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13. ABSTRACT (Maximum 200 Words) Our objective was to use X-ray crystallography to determine the basis for specific interactions between the Androgen receptor and its DNA targets, in order to understand how the receptor can recognize two different bipartite DNA response elements with diametrically opposing arrangements. Our research has identified a variant of the AR DNA binding domain that yields large, single lattice crystals when bound to a direct repeat response element. The 3-dimensional crystal structure of the androgen receptor (AR) DNA binding domain (DBD) bound to a selective ADR3, determined at 3.1 Å resolution, reveals an unexpected head-to-head arrangement of the two protomers, rather than the expected head-to-tail arrangement seen in nuclear receptors bound to response elements of similar geometry. Compared to the glucocorticoid receptor (GR), the DBD dimer interface of the AR has additional interactions that stabilize the AR dimer and increase the affinity for non-consensus response elements. This increased interfacial stability compared to the other steroid receptors may account for the selective binding of AR to ADR3 response elements.				
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Introduction

The androgen receptor (AR) is a member of the steroid and nuclear receptor superfamily and it plays a central role in the development and progression of prostate cancers. The class I steroid receptors (androgen (AR), glucocorticoid (GR), mineralocorticoid (MR), progesterone (PR)) have identical palindromic consensus response elements and are nearly identical in their core DNA binding domains. Recent work has uncovered a second androgen response element that retains the canonical half site sequence, but has half sites that are arranged as a direct repeat (ADR-3) instead of a palindrome. Our objective was to determine the stereochemical basis for specific interactions between the Androgen receptor and DNA targets, to understand how the receptor can recognize two different bipartite DNA response elements with diametrically opposing arrangements, and to understand the stereochemical role that the C-terminal extension and hinge regions of the AR DNA binding domain (dbd) play in the recognition of specific DNA sequences. We have used X-ray crystallography to visualize complexes between the androgen receptor and specific DNA targets. From the solved structure, the protein-protein and protein-DNA contacts that lead to specific binding have been analyzed. These structures could be used to design novel classes of anti-androgens that disrupt the protein-protein or protein-DNA interactions of the androgen receptor. These compounds may be extraordinarily potent inhibitors of AR activation, since they would fundamentally de-couple the receptor from its interactions with target genes.

Body

Task 1: To solve the structure of the AR dbd bound to a direct-repeat type response element. During the first project period, we succeeded in optimizing our crystals of the AR dbd bound to direct repeat DNA targets and were able to grow reproducibly large, single lattice crystals of the AR dbd-ADR3 complex. During the second project period, we have solved the structure of this complex, using a combined MAD and molecular replacement approach. Briefly, crystals were gradually equilibrated into reservoir solution supplemented with 35% glycerol before being flash cooled in liquid nitrogen. Diffraction data were collected at -180°C on beamline 22ID at the Advanced Photon Source using a MarResearch CCD detector. Data were indexed and reduced using HKL2000. Initial sites for the zincs were found using SOLVE and data from the peak anomalous wavelength. Four zinc sites were located. Experimental phases were generated using these sites and in the anomalous difference Fourier maps, the four zinc sites had peaks of greater than 30σ , while the next highest peak was 3σ , indicating there was one AR dimer in the asymmetric unit. Visual inspection of the zinc sites revealed that the proteins were arranged in a palindromic orientation. This led to construction of a molecular replacement model using the ER DBD-IR3 structure (PDB code 1HCQ). Due to its higher sequence homology to AR, the ER DBD was replaced with the core GR DBD (PDB code 1GLU) using least squares fitting. A molecular replacement solution was obtained using MOLREP. MAD phases were calculated using the remote and peak wavelength data to $3.4\text{\AA}$ and used in all rounds of refinement. Refinement of the structure was done using CNS and the MLHL target at $3.1\text{\AA}$ resolution. Model building was done using O. Even at $3.1\text{\AA}$ resolution, the number of reflections used was 8-fold greater than the number of modeled atoms due to the very large ($>80\%$) solvent content of the crystal, allowing for restrained individual B-factor refinement in later rounds. An overview of the structure is presented in Figure 1.

Task 2: To determine the structure of the AR dbd bound to a palindromic response element. The discovery that the AR dbd C552A mutant yields well-diffracting crystals when bound to direct repeat response elements is an important milestone that has been exploited in the crystallization experiments using palindromic response elements. To date, small crystals of the AR dbd C552A in complex with an IR3 palindromic response element have been obtained, but these did not grow to a suitable size for diffraction analysis. In a parallel effort, we have also begun an NMR analysis of the interaction of the AR dbd with both ADR3 and IR3 response

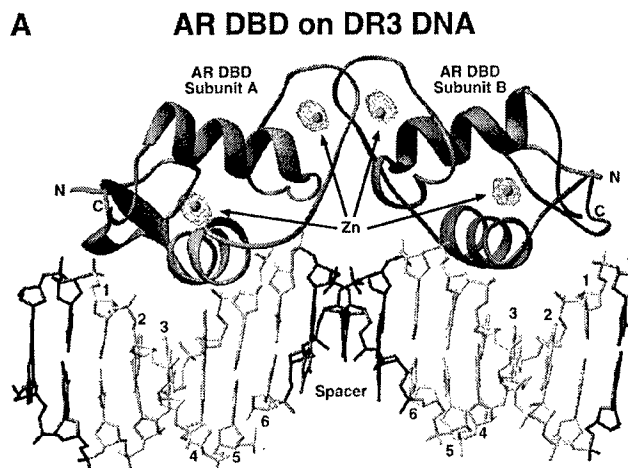


Figure 1. Overview of the structure of the AR dbd bound to ADR3 DNA. The two AR dbd subunits are shown in red and blue. The two DNA 1/2 sites are in gold, and the flanking and spacer sequences are in black. The model is superimposed on an electron density map calculated from MAD phases collected at the zinc edge, which, since they coincide with the Zn atoms, confirms the orientation of the two dbd subunits.

elements. The goal of these highly focused experiments is to use NOE analysis to observe the interaction between the methyl groups of Val465 of the AR dbd and the C5 methyl group of the T at position 4 of the AR response element. If the AR dbd is bound to an IR3 response element in an inverted repeat orientation, we expect to observe 2 distinct NOEs – one from each protein-DNA half-complex. On the other hand, if the AR dbd is bound as an inverted repeat (as seen in the crystal structure described above) to a direct repeat response element such as the ADR3, then we would expect to see only one NOE between the Val465 and T, corresponding to the specifically-bound complex. This should address both the question of how the AR dbd is bound to traditional inverted repeat response elements, as well as provide important confirmation of the results of the AR dbd –ADR3 crystallography experiments.

Task 3: Analysis and comparison of structures. As mentioned in the first year's report, the analysis of the VDR dbd-DR-3 complex has been completed and the work has been published (Shaffer, P.L. & Gewirth, D.T. (2002) "Structural Basis of VDR-DNA Interactions on Direct Repeat Response Elements." *EMBO J* 21, 2242-2252.). With the structure of the AR dbd-ADR3 complex solved we were able to make an informed comparison with both the VDR complex as well as the previously-determined structure of the glucocorticoid receptor bound to an inverted repeat response element. In summary, the structure of the AR dbd-ADR3 complex exhibits a more extensive dbd dimer interface than the corresponding interface in the GR dbd-DNA complex (Figure 2), and our hypothesis is that this stronger interface accounts in part for the ability of AR to bind to AR selective response elements.

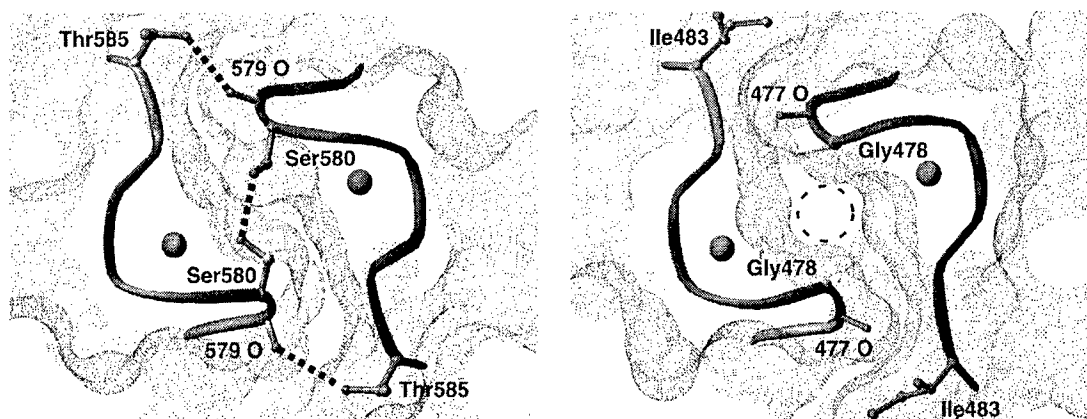


Figure 2. Comparison of key differences in the AR dbd (left) and GR dbd (right) dimer interfaces. The AR dbd dimer interface has hydrogen bond interactions between Thr585 and the carbonyl oxygen of G579, and reciprocal hydrogen bonds between Ser580. The GR dbd, on the other hand, exposes the side chain of I483 to solvent, and exhibits a "glycine hole" which makes no productive interactions between subunits. Zinc atoms are shown as grey spheres, and the two dbd subunits of the dbd dimer are shown in red and blue for both AR and GR.

In order to test these hypotheses derived from the structure, mutant variants of the AR dbd were designed and tested for function in collaboration with Dr. Frank Claessens, University of Leuven, Belgium. If the Ser580 and Thr585 interactions were the key discriminators, then mutation of these residues to their GR counterparts (S580 to G and T585 to I) should reduce the preference of AR for the direct repeat response element. Three AR dbd mutants were constructed: AR dbd S580G, AR dbd T585I, and AR dbd S580G/T585I. These mutants were tested for transcriptional activation on 4 different response elements: the TAT-GRE, an IR3 glucocorticoid response element, an idealized IR3 response element; PB-ARE2, a DR3-type androgen response element; and slp-HRE2, another DR3-type AR-specific response element. The results of these experiments, performed in the Claessens laboratory, revealed that the single or double point mutants of the AR dbd did not appreciably reduce the affinity for the PB-ARE2 or slp-HRE2 response elements. This would appear to indicate that the D-box residues that differ between AR and GR are not the discriminatory elements.

Key Research Accomplishments

- Discovery of an AR dbd mutational variant (C552A) that maintains DNA binding capability and results in single lattice crystals.
- Discovery of well-diffracting crystals of the AR dbd C552A-ADR3 complex.
- Determination of the structure of the AR dbd-ADR3 complex.
- Mutational testing of structural hypotheses with D-box mutants.

Reportable Outcomes

1. A paper describing the structure of the AR dbd-ADR3 complex has been published (Shaffer, P.L., Jivan, A., Dollins, D.E., Soldano, K.L., Claessens, F., & **Gewirth, D.T.** (2004) "Structural Basis for Androgen Receptor Interaction with Selective Response Elements." *Proc. Natl. Acad. Sci. USA* **101**, 4758-4763.)
2. A review article describing the structure of the AR dbd-ADR3 complex and analyzing the structure in the context of other data has been published as well (Claessens, F. & **Gewirth, D.T.** (2004) "DNA Recognition by Nuclear Receptors" *Essays in Biochemistry*, **40**, 59-72).
3. These results were presented on 8 October 2004 at the international meeting "Androgens 2004" held in Berlin, Germany.
4. A presentation of these results was given on 14 November, 2003 at the University of Leuven, Belgium, and on 5 February 2004 at Bowling Green State University, Bowling Green, Ohio.
5. The atomic coordinates of the AR dbd-ADR3 complex have been deposited with the Protein Data Bank (PDB), accession code 1R4I.
6. Ph.D. from Duke University awarded to Paul L. Shaffer, January 2005. Thesis title: "Structural Studies of Steroid and Nuclear Hormone Receptors Bound to DNA."

Conclusions

We have determined the structure of the AR DBD bound to an idealized steroid DR3 response element. Based on earlier studies of the Vitamin D receptor DBD (Shaffer and Gewirth, 2002), which also binds to a DR3-type response element, we expected the tandem arrangement of half-sites to direct head-to-tail binding of the AR DBD to the DNA. Surprisingly, however, the AR DBDs bind to the direct repeat response element as head-to-head symmetrical dimers (Figure 3). As a result of this mismatch between receptor dimer and response element arrangement, one AR DBD is bound to a high affinity cognate half-site, while the partner DBD is bound to a lower affinity half-site. This indicates that the energetic penalty incurred by binding to a less favored half-site sequence is more than offset by maintaining the preferred IR3-type dimer interface.

This is analogous to an earlier observation that the GR DBD maintains the IR3 dimer interface and spacing even when challenged with an IR4 response element (Luisi et al., 1991).

Both the AR and the GR exhibit similar interactions with steroid response elements, yet the AR exhibits consistently stronger binding to direct repeat type response elements than does the GR. Some of this difference in affinity may be attributable to differences in the CTE of each DBD. However, in the case of both GR and AR these regions were disordered in the crystal structures. Within the core of the DBD, however, the protein-DNA interactions are nearly identical for both receptor DBDs, and much of the difference in response element affinity is therefore likely to reside in the ability of each receptor to cooperatively form head-to-head dimers on bipartite response elements where the interaction with one or both hexameric half-sites is non-optimal.

The second zinc module has previously been shown to be necessary for AR to bind cooperatively to ADR3s (Schoenmakers et al., 1999). The steroid receptor DBD dimerization interface is contained within this module, and between AR and GR it differs at just four positions (Figure 4). The increased AR dimer affinity can be explained by two of these four substitutions: one in the D-box, and the other two residues beyond. In the D-box, AR is the only steroid receptor that has a Ser residue at the second position -- Ser580 -- and this serine packs into the core of the dimer interface, making both van der Waals interactions and a cross-subunit hydrogen bond (Figure 2). All other steroid receptors have a Gly at this position that leaves a void in the interface and lacks this additional hydrogen bond. Two residues beyond the D-box, an Ile to Thr substitution in AR allows both a favorable cross-subunit sidechain-to-backbone hydrogen bond, and removes the non-polar Ile side chain from exposure to solvent. Together these two substitutions appear to account for the stronger AR dimer interface. This in turn allows the receptor to bind to a more diverse set of response elements with higher affinity and cooperativity, compared to the GR.

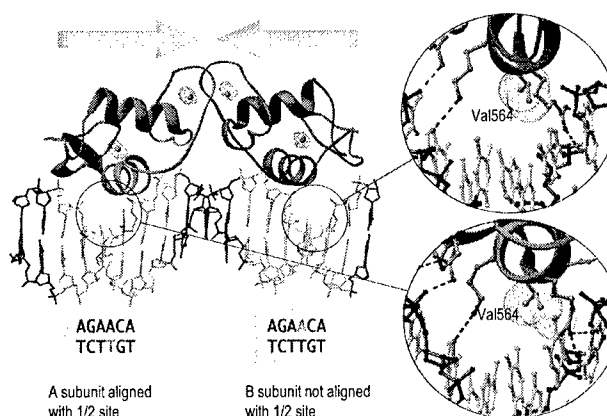


Figure 3. AR interactions on ADR3 response elements. One subunit (red) makes specific contacts via Val564, while the other subunit (blue), which is not aligned with the response element, lacks the specifying contact.

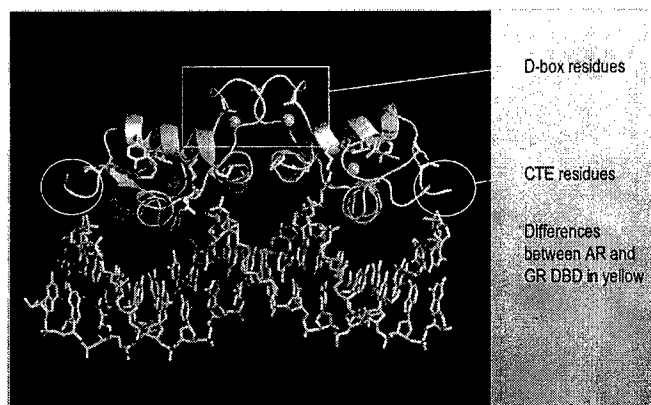


Figure 4. AR and GR differences mapped onto the AR-ADR3 structure. Differences between AR and GR are colored yellow. Areas of the structure that potentially contribute to specific DNA binding are boxed or circled.

Biochemical evidence for the increased cooperativity of the AR DBD dimer correlates with these structural observations. All the steroid receptors (MR, PR, GR, and AR) show a 5- to 10-fold lower affinity for the naturally occurring PB-ARE-2 DR3-type element compared to the C3(1) IR3-type element (Schoenmakers et al., 2000). Importantly however, the AR DBD binds 3- to 10-fold better to both elements relative to the other steroid receptors. Thus, the binding constant for AR on an apparent DR3 target (23 ± 5 nM) is the same as that of the other receptors for the more optimal IR3 element (average of other three is 23 ± 9 nM) (Schoenmakers et al., 1999). Since the concentration of individual steroid receptors in the cell is approximately nanomolar, differences in binding constants of this order are likely to be significant. AR substitutions in the GR dimerization interface show higher affinity binding to both DR3 and IR3 response elements, thus mimicking the behavior of the AR. Together with the structural data, these observations suggest a model where, due to the increased strength of the AR dimer interface, AR-selective gene activation arises from the ability of the AR to bind to IR3 response elements that have a greater deviation from the consensus half-site sequence (Figure 5). However, as the more recent mutagenesis data described above shows, the dimer interface may not be the only element that controls response element specificity. Other candidates include the CTE of the AR dbd, which differs significantly from the GR CTE. The mechanism by which the CTE, which has been disordered in all steroid receptor dbd crystal structures solved to date, influences the DNA target selectivity remains unknown.

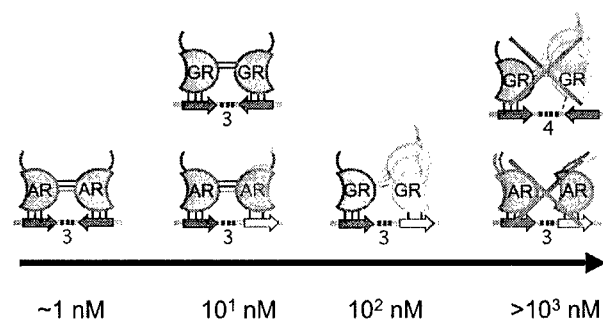


Figure 5. Model for AR response element selectivity. The AR and GR DNA binding domains are represented as lozenges, and DNA response elements are depicted by block arrows. In this scheme, the stronger AR DBD interface allows binding to a more diverse set of response elements, whereas the GR DBD interface only allows for high affinity binding to the IR3 element.

The structure of the AR DBD bound as an inverted repeat to a direct repeat response element highlights the fact that DNA target recognition by hormone receptors is strongly governed by the dimerization behavior of the two interacting protomers, even at the expense of losing specific interactions with the target DNA. To date no physiologically relevant dimerization interface within the steroid receptor DBDs other than the primary one has been observed in structural studies, and attempts to capture such potential alternative interfaces, as described in this report, and previously for GR (Luisi et al., 1991), have been unfruitful. This implies that selective hormone response elements that appear to have alternative arrangements of their hexameric half sites, such as the pemARE with a proposed 5 bp spacer between half-sites (Geserick et al., 2003), may instead simply be further examples of the ability of these receptors to exploit the strength of their DBD dimerization interfaces to accommodate sub-optimal protein-half site interactions. This is likely to be not only a mechanism of response element discrimination, but also an effective way of modulating transcription from different hormone responsive genes.

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