Selective Role of an NH₂-Terminal WxxLF Motif for Aberrant Androgen Receptor Activation in Androgen Depletion–Independent Prostate Cancer Cells

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Abstract

Systemic prostate cancer therapy requires androgen ablation, which inhibits the production or action of androgens. Prostate cancer ultimately relapses during androgen ablation, and an androgen depletion-independent (ADI) phenotype emerges. Aberrant androgen receptor (AR) activation underlies therapy resistance at this stage of the disease, and mounting evidence implicates the large and highly disordered AR NH₂-terminal domain (NTD) as a key mediator of this activity. In this study, we investigated the role of the NTD transactivation unit 5 (TAU5) domain in mediating AR transcriptional activity in cell-based models of prostate cancer progression. AR replacement and Gal4-based promoter tethering experiments revealed that AR TAU5 had a dichotomous function, inhibiting ligand-dependent AR activity in androgen-dependent prostate cancer cells, while enhancing ligand-independent AR activity in ADI prostate cancer cells. Molecular dissection of TAU5 showed that a WxxLF motif was fully responsible for its ligand-independent activity. Mechanistically, WxxLF did not rely on an interaction with the AR ligand-binding domain to mediate ligand-independent AR activity. Rather, WxxLF functioned as an autonomous transactivation domain. These data show that ligand-dependent and ligand-independent AR activation rely on fundamentally distinct mechanisms, and define WxxLF as the major transactivation motif within the AR TAU5 domain.

Introduction

Prostate cancer is the most common cancer affecting North American men (1). Activity of the androgen receptor (AR), a steroid-regulated nuclear transcription factor, is critical for the growth and survival of normal and cancerous prostate tissue (2, 3). Therefore, androgen ablation, a systemic treatment for locally advanced or metastatic prostate cancer, initially results in tumor regression. However, an untreatable manifestation of the disease, termed androgen depletion–independent (ADI) prostate cancer (4), develops after a median of 18 to 33 months (5). Although ADI prostate cancer is resistant to androgen ablation, a wealth of evidence supports the current paradigm that this stage of the disease remains AR dependent through various mechanisms of aberrant AR activation (3, 5, 6). Characterizing these modes of AR activation is of prime importance for developing new prostate cancer therapies.

The AR shares a modular organization with other steroid receptors, having an NH₂-terminal domain (NTD) harboring AR transcriptional activation function (AF)-1, a central DNA binding domain (DBD), and a COOH-terminal ligand-binding domain (LBD), which also harbors the AF-2 coactivator binding surface (7). Data from our laboratory and others have shown that the AR displays ligand-independent activity in a cell-based model of prostate cancer progression (8–10). Previously, we have shown that ligand-independent AR activity is resistant to antiandrogens such as bicalutamide due to a mechanism of activation that is independent of the AR LBD/AF-2 module (8). These observations support the hypothesis that a decoy peptide representing the entire AR NTD can inhibit AR activity and prostate cancer tumor growth, and thereby delay emergence of an ADI phenotype in a xenograft-based model of prostate cancer progression (11).

The AR NTD is highly flexible and displays intrinsic disorder in solution, which has hampered elucidation of its three-dimensional structure (7, 12–14). Moreover, specific NTD domains, through which coregulatory proteins may bind and regulate ligand-dependent or ligand-independent transcriptional activation, have not been fully described. Deletion analysis of ectopic AR in AR-null cell lines has shown that AF-1 in the AR NTD consists of two large domains, termed transactivation unit (TAU) 1 and TAU5, which participate in transcriptional activation (15). The core domain mediating TAU1 activity has been mapped to a discrete 178 KLDI motif, and its importance has been confirmed in various cell models (8, 15–17). However, the role or precise identity of AR TAU5 has not been established. The purpose of this study was to elucidate the role of TAU5 in mediating ligand-dependent and ligand-independent AR activation in cell-based models of prostate cancer progression. We show that TAU5 plays fundamentally different roles in androgen-dependent and ADI prostate cancer cells. In addition, we define the core sequence 435WHTLF439 as a novel AR transactivation motif that mediates TAU5 activity. Blocking the function of AR TAU5, via targeting 435WHTLF439, may represent a novel strategy to selectively inhibit aberrant AR activity in ADI prostate cancer cells.

Materials and Methods

Cell lines and culture conditions. LNCaP and ADI 22Rv1 cell lines were purchased from the American Type Culture Collection. The C4-2 cell line was purchased from UroCor. LNCaP, C4-2, and 22Rv1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. For androgen response experiments, cells were grown in serum-free medium for 48 h. Growth medium was replaced with serum-free medium containing 1 nmol/L mibolerone (Biomol), 10 µmol/L bicalutamide (Casodex, AstraZeneca), 10 µmol/L mibolerone (Biomol), and 10 µmol/L bicalutamide (Casodex, AstraZeneca),...
ethanol (vehicle control), or combinations of these compounds. Cells were cultured for an additional 24 h and harvested. For 22Rv1 small interfering RNA (siRNA) transfection experiments, culture medium was replaced for 24 h postransfection with serum-free, phenol red-free RPMI. After 48 h of growth, cells were harvested.

**Plasmid constructs.** Mouse mammary tumor virus (MMTV)-Luc was provided by Dr. Frank Claessens (Catholic University of Leuven, Leuven, Belgium). The pSHHAR-A plasmid was provided by Dr. Frank French (University of North Carolina, Chapel Hill, NC). The backbone for the enhanced green fluorescent protein (EGFP)-expressing AR replacement plasmid, pC5M5-Hip-EGFP, was provided by Dr. Dan Billadeau (Mayo Clinic, Rochester, MN). The SV40-Renilla luciferase and PG5-LUC reporter vectors were purchased from Promega and Clontech, respectively. PSAenh(ARE)-LUC, PSAenh(GAL4)-LUC, hARGal4, hARGal4ΔTAU5, and NTDGal4 have been described (8, 18). Plasmid construction details are included as Supplementary Materials and Methods.

**Transient transfections.** For siRNA transfections, 3 × 10^5 22Rv1 cells were mixed with 80 pmol of AR-targeted siRNA (AR siRNA sense: 5'-CAAGGGAGGUACACAAAUAU; ARsiRNA2 sense: 5'-GAAAUGAUUGCAUAAUGAUUU) or a nontargeted control siRNA (Dharmacon). The cell/siRNA mixture was transferred to electroporation cuvettes with a 4-mm gap-width (BTX) and subjected to a 350 V electrical pulse for 10 ms using a BTX ElectroSquare Electroporator. Following a 15-min recovery, cells were seeded in RPMI 1640 + 5% charcoal-stripped, steroid-depleted serum (CSS). For siRNA transfections coupled with rescue by hARR, 1 × 10^5 cells were seeded in 24-well dishes and transfected the following day with 375 ng of MMTV-LUC, 125 ng SV40-Renilla, 10 pmol ARsiRNA1 (or control siRNA), and 11.75 ng ARR, in complex with 2 µL of LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Following 6 h of transfection, the medium was aspirated and replaced with the appropriate medium containing 5% CSS. In addition to the LipofectAMINE 2000–based protocol, electroporation was also used as a mode of transfection for siRNA rescue experiments with hARR. For this approach, amounts of plasmids were increased by a factor of 24 from the LipofectAMINE 2000–based protocol. Therefore, for a standard electroporation, 3 × 10^5 cells from exponentially growing cultures were suspended in 350 µL RPMI + 5% CSS and mixed with 50 µL of a DNA/siRNA mixture containing 9 µg MMTV-LUC, 3 ng SV40-Renilla, 80 pmol ARsiRNA1 (or control siRNA), and 282 ng of hARR. The cell/DNA mixture was subjected to a 305 V (LNCaP, C4-2) or 350 V (22Rv1) pulse for 10 ms. Following a 15-min recovery, cells were seeded in RPMI 1640 + 5% CSS.

hARGal4ΔTAU5-based tethering assays were done in LNCaP and C4-2 cells as described (8) with minor modification. Briefly, 1 × 10^5 cells were seeded in 24-well dishes and transfected the following day using 2.5 µL of Superfect (Qigene) mixed with 375 ng of either PSAenh(ARE)-LUC or PSAenh(GAL4)-LUC as a reporter construct, 125 ng of SV40-Renilla, and 23.4 ng of hARGal4ΔTAU5. Transfections were done in the presence of 5% RPMI + 5% CSS. In addition to the Superfect-based protocol, electroporation was also used as a mode of transfection for hARGal4ΔTAU5-based tethering assays. For this approach, amounts of plasmids were increased by a factor of 24 from the Superfect-based protocol. Therefore, for a standard electroporation, 3 × 10^5 cells from exponentially growing cultures were suspended in 350 µL RPMI + 5% CSS and mixed with 50 µL of a DNA mixture containing 9 µg of a promoter-LUC reporter, 3 µg of SV40-Renilla, and 562 ng of hARGal4ΔTAU5. The cell/DNA mixture was electroporated at 305 V for 10 ms. Following a 15-min recovery, cells were seeded in RPMI 1640 + 5% CSS.

Overall transfection efficiency was optimized for each experiment by transfecting cells with GFP and assessing the number of fluorescent cells 24 h postransfection. Transfection efficiencies consistently ranged from 60% to 80%. For all reporter-based experiments, 24 h postransfection, medium was aspirated and replaced with phenol red–free RPMI medium containing 1 mmol/L mibohemone, 2 µmol/L to 1 mmol/L dihydrotestosterone (Sigma Aldrich), or ethanol (vehicle control). Cells were harvested after an additional 24 h and processed in a lysis buffer provided with a Dual Luciferase Assay Kit (Promega). Activities of the firefly and Renilla luciferase reporters were assayed in 96-well plates with a Dual Luciferase Assay Kit and detected with a Molecular Devices LMax luminometer. Transfection efficiency was addressed by dividing firefly luciferase activity by Renilla luciferase activity of samples cultured in the absence of androgens. Data presented represent the mean ± SE from at least three independent experiments, each done in duplicate.

**Cell sorting.** C4-2 cells were electroporated with AR replacement vectors expressing combinations of EGFP, AR-targeted short hairpin RNA (shRNA), and siRNA-resistant wild-type, ΔTAU5, or AHTAA mutant AR. EGFP-positive cells were collected using a FACS Vantage SE (Becton Dickinson).

**RNA extraction, Northern blot, and quantitative reverse transcription-PCR analysis.** Total cellular RNA was isolated via acid-guanidinium phenol/chloroform extraction as described (19). For Northern blots, equal amounts of RNA (15 µg per lane) were fractionated on denaturing formaldehyde-agarose gels. RNA was transferred to Hybond nylon membranes (Amersham), UV cross-linked, and hybridized with cDNA probes specific for prostate-specific antigen (PSA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) labeled with [α-32P]dCTP using a RadPrime labeling kit (Invitrogen). Autoradiography was done at −80°C using an intensifier screen (Kodak). Quantitative real-time PCR was done exactly as described (20) using primers specific for PSA (5'-AGGGCTTCCCTGTACACCCA and 5'-GTCTTGCCCTGTCATTCC), and GAPDH (Applied Biosystems). Relative quantitation was used to determine fold change in expression levels by the comparative Ct method using the formula 2ΔΔCt, where Ct is the threshold cycle of amplification.

**Chromatin immunoprecipitation.** Cells were electroporated with sPSAΔALL-Gal4 along with empty vector or wild-type, ΔTAU5, or AHTAA mutant versions of hARGal4 and cultured in RPMI medium containing 5% CSS for 48 h. Cells were switched to serum-free, phenol red-free RPMI medium for an additional 24 h. Formaldehyde was added to a final concentration of 1%, and cells were incubated for 10 min. Cells were harvested, lysed, and immunoprecipitations were done using a chromatin immunoprecipitation (ChIP) kit (Upstate Biotechnology) and antibodies specific for Gal4 (Santa Cruz Biotechnology), or nonspecific IgG. Immunoprecipitated DNA fragments were detected via PCR using forward and reverse primers specific for sPSAΔALL-Gal4 (forward 5'-AGTGGCAGGTGCCAgACAgATT; reverse: 5'-TTGTGAAAGCAGCATCCGTGC).

**Western blot analysis.** Cells were harvested directly in a loading buffer containing 65 mmol/L Tris-HCl (pH 7.0), 2% (w/v) SDS, 5% β-mercaptoethanol, 10% (v/v) glycerol, and 0.5% (w/v) bromophenol blue. Protein concentrations were determined using a kit based on a modified Lowry assay (Bio-Rad DC/BC assay). Equal amounts (typically 30 µg per lane) of protein were resolved in 10% SDS-polyacrylamide gels (Invitrogen), followed by transfer to nitrocellulose and membrane blocking. Blots were incubated with antibodies specific for AR (Santa Cruz Biotechnology, C-19 or N-20), extracellular signal-regulated kinase-2 (ERK-2; Santa Cruz Biotechnology, D-2), or Gal4 (Santa Cruz Biotechnology, RKS1C) at a final concentration of 100 ng/mL, washed, and probed with the appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) diluted 1:10,000. Membranes were immersed in chemiluminescence reagents (Pierce) and exposed to Kodak XAR film for signal detection.

**Results**

**Differential requirement of TAU5 for ligand-dependent and ligand-independent AR activity.** Our laboratory has been studying the LNCaP/C4-2 model of prostate cancer progression to study the mechanisms of AR transcriptional regulation in androgen-dependent and ADI prostate cancer cells (8, 21). The ADI C4-2 cell line was derived from the androgen-dependent LNCaP cell line through serial xenografting in castrated hosts (22). A key feature of this model is that LNCaP cells display ligand-dependent mRNA expression of the AR-regulated PSA4 gene, whereas C4-2 cells display constitutive PSA mRNA expression that can be further enhanced by androgens (Fig. 1A). Experiments using siRNA-mediated AR knockdown and ChIP have shown that constitutive PSA expression is due to constitutive, ligand-independent AR transcriptional activity on the PSA promoter in C4-2 cells (9, 10).
We recently described a promoter tethering system to study the structural and functional domains required by the AR to mediate this ligand-independent activity (8). This strategy involves swapping androgen response elements (ARE) in a PSA-based reporter construct [PSAenh(ARE)-LUC] with binding sites for the yeast Gal4 transcription factor [PSAenh(GAL4)-LUC] and concurrently swapping the zinc-finger AR DBD with the zinc-finger DBD of Gal4 to create chimeric hAR Gal4 (Fig. 1B, left). We have shown that these constructs serve as a sensitive monitoring system of AR activity in AR-expressing prostate cancer cells, which functions independent of the endogenous AR. Using this system, we showed that constitutive AR activity is independent of the AR AF-2 domain in C4-2 cells (8). Due to these observations, we have focused our attention on transcriptional activation domains within the unstructured AR NTD that may be responsible for mediating ligand-independent AR activity in ADI prostate cancer cells. A region of the AR NTD, encompassing amino acids 361 to 490, was previously termed TAU5 by virtue of its importance for full ligand-induced activity of ectopic AR in AR-null HeLa cells (15). To assess the role of TAU5 in mediating AR activity, we tethered wild-type and TAU5-deleted (ΔTAU5) versions of hARGal4 to PSAenh(GAL4)-LUC. GAL4 substitution of AREs in the PSA-based reporter
construct completely abolished androgen-induced promoter activity in both LNCaP and C4-2 cells (Fig. 1B). Importantly, GAL4 substitution of AREs also inhibited androgen-independent PSA promoter activity 4-fold, only in C4-2 cells (Fig. 1B; Supplementary Fig. S1). In LNCaP cells, hARGal4ΔTAU5 displayed over 2-fold higher androgen-induced transcriptional activity than wild-type hARGal4 (Fig. 1B). Conversely, ΔTAU5 hARGal4 displayed over 2-fold lower androgen-independent transcriptional activity when

Figure 2. TAU5 is required for ligand-hypersensitive AR activity in ADI prostate cancer cells. A, LNCaP and C4-2 cells were transfected with wild-type and ΔTAU5 versions of ARGal4 in conjunction with PSA enh (GAL4)-LUC. Cells were treated under serum-free conditions with 2 pmol/L to 0.2 nmol/L dihydrotestosterone (DHT) for 24 h. Luciferase activity was determined. Points, mean from at least three independent experiments, each done in duplicate; bars, SE. The activity of wild-type hARGal4 in response to 0.2 nmol/L dihydrotestosterone was arbitrarily set to 100%. B, endogenous AR expression was knocked down in LNCaP and C4-2 cells via AR-targeted siRNA. Cells were cotransfected with constructs encoding wild-type and ΔTAU5 versions of siRNA-resistant (AR sr) along with MMTV-LUC as indicated. Cells were treated and luciferase activity was determined as described for A. C, LNCaP and C4-2 cells were transfected with PSA enh (GAL4)-LUC along with wild-type, CTD-deleted (NTDsrGal4), or CTD/TAU5-deleted (NTDsrGal4ΔTAU5) versions of ARGal4 as indicated and treated with 1 nmol/L mibolerone or vehicle control (ethanol) for 24 h. Luciferase activity was determined as described for A. 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. Lysates were analyzed by Western blot for AR protein expression. The discrete protein species recognized by the NTD-targeted AR antibody are indicated.
compared with wild-type hAR\textsuperscript{Gal4} in C4-2 cells (Fig. 1B). Despite this observation, deletion of TAU5 had no effect on the full level of ligand-induced hARGal4 activity in C4-2 cells (Fig. 1B). These results show that TAU5 deletion selectively impairs ligand-independent hARGal4 transcriptional activity in ADI C4-2 cells.

To ensure that these findings did not represent an artifact of chimeric hARGal4, and were not restricted to a PSA-based reporter construct, we used an AR replacement strategy. To this end, we generated wild-type and ΔTAU5 forms of wild-type hAR that were resistant to an AR-targeted siRNA (hARsr) and tested their abilities to stimulate a MMTV promoter in LNCaP and C4-2 cells (Fig. 1C). AR-targeted siRNA completely attenuated androgen responsiveness of the MMTV promoter in LNCaP cells, but had no effect on ligand-independent MMTV promoter activity (Fig. 1C). Consistent with this finding, expression of hARsr restored androgen-dependent MMTV activity in LNCaP cells (Fig. 1C). Conversely, AR-targeted siRNA inhibited ligand-independent MMTV promoter activity by over 60% in C4-2 cells, which was restored upon expression of hARsr (Fig. 1C; Supplementary Fig. S2).

To examine whether TAU5 plays a role in mediating aberrant AR activity in other, non–LNCaP-based models of ADI prostate cancer, we used the ADI 22Rv1 prostate cancer cell line, which was derived from a CWR22 prostate cancer xenograft that relapsed after host castration-induced regression (23). In contrast to the T877A mutant AR expressed in LNCaP-derived cells, 22Rv1 cells harbor an AR with an in-frame duplication of exon 3, resulting in an extra second zinc finger in the AR DBD (24). Similar to C4-2 cells, we
determined that 22Rv1 cells displayed constitutively high PSA mRNA expression that resulted from ligand- and AF-2–independent AR activity (Supplementary Fig. S3). Indeed, AR-targeted siRNA inhibited androgen-independent MMTV activity in 22Rv1 cells (Fig. 1D). Moreover, similar to C4-2 cells, hAR ΔTAU5 deletion impaired androgen-independent MMTV activity 50% (Fig. 1D). Together, our findings show that TAU5 plays a selective role in mediating ligand-independent AR activity in ADI prostate cancer cells.

**TAU5 is required for AR ligand hypersensitivity in ADI prostate cancer cells.** Our data indicate that TAU5 could play a previously unrecognized role in mediating ligand-independent AR activation in ADI prostate cancer cells. However, although a complete androgen-depleted environment can be achieved in vitro, such a situation may not arise in vivo. For example, data from a rapid autopsy study showed that, despite androgen ablation, intraprostatic androgen levels in prostate cancer patients persist at levels sufficient to weakly transactivate the AR (25). Therefore, we explored the role of TAU5 in the AR transcriptional response to castrate levels of its natural ligand, dihydrotestosterone. In LNCaP cells, the transcriptional activity of hAR Gal4 ΔTAU5 was higher than hAR Gal4 at dihydrotestosterone concentrations higher than

![Figure 4.](image)

**Figure 4.** The AR TAU5 domain and 435WHTLF439 motif are required for ligand-independent transcriptional activation of the endogenous PSA gene in C4-2 cells. A, schematic of the plasmid used for AR replacement experiments. Key features of this plasmid include EGFP expression regulated by the SV40 promoter, AR shRNA expression regulated by the histone H1 promoter, and hAR sr expression regulated by the cytomegalovirus (CMV) promoter. B, C4-2 cells were transfected with versions of the AR replacement plasmid containing the indicated features. Cells were maintained under serum-free conditions for 48 h, and a sample was lysed for analysis of AR expression by Western blot. ERK-2 protein expression is shown as a control. EGFP-positive cells were collected by fluorescence-activated cell sorting, reseeded, and cultured in androgen-free medium for an additional 48 h. Cells were harvested and total RNA was isolated. RNA was subjected to two-step quantitative reverse transcription-PCR using primers specific for PSA and GAPDH. Results were analyzed using the \\( \Delta \Delta C_T \) method. Columns, mean from three experiments; bars, SE.
Figure 5. The AR 435WHTLF439 motif mediates ligand-independent TAUS transcriptional activity. A, C4-2 cells were transfected with wild-type, \( \Delta \)TAUS, and AHTAA mutant versions of hAR\(_{G4}^{TAUS}\), as well as \( \Delta \)TAUS versions of hAR\(_{G4}^{TAUS}\) with 21-amino-acid inserts encompassing wild-type or AHTAA mutant versions of the 435WHTLF439 domain as indicated. PSAenh(GAL4)-LUC was used as a reporter for these experiments. Cells were maintained 48 h in the absence of androgens. Luciferase activity was determined. Columns, mean from at least three independent experiments, each done in duplicate; bars, SE. 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. Lysates were analyzed by Western blot for hAR\(_{G4}^{TAUS}\) protein expression with Gal4-specific antibodies. B, C4-2 cells were transfected with wild-type, \( \Delta \)TAUS, or AHTAA versions of hAR\(_{G4}^{TAUS}\) along with PSAenh(GAL4)-LUC. Cells were grown under androgen-free conditions, cross-linked, lysed, and subjected to ChIP with antibodies specific for Gal4 or nonspecific IgG. Immunoprecipitated DNA fragments were amplified by PCR using primers specific for the PSAenh(GAL4)-LUC reporter as indicated in the schematic on the right. C, C4-2 cells were transfected with wild-type or \( \Delta \)TAUS versions of hAR\(_{G4}^{TAUS}\), or \( \Delta \)TAUS versions of hAR\(_{G4}^{TAUS}\) harboring one, two, or three copies of a 21-amino-acid insert encompassing the AR WHTLF motif. PSAenh(GAL4)-LUC was used as a reporter for these experiments. Assays were done as described for A, D, LNCaP cells were transfected with hAR\(_{G4}^{TAUS}\)-based constructs as described for A and B. Cells were treated under serum-free conditions with 1 nmol/L mibolerone or ethanol for 24 h (*P < 0.05).
0.1 nmol/L (Fig. 2A). Conversely, in C4-2 cells, hAR\textsuperscript{Gal4 ΔTAU5} transcriptional activity was ~50% lower than wild-type hAR\textsuperscript{Gal4} transcriptional activity over all dihydrotestosterone concentrations studied (Fig. 2A). When these experiments were done using an AR replacement strategy and MMTV-LUC as a reporter, a similar relationship was observed (Fig. 2B). In LNCaP cells, ΔTAU5 hAR\textsuperscript{sr} activity was higher than wild-type hAR\textsuperscript{sr} at dihydrotestosterone concentrations higher than 0.1 nmol/L (Fig. 2B). However, in C4-2 cells, hAR\textsuperscript{sr} displayed higher activity than hAR\textsuperscript{sr} ΔTAU5 at dihydrotestosterone concentrations lower than 10 pmol/L (Fig. 2B). At concentrations higher than 0.1 nmol/L dihydrotestosterone, TAU5 activity was not apparent in C4-2 cells (Fig. 2B). Together, these results suggest that TAU5 plays an important role in mediating AR activation, only under conditions of no/low androgens in ADI prostate cancer cells.

A consistent observation was that treatment of C4-2 cells with 1 nmol/L androgens abolished TAU5 activity in C4-2 cells (Figs. 2B; Supplementary Fig. S2), suggesting that full AR activity...
does not require TAU5. Previous studies have shown that deletion of the AR COOH-terminal domain (CTD) results in full AR activation through relieved inhibition of the AR NTD (8, 15–17). Therefore, we assessed the effect of TAU5 deletion in the context of a truncated version of hAR^Gal4 (NTD^Gal4; Fig. 2C). In both LNCaP and C4-2 cells, NTD^Gal4 was constitutively active and did not respond to androgens (Fig. 2C). Deletion of TAU5 further increased NTD^Gal4 activity in both cell lines, confirming that full AR activation does not require TAU5 activity (Fig. 2C). Together, these data show that TAU5 is inactive or inhibitory when the AR is fully activated.

NTD 435WHTLF439 is an important TAU5 motif that selectively mediates ligand-independent AR activity. Our results suggest the existence of a transactivation domain within AR TAU5 that could account for ~50% of the ligand-independent and/or ligand-hypersensitive AR activity in ADI prostate cancer cells. We hypothesized that patches of rigid secondary structure within this highly disordered domain could serve as important protein interaction sites. The neural network nnpredict secondary structure prediction algorithm (26) was therefore applied to the amino acid sequence representing AR TAU5. Two potential sites of extended secondary structure were identified (Fig. 3A). To test whether either of these regions of putative secondary structure was important for ligand-independent AR activity in C4-2 cells, each was independently deleted from hARGal4. Deletion of amino acids 420 to 449, but not amino acids 393 to 420, inhibited ligand-independent hARGal4 transcriptional activity to a similar degree as deletion of the entire TAU5 domain (Fig. 3B). Within this 420 to 449 region, a span of predicted secondary structure encompassed 435WHTLF439 (Fig. 3A), which was originally described as a short helical motif that can mediate androgen-dependent interaction with AF-2, the COOH-terminal AR coactivator binding surface (27–29). X-ray crystallography studies have shown that peptides containing a core WxxLF sequence adopt a helical conformation (29), which, as shown by a helical wheel (Fig. 3C, inset), would result in a hydrophobic surface created by the nonpolar side chains of W435, L438, and F439. To test the potential role of these hydrophobic residues within AR 435WHTLF439 in regulating ligand-independent AR transcriptional activity in ADI C4-2 cells, the hAR^Gal4-based tethering system was used (Fig. 3C). When the nonpolar W435, L438, and F439 residues were individually substituted with alanine, no effect on androgen-independent hAR^Gal4 transcriptional activity was observed (Fig. 3C). However, a W435A/L438A/F439A compound mutation, which eliminated all bulky hydrophobic side chains within 435WHTLF439, elicited an approximate 40% drop in androgen-independent hAR^Gal4 transcriptional activity (Fig. 3C). These results suggest that AR 435WHTLF439 could mediate selective TAU5 activity in ADI prostate cancer cells. To further test this possibility, we assessed the ligand-dependent and ligand-independent activities of wild-type and AHTAA mutant hAR⁴ in AR replacement experiments. Whereas hAR⁴ AHTAA exhibited reduced androgen-independent activity in C4-2 cells, there was no effect on ligand-dependent activity in LNCaP cells (Fig. 3D).

To test whether deletion of the TAU5 domain or mutation of the WHTLF motif also impaired ligand-independent AR activity in the context of chromatin, we transfected C4-2 cells with an AR replacement vector encoding EGFP, AR-targeted shRNA, and wild-type, deletion, or mutant versions of hAR⁴ (Fig. 4A). This approach led to effective replacement of the endogenous AR with ectopic versions of hAR⁴ in transfected C4-2 cells (Fig. 4B). Importantly, in EGFP-positive cells, ΔTAU5 and AHTAA mutant versions of hAR⁴ both displayed a 40% to 50% reduction in ligand-independent transcriptional activation of the endogenous PSA gene compared with wild-type hAR⁴ (Fig. 4C), thus confirming reporter-based findings.

To directly assess the relationship between TAU5 and 435WHTLF439, the transcriptional activities of ΔTAU5 and AHTAA versions of hAR^Gal4 were compared via promoter tethering assay in C4-2 cells (Fig. 5A). As shown previously, both TAU5 deletion and AHTAA mutation impaired hAR^Gal4 ligand-independent transcriptional activity ~50% (Fig. 5A). Importantly, a 21-amino-acid peptide containing the core 435WHTLF439, but not AHTAA, rescued full ligand-independent transcriptional activity of hAR^Gal4ΔTAU5 in C4-2 cells (Fig. 5A). ChiP analysis confirmed that wild-type, ΔTAU5, and AHTAA versions of AR^Gal4 effectively engaged with their cognate GAL4 DNA binding sites in these assays (Fig. 5B). These results show that the AR 435WHTLF439 motif plays a direct role in mediating TAU5 activity in ADI C4-2 cells.

The AR 435WHTLF439 motif functions as a transcriptional activation domain. Our results suggest that AR WHTLF could represent a novel NH2-terminal AR transactivation domain. Alternatively, aberrant AR activation could be facilitated by stabilization of the AR in the absence of ligand via an aberrant N/C interaction between 435WHTLF439 and AF-2. Indeed, structural and functional studies have shown that ligand-bound AR can be stabilized by an interaction between the AR AF-2 domain and 23FQNLE27 or 435WHTLF439 motifs in the AR NTD (27, 29–33). To differentiate between these scenarios, we added one, two, or three 435WHTLF439-containing peptides to hAR Gal4 ΔTAU5. Stepwise increases in ligand-independent hARGal4 transcriptional activity were observed in C4-2 cells with each 435WHTLF439-containing peptide inserted (Fig. 5C). When we did similar experiments in LNCaP cells, we were surprised to observe that insertion of two or three 435WHTLF439-containing peptides in hAR^Gal4 ΔTAU5 resulted in ligand-dependent superactivation of the AR (Fig. 5D).

Although these results were consistent with a transactivation role for 435WHTLF439, they did not rule out the possibility that multiple copies of 435WHTLF439 were mediating more efficient interactions with AF-2, resulting in stronger N/C stabilization and subsequent higher levels of AR activity. Therefore, we next abolished AF-2 function by incorporating three separate mutations in V716, K720, and E897, which are AR residues that comprise the conserved nuclear receptor AF-2 charge-clamp and mediate binding with AR 435WHTLF439 (28, 29). Mutations in these charge-clamp residues impair ligand-dependent AR transcriptional activity in LNCaP as well as other cell lines (8, 28, 30, 32, 34). However, in C4-2 cells, mutations in these charge-clamp residues did not disrupt the ligand-independent activity of wild-type hAR^Gal4, nor hAR^Gal4 ΔTAU5 with a 21-amino-acid insert harboring the AR 435WHTLF439 motif (Fig. 6A). These data argue against a role for 435WHTLF439 in mediating an aberrant N/C interaction in ADI prostate cancer cells, and strongly suggest that 435WHTLF439 represents a novel AR transactivation domain.

Finally, to directly assess a role for the AR 435WHTLF439 motif in transcriptional activation, one, two, or three copies of an AR-derived peptide with a core 435WHTLF439 sequence were tethered to the Ga4 DBD and tested for their ability to stimulate transcription from a GAL4-regulated luciferase reporter. In both LNCaP and C4-2 cells, a Ga4 DBD containing two 435WHTLF439 peptides mediated transcriptional activation (Fig. 6B). Of particular note, three tandem copies of 435WHTLF439 peptides activated...
transcription of the GAL4-driven promoter 600-fold in both cell lines. We thus conclude that the AR 435WHTLF439 motif is a novel transcriptional activation domain. Although this motif has the potential to stimulate transcription in both LNCaP and C4-2 cells (Figs. 5C and 6B), our data indicate that in the context of full-length AR, 435WHTLF439 selectively mediates transcriptional activation under no/low androgen conditions in ADI prostate cancer cells.

Discussion

The structures of the LBD and AF-2 folds of the AR have been determined by X-ray crystallography studies of peptides containing the AR CTD (29, 30, 35–37). However, the structure of the AR NTD is unknown, and the precise identity of functional domains within this region of the AR is currently limited. The AR NTD possesses potent transactivation potential, which is generally referred to by the all-encompassing term AF-1 (14). Conversely, AR AF-2 in isolation is a relatively weak transcriptional activation domain (32, 34, 38). Therefore, AR AF-1 is thought to be the major domain responsible for mediating AR transcriptional activity. The primary role for AF-2 may be to regulate this dominant AF-1 activity, in part by mediating a direct intramolecular interaction with FxxLF and/or WxxLF motifs in the NTD (27, 28, 31, 33, 39). TAU1 and TAU5 were identified originally as transactivation units within the AR NTD after functional deletion analysis of wild-type and CTD-deleted AR in AR-null COS-1 and HeLa cells (15). Subsequent studies defined the core sequence 178LKDIL182, resident within a discrete NTD helix, as the motif that actively mediates AR TAU1 transcriptional activity (16, 17). The importance of TAU5 has not been firmly established, due to conflicting reports describing TAU5 as either necessary (15–17) or dispensable (40, 41) for full AR transcriptional activity. Notably, the majority of these conflicting studies have been done in AR-null cell lines of nonhuman origin.

This study represents the first examination of TAU5 function in AR-dependent prostate cancer cells, where the AR is a disease-relevant therapeutic target. Using different promoters as reporters for a Gal4-based promoter tethering strategy as well as an AR replacement strategy, we showed a striking dichotomy of TAU5 function; TAU5 deletion enhanced ligand-induced AR transcriptional activity in androgen-dependent prostate cancer cells, but inhibited ligand-independent AR transcriptional activity in ADI prostate cancer cells. We further showed that TAU5 deletion inhibited AR transcriptional activation over a range of castrate prostate cancer cells. Indeed, short peptides containing 435WHTLF439 can mediate an N/C interaction with AF-2 (27–29), a dual-function role exists for this domain of the AR. Interestingly, a similar dual-function role has been shown for the NTD AR 22FQNLFL27 motif. For example, in addition to being the primary motif that binds AF-2 (29, 33, 37), AR 22FQNLFL27 is also able to bind directly to the X chromosome–linked melanoma antigen gene product MAGE-11, an androgen-dependent AR coactivator (42). Interestingly, the AR coactivator GRIP1/TIF-2/SRC-2 has been shown via yeast two-hybrid assay to bind the AR TAU5 domain, which may serve to bridge an interaction between the NLA and COOH termini of the AR (43, 44). However, our preliminary study has shown that siRNA-mediated knock down of GRIP1 has no effect on ligand-independent AR activity in C4-2 cells, which argues against a role for GRIP1 as a WHTLF-binding protein (data not shown).

In a previous study, we showed that the activity of hARGal4 in C4-2 cells was truly ligand independent, as mutations that abolished LBD or AF-2 function did not significantly affect basal transcriptional activity stimulated by saturating levels of androgens (3- to 4-fold versus >100-fold). However, comprehensive ChIP analysis has shown that the AR does transiently localize to the PSA locus at low levels in the absence of androgens in C4-2 versus LNCaP cells (10). This transient ligand-independent localization triggers major locus-wide histone acetylation and methylation, which significantly amplifies the overall transcriptional output of the PSA gene (10). It has also been proposed that a positive feedback loop exists between the AR and its target loci under ligand-free conditions in ADI prostate cancer cells, whereby AR-dependent alterations in the chromatin state facilitate subsequent AR binding and transcriptional activation (10). These mechanisms are likely to underlie AR ligand hypersensitivity as well (10), a property that we have shown to also require TAU5 (Fig. 2). Although our reporter-based studies do not take into account effects at the chromatin level (i.e., the “amplification”), they do provide a sensitive measure of AR transcriptional activity (i.e., the “trigger”). This is confirmed by our observation that ∆TAU5 and AHTAA mutant versions of the AR were impaired in their ability to mediate ligand-independent transcriptional activation of the endogenous PSA gene in C4-2 cells compared with wild-type AR (Fig. 4). Our current data thus add substantially to previous studies using this cell-based system of prostate cancer progression, and shows that aberrant 435WHTLF439 activity could be critical for AR-mediated triggering of AR target genes in ADI prostate cancer cells.

An important question arising from these studies is how ligand-independent 435WHTLF439 transcriptional activity is prevented in LNCaP cells. Indeed, short peptides containing 435WHTLF439 displayed a similar level of autonomous transcriptional activity in LNCaP and C4-2 cells (Fig. 5B). The finding that TAU5 deletion resulted in nearly 2-fold higher ligand-dependent AR activity in LNCaP cells indicates that negative regulatory element(s) exist within this domain, and may be dominant over the transactivating activity of the 435WHTLF439 motif (Fig. 1B and C). We are currently exploring the role and identity of this putative domain in regulating AR activity in prostate cancer cells. Our results also highlight the possibility of negative regulatory domain(s) existing outside of TAU5, because in the absence of TAU5, a single 435WHTLF439-containing peptide was not able to significantly elevate ligand-dependent AR transcriptional activity in a promoter tethering assay in LNCaP cells (Fig. 4C). However, multiple copies of
containing peptides inserted into TA5-deleted AR resulted in ligand-dependent superactivation in LNCaP cells (Fig. 4C). These data indicate that multiple negative regulatory elements, both inside and outside of TA5, function to prevent aberrant WHTLF activity in the absence of ligand. Therefore, we propose that the overall mechanism of AR activation in ADI prostate cancer is dependent on the adaptable and unordered nature of the NTD (13, 14), and a complex series of derepression and activation events are necessary for the AR to achieve a critical threshold of transcriptional activity in ADI prostate cancer cells. Overall, our data show that the AR TA5 domain in general, and the WHTLF motif in particular, play an important role in this mechanism, accounting for ~50% of aberrant AR activity observed in ADI prostate cancer cells. Thus, WHTLF could represent a novel interface through which aberrant AR activity could be targeted for therapy of ADI prostate cancer.

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References


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