

# Androgen Receptor Stabilization in Recurrent Prostate Cancer Is Associated with Hypersensitivity to Low Androgen<sup>1</sup>

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## ABSTRACT

The androgen receptor (AR) is highly expressed in androgen-dependent and recurrent prostate cancer (CaP) suggesting it has a role in the growth and progression of CaP. Previously proposed mechanisms for AR reactivation in recurrent CaP include altered growth factor signaling leading to protein phosphorylation and AR mutations that broaden ligand specificity. To further establish a role for AR in recurrent CaP, we compared several properties of AR in relation to the growth response to low levels of androgens in model systems of androgen-dependent and recurrent CaP. AR from all of the tumors and cell lines bound [<sup>3</sup>H]R1881 with similar high affinity (mean  $K_d$ , 0.12 nM). In the absence of androgen, AR in androgen-dependent LNCaP cells was unstable with a degradation half-time ( $t_{1/2}$ ) of 3 h at 37°C. In contrast, AR was 2–4 times more stable in recurrent CWR22 tumors ( $t_{1/2}$ , >12 h) and CWR-R1 or LNCaP-C4-2 cell lines ( $t_{1/2}$ , 6–7 h) derived from recurrent prostate tumors. In the recurrent CWR22 tumor and its CWR-R1 cell line grown in the absence of androgen, AR immunostaining was entirely nuclear, whereas under the same conditions AR in LNCaP-C4-2 and LNCaP cells was predominantly nuclear but was also detected in the cytoplasm. High level expression, increased stability, and nuclear localization of AR in recurrent tumor cells were associated with an increased sensitivity to the growth-promoting effects of dihydrotestosterone in the femtomolar range. The concentration of dihydrotestosterone required for growth stimulation in CWR-R1 and LNCaP-C4-2 cells was four orders of magnitude lower than that required for androgen-dependent LNCaP cells. The results suggest that AR is transcriptionally active in recurrent CaP and can increase cell proliferation at the low circulating levels of androgen reported in castrated men.

## INTRODUCTION

CaP<sup>3</sup> is initially dependent on androgen for growth. Once advanced, CaP is palliated by lowering circulating testosterone levels with medical or surgical castration. However, CaP cell growth recurs despite reduced androgen in essentially all of the cases of advanced disease. Previous studies (1) have shown that the AR in recurrent CaP is expressed at levels similar to androgen-dependent CaP, suggesting that the AR might contribute to recurrent cell growth. Thus, AR-mediated gene activation might stimulate CaP cell proliferation in the presence of low circulating androgen levels (2).

AR is a member of the superfamily of steroid/nuclear receptors that act as transcription factors. Androgen binding stabilizes AR against rapid degradation that occurs in the absence of androgen (3). In transient transfection studies, androgen binding causes AR nuclear localization (4, 5) and up-regulation of androgen responsive reporter

genes by interaction with androgen response elements (6–9). Activation of AR by low levels of androgen in addition to alternative signals involving growth factors, protein kinases, or other steroid hormones (10–16) may contribute to CaP progression.

The human CaP xenograft CWR22 propagated in nude mice maintains characteristics of human CaP, including an initial dependence on androgen for growth followed by recurrence several months after castration (17, 18). CWR22 tumor cells harbor a functional, mutated AR (H874Y) that displays broadened ligand specificity (19). The AR in LNCaP cells and in subline LNCaP-C4-2 derived from propagation of LNCaP cells in the absence of androgen contains a T877A mutation and is similar to wild-type AR in its activation by androgen, although it is less androgen specific (20–24).

In this study, we examined the properties of AR in the human androgen-dependent and recurrent CWR22 xenograft model, in a new cell line derived from recurrent CWR22 (CWR-R1), and in LNCaP and LNCaP-C4-2 cell lines. AR expression, androgen binding, stabilization, nuclear localization, and growth response to androgen are described. The results indicate that AR stability in the absence of androgen is greater in the recurrent tumors and cell lines derived therefrom. This increase in AR stabilization is associated with an acquired hypersensitivity of the cell lines to proliferation induced by very low levels of androgen.

## MATERIALS AND METHODS

**Materials.** LNCaP, DU145, PC-3, COS-1, and HeLa cell lines were purchased from the American Type Culture Collection (Rockville, MD), and LNCaP-C4-2 cells were purchased from UroCor, Inc. (Oklahoma City, OK). Fetal bovine serum was purchased from Hyclone Laboratories, Inc. (Logan, UT). Trans-<sup>35</sup>S label (L-methionine [<sup>35</sup>S], L-cysteine [<sup>35</sup>S]; 1000 Ci/mmol) was from ICN Biomedicals, Inc. (Costa Mesa, CA). [<sup>3</sup>H]Methyltrienolone ([17- $\alpha$ -methyl-<sup>3</sup>H]R1881; 80 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA). Pansorbin cells were from Calbiochem-Novabiochem International (La Jolla, CA). DHT, antibiotics, and cell culture media reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Casodex (bicalutamide) was provided by Zeneca Pharmaceuticals (Wilmington, DE).

**Transplantation of CWR22 Tumors.** Nude mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). CWR22 tumors were transplanted as dissociated cells in Matrigel (17, 18) into nude mice containing s.c. testosterone pellets (12.5 mg for sustained release of ~10  $\mu$ g testosterone/day; Innovative Research of America, Sarasota, FL) to normalize mice to serum testosterone levels of 4.0 ng/ml. Intact mice bearing androgen-stimulated tumors and castrated mice (testes and testosterone pellets were removed) carrying regressed (resected 1–6 days after castration) or recurrent (resected 150 days after castration) CWR22 tumors were exposed to methoxyflurane and sacrificed by cervical dislocation. Fresh tumor pieces were harvested, placed into RPMI 1640 medium, and processed for hormone binding and degradation studies or frozen in liquid nitrogen for protein lysate preparation.

**Generation of the CWR-R1 Cell Line.** Recurrent CWR22 xenograft tumors were harvested from nude mice 140–160 days after castration and dissociated as described (17, 18). Cells were placed in culture in PGM (25), comprised of Richter's Improved MEM (Irvine Scientific, Santa Ana, CA) supplemented with 10 mM nicotinamide, 20 ng/ml epidermal growth factor, 5  $\mu$ g/ml insulin, 5 ng/ml selenium, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.25  $\mu$ g/ml fungizone, and 2% fetal bovine serum. This new cell line

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<sup>3</sup> The abbreviations used are: CaP, prostate cancer; AR, androgen receptor; CX, castrate; DHT, dihydrotestosterone; PGM, prostate growth medium; DHEA, dehydroepiandrosterone.

designated CWR-R1 has been passaged >50 times and can be cryopreserved reliably in 15% DMSO. The epithelial cells grow in monolayers and show a diploid DNA content by flow cytometry. Cell cultures were negative for the basal cell-specific cytokeratin 34- $\beta$ -E12 (Sigma-Aldrich Co., St. Louis, MO) and positive for pancytokeratin when immunostained, demonstrating an epithelial phenotype. DNA sequencing demonstrated that CWR-R1 cells maintain the mutant H874Y AR found in CWR22 xenograft tumors and have no additional AR mutations.

**AR Stability, Androgen Binding, and Dissociation Assays.** Fresh tumor pieces (100–150 mg) of androgen-dependent CWR22, regressing tumors in castrated mice, or recurrent CWR22 tumors were incubated (equal wet weights of tumor tissue) in media lacking methionine for 30 min followed by incubation in the presence of Trans-<sup>35</sup>S label (100  $\mu$ Ci/tube) for 2 h at 37°C. Tumor pieces were centrifuged and washed once in PBS. Fresh culture medium (5 ml) was added, and tumors were incubated at 37°C for 0, 1, 3, or 6 h. Lysates were prepared from labeled tumor samples using radioimmunoprecipitation assay buffer containing protease inhibitors [1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.4), containing 0.5 mM phenylmethylsulfonyl fluoride, 80  $\mu$ M leupeptin, and 4  $\mu$ M aprotinin].

LNCaP cells were maintained in RPMI 1640 medium with 10% fetal bovine serum. LNCaP-C4-2 cells were grown in T media (DMEM:Ham's F-12 with 5% fetal bovine serum, 5  $\mu$ g/ml insulin, 13.65 pg/ml triiodothyronine, 5  $\mu$ g/ml apo-transferrin, 0.244  $\mu$ g/ml d-biotin, and 25  $\mu$ g/ml adenine), and CWR-R1 cells were grown in PGM. Cells ( $5 \times 10^5$ ) were plated in 10-cm dishes and allowed to grow for 48 h. LNCaP and LNCaP-C4-2 cells were then cultured in phenol red-free RPMI 1640 with 2% charcoal-stripped serum (Hyclone) and CWR-R1 cells in basal prostate medium (PGM without epidermal growth factor or phenol red with 2% charcoal-stripped serum). Cells were allowed to grow for 48 h. Cells were washed with PBS and incubated in Eagle's MEM without methionine with 2% charcoal-stripped serum for 20 min. Trans-<sup>35</sup>S label (100  $\mu$ Ci/dish) was added to the cells with or without hormone treatment and incubated for 2 h at 37°C. <sup>35</sup>S-labeled AR protein was immunoprecipitated from tumor and cell lysates using AR52 IgG and Pansorbin cells (Calbiochem-Novabiochem Corp., La Jolla, CA) and analyzed by SDS-PAGE. Autoradiograph signals were quantitated by densitometric scanning.

To determine specific androgen binding, cell lines or freshly dissociated tumor cells were labeled with [<sup>3</sup>H]R1881 at concentrations ranging from 0.025 to 2.5 nM for 2 h at 37°C in the absence and presence of 100-fold excess unlabeled R1881. Scintillation counting was performed to determine apparent equilibrium binding constants ( $K_d$ ) and binding capacity expressed as fmol AR/mg protein. The rate of dissociation of [<sup>3</sup>H]R1881 from AR was determined in cultured cells by labeling with 5 nM [<sup>3</sup>H]R1881 for 2 h, followed by competition with 50  $\mu$ M unlabeled R1881 for 15 min to 3 h. Transient transfections with wild-type and mutant pCMVhAR were performed as described previously (26). Scintillation counting was performed, and the dissociation rate of [<sup>3</sup>H]R1881 was determined.

**Northern and Southern Blot Analysis of AR.** Total RNA was prepared from cultured cells during logarithmic growth phase using TRIZOL Reagent (Life Technologies, Inc.) according to the manufacturer's instructions. RNA was prepared from frozen CWR22 tumor tissue as described previously (2). RNA (10  $\mu$ g) aliquots were fractionated on 0.8% agarose gels, transferred to nylon membranes, and hybridized with <sup>32</sup>P-labeled 18S rRNA or AR cDNA generated by PCR from pCMVhAR (exons E-G).

Genomic DNA was prepared as described (27) with slight modifications. Tumor tissue (100 mg) was pulverized under liquid nitrogen with mortar and pestle and resuspended in 1.5 ml of digestion buffer [100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K]. Samples were agitated at 37°C overnight. Samples were treated with 0.75 mg of RNase A and incubated for 4 h at 37°C. DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform, followed by precipitation with ethanol at -80°C overnight. DNA was collected by centrifugation at 13,000  $\times$  g for 10 min. The pellet was recovered in 0.1 mM EDTA, 10 mM Tris-HCl (pH 8.0) and incubated at 65°C for 30 min to dissolve the DNA. DNA (1  $\mu$ g) was digested with *Hind*III (Promega Corp., Madison, WI) for 2 h at 37°C followed by electrophoresis on a 0.75% agarose gel and capillary transfer to a positively charged nylon membrane. The membrane was hybridized with <sup>32</sup>P-labeled AR exon A fragment. Equal DNA loadings were determined by ethidium bromide staining of the gel.

**Immunoblot Analysis of AR.** Protein lysates were prepared from frozen tumors and from cultured cells during logarithmic growth at approximately 75% confluence and as described previously (2). Antihuman AR monoclonal antibody F39.4.1 (Biogenex, San Ramon, CA) was used at a 1:10,000 dilution. Secondary antibody goat-antimouse IgG conjugated to horseradish peroxidase (Amersham Corp., Arlington Heights, IL) was used for detection by enhanced chemiluminescence (DuPont, NEN Research Products, Boston, MA).

**Immunohistochemical Analysis of AR.** LNCaP, LNCaP-C4-2, and CWR-R1 cell lines were plated on ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA) in 10-cm dishes ( $5 \times 10^5$  cells/dish). Culture medium was replaced with phenol red-free RPMI 1640 containing 0.2% BSA (AlbuMAX I; Life Technologies, Inc.; Ref. 28) for LNCaP and LNCaP-C4-2 cells and phenol red-free Richter's Improved MEM with 2% charcoal-stripped serum for CWR-R1 cells for up to 4 days followed by the addition of 10 nM DHT or 5  $\mu$ M Casodex for 18 h. Slides were fixed in 95% ethanol for 10 min at -20°C, blocked in normal horse serum for 5 min at 37°C, and incubated with AR monoclonal F39.4.1 (Biogenex) at a 1:300 dilution for 30 min at 37°C. After a PBS wash, slides were incubated with goat-antimouse IgG conjugated to biotin at a 1:100 dilution for 10 min at 37°C and Vectastain ABC reagent (Vector Laboratories, Inc., Burlingame, CA) at 1:100 dilution for 10 min at 37°C. Positive signals were detected after incubation with diaminobenzidine tetrahydrochloride. Control slides were incubated with normal horse serum and showed no immunoreactivity.

**Cell Growth Assays.** Cells ( $1.5 \times 10^5$ ; LNCaP, LNCaP-C4-2, and CWR-R1) were plated in 12-well plates in the appropriate growth medium and allowed to grow for 48 h. Cells were washed with PBS and switched to phenol red-free medium with 2% charcoal-stripped serum (RPMI 1640 for LNCaP and LNCaP-C4-2 and basal prostate medium for CWR-R1). The following day, steroids were added, and triplicate wells were counted and represented day 0 of the growth assay. Triplicate wells were counted using a hemocytometer on days 2, 4, and 6 after the addition of steroids. Medium was changed on day 3, at which time fresh steroids were added.

## RESULTS

**AR mRNA and Protein Expression.** AR mRNA expression analyzed by Northern blot revealed a single 9.6-kb AR mRNA at similar levels in LNCaP, LNCaP-C4-2, and CWR-R1 cell lines and in CWR22 and recurrent CWR22 tumors (Fig. 1A). AR mRNA was not detected in PC-3 or DU145 or in HeLa or COS-1 cells. Protein lysates were prepared from cell cultures in logarithmic growth phase (4 days after plating). Immunoblot analysis revealed a  $M_r$  110,000 AR band in all of the cell lines with CWR-R1 and LNCaP-C4-2 cells showing 2–3-fold higher levels of AR than LNCaP cells (Fig. 1B). AR was detected at 3-fold higher levels in recurrent CWR22 tumors than in androgen-dependent CWR22, as reported previously (2). An additional AR band was evident at  $M_r$  84,000 in CWR-R1 cells and in CWR22 androgen-dependent and recurrent tumors, being stronger in the recurrent cell line and tumor. In studies not shown, the  $M_r$  84,000 band resulted from *in vitro* proteolysis of the AR during extraction and storage. Southern blot analysis revealed no evidence of AR gene amplification (Fig. 1C) in recurrent CWR22. These results were confirmed by competitive reverse-transcriptase PCR and by fluorescence *in situ* hybridization analysis (data not shown).

**AR Stability.** AR degradation rates in the LNCaP, LNCaP-C4-2, and CWR-R1 cell lines and in the CWR22 xenografts growing in the presence and absence of DHT were determined by pulse-chase labeling using [<sup>35</sup>S]methionine at 37°C. In the absence of DHT, AR was relatively stable in the cell lines derived after prolonged androgen withdrawal. The AR degradation half-time ( $t_{1/2}$ ) determined at 37°C was  $7.0 \pm 0.1$  h in LNCaP-C4-2 and  $6.0 \pm 0.2$  h in CWR-R1 cells (Fig. 2, B and C). AR degradation rates were at least 2 times slower in both these CaP cell lines compared with the degradation rate of AR in LNCaP cells ( $t_{1/2}$ ,  $3.0 \pm 0.1$  h; Fig. 2A).

AR in the androgen-dependent CWR22 tumor 1 and 6 days after castration was less stable ( $t_{1/2}$ ,  $6.0 \pm 0.3$  h) than was AR in the

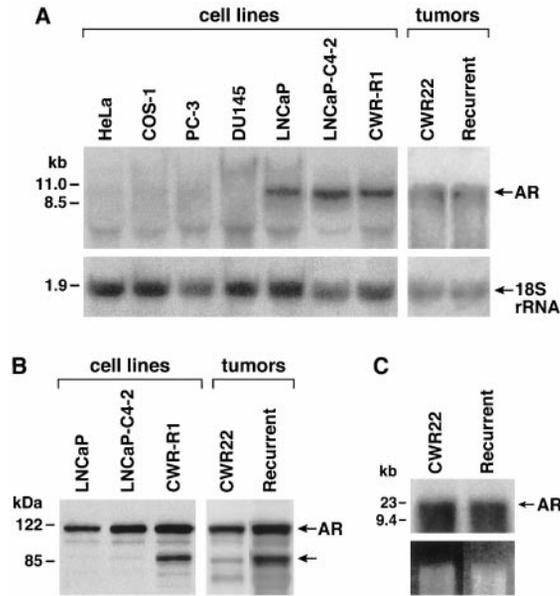


Fig. 1. AR mRNA and protein levels in CaP cell lines and CWR22 tumors. A, total RNA (10  $\mu$ g/lane) extracted from CaP cell lines and CWR22 tumors was analyzed by Northern blot analysis on 0.8% agarose gels using AR cDNA from ligand-binding domain exons E-G labeled with [ $^{32}$ P]dCTP. The position of molecular mass markers (kb) is indicated. 18S rRNA hybridization served as a loading control. B, immunoblot analysis of protein lysates (10  $\mu$ g/lane) of cell lines and CWR22 tumors were analyzed using monoclonal AR antibody F39.4.1. The position of molecular mass markers (kDa; shown in thousands) is indicated. Arrows, the migration of the full-length ( $M_r$ , 110,000) and truncated form ( $M_r$ , 84,000) of AR. C, Southern blot analysis of CWR22 genomic DNA using AR cDNA from exon A labeled with [ $^{32}$ P]dCTP. The position of molecular mass markers (kb) is indicated. Ethidium bromide staining of the gel in the bottom panel demonstrated equal loading of DNA. CWR22, tumors from intact male mice; recurrent, the CWR22 tumor that arises 5 months after castration, the growth of which is independent of testicular androgen.

recurrent CWR22 tumor obtained 150 days after castration ( $t_{1/2}$ , >12 h; Fig. 2D). In the absence of DHT, AR in the recurrent tumor was stable, with a degradation rate comparable with that for AR in androgen-dependent CWR22 in the presence of androgen. Treatment of cell lines and tumor tissue with the antiandrogen Casodex decreased the

stability of AR in the presence and absence of DHT (data not shown). These results are summarized in Table 1.

**Androgen Binding and Dissociation.** Androgen binding properties of AR in the cell lines and tumors were determined using [ $^3$ H]R1881, a tritium-labeled synthetic androgen methyltrienolone. AR in each of the CaP cell lines and CWR22 tumors displayed an apparent high equilibrium binding affinity of 0.07–0.16 nM (Table 1). Total AR-specific binding expressed as fmol/mg protein was higher in the CWR22 tumors and CWR-R1 cell line relative to LNCaP and LNCaP-C4-2 cells (Table 1), in agreement with the increased AR protein levels detected in CWR22 tumors and cell lines by immunoblot analysis (see Fig. 1).

Dissociation rates of bound [ $^3$ H]R1881 from AR in CaP cell lines were determined after the addition of a 10,000-fold molar excess of unlabeled R1881. AR in LNCaP, LNCaP-C4-2, and CWR-R1 cells had similar dissociation half-times ( $t_{1/2}$ , 83–110 min; Table 1). For comparison, transient transfection of COS-1 cells was performed with wild type and the H874Y and T877A mutant AR expression vectors, and [ $^3$ H]R1881 dissociation rates were determined. Wild-type AR had a dissociation half-time ( $t_{1/2}$ , 144  $\pm$  18 min), as reported previously (29). LNCaP and LNCaP-C4-2 T877A AR mutants were 1.6-fold slower ( $t_{1/2}$ , 228  $\pm$  12 min), and CWR22 H874Y AR mutant was 2.5-fold slower ( $t_{1/2}$ , 366  $\pm$  18 min) relative to wild-type AR (Table 1). Thus both recombinant mutant receptors had slower rates of dissociation of bound R1881 relative to the wild-type recombinant AR. The increased half-times of androgen dissociation could not be observed using the LNCaP, LNCaP-C4-2, or CWR-R1 cell lines, perhaps because of lower AR expression levels relative to the transient expression levels.

**AR Immunostaining.** Cells were grown without serum in the presence of 0.2% BSA for the removal of serum factors and low levels of steroid hormones present in serum. AR immunostaining was undetectable in the cell lines using nonimmune control serum (Fig. 3, A, E, and D). AR immunostaining in LNCaP cells cultured in the absence of DHT or serum for 2–4 days revealed cytoplasmic and nuclear AR immunoreactivity (Fig. 3B) and strong nuclear staining in the presence of 10 nM DHT (Fig. 3C). Treatment with 5  $\mu$ M Casodex alone for 18 h reduced nuclear and cytoplasmic AR staining to levels similar to those

Fig. 2. Degradation rates of endogenous AR. CaP cell lines in the presence and absence of 10 nM DHT or freshly isolated CWR22 tumors from intact mice or at 1, 6, or 150 (recurrent) days after castration were labeled with [ $^{35}$ S]methionine for 2 h at 37°C. Protein lysates were prepared at different times after incubation with unlabeled methionine and immunoprecipitated using AR polyclonal antibody AR-52. Immunoprecipitated AR was electrophoresed on SDS polyacrylamide gels, and autoradiographic signals were quantitated using densitometric scanning. Shown are representative experiments for AR degradation rates in (A) LNCaP, (B) LNCaP-C4-2, and (C) CWR-R1 cells and in (D) androgen-dependent CWR22 tumors (resected 1 day after CX), 6 day CX, and recurrent (tumor resected 150 days after CX). Degradation half-times at 37°C are indicated in parentheses.

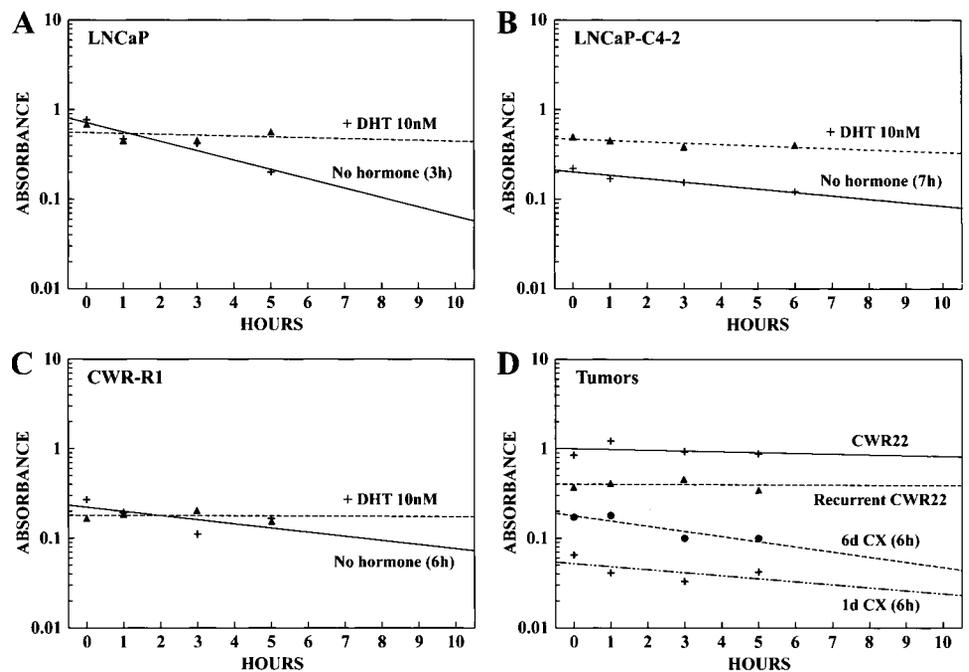


Table 1 AR biochemical characteristics in cell lines and tumors

AR apparent equilibrium binding constants ( $K_d$ ), total binding capacity (fmol/mg protein), dissociation times, and degradation half-times ( $t_{1/2}$ ) were determined in cell lines, CWR22 tumors, and with AR expression vectors in COS cells. All studies were performed in triplicate.

	$K_d$ (nM)	fmol/mg protein	R1881 dissociation ( $t_{1/2}$ , min)	Degradation ( $t_{1/2}$ h; -DHT)	Growth response (DHT; M) <sup>d</sup>
Cell line					
LNCaP	0.07 ± 0.02	290 ± 39	83 ± 7	3.0 ± 0.1	10 <sup>-9</sup>
LNCaP-C42	0.16 ± 0.07	295 ± 41	100 ± 40	7.0 ± 0.1	10 <sup>-14</sup>
CWR-R1	0.15 ± 0.03	471 ± 69	110 ± 20	6.0 ± 0.2	10 <sup>-14</sup>
AR expression vector					
Wild type	0.48 ± 0.25	<sup>b</sup>	144 ± 18	2.0 ± 0.1	
T877A	nd <sup>c</sup>	<sup>b</sup>	228 ± 12	1.8 ± 0.05	
H874Y	nd <sup>c</sup>	<sup>b</sup>	366 ± 18	1.8	
Tumor					
CWR22	0.1 ± 0.09	483 ± 102	nd <sup>c</sup>	>12	
Recurrent CWR22	0.1 ± 0.04	415 ± 23	nd <sup>c</sup>	>12	

<sup>a</sup> Minimum DHT concentrations (M) required to stimulate cell proliferation in cell lines are shown.

<sup>b</sup> Dependent on quantity of DNA transfected.

<sup>c</sup> nd, not determined.

seen in the absence of DHT. LNCaP-C4-2 cells also showed nuclear and cytoplasmic AR immunostaining in the absence of DHT or serum (Fig. 3F) and reduced cytoplasmic staining in the presence of 10 nM DHT (Fig. 3G). Treatment with 5  $\mu$ M Casodex increased AR cytoplasmic immunostaining in LNCaP-C4-2 cells, whereas nuclear levels remained similar to those observed in the presence or absence of DHT (Fig. 3H). CWR-R1 cells had nuclear AR immunoreactivity in the absence (Fig. 3I) and presence (Fig. 3K) of 10 nM DHT or with 5  $\mu$ M Casodex (Fig. 3L). There was no cytoplasmic staining detectable in any of the treatment conditions (Fig. 3, I-L). In summary, in the absence of DHT, intracellular localization of AR was predominantly nuclear with some variation between the cell lines. DHT treatment caused increased nuclear localization in all of the cell lines. The results agree with our previous results (2), demonstrating AR in the nucleus of both androgen-dependent CWR22 tumors growing in intact

mice and in recurrent CWR22 tumors in mice 150 days after castration.

**Androgen Dependence of Cell Proliferation.** To determine whether differences in AR stability and nuclear/cytoplasmic staining are reflected in functional differences in these cell lines, we measured the effects of androgen on cell proliferation. Basal growth rates of untreated cells were similar with cell doubling times of ~6 days. LNCaP cells have been shown to respond within a narrow range of 10<sup>-11</sup> to 10<sup>-9</sup> M DHT with 10<sup>-7</sup> M causing cell growth inhibition (30, 31). We observed LNCaP cell growth response at 10<sup>-9</sup> to 10<sup>-8</sup> M DHT and was inhibited by 10<sup>-7</sup> M Casodex in the absence or presence of DHT (Fig. 4A). In contrast, LNCaP-C4-2 and CWR-R1 cell proliferation was increased at 10<sup>-14</sup> to 10<sup>-10</sup> M DHT, indicating much greater androgen responsiveness of these cell lines derived after prolonged androgen withdrawal. Growth of LNCaP-C4-2 and

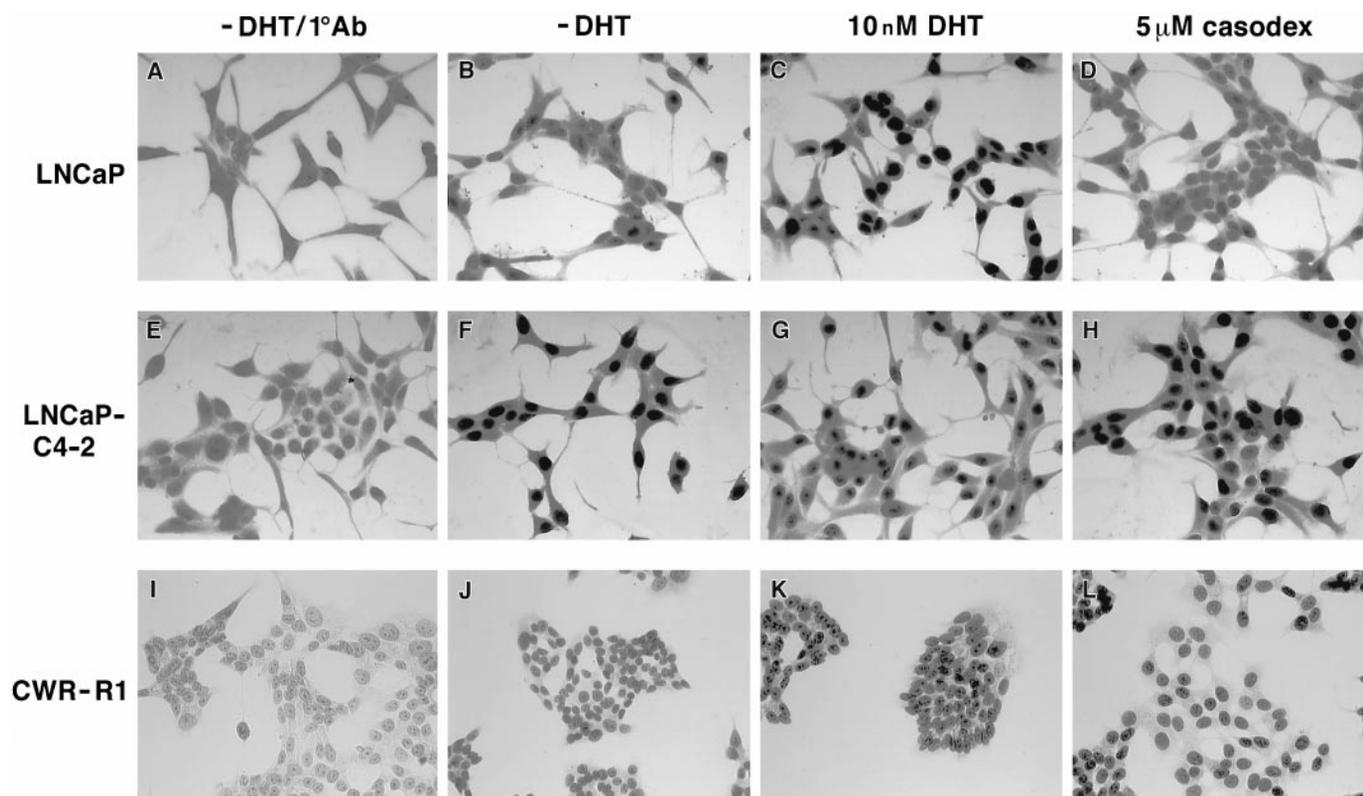


Fig. 3. Immunocytochemical localization of AR in cultured CaP cell lines. Immunostaining for AR was performed on ethanol-fixed slides of LNCaP (A-D), LNCaP-C4-2 (E-H), and CWR-R1 (I-L) cell lines. Cells were incubated in the absence of DHT and serum for 48 h before immunostaining with nonimmune serum (A, E, and I) or AR monoclonal antibody F39.4.1. A, B, E, F, I, and J, grown in the absence of DHT and serum for 48 h; C, G, and K, treated with 10 nM DHT for 18 h; D, H, and L, treated with 5  $\mu$ M Casodex for 18 h. Magnification,  $\times 200$ .

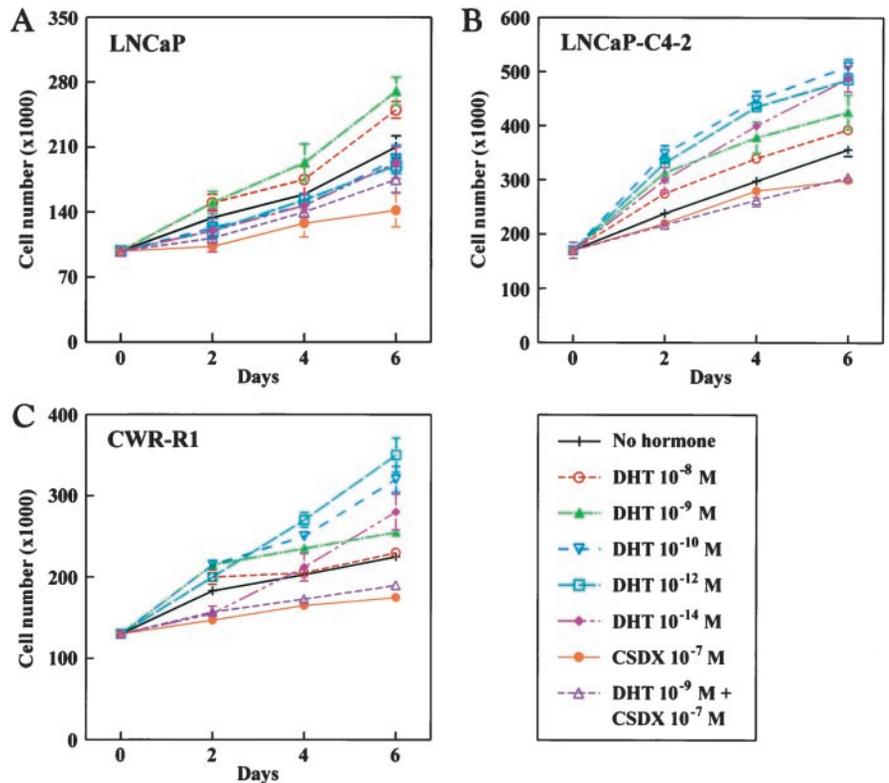


Fig. 4. Cell proliferation assay of CaP cell lines. LNCaP, LNCaP-C4-2, and CWR-R1 cell lines were plated in 12-well plates at  $1.5 \times 10^5$  cells/well and treated with increasing concentrations of DHT ( $10^{-14}$  to  $10^{-8}$  M) or  $5 \mu\text{M}$  Casodex. Cells in triplicate wells were counted with a hemocytometer on days 2, 4, and 6 after beginning hormone treatments. Results are representative of three to five separate experiments for each cell line. Bars, SE.

CWR-R1 was inhibited by Casodex in the presence and absence of DHT (Fig. 4, B and C).

## DISCUSSION

Castration of the adult male with CaP lowers the circulating testosterone concentration from 4.5 ng/ml ( $2.1 \times 10^{-8}$  M) to 0.28–1.3 ng/ml ( $1.3 \times 10^{-9}$ – $6.0 \times 10^{-9}$  M; Ref. 32), resulting in regression of CaP and involution of androgen-dependent tissues. In mice carrying androgen-dependent CaP xenografts, a decrease in serum testosterone from the normal level (4.0 ng/ml) to the castrate level ( $0.26 \pm 0.03$  ng/ml;  $n = 22$ ) also results in tumor regression (33, 34). During prolonged androgen withdrawal, the regressed CWR22 xenograft propagated in nude mice and the majority of human CaP tumors undergo recurrent growth. A major challenge is to establish the molecular mechanisms driving this recurrent growth under conditions of low circulating androgen. Two examples of recurrent CaP are the recurrent CWR22 and its derived cell line CWR-R1 and the LNCaP-C4-2 cell line derived from LNCaP after prolonged culture in the absence of androgen. AR is expressed at similar or higher levels in these recurrent CaPs but is more stable against degradation in the absence of androgen compared with AR in corresponding androgen-dependent tumors or cells. In comparing growth properties of the androgen-dependent and recurrent cell lines under study, a disparity was observed in the levels of androgen required for cell proliferation. The cell lines from recurrent CaP, CWR-R1 and LNCaP-C4-2, responded to an androgen concentration  $10^4$  times lower than required for the growth stimulation of androgen-dependent LNCaP, suggesting that the low levels of androgen in a male withdrawn from androgen by medical or surgical castration are sufficient to promote proliferation of recurrent CaP cells. This increased sensitivity to androgen was not associated with major increases in androgen binding affinity or in levels of AR.

Whether the increase in sensitivity to androgen relates to the AR

mutations in the LNCaP (T877A) and CWR-R1 (H874Y) cell lines is unclear. These mutations do not have a significant effect on the apparent equilibrium binding affinity for [ $^3\text{H}$ ]R1881 (19). They do result in a 2–3-fold slower dissociation rate of bound androgen for the recombinant full-length mutant AR expressed in COS cells, but this slower rate was less evident in the cell lines. A slower androgen dissociation rate appears to be critical for AR functions mediated by interaction of the  $\text{NH}_2$ - and  $\text{COOH}$ -terminal domains (35). However, a 2-fold reduction in the androgen dissociation rate was also observed when AR fragments containing the DNA-binding plus ligand-binding domains with the mutant sequences were expressed in the absence of the AR  $\text{NH}_2$ -terminal region (data not shown), suggesting a change in the structure of the ligand-binding domain by mutants, as suggested recently (36). Because increased sensitivity to androgen occurs during progression to androgen independence in the absence of AR gene mutations, changes in the structure of the ligand-binding domain by these mutations alone could not account for the increased sensitivity.

AR mutants in CaP usually maintain androgen-dependent transcriptional activity (37). The mutant AR T877A in LNCaP cells maintains androgen responsiveness similar to wild-type AR but has broadened ligand specificity (23, 24). AR H874Y in CWR22 xenografts is also transcriptionally activated by testosterone and DHT to an extent similar to wild-type AR and has broadened ligand specificity, being activated by estradiol, progesterone, androstenedione, and the adrenal androgen DHEA, as well as hydroxyflutamide (19). Recent AR modeling studies based on the crystal structure of progesterone receptor showed that T877 is in the ligand-binding pocket, and the LNCaP T877A substitution alters the binding space, resulting in altered ligand specificity (38, 39). It was predicted that residue 874 is not in direct contact with ligand, but a mutation at this site may affect the positioning of helix 12.

Recent studies suggest that AR-mediated signaling or gene expression has a key role in recurrent growth of CaP (2, 36). In addition to

causing a slower rate of AR degradation (40), androgen binding to AR results in dimerization of the receptor (41), interaction between the AR NH<sub>2</sub>- and COOH-terminal domains (35), and binding of AR to androgen response elements in androgen-regulated genes, leading to AR-induced transcriptional activation (6–9). Activation of AR in recurrent CaP cells mediates expression of androgen-regulated target gene networks (2, 42). Expression of androgen-regulated genes in the relative absence of androgens suggests that AR signaling is reactivated at the lower androgen levels present in recurrent CaP or that compensatory signaling mechanisms mediate target gene expression through divergent pathways involving protein kinases and growth factors (10–16).

Augmentation of the AR-mediated signal can occur by amplification of the AR gene itself. AR gene amplification was demonstrated in 7 of 23 (30%) recurrent CaP tumor specimens after androgen deprivation therapy (43). However, there was no evidence for AR gene amplification in androgen-dependent or recurrent CWR22 xenografts using Southern blot analysis, competitive reverse-transcriptase PCR, and fluorescence *in situ* hybridization analysis.<sup>4</sup> These results are in agreement with a recent report by Culig *et al.* (44) that the AR gene in the androgen-ablated LNCaP-abl cell line is not amplified. Thus, whereas AR gene amplification may occur in some recurrent CaPs, our studies present an alternative hypothesis that increased AR protein stability in CaP cell lines or tumor after prolonged androgen withdrawal contributes to tumor cell growth under conditions of low androgen.

Previous studies (4, 5, 45–48) in cell lines demonstrated that in the absence of ligand, steroid hormone receptors can be predominantly cytoplasmic or nuclear, depending on the receptor and hormone conditions. AR was localized in the perinuclear region of transfected COS cells (4) and in nuclei of LNCaP cells in the absence of DHT (49). Nuclear AR was observed in the absence of androgen in recurrent CaP tumors from xenograft models (2, 50) and humans (1, 51). In CWR22 xenograft tumors, AR localized to the nucleus 6–150 days after castration, and nuclear AR levels increased in recurrent tumor cells 120–150 days after castration (2). An increase in nuclear AR in recurrent CWR22 tumors growing in the absence of testicular androgen correlated with expression of a network of androgen-regulated genes (2). This finding suggested that nuclear AR is transcriptionally active in these recurrent tumors in the presence of low levels of androgen that occur after castration.

Increased AR stability may increase the AR nuclear retention time. The increased nuclear AR concentration could result in an increased biological response at lower DHT levels with no change in AR affinity as predicted by the spare receptor hypothesis (52). Our results suggest that progression to the recurrent state in cell lines or xenograft tumors increases the sensitivity of AR to low levels of androgen. The recurrent LNCaP-I and LNCaP-R cell lines developed by Kokontis *et al.* (53, 54) expressed 2-fold higher levels of AR mRNA and protein and had a 7-fold increase in chloramphenicol acetyltransferase reporter gene activity with R1881 compared with parental LNCaP cells. Culig *et al.* (44) reported a 10-fold increase in sensitivity of LNCaP-abl cells to R1881 compared with parental LNCaPs. Increased sensitivity to extremely low estrogen concentrations (10<sup>-14</sup> M) was reported in a cell line derived from a pituitary lactotroph tumor (55). We demonstrate growth responsiveness of LNCaP-C4-2 and CWR-R1 cell lines to DHT concentrations as low as 10 femtomolar, 10<sup>4</sup>-fold lower than required by androgen-dependent LNCaP cells. The androgen antagonist Casodex did not show agonist activity in LNCaP-C4-2 or CWR-R1 cells, as was reported by Culig *et al.* (44) in LNCaP-abl

cells but repressed growth of all of the cell lines, as was the case in LNCaP-R cells (54). This repression supported the possibility that AR was constitutively active in the absence of androgen and that the antagonist blocked its activation.

Growth factor signaling (10–16), up-regulation of androgen target genes (2, 36, 42), mutations in AR that alter ligand specificity (37), and AR gene amplification (43) have all been implicated as possible mechanisms for induction of recurrent CaP growth. Our results indicate that AR is highly expressed, stable, and localized to the nucleus in the absence of androgen in recurrent CaP cell lines and at very low androgen levels in recurrent CWR22 human tumor xenografts after prolonged periods of castration. This increased stability is associated with a hypersensitivity of AR to very low androgen levels. Conversion of the adrenal androgens androstenedione, DHEA, and DHEA-sulfate to testosterone occurs in the prostate (56, 57), and the data presented here suggest that low levels of androgen present in the prostates of men treated by androgen withdrawal may be sufficient to activate AR in androgen-independent CaP tumors.

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## Androgen Receptor Stabilization in Recurrent Prostate Cancer Is Associated with Hypersensitivity to Low Androgen

Christopher W. Gregory, Raymond T. Johnson, Jr., James L. Mohler, et al.

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