Hormone Status Selects for Spontaneous Somatic Androgen Receptor Variants That Demonstrate Specific Ligand and Cofactor Dependent Activities in Autochthonous Prostate Cancer*

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We have used the autochthonous transgenic adenocarcinoma of mouse prostate (TRAMP) model to investigate the relationship between somatic mutation in the androgen receptor (AR) and the emergence of androgen-independent prostate cancer. Here we report the identification, isolation, and characterization of distinct classes of AR variants from spontaneous prostate tumors in the TRAMP model. Using cDNA cloning, single stranded conformation polymorphism and sequencing strategies, 15 unique somatic mutations in the AR were identified in prostate tumors obtained from eight TRAMP mice between 24 and 29 weeks of age. At least one mutation was isolated from each mouse. All mutations were single base substitutions, 10 were missense and 5 were silent. Nine mutations in the AR were identified in tumors of four mice that were castrated at 12 weeks of age. Interestingly, the majority of mutations (seven out of nine, 78%) identified in the androgen-independent tumors colocalized in the AR transactivation domain. The remaining mutations colocalized in the AR ligand binding domain. In general, the AR variants demonstrated promotor-, cell-, and cofactor-specific activities in response to various hormones. All AR variants isolated in this study maintained strong sensitivity for androgens, and four AR variants isolated from castrated mice demonstrated increased activities in the absence of ligand. The K638M and F677S variants demonstrated increased activities in response to androgen, and K638M also demonstrated increased response to estradiol. In the presence of AR coactivator ARA70 the E231G variant demonstrated increased activity in response to both androgen and estradiol. However, in the presence of AR coactivator ARA160 the E231G variant was selectively responsive to androgen. Collectively these analyses not only indicate that somatic mutations in the AR gene occur spontaneously in TRAMP tumors but also how changes in the hormonal environment may drive the selection of spontaneous somatic mutations that provide a growth advantage.

Androgens are essential for the development and maintenance of the prostate and hormonal therapy. Androgen deprivation or blockade of androgens at the level of the androgen receptor (AR) remains the treatment choice for advanced prostate cancer (1). Although this therapy usually results in a favorable clinical response, a dramatic drop in serum prostate specific antigen level and tumor regression, most cases will eventually relapse with clinically defined androgen-independent disease (2). Understanding the molecular mechanisms governing progression of prostate cancer to androgen independence is critical to the development of new treatment modalities.

Androgens signal via the intracellular AR, a member of the superfamily of nuclear hormone receptors (3). Androgen binding to the AR transforms the receptor to an active conformation and initiates translocation to the nucleus, followed by binding to specific response elements in the promoter regions of target genes to modulate gene expression positively or negatively. Although it was initially thought that loss of AR expression was a mechanism of therapy failure, recent studies have demonstrated that despite hormone ablation therapy, the AR is expressed at substantive levels in a majority of hormone-refractory prostate cancers (4, 5). Subsequently, several hypotheses have been proposed to explain how changes in the androgen signaling axis may mediate the growth of hormone-refractory disease, including AR gene amplification (6, 7), ligand-independent activation of AR via cross-talk with growth factor receptor pathways (8, 9), and AR gene mutations (10).

Over the past several years a number of distinct AR gene mutations have been identified in clinical prostate cancer specimens (11–15).Possibly the most characterized of the AR variants are those identified in the prostate cancer cell lines LN-CaP (16) and MDA PCa 2b (17, 18). Remarkably, both the T877A variant in LNCaP cells and the L701H/T877A double variant in MDA PCa 2b cells are promiscuous receptors in that they are activated by steroid hormones other than androgens. Hence, these mutations have the potential to confer a selective growth advantage to cancer cells after hormone ablation therapy. This strongly supports the hypothesis that mutations in the AR gene which deregulate the androgen signaling axis can directly contribute to the development of hormone-refractory disease.

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Although AR gene mutations have been identified in human prostate cancer, it has been difficult to perform a comprehensive analysis of their incidence and nature because of the paucity of clinical samples representing the earliest form of the disease and the genetic and pathologic heterogeneity of the disease. We have, therefore, used the autochthonous transgenic adenocarcinoma of mouse prostate (TRAMP) model (19-21) to support further investigation of the relationship between mutations in AR and prostate cancer. The TRAMP model (22) was generated previously using minimal probasin (PB) → 426/+28 regulatory sequence to direct SV40 early gene (T and t antigen); Tag) expression to prostatic epithelium in a developmentally and hormonally regulated fashion. By 10–12 weeks of age, male TRAMP mice generally develop prostatic intraepithelial neoplasia and/or well-differentiated prostate cancer. All TRAMP males ultimately will develop prostate adenocarcinoma that metastasizes to distant sites, primarily the lymph nodes and lungs. This generally occurs by 24–30 weeks of age (21). After androgen ablation at 12 weeks of age, 70–80% TRAMP males will ultimately develop androgen-independent disease. These mice typically develop poorly differentiated adenocarcinoma and exhibit twice the incidence of metastasis as intact littermate (20). The parallel development of clinical prostatic cancer and tumors in TRAMP mice, the comparable emergence of hormone-refractory disease after androgen ablation, and the highly conserved nature of the AR suggested that the TRAMP model could be used to investigate the relationship between mutations in the AR gene and prostate cancer. Here we report the identification and characterization of distinct classes of AR variants isolated as spontaneous somatic mutations during prostate cancer progression in TRAMP. We demonstrate that hormonal status influences the nature of the mutations in AR and that these AR variants display unique functional properties. This study establishes in the TRAMP model a link between deregulation of the androgen signaling axis and prostate cancer progression.

**EXPERIMENTAL PROCEDURES**

**Transgenic Animals**—TRAMP mice, heterozygous for the PB-T antigen gene, were maintained in a pure C57BL/6 background. Female TRAMP mice were bred to nontransgenic male FVB mice (Harlan Sprague-Dawley) to obtain transgenic and nontransgenic (C57BL/6 × FVB) F1 males for this study. Isolations of DNA from mouse tails and polymerase chain reaction (PCR)-based screening assays to identify transgenic mice were performed as described previously (21). All TRAMP mice were randomly assigned to two cohorts. One cohort was anesthetized and castrated through a scrotal approach at 12 weeks of age. All mice were sacrificed between 24 and 29 weeks of age.

**Single Stranded Conformation Polymorphism (SSCP) Analysis**—Total RNA was isolated from tumor tissues by the cesium chloride method as described previously (23). Reverse transcription-PCR was performed with modifications of the procedure as previously described (24). Briefly, 1 μg of RNA was reverse transcribed for 1 h at 37°C using 300 ng of oligo(dt)12–18 primers (Amersham Pharmacia Biotec) and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in a 20-μl reaction containing 1 × first strand buffer (Life Technologies, Inc.), a 500 μM concentration of each of the four deoxyribonucleotide triphosphates (Life Technologies, Inc.), 1 mM dithiothreitol, 1.5 μCi of [α-<sup>32</sup>P]dCTP (3,000 Ci/mmol, ICN Biochemicals, Irvine, CA), 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1% Triton-100, 200 μM appropriate sense and antisense primer sets, and 0.5 unit of Vent DNA polymerase. After an initial denaturing step of 2 min at 94°C, the cycle parameters were as follows: 1 min at 94°C, 2 min at 65°C, and 3 min at 72°C. After amplification, the radiolabeled PCR products were diluted 1:10 in loading buffer that contained 95% formamide (v/v), 50 mM EDTA, 20 mM NaOH, and 0.05% each of xylene cyanol and bromphenol blue. Samples were denatured at 100°C for 10 min, quick frozen on dry ice, thawed slowly on wet ice, and fractionated on a nondenaturing 6% polyacrylamide gel (40:1 acrylamide:biacylamide) containing 10% glycerol in 0.5 × Tris borate-EDTA buffer at 4°C. The gels were transferred to filter paper and exposed to x-ray film (Kodak X-Omat AR5) at ~80°C. Point mutations were identified by a shift in the relative mobility of the PCR fragments compared with wild type AR controls. Testis RNA from each animal was used to control for germ line mutations.

**Sequence Analysis**—Recombinal plasmids harboring putative mutations identified by SSCP analysis were sequenced. Sequencing of AR mutations was performed by comparing the sequence of the HA tag, a consensus Kozak translation initiation sequence (see Fig. 2B). This was accomplished by inserting the mouse full-length AR cDNA and HA tag into CMV expression vector pcDNA3.1 (Invitrogen Corporation, Carlsbad, CA). To engineer the HA epitope to the amino terminus of the AR protein, the full-length mouse AR cDNA (kindly provided by Dr. Don Tindall, Mayo Clinic, Rochester, MN) was subcloned into the BamHI site of pcDNA3.1. The HA tag sequence was added to the amino terminus of the full-length AR cDNA by PCR using the primer 5′-CGGTATTACACCATGGATATATGATGCGGATTATGCCGAGGTGCAGTTAGGGCTGGA-3′ and the primer mAR-B (see Fig. 1A). The PCR product was then digested with the restriction enzyme for the sequence of subcloned AR cDNA with that of cDNA encoding 5′B57BL/6 mouse AR (GenBank X53779) (26) using the GCG Sequence Analysis Software Package provided through the Baylor College of Medicine Biocomputational Resource (Houston, TX).

**Expression Vectors**—The plasmid pcDNA3/HA-mAR was constructed to express full-length wild type mouse AR with an amino-terminal hemagglutinin antigen (HA; Santa Cruz Biotechnology, Santa Cruz, CA) tag sequence and a consensus Kozak translation initiation sequence (see Fig. 2B). This was accomplished by inserting the mouse full-length AR cDNA and HA tag into CMV expression vector pcDNA3.1 (Invitrogen Corporation, Carlsbad, CA). To engineer the HA epitope to the amino terminus of the AR protein, the full-length mouse AR cDNA (kindly provided by Dr. Don Tindall, Mayo Clinic, Rochester, MN) was subcloned into the BamHI site of pcDNA3.1. The HA tag sequence was added to the amino terminus of the full-length AR cDNA by PCR using the primer 5′-CGGTATTACACCATGGATATATGATGCGGATTATGCCGAGGTGCAGTTAGGGCTGGA-3′ and the primer mAR-B (see Fig. 1A). The PCR product was then digested with the restriction enzyme for the sequence of subcloned AR cDNA with that of cDNA encoding 5′B57BL/6 mouse AR (GenBank X53779) (26) using the GCG Sequence Analysis Software Package provided through the Baylor College of Medicine Biocomputational Resource (Houston, TX).

**Androgen Receptor Variants and Prostate Cancer**

Although AR gene mutations have been identified in human prostate cancer, it has been difficult to perform a comprehensive analysis of their incidence and nature because of the paucity of clinical samples representing the earliest form of the disease and the genetic and pathologic heterogeneity of the disease. We have, therefore, used the autochthonous transgenic adenocarcinoma of mouse prostate (TRAMP) model (19-21) to support further investigation of the relationship between mutations in AR and prostate cancer. The TRAMP model (22) was generated previously using minimal probasin (PB) → 426/+28 regulatory sequence to direct SV40 early gene (T and t antigen; Tag) expression to prostatic epithelium in a developmentally and hormonally regulated fashion. By 10–12 weeks of age, male TRAMP mice generally develop prostatic intraepithelial neoplasia and/or well-differentiated prostate cancer. All TRAMP males ultimately will develop prostate adenocarcinoma that metastasizes to distant sites, primarily the lymph nodes and lungs. This generally occurs by 24–30 weeks of age (21). After androgen ablation at 12 weeks of age, 70–80% TRAMP males will ultimately develop androgen-independent disease. These mice typically develop poorly differentiated adenocarcinoma and exhibit twice the incidence of metastasis as intact littermate (20). The parallel development of clinical prostatic cancer and tumors in TRAMP mice, the comparable emergence of hormone-refractory disease after androgen ablation, and the highly conserved nature of the AR suggested that the TRAMP model could be used to investigate the relationship between mutations in the AR gene and prostate cancer. Here we report the identification and characterization of distinct classes of AR variants isolated as spontaneous somatic mutations during prostate cancer progression in TRAMP. We demonstrate that hormonal status influences the nature of the mutations in AR and that these AR variants display unique functional properties. This study establishes in the TRAMP model a link between deregulation of the androgen signaling axis and prostate cancer progression.
appropiate plasmid DNA using Superfect reagent (Qiagen, Inc, Valencia, CA) according to the manufacturer's instructions. After 24 h, the cells were washed and fed with phenol-free Dulbecco's modified Eagle's medium containing 10% dextran-coated charcoal-stripped fetal bovine serum (Hyclone) containing hormones as required. 24 h later cells were washed twice with phosphate-buffered saline and lysed in 150 µl of lysis buffer containing 0.2 M Tris-HCl (pH 8.0), 0.1% Triton X-100. The luciferase and β-galactosidase activities were assayed using 20-µl aliquots of the extracts. The luciferase activity was corrected for transfection efficiency by normalization against β-galactosidase activity. The values shown represent the means of at least three independent experiments. Data processing was performed using the Microsoft Excel program, and statistical analyses were performed using two-way analysis of variance followed by post hoc comparisons with Fisher's protected least significant difference test.

Western Immunoblots—Cultured cells were lysed in 40 mM Tris-HCl (pH 7.0), 1 mM EDTA, 4% glycerol, 10 mM dithiothreitol, 2% SDS, and protease inhibitors. Protein concentrations were determined by the Bio-Rad protein assay. Proteins were resolved by electrophoresis through a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The membranes were probed with a mouse monoclonal antibody specific for HA and a goat anti-mouse IgG horseradish peroxidase conjugate. AR protein was displayed by a reaction with Supersignal Chemiluminescent Substrate using the ECL kit (Amersham Pharmacia Biotech).

Homology Modeling—A model of the human holo-AR LBD was constructed based on the crystal structure coordinates of the human progesterone receptor LBD in a complex with progesterone (30), as described elsewhere (31). The structure of the retinoid X receptor LBD (32) was used as the starting point for modeling the apo-AR LBD. Initially a threading based model was constructed in SPDV 3.5 (33) and a structural alignment generated. Portions of the receptor which aligned well with the retinoid X receptor were subjected to further minimization as for the holo-AR. Testosterone and the glucocorticoid receptor-interacting protein-1 (GRIP1) peptide were docked into the AR model by superimposing the AR LBD model onto crystal structures of the progesterone receptor LBD-progesterone (30) and estrogen receptor LBD-estrogen-GRIP1 peptide (34) complexes. An energy-minimized structure for testosterone was constructed using ISIS/Draw and Sculpt 3 (35) and aligned with progesterone by a Maximal Common Substructure Search (36). Electrostatic and van der Waals interactions of testosterone and the GRIP1 peptide with the AR LBD were minimized locally using Monte Carlo minimization (37); Van der Waals interactions were modeled using a modified Lennard-Jones function between atoms within 6 Å of each other, and electrostatic interactions were modeled with a distance-dependent dielectric between atoms within 10 Å.

Hormones—The synthetic androgen R1881 was purchased from NEN Life Science Products. 17β-Estradiol, progesterone, and dexamethasone were purchased from Sigma.

RESULTS

Spontaneous Mutation in AR in Primary Prostate Tumors—To investigate the possibility that somatic mutation in the AR occurred during the natural history of prostate cancer in the TRAMP model, full-length AR cDNA was first amplified from tumor tissue by reverse transcription-PCR using three overlapping primer sets and the error-free Vent DNA polymerase. The resulting AR cDNA fragments were then subcloned and analyzed by SSCP to detect mutations (38). Putative mutations in the AR cDNA fragments identified by the appearance of bands of altered mobility on the SSCP gels (Fig. 1B) were confirmed by DNA sequence analysis. In total, 15 somatic mutations in the AR were identified in prostate tumors isolated from eight individual TRAMP mice that ranged in age from 24 to 29 weeks. Of these, 9 mutations were identified in the tumors from castrated mice and 6 in tumors from intact mice. All mutations were single base substitutions. The majority of the mutations (10 out of 15; 67%) were missense mutations, and the remaining 5 were silent (Table 1). No frameshifts or deletions were observed, and no mutations in the AR DNA binding domain were identified. No mutations were detected in parallel reactions using RNA isolated from testis.

In this study, we chose to analyze 10 unique and independent clones for each AR cDNA fragment by SSCP. At least one variant AR was identified in each tumor examined. Hence it is reasonable to state that at least 10% of cells in each tumor likely harbored transcripts encoding a variant AR. Interestingly, nine of the AR variants were isolated from tumors of TRAMP mice that were castrated at 12 weeks of age, and the majority (seven out of nine; 78%) colocalized in the AR transactivation domain. In contrast, all six AR variants isolated from tumors of four intact TRAMP mice that were castrated at 12 weeks of age, and the majority (seven out of nine; 78%) colocalized in the AR transactivation domain. In contrast, all six AR variants isolated from tumors of four intact TRAMP mice localized to the AR LBD (Fig. 2A). These observations demonstrate that mutation of the AR is a spontaneous event in the natural history of prostate cancer in the TRAMP model. Furthermore, although the incidence of mutations in any given tumor is ~10%, the nature of the mutations appears to be a function of the hormonal status of the animal.

Transcriptional Activity of the AR Variants in PC3 Cells—To determine the consequence of mutation in the AR gene, the transcriptional activities of the AR variants identified in the TRAMP tumors were characterized. To this end, an expression vector pcDNA3/HA-mAR was first constructed to carry a full-length wild type mouse AR cDNA modified with a consensus Kozak translation sequence and the HA epitope tag at the amino terminus (Fig. 2B). The missense mutations identified...
from TRAMP tumors and the T857A variant corresponding to the human AR in LNCaP cells were subsequently introduced into the pcDNA3/HA-mAR using in vitro site-directed mutagenesis. The identity and integrity of each construct were confirmed by DNA sequencing (data not shown). Western blot analysis was used to confirm that each construct encoded a full-length HA-tagged AR. No significant variability in the level of expression was observed for these constructs (Fig. 2C).

The ability of the AR variants to transactivate AR-responsive reporter gene constructs was first evaluated in PC3 cells, an AR-deficient human prostate cancer cell line. In these experiments, two androgen-regulated reporter constructs, PB-LUC and ARR3-LUC, were employed. PB-LUC carries the naturally occurring androgen-regulated rat complex probasin promoter sequence spanning +2286 to +228 fused to the luciferase reporter. This sequence represents the native chromosomal sequence immediately upstream of the rat probasin structural gene and has been shown to impart both tissue and hormone specificity (39). ARR3-tk-luciferase (ARR3-LUC) carries three repeats of the rat probasin AR response element-1 and -2 region (+244/-96) ligated in tandem upstream of the minimal thymidine kinase (tk) enhancer element fused to the luciferase reporter. 

**FIG. 2.** Hormonal status influences the nature of somatic AR mutations in primary prostate cancer. Panel A, shown is a schematic structure of the mouse AR indicating the location of the mutations identified from TRAMP mice. The majority of mutations (seven out of nine; 78%) detected in tumors obtained from the mice castrated at 12 weeks of age were colocalized in the AR transactivation domain, and the majority of mutations (six out of eight; 75%) in the LBD clustered in the exon 4 encoded region. Light boxes, intact mice; boldface boxes, castrated mice. Panel B, a full-length cDNA encoding the wild type C57BL/6 mouse AR (mAR) was fused in-frame to the HA sequence carrying a consensus Kozak translation initiation signal and inserted into the KpnI-BamHI sites of pcDNA3.1 vector. The integrity of the construct was confirmed by automated DNA sequence analysis. MCS, multiple cloning site. Panel C, recombinant wild type (wt) and mutated androgen receptor were transfected into PC3 cells. Cell extracts were prepared and fractionated by SDS-polyacylamide gel electrophoresis, transferred to nylon membranes, and probed with anti-HA antibody. PC3 cells transfected with pcDNA3.1 vector alone were used as a control.

**TABLE I**

Mutations identified in the AR gene in TRAMP tumors

| Mouse | Age | Status  | Mutation | Consequence | Comment |
|-------|-----|---------|----------|-------------|---------|
| 8108  | 24  | Castrated | GCC → ACC | A229T | Missense |
|       |     |          | GAG → GGG | E231G | Missense |
|       |     |          | GCG → GCA | A394A | Silent |
| 8106  | 24  | Castrated | GAG → AAG | E206K | Missense |
| 8772  | 24  | Castrated | CCA → CCG | P140P | Silent |
|       |     |          | AAC → AGC | N384S | Missense |
|       |     |          | AAG → ATG | K638M | Missense |
| 8748  | 24  | Castrated | GTT → GCT | V487A | Missense |
|       |     |          | TTT → TTC | F653F | Silent |
| 5216  | 24  | Intact   | CGC → CAC | R754H | Missense |
|       |     |          | GAG → GGG | E877G | Missense |
| 5939  | 24  | Intact   | TTT → TCT | F677S | Missense |
| 1417  | 29  | Intact   | TTT → ATT | F653I | Missense |
| 5940  | 24  | Intact   | GAT → GAC | D644D | Silent |
The semisynthetic ARR3-LUC has been demonstrated previously to be more sensitive to AR signals than PB-LUC. As shown in Fig. 3, the basal transcriptional activities of the E206K, A229T, E231G, N384S, and T857A variants were about 1.5–2-fold over that observed for the wild type on the complex reporter PB-LUC \((p < 0.001)\) (Fig. 3A). Note that, except for T857A, all of these variants were isolated from tumors of castrated mice. Surprisingly, when the semisynthetic ARR3-LUC reporter was employed, only the T857A variant demonstrated an elevated basal transcriptional activity (Fig. 3F).

The ligand specificity of each AR variant was next examined by transactivation analysis. Remarkably, the wild type AR and all AR variants except for T857A exhibited a similar induction by 1 nM R1881 when assayed with PB-LUC (Fig. 3B). In contrast, the K638M and F677S variants showed 1.5–2-fold better response than wild type AR \((p < 0.001)\) in the presence of 1 nM R1881 (Fig. 3G) when assayed on ARR3-LUC, whereas T857A demonstrated a significantly lower response than wild type AR \((p < 0.001)\) on either PB-LUC or ARR3-LUC (Fig. 3B and G). No induction of any murine AR variant was detected in response to stimulation with 10 nM dexamethasone using either reporter constructs (Fig. 3C and H). As predicted from a previous study (11), the T857A variant demonstrated a 2–3-fold greater induction than wild type AR \((p < 0.001)\) in response to 10 nM progesterone with PB-LUC and ARR3-LUC (Fig. 3E and J). In response to 10 nM 17b-estradiol, all of the AR variants except F677S induced expression of both PB-LUC.

**Fig. 3.** Ligand specificity and transcriptional activities of the AR variants in PC3 cells. PC3 cells were transfected with a DNA mixture containing 0.5 \(\mu\)g of the mutant or the wild type AR (wtAR), 1.0 \(\mu\)g of PB-LUC or ARR3-LUC, and 0.5 \(\mu\)g of CMV-\(\beta\)-galactosidase plasmid as an internal reference. 24 h after transfection, the medium was changed to contain 10% charcoal-stripped serum with or without the hormones tested as indicated. The LUC activity was normalized against the \(\beta\)-galactosidase activity and is presented as the fold increase over the basal level. The values are the means ± S.D. of at least three independent experiments.
and ARR3-LUC, and the K638M and T857A variants induced ARR3-LUC constructs to a significantly greater extent than the wild type (p < 0.001). It was interesting to note that the F677S variant did not respond to 10 nM 17β-estradiol on either PB-LUC or ARR3-LUC (Fig. 3, D and I), suggesting that this mutation likely defines a region of the AR required for estrogen stimulation. These data clearly demonstrate that the AR variants display specific and unique deviation from the activity of the wild type receptor.

Transcription Activity of the AR Variants in TRAMP Cells—Three TRAMP cell lines, C1A, C1D, and C2G, were used to address the possibility that cellular context could influence AR function. These cell lines were all derived from a single primary TRAMP tumor. Whereas the C1A cells are not tumorigenic but not metastatic, the C2G cells are both tumorigenic and metastatic. Although endogenous AR can be detected in these cells by Western blotting, the level of AR is insufficient to drive expression of transfected PB-LUC or ARR3-LUC reporter genes (data not shown).

In these experiments, the activity of three AR variants, E231G, K638M, and T857A, were compared with exogenous wild type receptor. As shown in Fig. 4, consistent with the study in PC3 cells and a previous report (11), the T857A variant was found to activate ARR3-LUC by different hormones regardless of cell context (Fig. 4, A–C). In contrast, the E231G variant exhibited 3-fold higher induction than wild type AR (p < 0.001) in C1D cells in the presence of 1 nM R1881 (Fig. 4C) but lower induction than wild type AR in both C1A and C2G cells (Fig. 4, A and B). In response to R1881, the K638M variant exhibited a 2-fold increase in activity compared with wild type AR (p < 0.001) in C1A cells but lower activity than wild type AR in C1D cells (Fig. 4, A and C). No difference in activity was observed between wild type AR, E231G and K638M variants in response to stimulation by progesterone and 17β-estradiol on ARR3-LUC in the three TRAMP cell lines (Fig. 4, A–C).

Based on these data it was clear that cell context could influence the function of the AR variants. In all three cell lines, both E231G and K638M variants exhibited induction similar to that of wild type AR in the presence of 1 nM R1881 when ARR3-LUC was replaced by PB-LUC. However, T857A exhibited a 2–5-fold increased induction compared with wild type in response to 1 nM R1881, 10 nM progesterone, and 10 nM 17β-estradiol in C1A cells but not in C2G and C1D cells (Fig. 4, D–F). Therefore, cellular context was found to influence the function of the well characterized LNCaP T857A variant. Collectively, the E231G, K638M, and T857A variants exhibited multiple activity profiles in the three different TRAMP cell lines using the two different but related promoter systems. These observations suggest that these cell lines may express different complements of coactivators or corepressors that subsequently and profoundly influence the transcriptional activities of the AR variants.

Functional Interaction of the AR Variants with Known AR Cofactors—To investigate the consequence of mutation on the interaction between AR with known cofactors, we performed an additional series of transactivation experiments in PC3 cells. In these studies, the abilities of the E231G, K638M, and T857A variants and wild type AR to transactivate PB-LUC in presence of ARA70 and ARA160 were examined. Both cofactors have previously been shown to interact physically with wild type AR and functionally enhance transactivation activity of the AR in the presence of certain ligands (40). As shown in Fig. 5, ARA70 enhanced transcriptional activity of wild type AR and AR variants to 6–12-fold over base line in the presence of 1 nM R1881. Remarkably, the E231G variant exhibited 2-fold greater activity than wild type AR (p < 0.001) in the presence of ARA70 (Fig. 5A). ARA70 has also been reported to be the mediator of AR response to estrogen stimulation (41), and our transactivation data confirmed this observation. Upon transfection of wild type AR or AR variants in the absence of ARA70, only marginal induction was detected in the presence of 10 nM 17β-estradiol. The addition of ARA70, however, further enhanced the transcriptional activity of wild type AR and AR variants to 5–20-fold (Fig. 5B). It was interesting to note that the addition of ARA70 resulted in a higher induction on the E231G variant compared with wild type (p < 0.001). In contrast, K638M and T857A variants exhibited 4- and 2-fold less induction than wild type AR (p < 0.001) in the presence of ARA70 and 10 nM 17β-

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estradiol, respectively (Fig. 5B). When ARA70 was replaced by ARA160, the enhancement of AR activity was minimal except as observed for the E231G variant in the presence 10 nM R1881 (Fig. 6A). ARA160 did not enhance the activity of either wild type or the AR variants in the presence of 10 nM 17β-estradiol (Fig. 6B). Surprisingly, the E231G variant, isolated from a tumor of a castrated mouse, exhibited 3-fold more activity in response to androgen in the presence of ARA160 and 2-fold more activity in response to either androgen or estrogen in the presence of ARA70. Hence, the E231G variant was able to distinguish R1881 from 17β-estradiol (Fig. 3, B and C). For the most part, this can be attributed to variability in the disposition of these ligands in the pocket were observed (Fig. 7, D and E). These differences suggest that the effect of the phenylalanine residue in controlling ligand escape will depend on the nature of the bound ligand. The effect of the F677S mutation on ligand binding kinetics of these and other ligands will be determined in subsequent studies. Docking of the nuclear receptor box LXXLL peptide of the p160 coactivator GRIP1 to the AR in the homology model showed that neither the T857A nor F677S mutation is likely to influence the ability of the AR to interact with this class of coactivators. However, as shown by the coactivator analysis for several mutations (Figs. 5 and 6), the precise effect of these mutants may only be apparent in the presence of the appropriate cofactor. Unfortunately, as the crystal structure of the amino-terminal transactivation domain has not been solved for the AR, or indeed any of the nuclear receptors, predictive modeling was only possible for mutations occurring in the LBD.

**DISCUSSION**

Naturally occurring somatic mutations have been identified in human AR isolated from prostate cancer specimens (42). However, there is considerable debate in the literature concerning the incidence and frequency of mutation in the AR gene (43) For the most part, this can be attributed to variability in
analytic methodology and the heterogeneity of the clinical population and their clinical history. Generally, these studies have focused on the LBD of the AR. However, we (44) have examined all eight exons of the AR gene in 25 primary prostate cancer patients and discovered mutations in 13 (52%) of the tumors. Surprisingly, one-half (50%) of the mutations were located in exon 1. This not only demonstrates that the transactivation domain of the AR is a target for somatic mutation, but also provides a mechanism to explain the low frequencies of mutation observed in other reports. In this study we report identification of 15 somatic mutations in the AR gene from primary prostate tumors of TRAMP mice between 24 and 29 weeks of age. Of these, 7 mutations (47%) were also colocalized in exon 1. Clearly, these data confirm that exon 1 encoding the transactivation domain of the AR is indeed a target for somatic mutations in both human and murine primary prostate cancer.

**Fig. 7. Homology modeling of the AR LBD.** Shown are MOLSCRIPT/Raster3D ribbon diagrams of the AR LBD with the ligand binding pocket cavity represented in grid format, one grid point representing 1.4 Å and a similar probe size. Panel A, structure of the wild type (wt) AR LBD with testosterone docked in the ligand binding pocket. The side chains of amino acids residues 857 and 677 are shown in stick form. Panel B, structure of the LBD and ligand binding pocket of the testosterone bound T857A (LNCaP) AR variant. The side chains of amino acids residues 857 and 677 are shown in stick form. Panel C, comparison of the ligand binding pocket and associated structures of the apo-AR LBD (red) superimposed on the holo-AR LBD (blue) bound to testosterone (colored according to Corey-Pauling-Koltun). The phenylalanine residue at position 677 is depicted in stick form. Panels D and E, disposition of the synthetic androgen R1881 and testosterone, respectively, in the ligand binding pocket of the wild type AR LBD. mut., mutant.
There is evidence that the incidence of mutation in AR may be higher in the advanced stages of prostate cancer (45), supporting the clonal selection hypothesis that cells harboring distinct mutations in the AR would have a growth advantage under certain selective pressures. Taplin and co-workers (46) were able to detect mutations in the AR in 5 of 16 (31%) patients who were treated by androgen ablation and the anti-androgen flutamide. All 5 mutations in the AR were in exon 877, and these AR variants were strongly activated by flutamide but demonstrated sensitivity to the anti-androgen bicalutamide. In contrast, only one mutation in exon 890 of the AR was isolated from 17 patients (6%) treated with androgen ablation alone, and this AR variant was not stimulated by flutamide. These data not only demonstrate that mutations in the AR are detected following flutamide treatment, but also suggest that the selective pressure of flutamide treatment may determine the nature of mutation in the AR. In our studies, the majority (7 out of 9, 78%) of the AR variants isolated from castrated TRAMP mice colocalized in the AR transactivation domain (i.e. exon 1), whereas all of the mutations from the intact TRAMP mice were colocalized in the AR LBD. This indicates that the hormonal status influences the nature of the somatic mutations in AR in primary autochthonous prostate tumors. Furthermore, these data suggest that some mutations in the AR detected in tumors from castrated TRAMP mice might have originated in cells at a time when they were still androgen dependent. In this study, all mutations in the AR exon 1 were isolated from androgen-independent tumors, suggesting that although the patients did not receive androgen ablation in the clinical study (44), it is possible that the cells harboring mutations in exon 1 were already androgen-independent. It is also possible that castration by orchectomy may apply different selective pressures from castration by anti-androgens such as flutamide, bicalutamide, and luteinizing hormone-releasing hormone agonist treatment. These data underscore the need to undertake a comprehensive molecular study of the consequences of alternative hormonal therapies to understand better how mutations in the AR mechanistically contribute to the emergence of androgen-independent disease.

Alteration of the ligand specificity or affinity is one possible mechanism through which the AR variants may functionally contribute to the emergence of androgen-independent prostate cancer. In fact, most AR variants isolated from clinical prostate cancer demonstrated broadened ligand specificity that can significantly increase transcriptional capacity in response to estrogen, progesterone, or even paradoxically anti-androgens (11, 16). In this study we have confirmed that the LNCaP T857A variant could be activated by estrogen and progesterone, and we demonstrated that this activity was independent of the promoter context. We also found that the K638M variant isolated from an androgen-independent tumor demonstrated greater response to both androgen and estradiol stimulation compared with the wild type AR. In contrast to T857A and K638M variants, the F677S variant lost the response to estrogen but increased the response to androgen. The model of the holo-AR LBD clearly demonstrates that these mutations result in AR molecules with significant conformational changes in the LBD which likely function in ligand dependent manner and provides a mechanism to explain the altered response of the AR variants to androgenic and nonandrogenic ligands.

Ligand binding specificity or affinity does not alone reflect the functional capability of the AR. Recently, the “ligand-receptor cofactors” tripartite system has been proposed to explain the molecular interactions of steroid receptors (47, 48). It is now generally accepted that interactions between ligand-receptor complexes and cofactors are essential for AR function, selectivity, and sensitivity. In this study we demonstrated that the LNCaP T857A variant exhibited cell-specific activities (Fig. 5). We also demonstrated that ARA70 could synergistically enhance the AR transcription activity more than 10-fold in the presence of either R1881 or 10 nM 17β-estradiol; and the K231G, K638M, and T857A variants demonstrated differential activities in a ligand-dependent fashion in the presence of ARA70. Whereas ARA160 could only slightly increase the AR transcriptional activity in the presence of R1881, this was not observed in the presence of 17β-estradiol. Remarkably, the E231G variant exhibited increased activity in response to androgen but not to estrogen in the presence of ARA160. It was important to note that the E231G variant could discriminate between androgen and estrogen in the presence of cofactor. This suggests that selective cofactors, such as ARA160, can determine ligand specificity of AR variants.

As evident from our observations, the specificity and biological diversity of the steroid hormone receptor family can be generated at the level of cofactors. In addition, these observations suggest that structural or functional changes of AR co-regulators could also modulate the androgen signaling axis and may contribute to the progression of prostate cancer. In fact, amplification and/or overexpression of steroid hormone receptor cofactors, such as AIB1 (49) and ASC-2 (50), have been reported recently in breast and other human cancers. Hence, concomitant changes in coactivators or corepressors in addition to AR are also likely to be important molecular events in prostate cancer progression. It will therefore be necessary to develop suitable autochthonous models that carry specific complements of cofactors and AR variants. Such models can then be used to assess comprehensively the contribution of a specific AR variant to the initiation, progression, and metastasis of prostate cancer under various hormonal pressures to a better understanding the causal relationships between AR variants and prostate cancer.

Some AR mutations were identified to be silent in this study. These mutations, like the missense mutations, were located in either the AR transactivation domain or the LBD. Silent mutations have been reported in clinical prostate cancer at high frequency (44). It will be interesting in later studies to examine the functional consequence of these silent mutations and address the possibility that these mutations could influence AR mRNA stability or translational regulation.

The TRAMP model is the first transgenic prostate cancer model to display somatic mutations in AR. Interestingly, many of these mutations map to the regions known to be a “hot spot,” such as exon 4, in strong correlation with data for the human AR gene. This indicates that the TRAMP model is uniquely suited to elucidate how deregulation of the androgen signaling axis mechanistically contributes to the progression of prostate cancer. We anticipate that ongoing studies with the TRAMP model will elucidate the molecular nature whereby the AR can facilitate prostate cancer progression.

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Hormone Status Selects for Spontaneous Somatic Androgen Receptor Variants That Demonstrate Specific Ligand and Cofactor Dependent Activities in Autochthonous Prostate Cancer

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