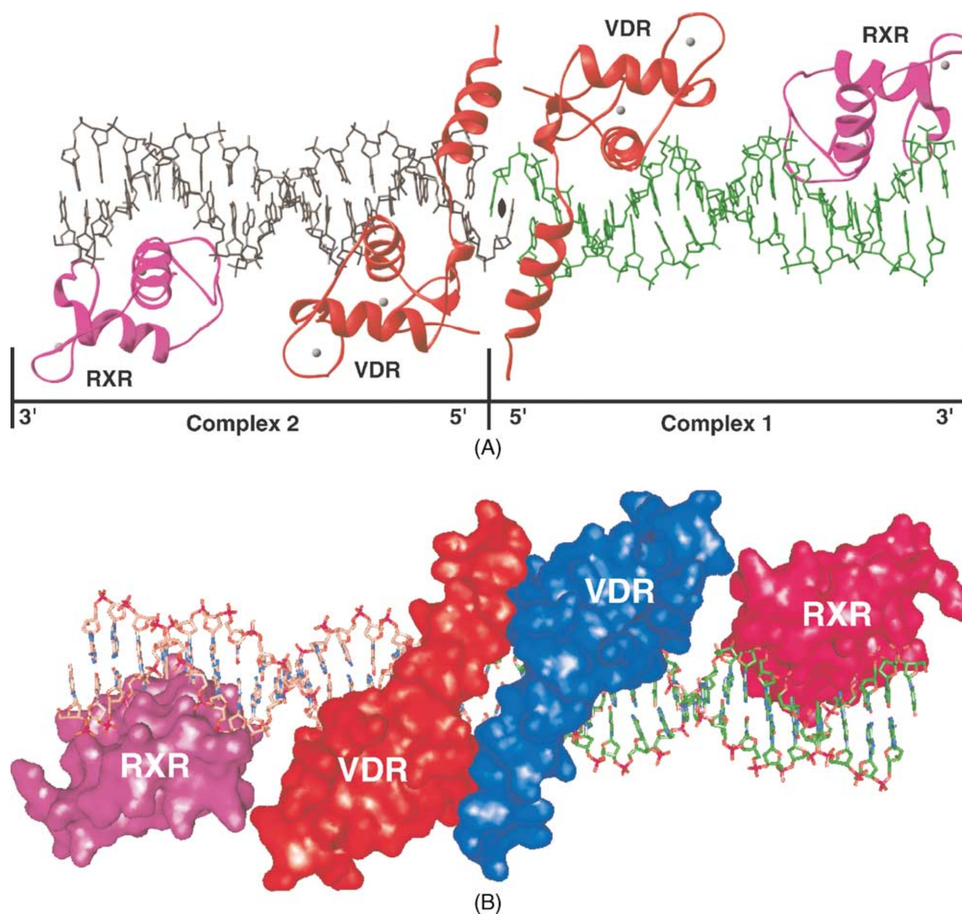

Fig. 2. Overall structural architecture of symmetry-related RXR DBD–VDR DBD–DR3 complexes. (A) Ribbon diagram showing two asymmetric crystal units of the complex. (B) Molecular surface representation of the four protomers on two DNA duplexes.

of other DBD–DNA complex structures previously solved shows that they still share the common core protein structure and mode of binding to hexameric half-site hexameric DNA [18,22,23].

The VDR and RXR DBDs in the heterodimer do not contact each other across the dimer interface, which is not surprising given that their putative dimer surfaces are not correctly juxtaposed. Instead, extensive contacts are observed between symmetry-related VDR molecules that span the junction between DNA duplexes (Fig. 2). These additional protein interactions are mediated by the VDR C-terminal extension. One face of the CTE helix of VDR (residues 102, 103, 106, 107, 109, and 110) packs against the DBD core of the adjacent symmetry mate (residues 34, 37, 90, 92, and 93) and is stabilized primarily by non-specific van der Waals contacts. Although the total buried surface area for the proteins is greater than 1000 $Å^2$, this alternate VDR DBD interface is likely to be non-physiological because it requires a non-continuous $5'$–$5'$ junction of DNA duplexes to precisely position the opposing VDR protomers.

4. Discussion

Classically, the DBD and LBD interfaces of the nuclear receptors are thought to have distinct and separable roles. Partner selection and transcriptional activation activities reside in the LBD, while DNA target discrimination and binding are accomplished by the DBD. However, isolated wild type VDR and RXR DBDs do not recapitulate the behavior of their full-length receptors: DBD heterodimers are not formed in the absence of the strong LBD mediated interface. This indicates that the cooperativity of binding of the heterodimer is less than that of the VDR homodimer. Experimentally, this problem was overcome by structure-based mutations that disfavor only VDR homodimer formation, thus forcing the equilibrium towards the formation of heterodimeric species. The structure presented here indicates that crystal packing forces, which are typically considered weak, are also sufficient to cause disruption of the physiological RXR–VDR DBD complex. We note that the change in polarity of the proteins observed in the crystal is not indicative of a loss of function caused by the mutations of the VDR molecule, since these mutants do not affect in vivo transcriptional activation. We are forced to conclude from these experiments that the RXR–VDR DBD interface is very weak (weaker than the homodimeric and crystal packing interactions) or non-existent. This leads to a conundrum, however, because DR3 response element identification must reside in the DBDs and/or the DBD/LBD linker. Chimeric proteins that contain only the extended VDR DBD retain preferential binding to DR3 VDREs [14]. Therefore, there must be sufficient cooperativity of binding to energetically favor and preferentially bind to DR3 elements contained in these portions of the molecules or through higher-order quaternary interactions that require an attached LBD. Further structural studies of RXR–VDR–DNA complexes should reveal the nature and extent of these interactions.

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