Identification of a Highly Conserved Domain in the Androgen Receptor That Suppresses
the DNA-Binding Domain-DNA Interactions

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Running title: Identification of an Inhibition Domain of AR

Key Words: androgen receptor, DNA-binding domain, inhibition domain, DNA binding,
nuclear receptor, transcription

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SUMMARY

The androgen receptor (AR) is a ligand-regulated and sequence-specific transcription factor that activates or represses expression of target genes. Here, we show that the N-terminus of AR contains an inhibitory domain located in an 81-amino acid segment lying upstream of the DNA-binding domain (DBD). The inhibitory domain interacted directly with DBD and repressed DBD binding to the androgen response element. Mutations of the conserved amino acid residues (K520E and R538E) within the inhibitory domain decreased its inhibiting ability in vitro and increased AR transactivation in vivo. These data demonstrate the existence of a novel inhibitory domain in the N-terminal part of AR, which might play important roles in the regulation of AR transactivation.
INTRODUCTION

The androgen receptor (AR) mediates androgen functions in the differentiation and maturation of the male reproductive organs and in the development of male secondary sex characteristics (1). Mutations in the AR gene are associated with the androgen insensitivity syndrome (AIS) (2,3). Numerous somatic mutations in the AR gene have been reported among prostate cancer patients and as well as in prostate cancer cell lines and xenografts (3,4). Most of these mutations have been detected in tumor tissues of late-stage prostate carcinoma, indicating that somatic mutation of the AR gene might be involved in the progression and aggressiveness of prostate cancer.

The AR is a member of the nuclear receptor (NR) superfamily (5). These receptors are characterized by distinct functional domains: an N-terminal part, involved in ligand-independent transcription activation (AF1), a DNA-binding domain (DBD), and a C-terminal part (LBD) involved in ligand binding and ligand-dependent transcription activation (AF2) (6). As for other steroid receptors, ligand binding is generally believed to result in a conformational charge in AR with consequent dissociation of heat shock proteins/chaperones (7), dimerization and binding to cognate androgen response elements (AREs) in target genes, and, (through its AF1 and AF2 domains) interactions with various coactivators that facilitate transcription by the general transcriptional machinery (8). The DBD encompasses two Zn-finger-like modules and binds as dimers to two hexameric sequences orientated as direct or inverted repeats (9,10). Although the DBD and LBD of steroid hormone receptors are highly conserved, there is much less homology among steroid hormone receptors in their N-terminal parts. The AR has a long N-terminal part with a strong autonomous AF1 and interacts directly with AF2 in the C-terminal part (11,12). The N- and C-terminal interactions are important for androgen-induced gene
regulation, and disruption of these interactions may be linked to AIS (13,14). The conserved FXXLF and WXXLF motifs within the N-terminal part seem to be involved in pairwise interactions between AF1 and AF2 (15). The N-terminal part contains stretches of glutamines (coded by CAG) and glycine (coded by GGN) (16). Expansion of the CAG repeats is associated with X-linked spinal and bulbar muscle atrophy (17). A shorter CAG repeat is associated with an increased transactivation of AR (18,19), but the biological role of GGN repeats is less clear.

In this study, we demonstrated that AR contains a highly conserved inhibitory domain within the N-terminal region. The inhibitory domain interacted directly with DBD and inhibited the DBD-DNA interactions. The mutations in the inhibitory domain result in decreased inhibitory ability and increased AR transactivation activity, indicating that this domain might play important roles in the regulation of AR function.
EXPERIMENTAL PROCEDURES

Production and Purification of Recombinant Proteins - The human full-length AR was expressed in Sf9 cells via the baculovirus expression vector pVL1393 (Pharmingen), and the recombinant AR was purified as described (20). All AR and GR cDNA fragments were amplified by PCR with specific oligonucleotides, cut with NdeI and BamHI, and subsequently cloned in the corresponding restriction sites of the vectors pET15d (Novagene), pGEX-2TL (Pharmacia), and pcDNA3.1 (Invitrogen). The fragments were expressed as 6His-tagged (via pET15d) or GST-fusion (via pGEX-2TL) proteins in \textit{E. coli} BL21 and purified through NTA Ni$^{2+}$ agarose or glutathione Sepharose columns, respectively. Point mutations were generated by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer’s instructions and confirmed by DNA sequencing analysis. The mutated proteins were expressed and purified similarly.

Gel Shift Assay – Two pairs of oligos (AGCTTTTGCAGAACAGCAAGTGCTAGCTG and AAATTCAGCTAGCAGGCTTTTGCTAGCTG) derived from the prostate specific antigen (PSA) gene (-152 to –174) were synthesized, annealed, and subcloned into HindIII and EcoRI sites of the vector pBluescript II (Stratagene). The underlined bases were mutated from their corresponding bases in the wild-type PSA gene sequence. The wild-type and mutant ARE probes were made by cutting these constructs with XhoI and XbaI, and purification of fragments from agarose gel. Probes were labeled with $\alpha$-$^{32}$P-dCTP by a fill-in reaction with the Klenow enzyme. In gel shift assays, 20 µl reaction contains 20 mM HEPES, pH 7.9, 70 mM KCl, 1 µg of poly(dI-dC), 1 mM DTT, 0.1% NP-40, 100 µg/ml of BSA, and various proteins. The reaction
mixture was incubated for 20 min at room temperature, and the binding reaction was initiated by the addition of the labeled probes (20,000 cpm) and then incubated for an additional 30 min at room temperature. The reaction mixture was loaded directly onto a 4% (37.5:1, acrylamide:bisacrylamide) nondenaturing polyacrylamide gel with 0.25 x TBE and run at 150 V for 2 h at room temperature.

Cell Culture and DNA Transfection - PC3 cells were cultured in RPMI1640 medium containing 10% fetal bovine serum. Cells (5 X 10^5) were plated in each well of 24-well plates and transfected with 100 ng of 4xARE-E4-luc reporter plasmid, 2.5 ng of control plasmid pRL-CMV, and various amounts of expression plasmids. Cells were grown in the presence of 10 nM R1881 for 48 h after transfection and harvested for dual-luciferase activity assay (Promega).

Protein-protein Pull-down Assay - GST and GST-DBD(AR537-644) were expressed in bacteria and immobilized on glutathione Sepharose beads. Beads (10 µl) containing 100 ng of GST or GST-DBD proteins were incubated with 5 µl of TNT rabbit reticulocyte lysates containing ^35S-labeled AR477-538 in BC150-0.1% NP-40 for 2 h at 4 °C. After being washed with the incubation buffer, beads were boiled with SDS sample buffer and subjected to SDS-PAGE followed by autoradiography.
RESULTS

The Full-length AR Interacts with the Androgen Response Element More Weakly than the DNA-binding Domain - The ligand-dependent interaction of AR with the ARE has been demonstrated in vitro with crude AR-containing cell extracts (21). However, the AR-DNA interactions have not been studied with the highly purified recombinant AR. To this end, the FLAG epitope-tagged human AR was expressed in Sf9 cells and immuno-purified under high salt conditions (500 mM KCl) to strip off heat shock proteins associated with the unliganded AR. The recombinant AR preparation is near homogeneity (Fig. 1B, lanes 2 and 3) and contains two bands that migrated near the 110-kDa position. The top band might be the phosphorylated form of AR (22). Two minor polypeptides (70 and 55 kDa, indicated by stars on the right) were recognized by the anti-FLAG monoclonal antibody (data not shown), indicating that they are degraded products of the full-length AR. A DNA probe containing the ARE derived from the PSA promoter (-152 to –174) (23) (Fig. 1D) was used for a gel shift assay. The recombinant AR (0.9 pmol) shifted the probe (Fig. 1C, lane 2), while there was no ligand (androgen) dependency (lane 3 versus lane 2). The band of the AR-ARE complex (indicated by an arrow on the left) is quite broad. However, mutations of the nucleotides in the probe that are critical for AR-ARE interaction (24) (Fig. 1D) dramatically decreased the density of the AR-ARE band (Fig. 1C, lanes 7 and 8 versus lanes 2 and 3), indicating that the shifted band is specific. The DBD of AR (amino acid residues 537 to 644) (Fig. 1A) was expressed as a 6His-tagged fusion protein and purified through an NTA Ni\(^{2+}\) agarose affinity column (Fig. 1B, lane 4). In the same assay, 0.3 pmol of AR537-644 almost completely shifted the probe (Fig. 1C, lane 4). The band of the AR537-644-ARE complex (indicated by an arrow on the left) is much sharper (Fig. 1C, lanes 4 and 5), and mutations in the probe (Fig. 1D) completely diminished the formation of the AR537-
644-ARE complex (Fig. 1C, lane 9 and 10). The results indicate that the binding affinity of DBD to the ARE is much stronger than that of the purified full-length AR to the same ARE.

_A Domain within the AR N-Terminus Inhibits DBD-ARE Interactions_ - A C-terminal extension (CTE) of the DBD of AR was found to be required for specific and high-affinity interactions of DBD with ARE (25). To investigate whether the sequences surrounding DBD would affect DBD-DNA interactions, AR537-662 and AR477-644 (Fig. 1A) were expressed in and purified from bacteria (Fig. 1B, lanes 5 and 6). AR537-662 strongly interacted with the probe, similar to AR537-644 (Fig. 2A, lanes 2-4). However, AR477-644 completely lost the ability to interact with the ARE probe even though much more protein (up to 1.6 pmol) was used in the binding reaction (lanes 5-7). The N-terminal extension of DBD (amino acid residues 477-558) was expressed and purified (Fig. 2B, lane 1). Its molecular weight as determined by SDS-PAGE (16 kDa) is much bigger than the calculated weight (10 kDa), and it was heavily degraded (Fig. 2B, lane 1). This region contains 20% charged amino acids and 16% proline residues, which may be responsible for this aberrant mobility of the protein. When AR477-558 was added to the binding reaction that contained the fixed amount (0.3 pmol) of AR537-644, the density of the DBD-ARE complex dramatically decreased (Fig. 2C, lanes 3-8). These results indicate that AR477-538 specifically inhibits the DBD-ARE interactions _in trans_ as well as _in cis_. We noticed that different preparations of AR477-538 contained various amounts of the full-length protein and that amounts of the full-length protein (Fig. 2B, lane 1, indicated by the top arrow on the right) were correlated with the inhibition ability of AR477-538. As negative controls, the recombinant PAR-4 (26), TIP30 (27) and RPC39 (28) expressed and purified similarly did not significantly affect the DBD binding to the ARE probe (Fig. 2D).
The Inhibitory Domain Interacts with DBD and Inhibits AR Transactivation – The protein-protein pull-down assay was performed to investigate whether the inhibitory domain (ID) interacts directly with DBD. GST and GST-DBD(AR537-644)-fusion protein were expressed in bacteria and immobilized on glutathione Sepharose beads (Fig. 3A, lanes 2 and 3). The *in vitro* translated $^{35}$S-AR477-558 (lane 4) bound to GST-DBD (lane 6) and not to GST (lane 5). This result indicates that the inhibitory domain interacts directly with DBD.

We then investigated the effect of the ID on AR transactivation by performing transient transfection assays. A luciferase reporter containing four tandem copies of the same ARE used for the gel shift assay upstream of the minimal adenovirus E4 promoter was cotransfected with expression vectors for AR, AR477-558, or both into prostate cancer PC3 cells in the presence of the synthetic androgen R1881. As shown in Fig. 3B, AR activated the reporter gene about 25-fold and coexpressed AR477-558 showed a strong (62%) inhibition of this activity. Coexpression of AR477-558 did not influence reporter gene activity driven by p53, indicating that the inhibiting effect of AR477-558 was specific for AR. Western blot analysis revealed that the AR protein levels in the absence and presence of AR477-558 were comparable (Fig. 3C, lane 3 versus lane 2). On the basis of *in vitro* studies (Fig. 2), the ID inhibited AR transactivation, most likely by blocking the interaction of the AR with the ARE.

The Inhibitory Domain Is Specific for AR - The DNA-binding domains of AR, glucocorticoid receptor (GR), progesterone receptor (PR), and mineralocorticoid receptor (MR) are highly conserved (29). Not surprisingly, they bind to the same consensus DNA site (GGTACAnnnTGTTCT) and can be considered a subfamily of the NR superfamily. However, the inhibitory domain of AR is not conserved in the other receptors (Fig. 4A). GR418-525 and GR358-525 were expressed and purified (Fig. 4B, lanes 1 and 2). Gel shift assay demonstrated
that GR358-525 and GR418-525 bound the ARE probe similarly (Fig. 4C, lane 3 versus lane 2). The lower band (Fig. 4C, lane 3, indicated by a star on the right) might contain a monomer of GR358-525. These results indicated that the ID in AR is not conserved in GR, and thus the inhibitory domain is specific for AR.

**Mutations in the ID Enhance AR Transactivation** - Sequence alignment shows that the ID of AR is highly conserved through evolution (Fig. 5A). To further characterize the biological effects of this region, we mutated two conserved residues (K520 and R538) in the ID and cDNAs encoding the mutated AR (K520E and R538E) were transiently transfected in PC3 cells with the luciferase reporter plasmid. The mutated AR had elevated transactivation activity compared with the wild-type AR (Fig. 5B), although the mutated and wild-type AR were expressed at the same level in the transfected cells (Fig. 5C, lanes 2-4). The ID (AR477-558) from the mutated AR (K520E and R538E) were expressed and purified (Fig. 2B, lanes 2 and 3). The gel shift assay revealed that mutations of K520E and R538E decreased the inhibitory ability of ID (Fig. 5D, lanes 9-12 and 13-16 versus lanes 5-8). Ten nanograms of the wild-type AR477-558 almost completely blocked AR537-644 binding to the ARE probe (Fig. 5D). However, the same amount of the mutated (K520E and R538E) AR477-558 inhibited the DBD-ARE interaction only 65% and 35%, respectively (Fig. 5D). Thus, enhancement of AR transactivation by mutations of K520E and R538E correlates with a decrease in the inhibitory effect of ID on DBD-ARE interactions.
DISCUSSION

The N-terminal parts of NRs are the most divergent among members of this superfamily of proteins, suggesting that each receptor will take on a unique N-terminal conformation to determine its specificity. This paper describes a highly conserved novel inhibitory domain, designated ID, which lies in the N-terminal 81-amino acid residues upstream of the DBD of AR. ID interacts directly with DBD and strongly inhibits the DBD-ARE interactions \textit{in vitro} and AR transactivation \textit{in vivo}.

Much of the work devoted to understanding regulation of transcription by the AR has focused on the N-terminal AF1 and the C-terminal AF2 (30). However, transcriptional inhibition may be equally important as a way of preventing activation. Studies that deal with inhibition of AR-dependent transcription have focused on silencing mechanisms through recruitment of corepressors to the target promoters and through receptor occupancy at one DNA site interfering with transcription by an activator at an adjoining site (5,31). We have now demonstrated that negative function element exists in the AR molecule itself and markedly suppresses the DNA-binding activity of DBD. The ID function is similar to that of the N-terminal region of TAF250 [the 250 kDa TATA box binding protein (TBP)-associated factor 1], which forms a DNA-like structure, interacts with the DNA-binding surface, and inhibits the DNA-binding activity of TBP (32). In contrast, the direct interactions between the ID and DBD suggest that perhaps ID acts through intramolecular contacts. In this respect, ID is similar to p53, which exists in a latent DNA-binding form as result of the C-terminal tail-DNA-binding domain interactions (33,34). Phosphorylation of lysine residues in the C-terminal region leads to the disruption of interactions between the C-terminal domain and the core DBD, thus allowing the DBD of p53 to adopt an active conformation. It is important to know whether modifications
in the ID of AR or interactions of this domain with the other proteins might regulate the DNA-binding activity of AR. A study on the rat AR indicated that the unknown protein could enhance the DNA-binding activity of the protein fragment containing the DBD in a gel shift assay (35). Another study has demonstrated that mutations on $^{668}$QPIF$^{671}$ at the boundary of the hinge and ligand-binding domain of AR, resulting in receptors that exhibit 2- to 4-fold increased activity compared with the wild-type AR in response to dihydrotestosterone, and these mutations have been detected in prostate cancer patients (36). However, the molecular mechanism for this phenomenon is unclear.

Several mutations found in men with prostate cancer (37) and in men with the AIS (38,39) localize in ID (Fig. 5A). These mutations might change the function of ID, therefore affecting AR transactivation. D528G mutation was detected in a patient with prostate cancer (37) and we found that AR with D528G mutation was more active (>3-fold) than the wild-type AR in transient transfection assays (data not shown). However, this mutation enhanced the stability of AR in PC3 cells (data not shown). Currently, we are investigating whether the enhanced AR transactivation is due to the enhanced AR stability, the decreased ID function, or both. Thus, ID may play an important regulatory role in AR function, and dysfunction of ID may contribute to prostate cancer or AIS in some men.
Acknowledgments

We thank Michael S. Worley for his critical editorial review and Liliana DeGeus for expert assistance in the preparation of the manuscript. This work was supported in part by U.S. Department of the Army grant DAMS17-01-1-0097, CaP CURE, Cancer Center Support Core grant CA16672, SPORE in Prostate Cancer grant CA90270, SPORE in Ovarian Cancer grant CA93639, and SPORE in Head and Neck Cancer grant CA97007 from the National Cancer Institute, National Institutes of Health.
REFERENCES

FIGURE LEGENDS

FIG. 1. The recombinant androgen receptor (AR) interacts with the androgen-response element (ARE) weaker than the DNA-binding domain (DBD). A, Diagram of domains and truncations of AR. B, SDS-PAGE analysis of the recombinant AR and DBD. Proteins of 100 ng (lane 2) and 200 ng (lane 3) of the purified recombinant AR expressed in Sf9 cells and recombinant 6His-tagged truncations of AR expressed in bacteria (lanes 4-6) were subjected to SDS-PAGE with Coomassie blue R250 staining. Lane 1 is standard protein markers (Bio-Rad). C, The gel shift assay was performed using a DNA probe containing the wild-type ARE (lanes 1-5) or the mutant ARE (lanes 6-10). 0.9 pmol of AR (lanes 2, 3, 7 and 8) and 0.3 pmol (lanes 4 and 9) or 0.6 pmol (lanes 5 and 10) of AR537-644 were used in the binding reactions. The synthetic androgen R1881 (100 nM) was included in the reactions in lanes 3 and 8, and lanes 1 and 6 are probes only. D, Sequences of the wild-type (AREwt) and mutant (AREmt) ARE and the ARE consensus. The mutated bases in AREmt are underlined.

FIG. 2. AR477-558 blocks DBD binding to ARE. A, AR477-644 did not interact with the ARE. 0.15 pmol (lane 2), 0.3 pmol (lane 3) or 0.6 pmol (lane 4) of AR537-662 and 0.4 pmol (lane 5), 0.8 pmol ng (lane 6) or 1.6 pmol (lane 7) of AR477-644 were used in the binding reactions. Lane 1 is the probe-only control. B, SDS-PAGE of the purified recombinant wild-type (lane 1), K580E (lane 2), or R538E (lane 3) AR477-558. The full-length proteins (top arrow) and the main degraded products (bottom arrow) are indicated on the right. Nonspecific background bands are marked with a star on the right. C, AR477-558 inhibits AR537-643 binding to the ARE. The binding reactions contained 0.3 pmol AR537-644 (lane 2) or 0.3 pmol AR537-644 plus 0.0625 pmol (lane 3), 0.125 pmol (lane 4), 0.25 pmol (lane 5), 0.5 pmol (lane 6), 1 pmol (lane 7) or 2 pmol (lane 8) of AR477-558. D, The purified recombinant PAR-4,
TIP30, and RPC39 proteins do not affect the interaction of AR537-644 with ARE. The binding reactions contained 0.3 pmol AR537-644 (lane 2) or 0.3 pmol AR537-644 plus 0.225 (lane 3), 0.45 (lane 4), 0.9 (lane 5), 1.8 (lane 6) pmol Par-4 (lane 8) or 1.25 (lanes 7 and 10), 2.5 (lanes 8 and 11) or 5 (lanes 9 and 12) pmol TIP30 (lanes 7-9) or RPC39 (lanes 10-12), respectively.

FIG. 3. **AR477-558 interacted directly with DBD in vitro and inhibited AR transactivation in vivo.** A, AR477-558 interacted directly with DBD(AR537-644) in vitro. SDS-PAGE analysis of GST (lane 2) and GST-DBD (lane 3) expressed in bacteria and immobilized on glutathione Sepharose 4B beads. Bands corresponding to GST and GST-DBD-fusion protein are indicated by arrows on the right. The immobilized GST-DBD pulled down $^{35}$S-AR477-558 (lane 6). Lane 4 is 10% of $^{35}$S-AR477-558 input for the pull-down assay. B, AR477-558 inhibited AR transactivation in vivo. PC3 cells were transfected with 100 ng of the reporters PGL3-ARE-E4 or pGL3-GAL4-E4, 2.5 ng of the internal control reporter pRL-CMV, 20 ng of pcDNA-AR or pcDNA-GAL4-p53(1-53), and 18.5 ng pcDNA-AR477-538 as indicated. Cells were treated with 10 nM R1881 after transfection and harvested 48 h later for the dual luciferase assay. C, AR477-538 did not affect AR protein levels in the transfected cells. Western blot analysis of cells transfected with pcDNA3.1 (lane 1), pcDNA-AR (lane 2), or pcDNA-AR plus pcDNA-AR477-558 (lane 3) with the anti-AR antibody.

FIG. 4. **The DBD-containing fragments of GR bind to the ARE probe.** A, Sequence alignment of DBD and ID of AR with the corresponding regions of rat GR. B, SDS-PAGE analysis of the recombinant GR418-525 and GR358-525. 500 ng of 6His-tagged GR418-525 (lanes 1) and GR 357-525 (lane 2) expressed in bacteria were subjected to SDS-PAGE with Coomassie blue R250 staining. The bands corresponding to the full-length protein fragments are indicated by arrows on the right, and a nonspecific background band is marked by a star on the
left. The standard protein markers (Bio-Rad) are indicated on the left. C, GR417-525 and GR357-525 bind to the ARE. 0.3 pmol of GR417-525 (lane 2) or GR357-525 (lane 3) was used in the binding reactions. Lane 1 is the probe-only control.

FIG. 5. **Mutations in ID enhanced AR transactivation and decreased ID inhibitory activity.** A, Sequence alignment of ID and DBD of human (hAR), rabbit (rAR), mouse (mAR) and Xenopus (xAR) AR. Point mutations found in prostate cancer (PC) and in complete (CAS), mild (MIAS) or partial (PAIS) androgen insensitivity syndrome patients are indicated by arrows on the top. B, Mutations (K520E and R538R) enhanced AR transactivation in vivo. PC3 cells were transfected with 100 ng of the reporter PGL3-ARE-E4, 2.5 ng of the internal control reporter pRL-CMV, or 10 ng of pcDNA-wild-type AR or pcDNA-mutant (K520E or R538) AR as indicated. Cells were treated with 10 nM R1881 after transfection and harvested 48 h later for the dual luciferase assay. Each value represents the mean ± standard deviation of a representative experiment performed in triplicate. C, Western blot analysis of cells transfected with pcDNA3.1 (lane 1) or with wild-type (lane 2), K520E (lane 3), and R538E (lane 4) mutant AR. D, Mutations of K520E and R538E decreased ID inhibitory ability on DBD-ARE interactions. The binding reactions contained 0.075 (lane 2), 0.15 (lane 3), or 0.3 (lane 4) pmol of DBD alone or 0.3 pmol of DBD plus 0.625 (lanes 5, 9, 13), 0.125 (lanes 6, 10, 14), 0.025 (lanes 7, 11, 15), or 0.005 pmol (lanes 8, 12, 16) of wild-type (lanes 5-8), K520E mutant (lanes 9-12) or R538E mutant AR477-558 (lanes 13-16).
Figure 1

A

B

C

D

PSA AREwt: AGAACAGCAAGTGCT
PSA AREmt: AGAATAGCAAAATGCT
ARE Consensus: GGWACANNNTGTTCT
Figure 3
Figure 4

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J. Biol. Chem. published online February 17, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212229200

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