Analysis of Interdomain Interactions of the Androgen Receptor

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Abstract

High-affinity binding of testosterone or dihydrotestosterone to the androgen receptor (AR) triggers the androgen-dependent AR NH_2- and carboxyl-terminal (N/C) interaction between the AR NH_2-terminal F*XX*LF motif and the activation function 2 (AF2) hydrophobic binding surface in the ligand-binding domain. The functional importance of the AR N/C interaction is supported by naturally occurring loss-of-function AR AF2 mutations where AR retains high-affinity androgen binding but is defective in AR F*XX*LF motif binding. Ligands with agonist activity in vivo such as testosterone, dihydrotestosterone, and the synthetic anabolic steroids induce the AR N/C interaction and increase AR transcriptional activity in part by slowing the dissociation rate of bound ligand and stabilizing AR against degradation. AR ligand-binding domain competitive antagonists inhibit the agonist-dependent AR N/C interaction. Although the human AR N/C interaction is important for transcriptional activity, it has an inhibitory effect on transcriptional activity from AF2 by competing for p160 coactivator L*XX*LL motif binding. The primate-specific AR coregulatory protein, melanoma antigen gene protein-A11 (MAGE-A11), modulates the AR N/C interaction through a direct interaction with the AR F*XX*LF motif. Inhibition of AF2 transcriptional activity by the AR N/C interaction is relieved by AR F*XX*LF motif binding to the F-box region of MAGE-11. Described here are methods to measure the androgen-dependent AR N/C inter-domain interaction and the influence of transcriptional coregulators.

Keywords

Androgen receptor; steroid receptor; N/C interaction; mammalian two-hybrid assay; MAGE-11; MAGE-A11

1. Introduction

The androgen receptor (AR) is a ligand-dependent transcription factor essential for male sex development and a critical factor in prostate cancer. AR binds the two biologically active androgens, testosterone and dihydrotestosterone (DHT), with similar high affinity. Androgen binding causes AR to translocate to the nucleus, bind to DNA-response elements, and interact with coregulatory proteins to promote the transcription of androgen-dependent genes required for male sex development and reproductive function. Early studies investigating the kinetics of androgen binding demonstrated that DHT dissociates from AR more slowly than does testosterone, a property that contributes to the greater physiological potency of DHT (1, 2). The greater effectiveness of DHT during development is evident from the incomplete masculinization of genetic males deficient in DHT due to naturally occurring gene mutations in the 5α-reductase enzyme that converts testosterone to DHT (3).

Both testosterone and DHT dissociate with slower kinetics from full-length AR than from an AR carboxyl-terminal, ligand-binding domain (LBD) fragment that lacks the NH_2-termimal region (4). In the presence of androgen, an AR NH_2-terminal fragment interacts with an AR DNA and ligand-binding domain fragment, and the complex binds DNA and activates the prostate-specific antigen (PSA) enhancer/promoter (5). These observations, together with the results of mammalian two-hybrid interaction assays (6, 7), provided the first evidence for an androgen-dependent AR NH_2- and carboxyl-terminal (N/C) interaction.

The importance of the AR N/C interaction in male reproductive physiology is suggested by naturally occurring AR gene mutations in the LBD that disrupt the AR N/C interaction without altering equilibrium androgen-binding affinity (7–13). These LBD mutations cause the androgen insensitivity syndrome that results in partial or complete failure of masculinization in 46XY genetic males by disrupting the AR N/C interaction and p160 coactivator binding (6–8). Ligands that induce the AR N/C interaction display complete agonist activity in vivo, which indicates that the mammalian two-hybrid N/C interaction assay is a useful method to identify ligands that function as active androgens (14) (*see* **Note 1**). The N/C interaction contributes to agonist potency in part by slowing the ligand dissociation rate and stabilizing AR (6). The AR N/C interaction assay is also useful in conjunction with transcription assays to identify AR antagonists (14).

The androgen-dependent AR N/C interaction is mediated by the AR NH_2-terminal F*XX*LF motif [23]FQNLF[27] binding to a hydrophobic cleft in the LBD surface known as activation function 2 (AF2) (9, 15, 16). Both the AR F*XX*LF motif and AF2 are flanked by complementary charged amino acid residues that facilitate their interaction (17). Assays of the AR N/C interaction require the expression of an AR NH_2-terminal fragment that contains the AR-20–30 NH_2-terminal region with [23]FQNLF[27] sequence (17). The coexpressed interacting LBD fragment must contain AR LBD residues 658–919, an AR fragment that retains high-affinity androgen binding but displays rapid androgen dissociation kinetics when expressed alone (2). The two-hybrid AR N/C interaction assay is performed in mammalian cells (*see* **Note 2**) using GAL4 DNA-binding domain and VP16 activation domain fusion proteins, and a GAL4–luciferase reporter gene. Deletion of the hinge region from the carboxyl-terminal LBD fragment minimizes an inhibitory effect (*see* **Note 3**). The assay can be performed using AR NH_2-terminal and carboxyl-terminal fragments with an androgen-responsive luciferase reporter vector such as PSA-Enh-Luc (*see* **Note 4**).

The androgen-dependent AR N/C interaction promotes the expression of AR target genes (18, 19). However, not all androgen-responsive enhancer/promoter regions require the AR N/C interaction. Most notably, the mouse mammary tumor virus (MMTV) and sex-limited gene enhancer/promoters do not require the AR N/C interaction that was required for maximal induction of the PSA and other androgen-responsive genes (18).

The human AR N/C interaction inhibits p160 coactivator L*XX*LL motif binding to AF2, which decreases overall AR transcriptional activity derived from AF2 (20, 21). Competitive inhibition at the AF2 site in the LBD occurs between the AR F*XX*LF motif and the p160 coactivator L*XX*LL motifs that bind the same AF2 hydrophobic cleft on the LBD surface

(22). The ~10 fold higher binding affinity for the AR F*XX*LF motif relative to p160 coactivator L*XX*LL motifs imposes an inhibitory effect on AF2 activity. Through this mechanism, the AR N/C interaction shifts the dominant activation region from AF2 in the LBD to activation function 1 (AF1) in the human AR NH_2-terminal region. In normal physiology, AF1 is androgen-dependent because of the inhibitory effect of the unliganded LBD. An AR NH_2-terminal and DNA-binding domain fragment that lacks the LBD is constitutively active (23).

The AR N/C interaction is modulated by AR coregulatory proteins (*see* **Note 5**). One recently characterized AR coregulator that influences the AR N/C interaction is melanoma antigen gene protein-A11 (MAGE-11). MAGE-11 is a member of the *MAGE* gene family of cancer-testis antigens that evolved in a species-specific manner. MAGE-11 is expressed only in humans and other primates and is not expressed in rodents. MAGE-11 binds the AR F*XX*LF motif and directly recruits p160 coactivators to the AR NH_2-terminal region (24, 25). MAGE-11 binds the AR F*XX*LF motif through a MAGE-11 F-box that is post-translationally modified. MAGE-11 is phosphorylated at Thr-360 within the F-box (amino acid residues 329–369) by cell cycle checkpoint kinase Chk1 in response to epidermal growth factor (EGF). MAGE-11 is phosphorylated at Ser-174 outside the F-box by MAP kinase in response to serum stimulation (25). In contrast to the minimal AR-20–30 amino acid region required to bind AF2 in the AR N/C interaction, a longer AR-16–36 region that contains the F*XX*LF motif is required to bind MAGE-11. Thus, overlapping AR F*XX*LF motif regions mediate interactions with AR AF2 in the AR N/C interaction and with MAGE-11. This suggests different flanking sequence requirements for AR F*XX*LF motif interactions. MAGE-11 interaction with AR also depends on the EGF-stimulated monoubiquitinylation of MAGE-11 outside the F-box that is triggered by phosphorylation at Thr-360 within the F-box (26). The recent evolutionary appearance of MAGE-11 as an AR coregulator in primates provides important and novel regulatory control on AR function.

Thus, the AR N/C interaction regulates AR function in response to agonists, is inhibited by AR antagonists, and modulated by coactivators. Two-hybrid assays of the AR N/C interaction in mammalian cells provide a measure of ligand potency for agonists or antagonists, and the functional effects of AR coregulators and naturally occurring and targeted AR mutations.

2. Materials

2.1. AR N/C Interaction Assay Reagents

1. HeLa epithelial cells derived from a human cervix adenocarcinoma (CCL-2; American Type Culture Collection, Rockville, MD) (*see* **Note 2**).

2. HeLa cell medium: Minimum essential medium with or without phenol red contains 10% fetal bovine serum (FBS); 2 mM L-glutamine (5.5 ml of 200 mM 100 × L-glutamine, added to 500 ml media); penicillin; and streptomycin (5.5 ml of 10,000 IU/ml 100× penicillin and streptomycin, added to 500 ml media).

3. Eukaryotic expression and reporter vectors:

GAL-AR-658–919 is a fusion protein of the GAL4 DNA-binding domain amino acid residues 1–147 and human AR carboxyl-terminal amino acid residues 658–919 that constitutes the AR LBD (2, 27).

VP-AR-1–660 is a fusion protein of VP16 transactivation domain amino acid residues 411–456 and human AR NH_2-terminal amino acid residues 1–660 that includes the DNA-binding domain (7).

5XGAL4Luc3 is a luciferase reporter vector with five copies of the GAL4 upstream enhancer element (17, 28).

4. Fugene 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) is stored at 4°C.

5. Phosphate buffered saline (PBS).

6. Testosterone, DHT (Steraloids, Inc., Newport, RI) and the synthetic androgen, methyltrienolone (R1881) (PerkinElmer, Waltham, MA) 10 mg/ml stocks, are prepared fresh each month in 100% ethanol and stored at −20°C. Dilutions of steroid stocks are prepared fresh each week in 100% ethanol.

7. Luciferase lysis buffer: 1% Triton X-100, 2 mM EDTA, and 25 mM Tris-phosphate, pH 7.8.

8. D-Luciferin, monopotassium salt (Thermo Scientific, Rockford, IL); 0.1 ml of 0.1 M D-luciferin is added automatically per well in 96-well assay plates in a luminometer.

9. Luciferase reading buffer: 25 mM Glycylglycine, 15 mM $MgCl_2$, 5 mM ATP, and 0.5 mg/ml bovine serum albumin, pH 7.8; 0.1 ml reading buffer is added automatically per well in 96-well assay plates in a luminometer.

10. Twelve-well treated nonpyrogenic polystyrene tissue culture plates (Corning, Inc., Corning, NY).

11. 96-Well, nontreated, flat-bottomed white polystyrene microtiter plates (Costar; Corning, Inc., Corning, NY).

12. 15-ml Sterile RNase/DNase-free nonpyrogenic polypropylene centrifuge tubes (Corning, Inc., Corning, NY).

13. Automated Lumistar Galaxy multi-well plate luminometer (BMG Labtech, Germany).

3. Methods

3.1. HeLa Cell Culture

HeLa cells (*see* **Note 2**) are propagated in MEM supplemented with 10% FBS, L-glutamine, penicillin, and streptomycin. Cells are passaged twice each week at 1:7 dilution. Cells are harvested by washing T150 flasks with 10 ml PBS, adding 2 ml of a 0.05% trypsin and 0.53 mM EDTA solution/flask, and incubating at 37°C for 5 min to release cell adhesion. MEM containing 10% FBS is added to inactivate trypsin. Cells are counted using a

hemocytometer, plated at 5×10^4 cells/well in 12-well plates, and transfected using FuGene 6 Transfection Reagent.

3.2. Experimental Design

The AR N/C interaction is performed as a mammalian two-hybrid assay. Cotransfection of an AR NH$_2$-terminal FXXLF motif-containing fragment with an AR carboxyl-terminal fragment that contains the LBD and AF2 binding surface increases reporter gene activity in the presence of an AR agonist. The AR N/C interaction requires the addition of an active androgen such as testosterone, DHT, or synthetic androgen R1881 (Fig. 8.1), mibolerone, or anabolic steroid (*see* **Note 1**). The concentration of steroid that increases luciferase light units by greater than fivefold is indicative of ligand potency and a ligand-dependent AR intramolecular and/or intermolecular interaction (*see* **Note 6**). The AR N/C interaction is not induced by antagonists such as hydroxyflutamide or Casodex (bicalutamide). These antagonists inhibit the agonist-induced AR N/C interaction in a dose-dependent manner (Fig. 8.2). The effects of naturally occurring AR mutations identified in patients with the androgen insensitivity syndrome, somatic mutations in prostate cancer tissue (21), or targeted mutations designed to establish the AR sequence requirements for the N/C interaction can be tested in the two-hybrid assay when mutations are introduced into the AR NH$_2$-terminal or LBD fragments. Assays are set in duplicate or triplicate with an agonist dose response range between 0.01 and 10 nM and an antagonist dose response range between 50 nM and 1 μM. The optimal dose response concentration for the AR N/C interaction in the two-hybrid assay is 10 nM testosterone, DHT, or synthetic androgen (Fig. 8.1). The N/C interaction assay has also been demonstrated for other steroid receptors using NH$_2$- and carboxyl-terminal fragments (*see* **Note 7**).

When the AR N/C interaction is performed using GAL4 and VP16 fusion vectors, a 5XGAL4Luc reporter vector is used. The AR N/C interaction may also be performed using the androgen-responsive luciferase reporter vector PSA-Enh-Luc, or the less active MMTV-Luc, transfected into HeLa cells with the AR DNA and ligand-binding domain fragment AR-507–919 and AR NH$_2$-terminal fragment AR-1–503 that lacks the DNA-binding domain (*see* **Note 4**). Experimental procedures for both assays are otherwise identical.

3.3. Transfection of HeLa Cells Using FuGene 6

DNAs are aliquoted into microfuge tubes and stored for not more than 1 week at −20°C using per well:

50 ng Gal-AR-658–919 (*see* **Note 3**)

50 ng VP-AR-1–660

0.1 μg 5XGAL4Luc3

Coregulator expression plasmid DNA (25–50 ng/well of 12-well plates) can be added to test for effects on the AR N/C interaction by including equivalent amounts of empty vector DNA added to controls (*see* **Note 5**).

Day 1: Plate 5×10^4 HeLa cells/well in 12-well plates with 1 ml HeLa cell medium containing phenol red and incubate overnight in a 5% CO_2 cell culture incubator.

Day 2:

1. Aspirate the medium and add 0.75 ml/well fresh HeLa cell medium containing phenol red using a sterile repeat pipette and return the plates to the cell culture incubator.

2. Calculate 43 μl × number of wells (include four extra wells in the calculation) of serum-free, phenol red-free medium and add to a 15-ml centrifuge tube.

3. Briefly warm the Fugene reagent and vortex for 1 s.

4. Add 0.6 μl Fugene reagent × total number of wells directly to the aliquoted serum-free media avoiding contact with the plastic tube.

5. Vortex for 1 s and incubate for 5 min at room temperature.

6. Thaw the previously aliquoted expression plasmid and luciferase reporter DNA.

7. Add sufficient Fugene cell medium solution for 43 μl/well to the aliquoted DNA.

8. Vortex each tube for 1 s and incubate for 15 min at room temperature.

9. Vortex briefly and add 40 μl of DNA–Fugene cell medium solution to each well.

10. Return the plates to the 37°C cell culture incubator and incubate overnight.

Day 3

1. Aspirate the medium and replace with 1 ml serum-free, phenol red-free HeLa cell medium with and without ligand.

2. Return the plates to the 37°C cell culture incubator and incubate overnight.

Day 4

1. Aspirate the media and wash each well with 1 ml PBS.

2. Aspirate PBS twice to dryness and add 0.25 ml luciferase lysis buffer using a repipetter.

3. Gently rock the plates on a platform shaker for 30 min at room temperature.

4. Aliquot 0.1 ml from each well into a 96-well microtiter assay plates and measure luciferase activity using an automated luminometer.

4. Notes

1. N/C interaction screen for AR agonists and antagonists**:** Androgens that induce the AR N/C interaction have agonist activity in vivo. The AR N/C interaction is inhibited by classical AR antagonists such as hydroxyflutamide and Casodex that bind the AR LBD with moderate affinity (14). Examples of AR agonists that induce the AR N/C interaction are testosterone, DHT, and the anabolic steroids oxandrolone and fluoxymesterone which bind with lower affinity than testosterone

or DHT but are potent agonists in vivo (14). These findings suggest that the AR N/C two-hybrid interaction assay can be used to identify agonists that increase AR transcriptional activity in vivo when performed in cells with suitable ligand uptake and lack of metabolism (*see* **Note 2**). The AR N/C interaction has been used to screen AR agonists and antagonists, establish ligand dependence and motif-binding specificity, and investigate *AR* gene mutations that cause the androgen insensitivity syndrome (10, 29). While AR antagonists competitively inhibit the agonist-induced N/C interaction (Fig. 8.2), high concentrations of an AR antagonist, e.g., 10 μM hydroxyflutamide, may have agonist activity with wild-type AR in transient transfection experiments, but only weakly induce the AR N/C interaction (6, 14, 30). Somatic AR mutations in prostate cancer can enhance the ability of antagonists to induce the AR N/C interaction and increase AR transcriptional activity.

2. *AR N/C interaction assay in other cell lines:* The AR N/C interaction has been performed in Chinese hamster ovary (CHO) (6, 7, 9, 15, 31), human hepatocellular carcinoma HepG2 (17, 32), and HeLa cells (2, 18, 21). HeLa cells are advantageous because they contain low levels of steroid-metabolizing enzymes. This is evident by the similar activities of the naturally occurring androgens testosterone and DHT, and the synthetic androgen methyltrienolone (R1881), which is less susceptible to metabolism than are naturally occurring androgens. HepG2 cells derive from a human hepatocellular carcinoma and thus may express liver-derived, steroid-metabolizing enzymes. This is supported by the weaker activities of testosterone and DHT compared to synthetic androgens when assayed in HepG2 cells. The AR N/C interaction has also been performed in yeast (33). In this case, consideration should be given to ligand uptake and metabolism. While a variety of cell lines can be used to perform the AR N/C interaction assay, possible complications to be considered are ligand uptake and metabolism, and the influence of endogenous transcriptional coregulators that can differ between cell lines.

3. Inhibition of the AR N/C interaction by the hinge region: Human AR has 919 amino acids that include the NH_2-terminal residues 1–558, DNA-binding domain residues 559–624, hinge region residues 625–676, and LBD residues 677–919 (34). However, the number of amino acid residues in human AR varies between individuals depending on the length of the polymorphic NH_2-terminal glutamine repeat that begins at amino acid residue 58. Length of the AR CAG-encoded glutamine repeat influences AR transcriptional capacity through mechanisms that are not completely understood (35–37).

    Initial experiments that identified and characterized the androgen-dependent AR N/C interaction made use of the AR LBD fragment AR-624–919, which contains the LBD and the entire hinge region (6, 7). It was shown subsequently that human AR hinge region residues 628–646, 624–658, or 629–636 have an inhibitory effect on the AR N/C interaction and AR transcriptional activity (2, 38, 39). The inhibitory region contains part of the bipartite AR nuclear-targeting signal at residues 617–633 (39, 40). However, inhibition may be independent of a detrimental effect on AR nuclear transport, since inhibition was also observed using a GAL4 DNA-binding domain–AR LBD fusion protein that has an

independent nuclear-targeting signal (2). The inhibitory effect of the AR hinge region appears to be mediated through the AR AF2 site (2). A naturally occurring AR-R629W hinge mutation that disrupts the AR N/C interaction caused severe androgen insensitivity without altering androgen-binding affinity and nuclear localization (12). These findings support a negative influence of the hinge region on the AR N/C interaction and AR transcriptional activity in vivo. The phenotypic expression of decreased AR transcriptional activity in the androgen insensitivity syndrome resulting from mutations that disrupt the AR N/C interaction supports the functional importance of the AR N/C interaction in vivo.

The inhibitory effect of the hinge region does not appear to result from structural artifacts associated with expression of the AR NH$_2$-terminal and LBD fragments, since inhibition was also observed in full-length AR. While the precise mechanism for AR hinge region inhibition of AF2 is not known, the inhibitory effect decreases AF2 binding of the AR F*XX*LF and p160 coactivator L*XX*LL motifs (2). This suggests that AR AF2 may have greater inherent transcriptional capacity than previously recognized, especially when p160 coactivator levels are increased as in castration-recurrent prostate cancer (41). In HeLa cells in the absence of overexpressed p160 coactivators, AR AF2 transcriptional activity was almost undetectable when GAL-AR-LBD was expressed in the presence of 10 nM DHT in the absence of an interacting AR NH$_2$-terminal fragment (Fig. 8.1). This reflects in part the genetic changes in the AR LBD AF2 site during evolution that have weakened the binding affinity for p160 coactivator L*XX*LL motifs and improved binding of the F*XX*LF motifs (22). However, in prostate cancer cells, such as CWR-R1 cells that have higher levels of coactivators, expression of GAL-AR-LBD alone can have significant activity (2).

Thus, the contribution of AF2 to AR transcriptional activity relative to AR NH$_2$-terminal AF1 is limited by the inhibitory effect of the AR N/C interaction, by genetic changes in AF2 that weaken p160 coactivator L*XX*LL motif binding relative to the F*XX*LF motif, through inhibitory mechanisms involving the hinge region, and through the complement of cell-specific coregulatory proteins.

4. *Promoter specificity of the AR N/C interaction*: Androgen-responsive enhancer/promoters differ in the requirement for the AR N/C interaction. For example, transcriptional activation of the PSA enhancer is increased through mechanisms that are increased by the AR N/C interaction (18, 19), whereas some androgen-responsive enhancers/promoters do not require the AR N/C interaction. The most notable example is MMTV which is activated to a similar extent in transient transfection assays whether or not the AR N/C interaction is disrupted by mutations in the AR F*XX*LF motif (18). The extent of activation of PSA-Enh-Luc or MMTV-Luc by the coexpression of AR-507–919 and AR-1–503 is influenced by the concentration of endogenous coregulators which differs between cell lines. The full complement of transcriptional regulators that influence the AR N/C interaction and AR transcriptional activity remains to be defined.

5. *AR FXXLF motif and influence of coregulators*: The effect of coregulatory proteins on the AR N/C interaction can be assessed in the mammalian two-hybrid assay. However, nonspecific inhibitory effects associated with the addition of increasing amounts of coactivator DNA require appropriate controls. Expression vector DNA concentrations should be kept to a minimum (100 ng or less/well in 12-well plates) to avoid nonspecific inhibition. Transient transfections may be performed in monkey kidney CV1 cells using calcium phosphate precipitation of DNA when assessing the effects of coregulatory proteins on AR transcriptional activity (24).

The AR N/C interaction assay measures the androgen-dependent AR NH_2-terminal F*XX*LF motif 23FQNLF[27] interaction with the AF2 site in the LBD. The AR F*XX*LF motif also serves as the principal interaction site for MAGE-11, an AR coregulator that increases AR transcriptional activity by relieving inhibition of p160 coactivator L*XX*LL motif binding at AF2 and through direct interactions with transcriptional coregulators (20, 25). The AR F*XX*LF motif was also implicated as a site involved in AR degradation by the proteasome (42). An NH_2-terminal W*XX*LF ([433]WHTLF[437]) contributes to ligand-independent AR transcriptional activity and to the AR N/C interaction through mechanisms that remain to be established (15, 18, 43).

F*XX*LF-like motifs that mediate androgen-dependent interactions with AR AF2 are present in the AR coregulatory proteins ARA54, ARA55, and ARA70 (32, 44–46). While the affinity of these F*XX*LF-related motifs has not been reported, their inability to slow the dissociation rate of bound androgen when inserted to replace the AR F*XX*LF motif suggests that AR AF2 affinity is weaker for these coregulator F*XX*LF motifs than for the AR F*XX*LF motif (32). Furthermore, MAGE-11 does not interact with F*XX*LF-related motifs present in these AR coregulators, indicating specificity for the AR F*XX*LF motif (24). MAGE-11 contains an F*XX*IF motif that serves as part of a recognition sequence for p160 coactivators. Interaction between the MAGE-11 F*XX*IF and an F-box-like region in transcriptional mediator protein 2 (TIF2), and AR F*XX*LF motif binding to the MAGE-11 F-box, suggests a novel protein–protein interaction paradigm of F*XX*LF motif binding to the F-box (25).

A number of AR coregulatory proteins are reported to influence the AR N/C interaction, some of which involve interactions with F*XX*LF-related motifs. Cyclin D1 binds the AR NH_2-terminal F*XX*LF motif and inhibits the AR N/C interaction and AR transcriptional activity independent of its ability to recruit histone deacetylases (47). p53 contains an α-helical F*XX*LF-like recognition motif in its activation domain and inhibits the AR N/C interaction (48, 49). hRAD9 contains a carboxyl-terminal F*XX*LF motif, inhibits the AR N/C interaction, and represses AR transcriptional activity (50). FoxO1 binding to the AR NH_2-terminal region inhibits the AR N/C interaction (51). Inhibition of the AR N/C interaction is caused by corepressor SMRT that interacts with the AR NH_2-terminal region (52, 53) and by N-CoR that interacts with the AR carboxyl-terminal region (54, 55).

MAGE-11 contains an F*XX*IF motif and increases AR transcriptional activity by competitively inhibiting AR F*XX*LF motif binding to AF2 in the AR N/C

interaction, and by directly recruiting p160 coactivators (24). c-Jun, although not reported to contain an F*XX*LF motif, enhanced the AR N/C interaction, AR binding to DNA, and AR transcriptional activity (56). Effects of AR coregula-tory proteins can be assessed in two-hybrid interaction assays as described here. Endogenous levels of these and other coregulators yet to be defined influence the AR N/C interaction. Experiments using small inhibitory RNAs to knock down endogenous protein expression can therefore provide additional evidence for the effects of coregulatory proteins in the AR N/C interaction and AR function.

6. Intramolecular versus intermolecular AR N/C interaction: The AR N/C interaction may occur as an intramolecular and an intermolecular interaction. Early studies on androgen insensitivity syndrome AR mutations suggest that an intermolecular AR N/C interaction facilitates AR binding to DNA as an anti-parallel dimer (5–7). Structural analysis using fluorescence resonance energy transfer (FRET) suggests an intermolecular AR N/C interaction in the nucleus and an intramolecular interaction in the cytoplasm (57). Additional FRET analysis suggests that the AR N/C interaction occurs when AR is mobile, but not when transiently bound to DNA (58), and an intramolecular N/C interaction for transcriptionally active AR bound to DNA (59). Under some conditions, an intermolecular AR N/C interaction could contribute to runaway domain swapping (60), in which amyloid-like fibrils are associated with the degenerative phenotype that results from glutamine expansion diseases, such as spinal bulbar muscular atrophy which is caused by an expanded AR NH_2-terminal glutamine repeat (61). However, the length of the polymorphic glutamine repeat in the human AR NH_2-terminal region has not been shown to influence the AR N/C interaction (6).

7. *N/C interaction in other steroid receptors*: A ligand-dependent N/C interaction has been reported for the progesterone receptor (PR) (62), estrogen receptor-α (63), and mineralocorticoid receptor (MR) (64), although none has been as extensively characterized as the AR N/C interaction. The glucocorticoid receptor (GR) does not undergo an agonist-induced N/C interaction. However, introducing a p160 coactivator L*XX*LL motif into the NH_2-terminal region to create a GR chimera slows the dissociation rate of dexamethasone ($t_{1/2}$ 168 min), a synthetic glucocorticoid, compared to wild-type GR ($t_{1/2}$ 31 min) (20). This demonstrates the ability of an N/C interaction to slow ligand dissociation and stabilize a receptor.

The longest PR-B contains an extended NH_2-terminal L*XX*LL-like motif upstream region absent from the shorter PR-A. This unique PR-B upstream region imparts greater transcriptional activity through cooperative interactions involving a PR-B N/C interaction when bound to DNA (65).

The MR N/C interaction displays striking ligand specificity, with strong induction by aldosterone and the synthetic agonist 9α-fludrocortisol, and weak or no interaction with other physiologically relevant ligands such as deoxycorticosterone or cortisol (64, 66). While the precise nature of the MR NH_2-terminal interaction motif has yet to be characterized, evidence suggests that like AR, the MR N/C interaction contributes to MR transcriptional activity by discriminating ligand-

specific effects in vivo. An MR corepressor recruited by agonist-bound MR inhibited the MR N/C interaction (67).

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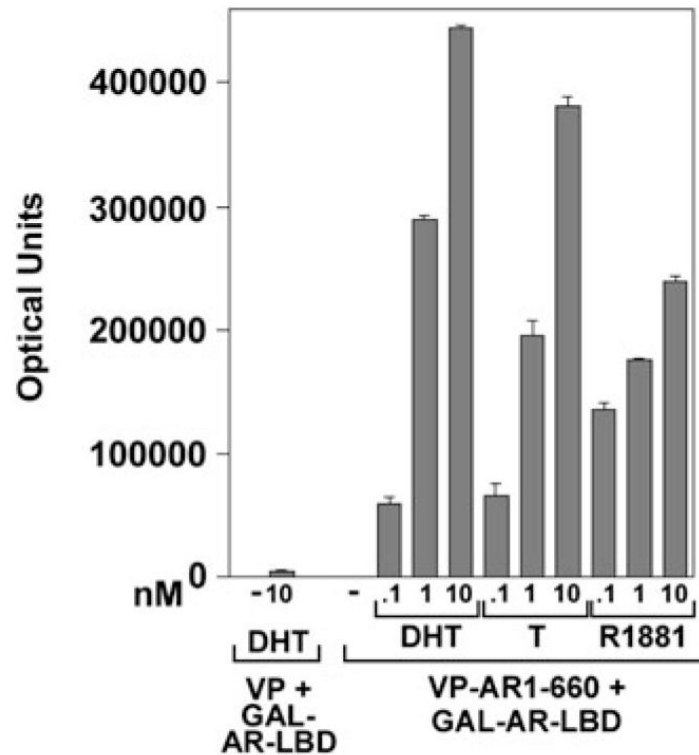
Fig. 8.1.

Androgen-dependent AR N/C interaction. HeLa cells (5×10^4/well of 12-well plates) were transfected using Fugene 6 with (per well) 0.1 μg 5XGAL4Luc3 reporter and 50 ng GAL-AR-658–919 (GAL-AR-ligand-binding domain (−LBD)) in the presence of 50 ng pVP16 empty vector (VP) or 50 ng VP-AR-1–660 that codes for the AR NH_2-terminal and DNA-binding domains. The day after transfection, the medium was exchanged with phenol red-free, serum-free medium in the absence and the presence of 0.1, 1, and 10 nM dihydrotestosterone (DHT), testosterone (T), or the synthetic androgen methyltrienolone (R1881), respectively. Cells were incubated overnight at 37°C and luciferase activity was determined. The data are representative of the androgen-dependent mammalian two-hybrid AR N/C interaction assay.
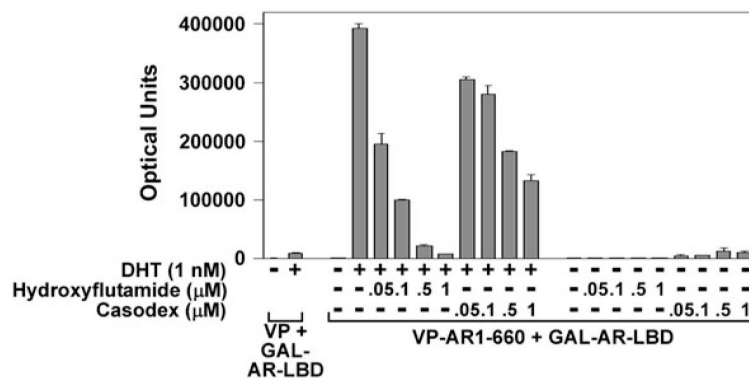
Fig. 8.2.
Inhibition of the DHT-induced AR N/C interaction by AR antagonists. HeLa cells were transfected with 5XGAL4Luc3 reporter vector and GAL-AR-658–919 (GAL-AR-LBD) with pVP16 empty vector (VP) or VP-AR-1–660 as described in Fig. 8.1. The next day, cells were incubated in phenol red-free, serum-free media in the absence and the presence of 1 nM dihydrotestosterone (DHT) with and without 0.05, 0.1, 0.5, and 1 μM hydroxyflutamide or Casodex (bicalutamide), and the same increasing concentrations of hydroxyflutamide or Casodex alone. The data show that the DHT-induced AR N/C interaction measured in a mammalian two-hybrid assay is inhibited in a dose-dependent manner by increasing concentrations of AR antagonists, hydroxyflutamide and Casodex, and that these antagonists alone do not induce the AR N/C interaction.