Ligand-independent Androgen Receptor Activity Is Activation Function-2-independent and Resistant to Antiandrogens in Androgen Refractory Prostate Cancer Cells*

Received for publication, May 24, 2006, and in revised form, July 17, 2006 Published, JBC Papers in Press, July 25, 2006, DOI 10.1074/jbc.M605002200

Scott M. Dehm and Donald J. Tindall

From the Departments of Urology and Biochemistry & Molecular Biology, Mayo Clinic College of Medicine, Rochester, Minnesota 55905

Androgen ablation inhibits androgen receptor (AR) activity and is as an effective treatment for advanced prostate cancer (PCa). Invariably, PCa relapses in a form resistant to further hormonal manipulations. Although this stage of the disease is androgen-refractory, or androgen depletion-independent (ADI), most tumors remain AR-dependent through aberrant mechanisms of AR activation. We employed the LNCaP/C4-2 model of PCa progression to study AR activity in androgen-dependent and ADI PCa cells. In this report, we show that the AR is transcriptionally inactive in androgen-dependent LNCaP cells in the absence of androgens. However, in ADI C4-2 cells, the AR displays a high level of constitutive, androgen-independent transcriptional activity. To study the mechanisms of ligand-dependent and ligand-independent AR activation in these AR-expressing cells, we generated a reporter system based on swapping the DNA binding domain of the AR with the DNA binding domain of the yeast Gal4 transcription factor. In androgen-dependent PCa cells, the well characterized C-terminal AR activation function-2 (AF-2) domain was critical for strong, ligand-dependent activity. Conversely, in ADI PCa cells, constitutive, ligand-independent AR activity was AF-2-independent but instead dependent on N-terminal AR domains. Importantly, the ligand- and AF-2-independent mode of AR activation observed in ADI PCa cells was completely resistant to the antiandrogen, bicalutamide. Our data thus demonstrate that the AR can inappropriately activate transcription in ADI PCa cells via mechanisms that are resistant to castration and AR antagonist, the two modes of androgen ablation used to treat advanced PCa.

Prostate cancer (PCa) is the most frequently diagnosed male cancer and second leading cause of cancer deaths in the United States (1). Androgens support the development and survival of normal and cancerous prostate tissue by binding and transcriptionally activating the androgen receptor (AR), a member of the nuclear receptor transcription factor superfamily (2). The treatment regimen for locally advanced or metastatic PCa includes AR antagonists and is achieved through surgical/chemical castration or administration of AR antagonists (3). This approach results in reduced expression of AR target genes, such as prostate specific antigen (PSA), and concomitant tumor regression (4). However, PCa invariably relapses in a form that is resistant to these hormonal manipulations, and further treatment is essentially palliative (5). This stage of the disease is referred to as androgen-independent, androgen-refractory, or androgen depletion-independent (ADI) (6). Significantly, although ADI PCa is resistant to androgen ablation, the AR remains critical for the growth and survival of most tumors (7). For example, most ADI PCa retains AR expression (8). Moreover, the PSA gene continues to be expressed at this stage of the disease (8). Indeed, targeted AR inhibition decreases PSA expression, cell proliferation, and survival in various cell-based models of ADI PCa (9–12). These findings suggest that ADI PCa cells continue to proliferate and survive through aberrant mechanisms of AR activation, and thus the AR remains a valid target for therapy.

The AR shares a modular organization with other nuclear receptors and contains a loosely structured N-terminal domain (NTD) consisting of the AF-1 transactivation function, a zinc-finger DNA binding domain (DBD), a hinge region, and a C-terminal domain (CTD) ligand binding (LBD)/AF-2 transactivation function module (13). In the absence of ligand, the AR CTD serves to inhibit the transcriptional activity of the NTD AF-1 module. Ligand binding, conversely, relieves this negative regulation on AR AF-1 and also allows the AR AF-2 domain to adopt a structure that can bind FXXLF or LXXLF motifs present in AR coactivators such as ARA70 and members of the p160 coactivator family (14, 15). Thus, both the AF-1 and AF-2 modules are able to recruit coregulatory proteins to genomic androgen response elements (AREs) encoded in the promoter and enhancer regions of target genes (16). These coregulators are responsible for mediating full transcriptional activation (17).
Mechanisms of Ligand-independent AR Activity

AR mutation or overexpression, altered expression or activity of coregulators, or cross-talk with activated signal transduction pathways can influence AR activity in ADI PCA cells (4, 5). These mechanisms can elicit ligand-independent activation of the AR, or allow inappropriate AR activation in response to low ligand concentrations, alternative steroids, or antiandrogens (4). These mechanisms can operate alone or in concert to mediate a critical level of AR activity in ADI PCA cells. Therefore, each plays a key role in the development of resistance to androgen ablation.

Before new anti-AR therapies can be developed and tested as treatments for ADI PCA, the domains of the AR that are relevant for targeting must first be identified. To date, all knowledge on mechanisms of AR transactivation comes from studies in AR-null, and usually non-epithelial, non-human cell lines (14, 15, 18–27). The purpose of this study was to assess the mechanistic role of the AR in a model relevant to PCA biology.

To this end, we developed a system to allow functional study of the AR in a human cell-based model of AR-dependent PCA progression. Our results show that AR activity is strictly ligand-dependent as well as AF-2-dependent in androgen-dependent PCA cells and can thus be blocked by the antiandrogen, bicalutamide. In ADI PCA cells, AR activity is constitutive, ligand-independent, AF-2-independent, and antiandrogen-resistant. We identified transcriptionally active domains in the AR NTD and found that transactivation unit (TAU)-1 was important for full ligand-dependent as well as ligand-independent AR activity in androgen-dependent and ADI cells. Together, these findings suggest that therapies targeted to the AR CTD may lack long-term effectiveness due to an inability to inhibit AF-2-independent AR activity. Thus, AR NTD modules such as TAU-1 may be more important and relevant targets for the development of novel anti-AR strategies in androgen-dependent and ADI PCA.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Androgen-dependent LNCaP cells were purchased from the American Type Culture Collection (ATCC). The ADI LNCaP-derived C4-2 cell line was purchased from UroCor (Oklahoma City, OK). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were maintained at 37 °C and 5% CO₂. LNCaP cells between passages 29 and 45 and C4-2 cells between passages 34 and 50 were used for experiments.

For androgen response experiments, LNCaP and C4-2 cells were grown in serum-free medium or medium supplemented with 10% charcoal-stripped (steroid depleted) serum (CSS) for 48 h. Growth medium was replaced with serum-free medium or medium plus 10% CSS containing 1 nM mibolerone (BIOMOL, Plymouth Meeting, PA), 10 μM bicalutamide (AstraZeneca) ethanol, or combinations of these compounds. Cells were cultured for an additional 24 h and then harvested. For C4-2 siRNA-transfection experiments, culture medium was replaced 24 h post-transfection with RPMI plus 5% CSS. After 48-h growth, cells were harvested.

Plasmid Constructs—The SV40-Renilla luciferase reporter vector and pG5-LUC were purchased from Promega (Madison, WI). All PSA promoter-based fragments contained the firefly luciferase reporter pG3-Basic backbone (Promega). The full-length PSA promoter luciferase reporter, −5746PSA–LUC, containing a 5.8-kb genomic fragment from the PSA locus was obtained from Dr. Charles Young (Mayo Clinic, Rochester MN). The −5746PSA–LUCΔEco/Pst construct was generated by digestion of −5746PSA–LUC with EcoRI/Pstl, generation of blunt ends, and re-ligation to create −5746PSA–LUCΔEco/Pst. sPSA–luc (28), referred to here as PSAenh–LUC, and pN/H (28), referred to here as PSACore–LUC, were gifts from Dr. Leland Chung (Emory University, GA). PSAenh(ARE)-E4–LUC and S-All (29), referred to here as PSAenh(GAL4)-E4–LUC, were gifts from Dr. Michael Carey (University of California, Los Angeles, CA). PSAenh(GAL4)-LUC has been described previously (30).

The Gal4 expression plasmid, pM, was purchased from Clontech. pSHbHAR-A, containing the full-length human AR cDNA cloned into the pCMV5 expression vector, was a gift from Dr. Frank French (University of North Carolina-Chapel Hill). hARGal4 was generated by first creating a Mmul site at the 5′-end of the AR DBD within pSHbHAR-A via QuikChange site-directed mutagenesis (Stratagene) using the mutagenic primers ARDBD5′mutFWD (5′-CCACCC-CAGAAGACGCCGTCGTAGCTGGAAGATGAAAGC) and ARDBD5′mutREV (5′-GGTCATCTCCACAGATAGCAG-CCGGTCTTCTGGGTTG). An SacII site was then created at the 3′-end of the AR DBD via mutagenesis using the mutagenic primers ARDBD3′mutFWD (5′-GGGATGACTCTGGGAC-CGGCGGAGCTGAAGAAACTTGG) and ARDBD3′mutREV (5′-CCAAGTTTTCTCAGTTCGCGGCCGCTCCAGATCATCCC). The Gal4 DBD was then amplified from pM using the primers GAL4DBDMMulFWD (5′-GAAAGACGCCGTCCTAGTGTCTTCTTTC) and GAL4DBDMSacIIREV (5′-AATTCCCCGCGTACA-GTCACAC), digested with Mmul/SacII, and cloned into the newly created Mmul/SacII sites flanking the AR DBD in p5HBHAR-A. This version of the AR contained 24 CAG repeats in the AR NTD; however, amino acid residues were numbered based on an AR containing 22 CAG repeats and a wild-type DBD to maintain consistency with the literature. For the generation of hARGal4 NTD deletions, successive QuikChange site-directed mutagenesis reactions were carried out to generate in-frame BssHII sites at positions 101 and 211, 211 and 253, 253 and 361, or 361 and 574. The resultant ARGal4 BssHII double mutants were subsequently digested with BssHII and re-ligated to generate the ARGal4 deletions Δ101/211, Δ211/253, Δ253/361, and Δ361/574. QuikChange site-directed mutagenesis was employed to create N705S, R752Q, V716R, K720A, and E897K mutant versions of hARGal4. Details of the primers used for these mutagenesis reactions will be made available upon request. All deletion and point-mutant constructs were sequenced to verify their integrity and tested for expression via transfection and Western blot with anti-Gal4 antibodies.

Transient Transfections—For experiments that directly compared the activities of reporter constructs in LNCaP versus C4-2, cells were transfected 1.5 μg of a promoter–LUC construct and 0.5 μg of SV40-Renilla, using 10 μl of SuperFect reagent (Qiagen). Cells were seeded the previous day at a density of 5 × 10⁵ cells per well in 6-well plates. Transfections were allowed to proceed for 2 h followed by the addition of 2 ml of...
Mechanisms of Ligand-independent AR Activity

fresh medium plus 5% CSS. For transfections using PSAenh-(ARE)-E4-LUC or PSAenh(GAL4)-E4-LUC as a reporter construct, the same protocol was followed, except transfection mixtures consisted of 1 µg of a promoter-LUC reporter, 0.5 µg of SV40-Renilla, and 0.2 or 0.5 µg of either PM or hARGal4 and pBluescript to a total DNA content of 2 µg. For transfections using PSAenh(ARE)-LUC, PSAenh(GAL4)-LUC, or pG5-LUC as reporters, transfection mixtures consisted of 0.875 µg of a promoter-LUC reporter, 0.25 µg of SV40-Renilla, and 13.7 or 54.7 ng of either PM or hARGal4 and pBluescript to a total DNA content of 2 µg. We also employed electroporation for LNCaP transfections with pG5-LUC and PSAenh(ARE)-LUC. For this approach, components were increased by a factor of 6 from the SuperFect-based protocol. Therefore, for a typical electroporation, 3 × 10^6 cells were suspended in 350 µl of medium plus 5% CSS and mixed with 50 µl of a DNA mixture containing 5.25 µg of a promoter-LUC reporter, 1.5 µg of SV40-Renilla, and 328 or 82 ng of either PM or hARGal4 and pBluescript to a total DNA content of 12 µg. The cell/DNA mixture was subjected to a 305-V electrical pulse for 10 ms in cuvettes with a 4-mm gap-width using a BTX ElectroSquare electroporator. For siRNA experiments, 100 µl of a pool of four siRNAs targeted to the AR (AR Smartpool, Dharmacon) or an AR DBD-targeted siRNA were added to mixtures prior to electroporation. Cells were subsequently plated in medium plus 5% CSS. For all experiments, 24 h post-transfection, medium was aspirated and replaced with serum-free, phenol red-free medium containing 1 nm mibolerone, 10 µm bicalutamide, ethanol (vehicle control), or combinations of these compounds. Cells were harvested after an additional 24 h. For both SuperFect- and electroporation-based protocols, transfection efficiency was consistently 60–80%. Firefly and Renilla luciferase reporter activities were assayed via a Dual Luciferase Assay (Promega). Activities were normalized by dividing firefly luciferase activity by Renilla luciferase activity of samples cultured in the absence of androgens. Data presented represent the mean ± S.E. from at least three independent experiments, each performed in duplicate.

For siRNA transfections in C4-2 cells, 1.8 × 10^6 cells were seeded on 10-cm dishes in antibiotic-free RPMI 1640 plus 10% fetal bovine serum. Cells were transfected 24 h later with 500 pmol of a pool of four siRNAs targeted to the AR (AR SmartPool, Dharmacon), or a pool of four non-targeted, control siRNAs (Dharmacon) using 25 µg of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

RNA Extraction and Northern Blot Analysis—Total cellular RNA was isolated via acid-guanidinium phenol/chloroform extraction as described (31). Equal amounts of RNA (15 µg per lane) were fractionated on 1% denaturing formaldehyde-agarose gels. RNA was transferred to Hybond nylon membranes (Amersham Biosciences), UV cross-linked, and then hybridized with cDNA probes specific for PSA or glyceraldehyde-3-phosphate dehydrogenase labeled with [α-32P]dCTP using a RadPrime labeling kit (Invitrogen). Autoradiography was performed at ~80 °C using an intensifier screen (Kodak).

Western Blot Analysis—Cells were harvested directly in a loading buffer containing 65 mM Tris-HCl (pH 7.0), 2% (w/v) SDS, 5% β-mercaptoethanol, 10% (v/v) glycerol, and 0.5% (w/v) bromophenol blue. Equal amounts (typically 30 µg per lane) of protein were resolved in 10% NuPAGE gels (Invitrogen), followed by transfer to nitrocellulose and membrane blocking. Blots were incubated with antibodies specific for AR (N-20, Santa Cruz Biotechnology, Santa Cruz, CA) ERK-2 (D-2, Santa Cruz Biotechnology), or Gal4 (RRK5C1, Santa Cruz Biotechnology) at a final concentration of 100 ng/ml, washed, and probed with the appropriate secondary antibody conjugated to horse-radish peroxidase (Santa Cruz Biotechnology) diluted 1:10,000. Membranes were immersed in chemiluminescence reagents (Pierce) and exposed to Kodak X-Omat Blue XB-1 film for signal detection.

Cell Viability Assays—C4-2 cells were seeded at a density of 3000 cells/well on 96-well plates in RPMI 1640 plus 5% CSS. Following 24-h growth, the medium was replaced with RPMI 1640 plus 5% CSS containing 10 µM bicalutamide or ethanol (vehicle control). Following 0, 24, 48, and 72 h of growth, the number of viable cells was determined by 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium reduction (Cell Titer 96 AQueous One, Promega), which was measured at 490 nm. Data presented represent the mean ± S.E. from quadruplicate experiments.

RESULTS

Constitutive AR Activity in ADI PCa Cells—We employed the isogenic LNCaP/C4-2 cell model of PCa progression to study regulated and de-regulated mechanisms of AR activation. C4-2 cells grow in an ADI manner in vivo and in vitro, and were derived from androgen-dependent LNCaP cells through serial passage as xenografts in castrated athymic male mice (32). To characterize the influence of serum-derived as well as autocrine growth factors on basal AR activity in this model of PCa progression, we cultured LNCaP and C4-2 cells for 72 h in medium supplemented with steroid-depleted serum or serum-free medium. In addition, we also tested the effect of a 6-h re-feeding regimen with serum-free medium, designed to minimize the accumulation of autocrine factors. Under all conditions tested, LNCaP cells expressed very low mRNA levels of the AR target PSA gene in the absence of androgens (Fig. 1A). Conversely, C4-2 cells displayed constitutive PSA expression in the absence of androgens, which was on average 3- to 4-fold higher than in LNCaP cells (Fig. 1A). In the presence of 1 nm mibolerone, LNCaP cells displayed an average 9.5-fold androgen induction of PSA expression, whereas C4-2 cells displayed an average 3.8-fold androgen induction (Fig. 1A). These results suggest that AR proteins have a relatively high level of constitutive activity but a lower degree of responsiveness to androgens in C4-2 versus LNCaP cells. To test the AR dependence of this apparent androgen-independent PSA expression in C4-2 cells, we employed siRNA. We observed a decrease in PSA mRNA expression when we compared C4-2 cells transfected with AR-targeted siRNA to cells transfected with non-targeted siRNA (Fig. 1B). This finding agrees with a previous study, which employed both AR-targeted siRNA and antisense oligonucleotides to demonstrate the AR dependence of constitutive PSA expression in C4-2 cells (33). These data demonstrate that the AR is aberrantly active in the absence of androgens in C4-2 cells.
To assess whether constitutive AR activity in C4-2 cells was due to an activating AR mutation(s), we generated AR cDNAs from LNCaP and C4-2 cells via reverse transcription-PCR. We sequenced several cDNA clones from each cell line and found that no sequence aberrations existed apart from the previously described AR T877A mutation (data not shown). These findings extend others’ sequencing efforts in these cell lines, which described an identical AR coding sequence throughout the DBD/CTD. These data suggest that the C4-2 cellular milieu is distinct from LNCaP cells and is supportive of aberrant AR activity.

To identify regions of the PSA promoter that mediate constitutive AR activity, LNCaP and C4-2 cells were transfected with an expression construct regulated by a 5.8-kb PSA promoter fragment (~5746 PSA-LUC) in the absence of serum and androgens. Transfection efficiencies were consistently 60–80% in both cell lines (data not shown). We observed 5-fold higher androgen-independent activity of ~5746 PSA-LUC in C4-2 compared with LNCaP cells (Fig. 1C). In agreement with a previous study (28), the upstream PSA enhancer core (AREc) mediated this elevated androgen-independent PSA promoter activity in C4-2 cells, and deletion constructs lacking this element did not display detectable activity (Fig. 1C). Based on these findings, we conclude that the enhancer core of the PSA promoter contains elements that are regulated by constitutively active AR in C4-2 cells.

An AR-Gal4 Fusion Strategy to Functionally Study the AR in AR-dependent Cells—To allow the study of AR structure/function relationships in LNCaP and C4-2 cells, we developed an AR-Gal4 fusion strategy. This strategy was based on the premise that swapping AREs in PSA enhancer-based reporter constructs with binding sites for the Gal4 yeast transcription factor would render these constructs responsive to hARGal4, a hybrid protein wherein the zinc-finger AR DBD was swapped with the zinc-finger Gal4 DBD (Fig. 2, A and B). A reporter construct regulated by the PSA enhancer, PSAenh(ARE)E4-LUC, displayed robust activation in response to 1 nM mibolerone (Fig. 2C), and replacement of the 4 AREs with GAL4 binding sites completely abolished this androgen response (Fig. 2C). Cotransfection with an expression construct encoding the Gal4 DBD alone failed to restore androgen responsiveness (Fig. 2C). However, cotransfection with hARGal4 restored this robust androgen response (Fig. 2C). hARGal4 was also able to activate pG5-LUC, a reporter construct containing...
five tandem GAL4 binding sites upstream of the adenovirus major late core promoter, only in the presence of androgen (Fig. 2C). Importantly, siRNA targeted to the wild-type AR DBD, which selectively knocked down endogenous AR expression in these cells, did not affect the activity of hAR\textsuperscript{Gal4} (Fig. 2D). To test whether this system was a sensitive measure of constitutive AR activity in C4-2 cells, the AREs in PSAenh(ARE)-LUC were replaced with GAL4 binding sites (Fig. 2A). Under androgen-free conditions, replacement of AREs with GAL4 binding sites significantly inhibited promoter activity in C4-2 (Fig. 3A) but not LNCaP cells (Fig. 3B). Cotransfection with hAR\textsuperscript{Gal4} resulted in robust androgen-independent activation of PSAenh(GAL4)-LUC in C4-2 (Fig. 3A) but not LNCaP cells (Fig. 3B). To test whether these observations were restricted to the PSA gene, or represented a more general indication of AR activity in these cells, we tested the ability of hAR\textsuperscript{Gal4} to activate pG5-LUC in an androgen-independent manner. Indeed, hAR\textsuperscript{Gal4} was able to activate pG5-LUC in C4-2 (Fig. 3A) but not LNCaP cells (Fig. 3B) in the absence of androgens. Overall, the GAL4-based reporter system (Fig. 3C) mirrored the most salient properties of endogenous AR (Fig. 1A) in these cell lines. For example, although endogenous AR and hAR\textsuperscript{Gal4} displayed a higher level of constitutive activity and overall activation in C4-2 versus LNCaP cells, the overall androgen induction of endogenous AR and hAR\textsuperscript{Gal4} was much lower in C4-2 cells (Figs. 1A and 3C). These results demonstrate that the AR-Gal4 fusion strategy is ideally suited for functional studies of AR transcriptional activation in PCa cells that express and are dependent on functional AR.

**Mechanisms of Ligand-independent AR Activity**

Constitutive hAR\textsuperscript{Gal4} Activity Is Independent of the C-terminal Ligand Binding and AF-2 Domains—Androgen depletion inhibits AR activity through the CTD of the AR protein. Our
analysis of endogenous AR and hAR\textsuperscript{Gal4} suggests that constitutive AR activity in C4-2 cells may be completely ligand-independent and thus resistant to androgen depletion. To directly test this hypothesis, we analyzed two naturally occurring AR mutations, N705S and R752Q, which have been found in the germline of a subset of patients with complete androgen insensitivity syndrome and partial androgen insensitivity syndrome, respectively (34). The Asn-705 and Arg-752 residues have structural homologues in the binding pockets of other nuclear receptors (Fig. 4A), and contact the 17β-OH and O-3 positions of the steroidal ligand (34). The N705S and R752Q mutations impair AR ligand-binding activity to a degree that is directly proportional to the severity of androgen insensitivity with which they are associated (35). Indeed, the complete androgen insensitivity syndrome-associated N705S mutation nearly completely abolished androgen-induced hAR\textsuperscript{Gal4} activity in LNCaP cells, whereas the partial androgen insensitivity syndrome-associated R752Q mutation inhibited androgen-induced hAR\textsuperscript{Gal4} activity ~60% (Fig. 4B). In contrast, neither of these mutations was able to abolish constitutive hAR\textsuperscript{Gal4} activity in the absence of androgens in C4-2 cells (Fig. 4B). These findings indicate that hAR\textsuperscript{Gal4} activity in C4-2 cells is independent of the AR LBD. To further substantiate this finding, we tested the requirement of AR AF-2 for ligand-independent hAR\textsuperscript{Gal4} activity in C4-2 cells. Previous studies have suggested that the development of ADI PCa could be the result of overexpression of LXXLL- and/or FXLL-containing coactivators such as ARA70, SRC-1, and TIF2, which could facilitate AR activation through AF-2 despite low ligand concentrations (14, 15, 36, 37). To disrupt binding of LXXLL- and FXLL-containing coactivators, we generated specific point mutations based on the recently reported x-ray crystal structures of the AR AF-2 domain in complex with coactivator-derived peptides (14, 15). Amino acid residues Glu-897, Val-716, and Lys-720 are important components of the charge clamp and hydrophobic groove of the AR AF-2 domain and have structural homologues in the AF-2 domains of other nuclear receptors (38) (Fig. 4A). We chose to study E897K, V716R, and K720A mutations because a previous study demonstrated that these amino acid substitutions selectively abrogate AR AF-2 without affecting ligand binding affinity (39). Indeed, when compared with wild-
type hAR\textsuperscript{Gal4}, V716R, K720A, and E897K substitutions compromised androgen-induced hAR\textsuperscript{Gal4} activity in LNCaP cells (Fig. 4C). Conversely, these AF-2 mutations did not have a severe impact on ligand-independent hAR\textsuperscript{Gal4} activity in C4-2 cells (Fig. 4C). To ensure that this was specifically due to AF-2 independence of ligand-independent hAR\textsuperscript{Gal4} activity, and not a general impairment of AF-2 function in C4-2 cells, we also tested the effects of the V716R and E897K mutations on ligand-induced hAR\textsuperscript{Gal4} activity in C4-2 cells. As demonstrated in Fig. 4D, V716R and E897K selectively abrogated ligand-induced, but not ligand-independent hAR\textsuperscript{Gal4} activity in C4-2 cells. Taken together, these results demonstrate that constitutive hAR\textsuperscript{Gal4} activity is independent of the C-terminal LBD and AF-2 motifs in ADI C4-2 cells.

Ligand-independent AR\textsuperscript{Gal4} Activity Is Resistant to Antiandrogens—In addition to androgen depletion, AR antagonists inhibit AR activity through the CTD of the AR protein. Based on our findings, we hypothesize that antiandrogens such as bicalutamide may effectively repress androgen-induced AR activity in androgen-dependent PCa cells by targeting the AR functional requirement for the AF-2 domain. Our data also raise the possibility that bicalutamide may be ineffective at blocking ligand-independent AR activity in ADI PCa cells due to the AF-2-independent nature of this mode of activation. To probe this hypothesis, we tested the effect of bicalutamide on androgen-dependent and ligand-independent AR activity in LNCaP and C4-2 cells, respectively. In LNCaP cells, 10 μM bicalutamide inhibited the androgen-dependent induction of PSA expression (Fig. 5A). However, in C4-2 cells, constitutive PSA expression was unaffected, or slightly increased in the presence of 10 μM bicalutamide (Fig. 5A). Similarly, in LNCaP cells, bicalutamide inhibited the androgen-induced activity of hAR\textsuperscript{Gal4} (Fig. 5B). Conversely, in C4-2 cells, bicalutamide had no effect on, or
FIGURE 5. Ligand-independent AR activity is resistant to the antiandrogen, bicalutamide. A, LNCaP and C4-2 cells were cultured in medium containing 5% CSS for 24 h, and then shifted to serum-free medium containing 1 nM mibolerone (Mib), 10 μM bicalutamide (bic), vehicle control, or combinations of these compounds for an additional 24 h. PSA mRNA levels were determined by Northern blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels and ethidium bromide staining of ribosomal RNA subunits are shown as controls. AR levels were concurrently determined by Western blot. ERK-2 levels are shown as a control.

B, LNCaP and C4-2 cells were transfected with PSAenh(GAL4)-LUC as a reporter and hARgal4. Cells were cultured in medium containing 5% CSS for 24 h following transfection, and then switched to serum-free, phenol red-free medium containing 1 nM mibolerone (Mib), 10 μM bicalutamide, ethanol (EtOH, vehicle control), or combinations of these compounds for an additional 24 h. Luciferase activity was subsequently determined.

C, LNCaP cells were transfected with PSAenh(GAL4)-LUC as a reporter and wild-type, V716R, or E897K versions of hARgal4 exactly as described in B. D, C4-2 cells were cultured in the medium containing 1 nM Mib, 1 nM Mib plus 10 μM bic, or vehicle control as described in A and harvested. Lysates were analyzed exactly by Northern blot as described in A. E, C4-2 cells were transfected exactly as described in B to assess the effect of bicalutamide on androgen-induced hARgal4 function in ADI PCa cells. Data represent the mean ± S.E. from at least three independent experiments, each performed in duplicate. For each panel, values are shown relative to the activity of the GAL4-based reporter construct in the absence of androgens and transactivator, which was arbitrarily set to 1. F, C4-2 cells were cultured in medium containing 5% CSS on 96-well plates in the presence of either 10 μM bicalutamide (bic) or ethanol (EtOH, vehicle control). The relative number of viable cells 24, 48, or 72 h following exposure to these compounds was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Data represent results from quadruplicate assays.
Mechanisms of Ligand-independent AR Activity

slightly increased, androgen-independent hARGal4 activity (Fig. 5B). These data suggest that bicalutamide is unable to inhibit AF-2-independent AR activity. To further test this hypothesis, we assessed whether the AR AF-2 mutants V716R and E897K could be further repressed by bicalutamide in LNCaP cells. As observed previously, these hARGal4 mutants with abrogated AF-2 function were able to weakly support androgen-dependent transcription (Fig. 5C). However, this residual androgen-induced hARGal4 activity was not blocked by 10 μM bicalutamide (Fig. 5C). To verify that the lack of effect of bicalutamide on ligand-independent AR activity was specifically due to AF-2 independent activity, and not a general resistance of C4-2 cells to antiandrogens, we tested whether bicalutamide could inhibit ligand-induced, AF-2-dependent AR activity in ADI C4-2 cells. As demonstrated in Fig. 5D, bicalutamide was able to effectively inhibit androgen-induced PSA mRNA expression in C4-2 cells. Similarly, when the AR-Gal4 system was employed, bicalutamide effectively inhibited the component of hARGal4 activity that resulted from androgen induction but was unable to affect the constitutive, AF-2-independent component of hARGal4 activity in C4-2 cells (Fig. 5E). C4-2 cells are able to proliferate in vitro in an AR-dependent manner in the absence of exogenous androgens (12, 32). Because our results demonstrate that bicalutamide selectively affects AF-2-dependent/ligand-dependent versus AF-2 independent/ligand-independent AR activity in C4-2 cells, we used bicalutamide to test whether ADI C4-2 growth was indeed AF-2-independent and ligand-independent. Under conditions that mimic a complete androgen blockade, we observed no effect on the growth of C4-2 cells compared with androgen-depletion alone (Fig. 5F). These data suggest that C4-2 cells are ADI due to constitutive, ligand-independent, and AF-2-independent AR activity.

Activated AR NTD Motifs Mediate Ligand-independent AR Activity—In addition to harboring ligand-binding and AF-2 functions, the AR CTD also serves to repress the inherently strong transcriptional activity of the AR NTD in the absence of ligand. Therefore, constitutive, ligand-independent AR activity in C4-2 cells could result from decreased inhibition of the AR NTD by the CTD (NTD re-expression), or alternatively, increased activity of the AR NTD, which could override CTD-mediated inhibition (NTD activation). We hypothesize that NTD activation is the most likely mechanism of ligand-independent AR activation in C4-2 cells, because full, androgen-stimulated PSA expression is higher in C4-2 than in LNCaP cells (Fig. 1A). In addition, overall hARGal4 activity is higher in C4-2 cells than in LNCaP cells (Fig. 3C). To directly test AR NTD activity in LNCaP and C4-2 cells, we deleted the CTD from hARGal4. In both cell lines, we observed that the constitutive activity of the isolated NTDGal4 was higher than the androgen-induced activity of intact hARGal4, despite comparable expression levels of these Gal4-based proteins (Fig. 6A). In addition, NTDGal4 was able to activate the reporter to a greater degree in C4-2 versus LNCaP cells (110 versus 60 activation). These findings indicate that the AR NTD has higher intrinsic activity in C4-2 cells. To gain insight into the AR NTD domains that mediate constitutive AR activity in C4-2 cells, we tested the effects of NTD deletions on ligand-dependent and ligand-independent AR activity in LNCaP and C4-2 cells, respectively. TAU-1 and TAU-5 are the primary transactivation domains that have been identified in the AR NTD (20). Two discrete domains, termed AF-1a and AF-1b, have been identified within the TAU-1 core (18). Indeed, deletion of AF-1a (Δ101/211) or AF-1b (Δ253–361) impaired both ligand-dependent and ligand-independent hARGal4 activity (Fig. 6B). Deletion of the region separating AF-1a and AF-1b (Δ211–253) resulted in a 2-fold increase in both ligand-dependent and ligand-independent AR activity (Fig. 6B). However, this increase in activity was associated with a higher overall level of protein expression as assessed by immunoblotting with anti-Gal4 antibodies (Fig. 6B). This overall increase in hARGal4Δ211/253 expression was a consistent finding and did not result from different DNA content in transfection mixtures, suggesting that this region of the AR NTD could play a role in mediating AR protein stability. Surprisingly, deletion of TAU-5 (Δ361–490), resulted in a 2-fold increase in ligand-dependent hARGal4 activity but a 2-fold decrease in ligand-independent hARGal4 activity (Fig. 6B). From these findings, we conclude that an activated AR NTD, via AF-1a, AF-1b, and TAU-5, mediates ligand- and AF-2 independent, antiandrogen-resistant AR activity in ADI C4-2 cells.

DISCUSSION

This study represents the first mechanistic examination of AR transcriptional activation in AR-dependent PCa cells. Although previous studies in AR-null cells have provided detailed information on the mechanisms of ligand-induced AR activation (14, 15, 18, 19, 22, 23, 25–27, 40, 41), they have not addressed whether these mechanisms are operational in PCa cells, where the AR is a disease-relevant therapeutic target. To address this issue, we designed a novel Gal4-based reporter system, which we demonstrated was completely autonomous of endogenous AR. Importantly, hARGal4 was able to recapitulate the most salient features of endogenous AR activity in a cell-based model of PCa progression: 1) both endogenous AR and hARGal4 displayed constitutive activity in ADI C4-2 cells, but not androgen-dependent LNCaP cells; 2) both endogenous AR and hARGal4 displayed higher -fold induction by androgens in LNCaP cells than C4-2 cells; 3) the overall level of activity achieved by both endogenous AR and hARGal4 in the presence of androgens was higher in C4-2 cells than LNCaP cells, likely due to the cumulative effect of constitutive and ligand-induced activity; and 4) the constitutive activity of both endogenous AR and hARGal4 was resistant to bicalutamide. Based on these findings, we conclude that the mechanistic properties of hARGal4 can be accurately extrapolated to endogenous AR in this cell-based model of PCa progression.

In this study, we provide strong evidence in support of an important functional role for ligand-independent AR activity in ADI C4-2 cells. For example, through quantitation of endogenous PSA expression as well as hARGal4 activity on a PSA based, GAL4-regulated promoter, we have shown that the level of constitutive AR activity in C4-2 cells is roughly 30–50% of the level of AR activity induced by 1 nM mibolerone in LNCaP cells. Our results further demonstrate that this constitutive activity is ligand-independent, and not the result of residual androgens in the media. In both LNCaP and C4-2 cells, we observed that
bicalutamide selectivity inhibits ligand-dependent AR activity and has no effect on ligand-independent AR activity. Importantly, bicalutamide did not block the growth of C4-2 cells in androgen-depleted medium, thus indicating that the growth of these cells is ligand-independent. This finding agrees with the initial characterization of these ADI cells, which demonstrated they do not display a growth response to dihydrotestosterone over a range of 10 pM to 1 nM (32). Nevertheless, direct AR targeting via siRNA induces cell-cycle arrest and apoptosis in this cell line (11). Therefore, although androgens can further enhance AR activity in C4-2 cells, we propose that C4-2 cells achieve a critical level of AR activity through ligand-independent mechanisms, which is sufficient to support growth and survival.

If surgery or radiation does not cure patients with PCa, then androgen ablation therapy is the standard systemic therapy for locally advanced, relapsed, or metastatic disease. Both forms of androgen ablation therapy, chemical/surgical castration and AR antagonism, are targeted to the CTD of the AR protein. For example, castration results in decreased ligand availability, thus preventing the AR LBD from activating the receptor. Antian- drogens such as bicalutamide compete with androgens for the AR LBD (42), resulting in an inactive receptor complex at the promoter regions of AR-responsive genes such as PSA (43). Our data show that C4-2 cells, which were rendered ADI in vivo through serial passage in castrated hosts (32), are resistant to both of these forms of androgen ablation. Mechanistically, we demonstrated that the CTD AF-2 and LBD modules of the AR are functional in C4-2 cells. However, we further demonstrated that these CTD modules are not necessary for constitutive, ligand-independent AR activity in these cells, which explains their cross-resistance to androgen ablation and AR antagonism. Therefore, the mechanisms of ligand-dependent and ligand-independent AR activation are fundamentally distinct in PCa cells. These results suggest that therapies targeted to the AR CTD LBD/AF-2 module may, as a whole, be ineffective long term modes of AR inhibition for PCa due to their shared inability to block ligand-independent AR activity. This possibility has important ramifications, because there is a concerted effort underway to identify or design novel AR antagonists or selective AR modulators, which are dependent on targeting the AR LBD (42, 44–46). The Gal4-based assay system we have developed could serve as a critical tool for evaluating the ability of AR-targeted compounds to inhibit both ligand-dependent and ligand-independent AR activity.

If LBD/AF-2-targeted compounds are likely to be ineffective long term AR inhibitors due to their inability to block ligand-independent AR activity, are there more suitable targets for AR inhibition? hARGal4 deletion studies revealed that constitutive AR NTD activity is inherently stronger in C4-2 than LNCaP...
Mechanisms of Ligand-independent AR Activity

cells. This finding likely explains why the AR can achieve a higher overall level of activity in the presence of ligand in C4-2 cells than can be achieved in LNCaP cells. Based on this finding we suggest a mechanism by which elevated NTD activity in C4-2 cells is able to override the strong negative regulation imparted by the CTD. In LNCaP cells, AR activity is also mediated in large part by the AR NTD but in a manner that is dependent on ligand as well as functional LBD and AF-2 modules. Therefore, a critical finding is that, despite the fundamental differences in the mechanisms of ligand-dependent and ligand-independent AR activation in PCa cells, not only are LNCaP and C4-2 cells AR-dependent (9–12), both cell lines appear to be AR NTD-dependent. For example, we demonstrated that both ligand-dependent and ligand-independent mechanisms of AR activation are reliant upon the NTD AF-1a and AF-1b activation domains, which comprise TAU-1 of the AR (18). Therefore, our data suggest that novel anti-AR strategies based on blocking AR NTD function, for example by inhibiting AF-1a or AF-1b, could serve to effectively inhibit both ligand-dependent and ligand-independent AR activity in both androgen-dependent and ADI PCa cells.

Interestingly, we observed that TAU-5, which has also been described as an important transactivation motif within the AR NTD, was necessary for full ligand-independent hAR(C) activity but inhibited ligand-dependent hAR(C) activity. The strong transactivation function of TAU-5 was first demonstrated following AR transfection studies with glucocorticoid response element-regulated reporters in CV-1, COS-7, and HeLa cells (19, 20). However, similar transfection experiments in 293 cells demonstrated that TAU5 is dispensable for ligand-induced AR transactivation (47). Therefore, TAU5 is likely to mediate context-specific effects on AR transactivation. Indeed, although TAU5 deletion does not affect ligand-induced AR activation in 293 cells, TAU5 deletion does prevent activation of the AR by overexpression of the RhoA effector protein kinase C-related kinase, PRK1, in this cell line (47). Our findings that TAU-5 plays a differential role in the regulation of ligand-dependent and ligand-independent AR activity in PCa cells lends further support for a context-specific role for TAU-5. Further studies are warranted to define the precise role of TAU-5 in AR transcriptional activation in PCa and other cell types.

In summary, we created a Gal4-based system to study AR function in a cell-based model of AR-dependent PCa progression. Our data reveal disease-relevant targets for novel anti-AR strategies in androgen-dependent and ADI PCa. In androgen-dependent LNCaP cells, AR NTD transactivation domains display a strict dependence on ligand, AF-2, and LBD function for activity. In ADI C4-2 cells, AR NTD transactivation domains display constitutive, ligand-independent activity. Our data also demonstrate that the antibiandrogen, bicalutamide, displays selectivity for ligand-dependent, AF-2-dependent AR activity in both androgen-dependent and ADI PCa cells. An important priority will be to identify specific coregulatory molecules in androgen-dependent and ADI PCa cells that interact with transcriptionally active AR NTD domains. This will permit the design of small molecules or peptidomimetics that could directly interfere with the function of these domains and thus enhance the arsenal of AR inhibitors for PCa therapy.
Chem. 274, 25756–25768
30. Debes, J. D., Comuzzi, B., Schmidt, L. J., Dehm, S. M., Culig, Z., and Tindall, D. J. (2005) Cancer Res. 65, 5965–5973
31. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
32. Wu, H. C., Hsieh, J. T., Gleave, M. E., Brown, N. M., Pathak, S., and Chung, L. W. (1994) Int. J. Cancer 57, 406–412
33. Agoulnik, I. U., Vaid, A., Bingman, W. E., 3rd, Erdeke, H., Frolov, A., Smith, C. L., Ayala, G., Ittmann, M. M., and Weigel, N. L. (2005) Cancer Res. 65, 7959–7967
34. Matias, P. M., Donner, P., Coelho, R., Thomaz, M., Peixoto, C., Macedo, S., Otto, N., Joschko, S., Scholz, P., Wegg, A., Basler, S., Schafer, M., Egner, U., and Carrondo, M. A. (2000) J. Biol. Chem. 275, 26164–26171
35. Chen, C. D., Welsbie, D. S., Tran, C., Baek, S. H., Chen, R., Vessella, R., Rosenfeld, M. G., and Sawyers, C. L. (2004) Nat. Med. 10, 33–39
36. Gregory, C. W., He, B., Johnson, R. T., Ford, O. H., Mohler, J. L., French, F. S., and Wilson, E. M. (2001) Cancer Res. 61, 4315–4319
37. Culig, Z., Comuzzi, B., Steiner, H., Bartsch, G., and Hobisch, A. (2004) J. Steroid Biochem. Mol. Biol. 92, 265–271
38. Warnmark, A., Treuter, E., Wright, A. P., and Gustafsson, J. A. (2003) Mol. Endocrinol. 17, 1901–1909
39. He, B., Kemppainen, J. A., Voegel, J. I., Gronemeyer, H., and Wilson, E. M. (1999) J. Biol. Chem. 274, 37219–37225
40. Alen, P., Claessens, F., Verhoeven, G., Rombauts, W., and Peeters, B. (1999) Mol. Cell Biol. 19, 6085–6097
41. He, B., Kemppainen, J. A., and Wilson, E. M. (2000) J. Biol. Chem. 275, 22986–22994
42. Bohl, C. E., Chang, C., Mohler, M. L., Chen, J., Miller, D. D., Swaan, P. W., and Dalton, J. T. (2004) J. Med. Chem. 47, 3765–3776
43. Zhu, P., Baek, S. H., Bourk, E. M., Ohgi, K. A., Garcia-Bassets, I., Sanjo, H., Akira, S., Kotol, P. F., Glass, C. K., Rosenfeld, M. G., and Rose, D. W. (2006) Cell 124, 615–629
44. Gao, W., Kearbey, J. D., Nair, V. A., Chung, K., Parlow, A. F., Miller, D. D., and Dalton, J. T. (2004) Endocrinology 145, 5420–5428
45. Marhefka, C. A., Gao, W., Chung, K., Kim, J., He, Y., Yin, D., Bohl, C., Dalton, J. T., and Miller, D. D. (2004) J. Med. Chem. 47, 993–998
46. Yin, D., Gao, W., Kearbey, J. D., Xu, H., Chung, K., He, Y., Marhefka, C. A., Veverka, K. A., Miller, D. D., and Dalton, J. T. (2003) J. Pharmacol. Exp. Ther. 304, 1334–1340
47. Metzger, E., Muller, J. M., Ferrari, S., Buettner, R., and Schule, R. (2003) EMBO J. 22, 270–280

Mechanisms of Ligand-independent AR Activity