Characterization of a Novel Androgen Receptor Mutation in a Relapsed CWR22 Prostate Cancer Xenograft and Cell Line

Clifford G. Tepper, David L. Boucher, Philip E. Ryan, Ai-Hong Ma, Liang Xia, Li-Fen Lee, Thomas G. Pretlow, and Hsing-Jien Kung

ABSTRACT

CWR22 has been a valuable xenograft model for the study of prostate cancer progression from an androgen-dependent tumor to one that grows in castrated animals. Herein, we report the identification and characterization of a novel androgen receptor (AR) mutation occurring in a relapsed tumor (CWR22Rv1/2152) and in the CWR22Rv1 cell line established from it. The mutation was not detected in the original, hormone-dependent CWR22 xenograft, indicating that this change occurred during the progression to androgen independence. It is characterized by an in-frame tandem duplication of exon 3 that encodes the second zinc finger of the AR DNA-binding domain. Accordingly, immunoblot analyses demonstrated the expression of an AR species having an approximately 5-kDa increase in size relative to the LNCaP AR. This was accompanied by a COOH-terminally truncated AR species migrating with a relative mass of 75–80 kDa, referred to as ARΔLBD because it lacks the ligand-binding domain. By recreating the exon 3 duplication mutation in a wild-type AR expression construct, the generation of ARΔLBD could be recapitulated. Whereas ARΔLBD exhibited constitutive nuclear localization and DNA binding, these functions in the full-length AR remained androgen dependent. The CWR22Rv1/AR repertoire displayed dose-dependent, androgen-responsive transcriptional transactivation in reporter assays, albeit to a lesser extent in comparison with LNCaP. This cell line also expressed low levels of prostate-specific antigen mRNA and did not express or secrete detectable levels of prostate-specific antigen protein in androgen-depleted medium or in response to physiological androgenic stimulation. In summary, the CWR22Rv1 cell line displays both androgen-responsive and androgen-insensitive features due, at least in part, to a novel insertional mutation of the AR.

INTRODUCTION

A serious clinical complication of CaP is the development of disease refractory to hormonal therapy. Most CaPs initially present as androgen-dependent neoplasms (1) and respond well to androgen ablative therapy. However, they commonly relapse as tumors resistant to hormonal therapy as well as standard chemotherapeutic interventions (2). As such, much research has been directed toward understanding the molecular mechanisms underlying this phenotypic transition.

6606
progression to a hormone-refractory state because it possesses features of clinical advanced disease: AR expression; AI proliferation; and androgen-responsiveness. As such, it serves as a model presenting an intermediate, or transition, phenotype between that of hormone-sensitive, AR-positive cell lines (e.g., LNCaP) and AI, AR-negative cell lines (e.g., DU-145 and PC-3). In this report, we describe a novel AR mutation and the expression of novel AR protein products present in CWR22Rv1. The impact of this novel receptor upon androgen-responsiveness in this system has also been investigated by cloning and functional analysis of this mutation. The results of this study suggest that this AR mutation potentially represents a novel step in a multistep process of progression toward hormone-refractory disease.

MATERIALS AND METHODS

Cell Lines and Culture. LNCaP, DU-145, and PC-3 human CaP cell lines were obtained from the American Type Culture Collection (Manassas, VA). The CWR22Rv1 cell line was established from a CWR22R prostate cancer xenograft, which was derived from the parental androgen-dependent CWR22 tumor that had been serially transplanted after castration-induced regression and relapse (18, 20). All cell cultures were maintained in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified environment of 5% CO2 in air. For experiments simulating conditions of androgen withdrawal, cells were washed twice with Dulbecco’s PBS and then cultured continuously in RPMI 1640 (without phenol red; Life Technologies, Inc.) supplemented with 10% CDT FBS (Hyclone). This will be referred to as AD medium.

Tumor Xenografts. The CWR22 primary human CaP xenograft and relapsed CWR22R tumors were propagated and serially transplanted in nude mice as described previously (18, 21).

Reagents. AR antibodies used in these experiments included AR441 mouse monoclonal antibody (Ab-1; Lab Vision Corp., Fremont, CA) and the rabbit polyclonal antibodies PG-21 (Upstate Biotechnology, Inc., Lake Placid, NY) and AR C-19 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Additional monoclonal antibodies used were anti-NSE (clone E27, IgG1; Lab Vision Corp.) and anti-PSA (clone A67-B/E3, Ab-1; Lab Vision Corp.). DHT and flutamide were purchased from Sigma (St. Louis, MO), and mibolerone was obtained from New England Nuclear Life Science Products Inc. (Albany, MA). Casodex (bicalutamide) was the generous gift of AstraZeneca, Inc.

Immunoblot Analysis. Cell lysates were made in NP40 lysis buffer [30 mM Tris-Cl (pH 7.5), 150 mM EDTA, 150 mM NaCl, and 1% NP40 supplemented with 1 μg/ml each of aprotinin, leupeptin, and pepstatin A] and phosphatase (1 mM sodium fluoride and sodium orthovanadate). Nuclei and cellular debris were removed by centrifugation at 14,000 × g for 15 min at 4°C. The protein content of the supernatant was then quantitated with the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Proteins (50 μg of lysate) were separated by denaturing SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). Membranes were blocked for at least 1 h with 5% nonfat dry milk or BSA in Tris-buffered saline containing 0.05% Tween 20 (TBST). Primary antibodies were diluted in blocking buffer according to the manufacturers’ protocols and subsequently incubated with the blots for 2 h at room temperature or overnight at 4°C. The membranes were washed three times with TBST and incubated with a 1:2,000 dilution of the appropriate horseradish peroxidase-conjugated anti-IgG in blocking buffer for 1 h at room temperature. After washing, the blots were developed with enhanced chemiluminescence (Amer sham) and exposed to BioMax X-ray film (Kodak).

Immunoprecipitation Analysis. Immunoprecipitation was performed according to standard protocol. Briefly, 5 μg of polyclonal C-19 antibody or monoclonal AR441 were used to immunoprecipitate the AR from 250 μg of nuclear-free NP40 lysates made from LNCaP and CWR22Rv1 cells. Rabbit antimouse IgG was added as bridging antibody for the AR441 precipitation, and all immunocomplexes were precipitated by the addition of protein A-agarose. Immunoblot analysis was performed as described above.

Preparation of Nuclear and Cytosolic Extracts. Nuclear and cytosolic extracts were prepared according to the protocol of Dignam et al. (22). Briefly, LNCaP and CWR22Rv1 cells (1 × 10⁶) were harvested, resuspended in hypotonic buffer [10 mM HEPES (pH 7.9 at 4°C), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride], and lysed by disruption with a Dounce homogenizer. Nuclei were isolated by centrifugation at 3,300 × g for 15 min at 4°C. The nuclear extract was prepared by incubation of the nuclear pellet in an equivalent volume of high-salt buffer [20 mM HEPES (pH 7.9 at 4°C), 25% glycerol, 1.5 mM MgCl2, 300 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride], followed by centrifugation to remove the extracted nuclei. The cytosolic extract was then prepared by clarification of the supernatant of the nuclear isolation by centrifugation at 16,000 × g.

RT-PCR Mapping and Sequencing Analysis of the AR mRNA. Total cellular RNA was isolated from proliferating LNCaP and CWR22Rv1 cells by the acid guanidinium thiocyanate-phenol-chloroform method. Alternatively, first-strand cDNA was synthesized from 5 μg of RNA with the cDNA preamplification system (Life Technologies, Inc.) using SuperScript II reverse transcriptase and an oligo(dT)15 primer and used as the template in standard PCR reactions using AmpliTag DNA polymerase (Perkin-Elmer Life Sciences, Boston, MA). Amplification products were analyzed on 2% TAE agarose gels made with MetaPhor BioProducts, Rockland, ME) and photographed under UV illumination. DNA ladders of 25 and 100 bp (Life Technologies, Inc.) were used as size standards.

To analyze the AR mRNA, primer pairs were designed to amplify overlapping segments encompassing the entire AR coding region plus 5'- and 3'-untranslated sequences. Primers (custom synthesized; Integrated DNA Technologies, Coralville, IA) were designed based on the human AR mRNA reference sequence (NG_000440) downloaded from the GenBank database (National Center for Biotechnology Information, Bethesda, MD). Primer designations in each primer name specify the mRNA location of the 5’ nucleotide of each primer: segment I (hAR 921 For 5'-TGTCGAGACCTTGAGGCTGTC-3' and hAR 1663 Rev 5'-CAGGTATCCTTATTTAGGTCGCGG-3'); segment II (hAR 1182 For 5'-TCCGAGAATCTGTTCCAGAG-3' and hAR 1925 Rev 5'-AAATGGGCGGCTACATGCATC-3'); segment III (hAR 1695 For 5'-AACACAGACGGAGAACGATCC-3' and hAR 2426 Rev 5'-TGAAGAGAGTGCCGAGTAG-3'); segment IV (hAR 2042 For 5'-TTCAAGAGGAGGTTACCCAACAGG-3' and hAR 2788 Rev 5'-GTGCTTCTGGGTGGTAAAGTAAG-3'); segment V (hAR 2756 For 5'-CCATTGACTATTACTTTTCCACCC-3' and hAR 3490 Rev 5'-TTGAGAGGTGGCTTCTGAGCG-3'); and segment VI (hAR 3386 For 5'-AATCTCAGAGTGCTCTTTCCGC-3' and hAR 4118 Rev 5'-CAATGGGAAGAGAGGACTGAGG-3'). The major CAG trinucleotide repeat (nucleotides 1286–1345) was amplified with a primer pair flanking this region and has been described previously (23).

Automated DNA sequencing (Davis Sequencing, LLC, Davis, CA) was performed on both strands of purified RT-PCR products (Qiagen, Valencia, CA). GeneTool Lite (version 1.0; DoubleTwist, Inc., Oakland, CA) and MacVector (version 7.0; Genetics Computer Group, Madison, WI) software packages were used for DNA sequence analysis, and alignment of the AR sequences was performed using the ClustalW algorithm (24).

Cell Proliferation Assay. LNCaP and CWR22Rv1 cells (2 × 10⁵) were seeded into 10-cm dishes in androgen-containing medium and allowed to attach for 24 h. The medium was then replaced with fresh medium containing FBS (androgen-containing medium) or CDF FBS (AD medium), and each cell line was cultured for up to 5 days. At each time point, cells were enumerated with a MicroWell counter.

Transfections and Luciferase Reporter Assays. LNCaP and CWR22Rv1 cells in 6-well plates were cotransfected (Effectene; Qiagen) with the pGL3-E-Probasin firefly luciferase reporter plasmid in combination with the pRL-SV40 Renilla luciferase plasmid (Promega, Madison, WI). pGL3-E-Probasin contains a 267-bp fragment of the rat probasin gene promoter (base positions –256 to +11) inserted into the pGL3-Enhancer vector (Promega) and was provided by Dr. Zoran Culig (University of Innsbruck, Innsbruck, Austria). For all transfections, a total of 200 ng of plasmid DNA was used in a 9:1 ratio of experimental:control plasmids. Sixteen h after transfection, the cells were shifted to AD medium and treated with 0.1, 1.0, and 10.0 nM DHT or left untreated (ethanol vehicle control), for an additional 24 h. Cell lysates were

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harvested and analyzed for firefly and Renilla luciferase activities with reagents from the Dual-Luciferase Reporter Assay System (Promega) using an EG & G Berthold LB96V MicroLumatPlus microplate luminometer (Perkin-Elmer-Wallac Inc., Gaithersburg, MD).

Alternatively, PC-3 cells were seeded into 24-well plates and transfected with AR expression constructs in combination with pGL3E-Probasin and pRL-SV40 luciferase reporters in a ratio of 4:5:4:5:1, respectively. A total of 200 ng of plasmid DNA was used. Treatments and analyses were performed essentially as described above.

**Determination of PSA Concentration.** LNCaP and CWR22Rv1 cells (2 x 10⁶) were seeded into 10-cm dishes and allowed to attach for 24 h. The medium was replaced with 10 ml of AD medium, and the cells were then cultured in the absence or presence of 0.1 or 1 nM DHT for 48 and 72 h. At each time point, secreted PSA was quantitated by analysis of culture supernatants with a PSA enzyme immunoassay kit (Medicorp, Montreal, Quebec, Canada) according to the manufacturer’s protocol. For the analysis of LNCaP PSA secretion, a 5-fold dilution in fresh medium was made to remain within the quantitative limits of the assay. Absorbance was measured at 414 nm with a microplate reader (Molecular Devices Corp., Sunnyvale, CA).

**Construction of pcDNA3.1-hAR-E3DM.** A DNA fragment containing the CWR22Rv1 AR exon 3 duplication mutation (E3DM) was generated from CWR22Rv1 first-strand cDNA by PCR using the BstEII/EcoRI primers and the Expand High Fidelity PCR system (Roche). Gel-purified amplification products were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and sequence verified. The BstEII/EcoRI CWR22Rv1 AR fragment (1257 bp) from this plasmid was then ligated into similarly digested pcDNA3.1-hAR-wt to produce pcDNA3.1-hAR-E3DM.

**DNA Pull-down Assay.** Oligonucleotides corresponding to the PSA ARE and ARR were synthesized according to published sequences (25). Sequences of the oligonucleotides were as follows: PSA ARE (-170) For, 5′-biotin-GATCCCTGGTGGTTCGCAATAGTGATG-3′; PSA ARE (-170) Rev, 5′-GATCCAGCTGACCTTGTGCCTGACAG-3′; PSA ARR (-400) For, 5′-biotin-GATCCCTGGTGGTTCGCAATAGTGATG-3′; and PSA ARR (-400) Rev, 5′-GATCCAGCTGACCTTGTGCCTGACAG-3′. The “forward” oligonucleotide of each probe was biotinylated at the 5′-end. Double-stranded probes were made by annealing a 50 μM mixture of complimentary oligonucleotides in TNE [10 mM Tris-Cl, 50 mM NaCl, and 1 mM EDTA (pH 8.0)] by heating to 95°C for 5 min followed by slowly cooling to room temperature. Nuclear extracts from CWR22Rv1 cells were prepared according to the protocol described above. For pull-down assays, 30 μg of nuclear extract were incubated in a 25-μl reaction mixture consisting of 10 μM probe and 1X binding buffer containing 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA (pH 8.0), 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC)-poly(dI-dC) (Promega). Incubations were for 30 min at room temperature, after which the reaction volume was increased to 0.5 ml with modified binding buffer [i.e., which does not contain poly(dI-dC)-poly(dI-dC)]. To capture the complexes, streptavidin-agarose (Santa Cruz Biotechnology) was added, and the incubation continued for 1 h at 4°C. The complexes were collected by pulse centrifugation in a microcentrifuge, washed three times with modified binding buffer, and eluted from the beads by the addition of 2X Laemmli buffer and heating to 94°C for 5 min. Proteins were then separated by 8% SDS-PAGE and analyzed by immunoblot analyses as described above.

**RESULTS**

**Expression of Novel Full-length and Truncated Forms of the AR in CWR22Rv1.** A survey of AR expression in a panel of commonly used CaP cell lines demonstrated the expression of full-length AR in the LNCaP and CWR22Rv1 cell lines, whereas AR could not be detected in the AI cell lines DU-145 and PC-3 (Fig. 1A). The LNCaP AR has a relative electrophoretic mobility of approximately 80-kDa species represented a COOH-terminally truncated AR because it could easily be recognized by the AR441 and PG-21 antibodies, but not by the COOH-terminus domain-specific C-19 antibody. Taken together, bands, in the range of 75–80 kDa. Interestingly, the larger full-length and smaller AR proteins have not been described previously in the original CWR22 xenograft (17), relapsed tumors (10), or in the original description of the CWR22Rv1 cell line (20). These novel findings prompted further investigations.

Initially, we pursued a biochemical characterization of the 75–80-kDa species using an antibody mapping approach. Antibodies recognizing the NH₂-terminus (PG-21; amino acids 1–21 (26)), a central region of the transactivation domain (AR441; amino acids 299–315), and an epitope in the COOH-terminus (C-19) of the AR were used (Fig. 1B). The results demonstrated that the 75–80-kDa species represents a COOH-terminally truncated AR because it could easily be recognized by the AR441 and PG-21 antibodies, but not by the COOH-terminus domain-specific C-19 antibody. Taken together,
AN AR MUTATION OCCURRING DURING RELAPSED GROWTH OF CWR22R

these data implied that the truncated AR contains intact transactivating and DBDs but lacks the LBD (Fig. 1B). From this point onward, we will refer to the truncated AR as ARΔLBD.

The cellular distribution of the CWR22Rv1 AR protein isoforms was determined by immunoblot analysis of cytosolic and nuclear extracts prepared from cells cultured for 3 days in the presence or absence of androgen (Fig. 2A). In androgen-containing medium, full-length CWR22Rv1 AR and ARΔLBD were easily detected in both the cytosol and the nucleus (Fig. 2A). CWR22Rv1 FBS/Lanes C and N). Notably, a higher proportion of the total cellular content of ARΔLBD, as compared with the full-length receptor, was localized to the nucleus. In the absence of androgen, nuclear localization of ARΔLBD was sustained, whereas that of the full-length 114-kDa AR was diminished (Fig. 2A. CWR22Rv1/lanes C and N). In addition, nuclear ARΔLBD migrated as a doublet, with the upper band becoming more distinctive (i.e., in terms of resolution and content) during androgen withdrawal.

Data obtained from antibody mapping did not provide absolute confirmation of the presence of a DBD in ARΔLBD. We next wanted to determine this by using a functional approach with a DNA pull-down assay. Nuclear extracts from androgen-deprived CWR22Rv1 cells were incubated with biotinylated, double-stranded oligonucleotide probes specific for the ARE and ARR at positions −170 and −400 of the PSA promoter, respectively (25). Under these conditions (3 days of androgen withdrawal), ARΔLBD is the predominant AR in

the nucleus (Fig. 2A). DNA-protein complexes were captured with streptavidin-agarose beads and analyzed by immunoblot analysis for the presence of AR forms. As shown in Fig. 2B, ARΔLBD was indeed able to interact with both promoter elements in the absence (−lanes) or presence (+lanes) of androgen. By contrast, binding of the full-length CWR22Rv1 AR only occurs in the presence of androgen, consistent with it being translocated to the nucleus under such conditions. Taken together, the results indicate that ARΔLBD contains a functional DBD and displays ligand-independent nuclear localization and promoter binding.

To determine whether ARΔLBD interacted with the full-length form in cells cultured in the presence of androgen, immunoprecipitation analysis was performed. Clarified whole cell lysates from LNCaP and CWR22Rv1 cells were immunoprecipitated with the COOH terminus-specific C-19 antibody followed by immunoblot analysis with the AR441 antibody (Fig. 2C). In this assay, we did not observe an interaction between the two forms of the receptor because C-19 precipitated the full-length AR, but immunocomplexes did not contain ARΔLBD. Thus, ARΔLBD appears to be able to function independently.

The CWR22Rv1 mRNA Transcript Contains an In-Frame Tandem Duplication of Exon 3. The results described above clearly demonstrate the expression of a larger form of the AR in CWR22Rv1 cells that contains at least three epitopes in common with the wild-type AR. We next wanted to determine whether there was a molecular basis for the generation of the CWR22Rv1 ~114-kDa full-length receptor, such as being encoded by a variant transcript. To that end, an initial characterization of the CWR22Rv1 AR transcript was performed by RT-PCR mapping using primer pairs designed to amplify overlapping AR mRNA segments of approximately 700 bp in length and spanning the entire length of the AR coding sequence. The exons and the 5′- and 3′-untranslated sequences amplified by each of the primer sets are depicted in Fig. 3A. In addition, the organization of the domains and various repeat regions of the AR polypeptide are presented in relation to the exon organization of the AR transcript. RT-PCR reactions performed with RNA samples isolated from both LNCaP and CWR22Rv1 yielded amplification products for segments I–IV and VI with the expected sizes of 743, 744, 732, 747, and 733 bp, respectively (Fig. 3B). The presence of an expanded CAG trinucleotide repeat region was ruled out because primer pairs flanking this region, as well as those for segments I and II, generated products with the expected sizes and did not exhibit increased sizes relative to those from LNCaP (Fig. 3B. segments I, II, and CAG lanes). Whereas LNCaP mRNA yielded the expected 735-bp amplicon for region V, the CWR22Rv1 product displayed an obvious shift in mobility corresponding to an approximately 100-bp increase in size (Fig. 3B). Segment V encompassed AR mRNA nucleotide positions 2756 through 3490 (i.e., 1642–2376 of the cDNA sequence) and spans a region beginning within exon 2 and extending into exon 6, corresponding to the DBD, hinge region, and LBD (Fig. 3A).

Purified segment V amplification products from both LNCaP and CWR22Rv1 were analyzed by automated DNA sequencing followed by ClustalW-driven pairwise alignment of the sequences with each other and with the reference AR cDNA sequence (locus/accession number NM_000044) in GenBank (National Center for Biotechnology Information). This immediately demonstrated an insertion of 117 bp in the CWR22Rv1 sequence occurring between the 3′-end of exon 3 and the 5′-end of exon 4 (Fig. 3C). BLAST analysis of the inserted sequence against the nonrandom GenBank database revealed 100% identity with exon 3 of AR. This represents a tandem duplication, and the inserted sequence will be referred to as exon 3′ (Fig. 3C). In addition, this insertion is in-frame and encodes an additional 39 amino acids and 4–5 kDa of protein mass, thus accounting for the observed

Fig. 2. Cellular distribution, DNA binding, and interaction of the CWR22Rv1 AR isoforms. A, nuclear (N) and cytosolic (C) extracts were prepared from LNCaP and CWR22Rv1 cells grown for 3 days in medium supplemented with FBS or in AD medium supplemented with CDT FBS (CDT). AR immunoblot analysis was performed with the AR441 monoclonal antibody on 8% SDS-PAGE-separated proteins from 50 μg of cytosolic extract and the cell-equivalent amount of nuclear extract for each sample (top panel). The integrity of nuclear/cytosolic fractionation was confirmed by partitioning of NSE to the cytosolic extract (bottom panel). B, DNA binding ability of full-length and truncated AR in CWR22Rv1 cells. Nuclear extracts were prepared from CWR22Rv1 cells cultured in the absence of androgen for 3 days (−) and stimulated with DHT (1 nM) for v1

LNCaP AR (Fig. 2A). CWR22Rv1/lanes C and N). In addition, nuclear ARΔLBD migrated as a doublet, with the upper band becoming more distinctive (i.e., in terms of resolution and content) during androgen withdrawal.

Data obtained from antibody mapping did not provide absolute confirmation of the presence of a DBD in ARΔLBD. We next wanted to determine this by using a functional approach with a DNA pull-down assay. Nuclear extracts from androgen-deprived CWR22Rv1 cells were incubated with biotinylated, double-stranded oligonucleotide probes specific for the ARE and ARR at positions −170 and −400 of the PSA promoter, respectively (25). Under these conditions (3 days of androgen withdrawal), ARΔLBD is the predominant AR in

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increase in size of the AR in CWR22Rv1. Exons 2 and 3 each encode zinc fingers comprising the AR DBD. Therefore, the ∼114-kDa full-length CWR22Rv1 described herein contains an additional zinc finger.

**The AR Exon 3 Duplication Originated in a Relapsed CWR22R Xenograft.** The only AR mutation described previously in the CWR22 xenograft had been the H874Y mutation (27). Because the mutation described herein was identified in a cell line established from a relapsed tumor, we wanted to determine whether it was a somatic mutation originating in the tumor or during the establishment and propagation of this cell line. To this end, we performed RT-PCR analysis of RNA isolated from two separate passages of the original CWR22 xenograft (4152 and 6568) and four separate relapsed CWR22R tumors (i.e., strains 2152, 2272, 2274, and 2524; Fig. 4). In this experiment, RT-PCR analysis was directed to segment V of the AR (Fig. 3A). As expected, the original CWR22 xenografts did not contain the inserted sequence, as demonstrated by their AR amplification products having identical sizes with that of LNCaP. Analysis of samples from the relapsed tumors revealed that one tumor (CWR22R-2152) was indeed positive for the exon 3 duplication observed in CWR22Rv1. CWR22R-2152 is also the tumor from which the CWR22Rv1 cell line was established (20).

**CWR22Rv1 Possesses Androgen-responsive and Androgen-insensitive Features.** The next set of experiments was designed to define the influence of the combinatorial activity of the full-length and truncated AR species on several biological properties of CWR22Rv1, namely, growth, androgen-induced transcription, and PSA secretion. LNCaP and CWR22Rv1 cells proliferated with similar rates in androgen-containing medium over a 5-day period (Fig. 5A). However, these two cell lines displayed markedly contrasting growth properties when they were subjected to androgen deprivation. Whereas LNCaP cells underwent a complete growth arrest by day 3, CWR22Rv1 maintained a proliferative rate indistinguishable from that observed in the presence of androgen, having undergone approximately 4.4 population doublings over 5 days (Fig. 5A). Therefore, proliferation of CWR22Rv1 is not dependent on or enhanced by a physiological concentration (∼0.1 nM) of testosterone present in medium supplemented with 10% FBS. The effects of androgens and antiandrogens were examined more directly by assessing their activities in AD medium (Fig. 5B). Similar to the results in Fig. 5A, CWR22Rv1 proliferation was not stimulated by a physiological concentration of DHT, but it was inhibited by approximately 32% by a supraphysiological level (100 nM). Whereas cell growth was also not modulated by the antiandrogen flutamide, an inhibitory effect upon CWR22Rv1 proliferation could be detected by exposure to concentrations of Casodex as low as 1 nM.

![Fig. 3. Molecular analysis of the CWR22Rv1 AR mRNA. A, the AR mRNA structure was analyzed by a RT-PCR mapping strategy. Primer pairs (“Materials and Methods”) were designed to amplify overlapping regions (segments I–VI) spanning the entire AR cDNA sequence. These segments are depicted relative to schematic representations of the AR mRNA and polypeptide. Exons 1–8, major protein domains, and poly-aminio acid stretches are indicated. B, RT-PCR reactions were performed on total RNA isolated from LNCaP (L) and CWR22Rv1 (C) cells using the primer pairs for the segments described in A. In addition, the 5′-most trinucleotide repeat region (CAG) encoding the first polyglutamine tract was analyzed. Reaction products were analyzed by agarose gel electrophoresis in 2% agarose-TAE gels containing ethidium bromide and photographed under UV illumination. The AR mRNA segments analyzed are indicated by Roman numerals (I–VI) at the top of the gel. The positions of the 100-bp DNA ladder size standards are annotated on the left. C, automated DNA sequencing was performed on purified segment V amplification products from both LNCaP and CWR22Rv1. Sequences were aligned using the ClustalW program, and the CWR22Rv1 insertion sequence was subsequently identified by BLAST analysis. Regions of homology between the CWR22Rv1, LNCaP, and reference human AR sequences are darkly shaded. The CWR22Rv1 insertion (Exon 3) is highlighted with light shading. The resulting gaps in the aligned reference and LNCaP sequences are denoted by dashes above the inserted sequence.

![Fig. 4. RT-PCR analysis of AR in CWR22 and CWR22Rv1 xenografts. Top panel, two separate passages of the original CWR22 xenograft and four relapsed tumor strains were analyzed by RT-PCR using the primer pair for AR segment V (Fig. 3A). RNA isolated from LNCaP and CWR22Rv1 cell lines served as references. Arrows on the left indicate AR amplification products. Bottom panel, the same samples were analyzed for PSA expression.](cancerres.aacrjournals.org)
AN AR MUTATION OCCURRING DURING RELAPSED GROWTH OF CWR22R

A

B

C

Fig. 5. Effects of androgen and antiandrogens on the proliferation of CWR22Rv1 cells. A. LNCaP (■) and CWR22Rv1 (○) cells were cultured in the presence (filled symbols) or absence (open symbols) of androgen as described in “Materials and Methods.” Cells were plated at a density of 2 × 10^4 cells/dish. At the indicated times, cells were enumerated with a hemocytometer. Results are presented as the total number of cells/dish and represent the mean ± SD of three separate determinations for each sample. B. CWR22Rv1 cells were seeded into 24-well plates at a density of 1 × 10^4 cells/well in AD medium. The cells were left untreated (open bars) or exposed to increasing concentrations of DHT (1, 10, and 100 nM), Casodex (1, 10, and 100 nM), and flutamide (0.2, 0.5, and 1.0 µM) as indicated by right-hatched, horizontally lined, and filled bars, respectively. After 5 days, the total number of cells/dish was determined with a Coulter counter. For each set of treatments, results are presented as the mean fractional change in growth ± SD as compared with the untreated controls. C. CWR22Rv1 cells were cultured in androgen-containing medium (FBS) or in AD medium for a duration of 0 (4 h) to 10 days. Cells were harvested daily for immunoblot analysis of AR and NSE expression as described in “Materials and Methods.” The AR full-length and ARΔLBD isoforms are indicated.

AR expression is autoregulated by both transcriptional and translational mechanisms (reviewed in Ref. 28). Whereas AR gene transcription is suppressed by androgen in LNCaP cells, AR protein expression is up-regulated. In CWR22 xenografts, AR protein expression has also been demonstrated to be positively regulated by androgen, decreasing in response to castration-induced androgen withdrawal and returning in the relapsed tumor (10). Although androgen withdrawal did not influence CWR22Rv1 proliferation, we wanted to determine whether or not androgen withdrawal impacted AR expression.

CWR22Rv1 cells were continuously cultured in the absence of serum hormones, and samples for immunoblot analyses were harvested daily for 10 days. AR expression was unchanged throughout the course of the experiment (Fig. 5C). In our studies, both the 114-kDa and the 75–80 kDa AR proteins in lysates obtained from cells cultured in androgen-containing medium migrated as single bands (Fig. 5C, FBS lane). However, shortly after androgen withdrawal (1 day), ARΔLBD began to migrate as a doublet, with the upper band becoming more distinctive (i.e., in terms of resolution) as the duration of androgen withdrawal increased. In fact, the band intensity of the upper species increased until the two proteins comprising the doublet were of comparable content.

The responsiveness of the CWR22Rv1 AR repertoire was investigated by determining the ability of endogenous AR to transactivate an androgen-responsive promoter in a standard luciferase-based reporter assay. LNCaP and CWR22Rv1 cells were transfected with a reporter construct containing the entire ARR of the rat probasin gene promoter [base positions –256 to +11 (29); pGL3E-Probasin] and cultured in the absence or presence of DHT. In both cell lines, DHT induced a dose-dependent stimulation of luciferase activity compared with that of untreated controls (Fig. 6A). Whereas the activity induced by 0.1 nM DHT was similar for both cell lines, clear differences were observed at the highest DHT concentrations. At the 1.0 nM concentration, CWR22Rv1 had approximately 30% less activity than LNCaP (i.e., 14.4-fold versus 20.5-fold, respectively). Furthermore, attenuation of AR activity in CWR22Rv1 was even more apparent after treatment with 10 nM DHT. In contrast to the 62.6-fold induction observed in LNCaP cells, the AR activity in CWR22Rv1 (i.e., 24.2-fold induction) was 61% less than that observed for LNCaP (Fig. 6A). These results demonstrate that although CWR22Rv1 is indeed androgen responsive, AR-mediated transcriptional activation is markedly attenuated (at least in the context of this assay).

Hormone-dependent CWR22 xenografts and relapsed tumors (CWR22R) are known to secrete PSA (18). Therefore, it was critical to determine whether the CWR22Rv1 cell phenotype was also characterized by AI and/or androgen-responsive PSA secretion. This was determined by measurement of PSA secreted into the culture medium using a standard clinical assay technique. LNCaP cells demonstrated a dose- and time-dependent secretion of PSA (Fig. 6B). In response to 1 nM DHT, PSA levels accumulated to concentrations of 101 and 143 ng/ml in the medium after 48 and 72 h, respectively. In contrast, CWR22Rv1 lacked detectable levels of secreted PSA under basal culture conditions (AD medium) and in response to DHT (Fig. 6B). Consistent with these results, PSA protein expression was not detected by immunoblot analysis of lysates from androgen-stimulated CWR22Rv1 cells, whereas it was clearly induced in LNCaP cells exposed to 1 and 10 nM mibolerone (Fig. 6C). In addition, PSA message level was reduced in CWR22Rv1 and the parental CWR22-2152 tumor in comparison with LNCaP and the CWR22 xenografts, respectively (Fig. 4).

Regulation of CWR22Rv1 AR expression in response to exogenous androgen was also examined (Fig. 6C). In LNCaP cells, AR levels decreased in the complete absence of androgen and in the presence of 1 pm mibolerone. Mibolerone concentrations from 10 pm to 1 nM not only restored AR protein levels but augmented them above that observed in cells cultured in the presence of FBS. The expression of the full-length 114-kDa AR in CWR22Rv1 displayed a similar trend to that of the LNCaP AR in response to treatment with the three highest mibolerone concentrations, demonstrative of androgen resistance at least at the level of AR translation and/or protein stability (Fig. 6C). Of note, the appearance of the ARΔLBD doublet was reduced by concentrations of mibolerone that increased AR expression (i.e., 10 pm to 1 nM), but was enhanced in androgen-deprived medium (i.e., 0 and 1 pm mibolerone). These results are consistent with the data presented in Fig. 2A and illustrate the presence of one mode of androgen responsiveness in this model.
AN AR MUTATION OCCURRING DURING RELAPSED GROWTH OF CWR22R

Fig. 6. Androgen responsiveness of the CWR22Rv1 cell line. A, LNCaP and CWR22Rv1 cells were cotransfected with the pGL3E-Probasin androgen-responsive reporter construct plus a Renilla luciferase construct (pRL-SV40). Twenty-four h later, the medium was replaced with AD medium, and the cells were incubated for an additional 24 h in the absence of androgen (open bars) or the presence of 0.1 (right-hatched bars), 1.0 (horizontally lined bars), and 10 nM (filled bars) DHT. RLU contained in the cell lysates were determined with a dual-luciferase reporter assay. Instrument background was controlled in all experimental readings by subtracting the RLU values obtained with lysates from nontransfected cells. Results are expressed as the fold induction in luciferase activity (relative to untreated control cultures) calculated from the ratios of firefly:Renilla luciferase activities in each sample. Data points represent the mean ± SD of three separate determinations for each sample. B, secretion of PSA by LNCaP and CWR22Rv1 cells was assessed by immunomassays of culture supernatants from cells cultured in the absence (open bars) or presence of 0.1 (right-hatched bars) or 1 nM DHT (filled bars) for 48 and 72 h. Results are expressed as the concentration of PSA (ng/ml) in the culture medium and represent the mean ± SD of three separate assays for each sample. C, LNCaP and CWR22Rv1 cells were cultured in medium supplemented with PBS or in AD medium supplemented with 10-fold increasing concentrations of mibolerone for 3 days. Cells were harvested daily for immunoblot analysis of PSA and AR expression as described in “Materials and Methods.”

Exon 3 Duplication Promotes the Generation of ARΔLBD and Alters AR Activity. Having characterized the biochemical and functional properties of the CWR22Rv1 full-length AR and ARΔLBD in the context of CWR22Rv1 cells, we next wanted to investigate the impact of the exon 3 duplication mutation (E3DM) on AR function. Specifically, we wanted to determine whether it could promote the generation of a truncated AR species from the extended full-length receptor and whether it altered AR transcriptional activity. To this end, we generated a hAR-E3DM expression construct by replacing the region spanning exon 3 in a wild-type AR expression construct (hAR-wt) with the region containing E3DM from the CWR22Rv1 AR receptor sequence. 293T cells were transiently transfected with the hAR-wt and hAR-E3DM expression constructs followed by immunoblot analysis of the detergent lysates. As shown in Fig. 7A, expression of the 114-KDa full-length hAR-E3DM was indeed accompanied by the appearance of ARΔLBD, albeit not to the same extent as seen in CWR22Rv1 cells. The influence of E3DM on the androgen-induced transcriptional activity of the AR was examined in transiently transfected PC-3 cells. Whereas hAR-wt displayed a 44.2-fold induction in luciferase activity by 1 nM DHT, hAR-E3DM inducibility was attenuated by approximately 33%, exhibiting only a 29.6-fold induction (Fig. 7B). In these experiments, normalized luciferase activities in unstimulated cells from both transfections were similar (i.e., 3641 and 3021 RLU, respectively), indicating that the E3DM causes a genuine decrease in AR activity. In summary, duplication of exon 3 could recapitulate properties of the original CWR22Rv1 AR, namely, generation of ARΔLBD and diminished AR transcriptional activity.

DISCUSSION

In this report we have identified a novel AR mutation in the CWR22Rv1 cell line and in the serially transplanted relapsed CWR22R-2152 xenograft tumor from which it was established (Figs. 3B and 4). This insertion mutation presumably originated during relapsed tumor growth after castration-induced regression or during serial passage of the CWR22R-2152 tumor because the original, hormone-dependent CWR22 xenograft did not contain this mutation (17). The occurrence of the exon 3 mutation in only one of the serially transplanted CWR22R tumors examined highlights the heterogeneity of CWR22Rv1 cells. This is consistent with previous cytoge-
netic studies of the same CWR22R tumor panel, which demonstrated that each tumor had a distinctive karyotype (21). Based on their results, Kochera et al. (21) suggested that each relapsed tumor occurred as a separate event or was derived by selection for a subpopulation of cells present in the patient’s original, primary tumor. After surveying the AR Gene Mutations Database, this appears to be the first CaP-associated exon duplication and insertion event and is only the second reported CaP-related AR mutation involving exon 3, although numerous exon 3 mutations have been described in AR genes associated with complete or partial androgen insensitivity syndromes (8, 30, 31). During the preparation of this manuscript, Chlenski et al. (32) independently identified the same mutation. Our results not only confirm their data but also describe in vivo, biochemical, and functional properties of the CWR22Rv1 AR. Together, our studies provide insight into the potential influence of this mutant AR on the phenotypic properties of this cell line and its parent tumor.

The expression of the 114-kDa full-length AR observed in CWR22Rv1 lysates is consistent with the duplication and/or insertion of an entire exon 3 sequence (i.e., 117 bp; Figs. 1 and 3). In light of the exon 3 duplication, elucidation of the exact composition of ARΔLBD is a critical issue from a functional standpoint. We calculated that the truncated CWR22Rv1 ARΔLBD is approximately 672 amino acids in length. This was obtained using 80 kDa for the mass of the truncated AR, 110 kDa for the mass of the wild-type AR, and 920 amino acids as the length of the wild-type AR polypeptide. This suggests that this AR contains intact transactivation and DBDs but lacks the LBD. The results of our antibody mapping and DNA binding experiments support this (Figs. 1B and 2B). We have been unable to clone a variant transcript solely encoding ARΔLBD. The resolution obtained during our SDS-PAGE analyses has not enabled an accurate assignment of molecular masses to the truncated AR (Figs. 1 and 2).

Without this information, we favor referring to the mass of this novel clone a variant transcript solely encoding AR

LBD. The resolution
B lacks the LBD. The results of our antibody mapping and DNA binding experiments support this (Figs. 1B and 2B). We have been unable to clone a variant transcript solely encoding ARΔLBD. The resolution obtained during our SDS-PAGE analyses has not enabled an accurate assignment of molecular masses to the truncated AR (Figs. 1 and 2). Without this information, we favor referring to the mass of this novel species as 75–80 kDa because a difference of 5 kDa literally translates into an AR polypeptide being encoded by a single exon 3 (~76 kDa, 633 amino acids) or the tandemly duplicated exon 3 and exon 3′ (~80 kDa, 672 amino acids). The latter case accommodates the inclusion of a complete bipartite nuclear localization signal at the COOH-terminal boundary of the DBD and the hinge region of the AR (Fig. 3C; Ref. 33).

The potential functional significance of ARΔLBD is indicated from previous studies by other investigators that demonstrated that AR forming the LBD retain the ability to bind androgen-responsive DNA elements and have constitutive transactivational transactivation capacity (34–36). Although not yet confirmed, the conservation of the nuclear localization signal (NLS) in ARΔLBD would enable constitutive nuclear localization and DNA binding and is consistent with our findings (Fig. 2, A and B). It is interesting that this AR isoform binds the ARE and ARR in the absence of ligand. Indeed, under androgen-ablative conditions, the nuclear AR component that binds ARE and ARR is comprised almost entirely by the truncated version.

The derivation of the truncated AR species has not been ascertained, but premature termination of translation and/or proteolytic degradation are possible mechanisms and have been proposed previously for the CWR22Rv1 model (32). A truncated AR product (84 kDa) has been observed in another CWR22R-derived cell line (CWR-R1), but the full-length AR in CWR-R1 appeared to be similar in size to both CWR22 and LNCaP (37). The generation of the 84-kDa AR has been attributed to in vitro proteolysis during lysate preparation. Taken together, the generation of a truncated AR in CWR22R tumors and cell lines seems to be a phenomenon that might be independent of the exon 3 insertion. However, insertion of the additional 39 amino acids placed adjacent to the hinge region could certainly render the CWR22Rv1 AR unstable or more susceptible to proteolytic degradation due to alteration in its conformation. Data from our experiments with the recombinant hAR-EDSM construct support this (Fig. 7A) and suggest that levels of cleavage might be modulated by the combined influences of altered AR structure and cellular milieu. We have also investigated the possibility of in vivo proteolytic processing of the full-length AR mediated via caspases and/or proteasomal proteases. The inclusion of specific inhibitors of both classes of activities (caspases, z-VAD-FMK and z-DEVD-FMK; proteasome, lactacystin and MG-132) in the culture medium of the CWR22Rv1 cells neither prevented nor decreased the level of expression of ARΔLBD in comparison with untreated controls that received only vehicle (data not shown).

Based on our data, CWR22Rv1 exhibits a partial androgen-insensitive phenotype. Whereas this cell line exhibits hormone-independent proliferation (Fig. 5A) and expresses an AR capable of activating transcription of a transfected exogenous gene (albeit with reduced efficiency; Figs. 6A and 7B), the ability to activate expression of an endogenous androgen-responsive gene (i.e., PSA) was compromised, and protein expression was absent (Figs. 4 and 6, B and C). A similar scenario of steroid hormone insensitivity in the presence of functional steroid receptors has been described in the S115 mouse mammary tumor model (38). Although an ectopic ARE-containing construct might serve as a suitable promoter for AR-mediated transcription (Fig. 6A), the context (e.g., methylation) of the endogenous PSA promoter might have changed during the progression of CWR22Rv1 (and possibly CWR22R-2152) toward androgen independence and may no longer be suitable to drive AR-mediated transcription. Indeed, methylation of CpG islands in the AR gene promoter and concomitant transcriptional silencing have recently been reported to occur in AR-negative, hormone-refractory CaPs and cell lines (39). Reduced PSA expression and attenuated androgen-responsive transcription have also been described in the androgen-repressed ARCaP cell line that was established from metastatic CaP cells isolated from the ascites fluid of a patient with hormone-refractory disease (40). Furthermore, decreased PSA expression in the CWR22R tumors and cell line (Fig. 4) is consistent with recent microarray expression studies conducted with the CWR22 xenograft model that demonstrated an incomplete reactivation of the AR-induced repertoire of genes during acquisition of androgen independence (9).

Progression of CaP to a hormone-refractory state is characterized by the accumulation of molecular alterations ultimately translating into phenotypic alterations. The results from this study further illustrate the paradigm for the development of hormone-refractory disease mediated by mutations of the AR and/or augmentation of its activity. Furthermore, the AR mutation and its altered forms, as well as the complex phenotype of CWR22Rv1, suggest it depicts a novel or variant stage in CaP tumor progression. That is, an AI phenotype characterized by: (a) an androgen-responsive, full-length form of the AR containing an exon 3 duplication; (b) a COOH-terminally truncated AR that displays AI nuclear localization and constitutive DNA binding and transactivation capacities; (c) attenuated androgen-responsive transcription; and (d) the absence of androgen-induced PSA expression and secretion. The presence of both full-length and truncated AR within the same cell is of potential importance to the evolution of androgen independence. ARΔLBD might function to promote AI growth by constitutively binding to AREs (ARE and ARR) and transactivating androgen-responsive gene transcription, albeit less efficiently (Figs. 2B and 4). It might simultaneously reduce androgen responsiveness by competing with ligand-activated full-length AR for binding to ARE sites. Alternatively, the full-length

8 http://www2.mcgill.ca/androgendb/.
CWR22Rv1 AR containing the duplicated exon 3 (and therefore containing an additional DBD zinc finger) might possess altered transactivation capability (Fig. 7B). In any event, ARΔLBD might serve to assist the cell in achieving androgen independence via bypassing the requirement for androgen while molecular alterations necessary for complete independence from AR signaling are accumulating within the cell. Taken together, the results of our study might be extended to represent a novel mechanism for the generation of AR independence (as seen in the cell lines DU-145 and PC-3) via the acquisition of partial androgen insensitivity.

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