Splicing of a Novel Androgen Receptor Exon Generates a Constitutively Active Androgen Receptor that Mediates Prostate Cancer Therapy Resistance

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Abstract

The standard systemic treatment for prostate cancer (PCa) is androgen ablation, which causes tumor regression by inhibiting activity of the androgen receptor (AR). Invariably, PCs recur with a fatal androgen-refractory phenotype. Importantly, the growth of androgen-refractory PCs remains dependent on the AR through various mechanisms of aberrant AR activation. Here, we studied the 22Rv1 PCa cell line, which was derived from a CWR22 xenograft that relapsed during androgen ablation. Three AR isoforms are expressed in 22Rv1 cells: a full-length version with duplicated exon 3 and two truncated versions lacking the COOH terminal domain (CTD). We found that CTD-truncated AR isoforms are encoded by mRNAs that have a novel exon 2b at their 3′ end. Functionally, these AR isoforms are constitutively active and promote the expression of endogenous AR-dependent genes, as well as the proliferation of 22Rv1 cells in a ligand-independent manner. AR mRNAs containing exon 2b and their protein products are expressed in commonly studied PCa cell lines. Moreover, exon 2b-derived species are enriched in xenograft-based models of therapy-resistant PCs. Together, our data describe a simple and effective mechanism by which PCs can synthesize a constitutively active AR and thus circumvent androgen ablation.

Introduction

Prostate cancer (PCa) is the most commonly diagnosed malignancy in U.S. males and the second leading cause of male cancer mortality (1). In the event that surgery and/or radiation do not cure PCs, systemic therapy is based on inhibiting the androgen receptor (AR). The AR is a 110-kDa steroid receptor transcription factor, which promotes the growth and survival of normal and cancerous prostate cells. Androgen ablation, which blocks the production and/or activity of androgens, initially results in a favorable clinical response. However, PCs invariably recur in a fatal manifestation that is resistant to androgen ablation, as well as further treatments. Importantly, clinical and experimental evidence has shown that this stage of the disease, termed androgen refractory, is associated with mechanisms of aberrant AR activation (2, 3).

The AR shares a modular organization with other steroid receptors and consists of an NH2 terminal domain (NTD), a central DNA-binding domain (DBD), and a COOH terminal domain (CTD). The amorphous NTD makes up nearly 60% of the AR protein and has been resistant to structural determination. The remaining 40% is composed of the DBD and CTD, both of which have been extensively studied structurally and functionally (4–7). The AR NTD and CTD possess separate transcriptional activation domains, termed AF-1 and AF-2, respectively (8). AF-2 maps to a ligand-induced coactivator binding pocket, which is structurally conserved among steroid receptors (6). In contrast to the CTDs of other steroid receptors, the AR CTD in isolation displays very weak transcriptional activity (9). The AR NTD shares virtually no sequence identity with other known proteins and functions as a potent transcriptional activator in isolation (6, 10, 11). The relative role of these domains in mediating the transcriptional activation of AR-regulated genes and the growth and progression of PCs is a critical unanswered question. We have shown that androgen refractory PCs may circumvent androgen ablation through an aberrant, NTD-dependent, but CTD-independent, mode of AR activity (10, 11). This suggests that activation of the AR NTD may be selected for during progression to androgen refractory disease. This hypothesis is supported by the finding that a decoy molecule derived from the AR NTD blocks the growth and progression of PCs in a xenograft-based model of PCa (12).

The androgen refractory 22Rv1 PCa cell line was derived from an androgen-dependent CWR22 PCa xenograft that relapsed during androgen ablation (13). 22Rv1 cells are AD and express the AR-regulated gene, prostate-specific antigen (PSA). AR mRNA in 22Rv1 cells contains a duplication of exon 3, which results in a larger AR protein (hereafter called AREx3dup), consisting of three zinc fingers in its DBD (14). AREx3dup does not exist in the parental CWR22 xenograft and has not been observed in any other PCa tumor or cell line (14). Interestingly, in addition to AREx3dup, a novel short AR isoform is also expressed in 22Rv1 cells that may result from proteolytic cleavage of full-length AREx3dup (14, 15). Antibody mapping has shown that this short AR isoform lacks most or all of the AR CTD but retains an intact NTD and DBD (14). This model therefore provides an excellent opportunity to study AR isoforms that either contain or lack the AR CTD. The purpose of this study was to understand the mechanism by which these AR isoforms regulate the androgen refractory phenotype of 22Rv1 cells.

Materials and Methods

Cell culture. PCs cell lines were purchased and cultured as described (11). LAPC4 cells were provided by Charles Sawyers (Memorial Sloan-Kettering Cancer Center).
**PCA xenografts.** AD and androgen-independent (AI) versions of LuCaP 23.1 and LuCaP 35 PCa xenografts have been described (16, 17). AD xenografts were propagated in BALB/c nu/nu mice and AI xenografts were propagated in SCID mice as described (16, 17). Animals were housed in the Mayo Clinic pathogen-free rodent facility, and all procedures performed were approved by the Mayo Clinic institutional animal care and use committee. When tumors reached ~100 mm³, they were excised and frozen on dry ice. Before RNA and protein extraction, tumor bios (27 mm³) were pulverized under liquid N₂ using a mortar and pestle.

**Plasmids.** Plasmid constructs for full-length AR (h5HBhAR) and MMTV-Luc have been described (10, 11). Plasmid 4XARE-E4-Luc was provided by Dr. Michael Carey (University of California at Los Angeles). Plasmid AP31/2/2b was generated by mutating h5HBhAR to generate an XbaI site within exon 2 using mutagenic primers FW: 5'-GGAAGCTTCAAGGTCTTC- TATGAAAAGACGCCTGAAGG and RV: 5'-CTTCCATTCCTCTTTTCTA- GAAGACCTGGCAGCCT and a Site Directed Mutagenesis kit (Stratagene). Two oligonucleotides were synthesized and annealed (FW: 5'-CTGAAAGAGCCGCGTGAAGATTTTTTCAAGATGAAACAAATTTAA- GAATCATAG and RV: 5'-CTAGC TTATGATTCTTTTAATTTGTTC). A cassette was generated which contained exon 2 sequence downstream from the XbaI site spliced to exon 2b. This cassette was phosphorylated and inserted into XbaI-cut h5HBhAR. The XbaI site within exon 2 was then converted back to wild-type sequence via site directed mutagenesis. The same strategy was used to generate AR2bF and AR2bR, but in this case, the mutagenic primers used were FW: 5'- GGAAGCTTCAAGGTCTTC-TATGAAAAGACGCCTGAAGG and RV: 5'- CTTCAGACCTGGCAGCCT and oligoC1-0001 used for cassette generation were FW: 5'-CTGAGGGATTATGAAATTTTAATTTGTTC- GAAACAAATTTAAAGATCATATT and RV: 5'-CTAGC TTATGATTCTTTTAATTTGTTC-

**Western blot.** PCA cell lines and xenografts were subjected to Western blot analysis exactly as described (11). Blots were incubated with antibodies specific for AR (Santa Cruz, X-20, A-442, or N-20) or extracellular signal-regulated kinase-2 (ERK-2; Santa Cruz, D-2) exactly as described (11).

**Cell viability and proliferation assays.** Electroporated 22Rv1 cells were seeded at a density of 3,000 per well on 96-well plates in RPMI 1640 + 5% CSS. After 24 h growth, medium was replaced with RPMI 1640 + 5% CSS. Activity and cell proliferation were determined by MTS reduction (Cell Titer 96 AQueous One, Promega) as described (10). Cell proliferation assays were performed using the exact same approach. BrdUrd incorporation was measured 24 h after DHT/vehicle treatment using a Cell Proliferation Assay kit (Promega) via manufacturer specifications.

**Results**

**Short AR isoforms mediate ligand-independent AR activity in 22Rv1 cells.** To assess whether exon 3 duplication imparts gain of function to the AR in 22Rv1 cells, we quantified expression of AR target genes via real-time reverse transcription–PCR (RT-PCR). For comparison, we used the VCaP PCA cell line, which harbors wild-type AR protein. After 24 h treatment with the synthetic androgen, mibolerone, the androgen-responsive PSA and TMPRSS2 (19) genes were strongly up-regulated in VCaP, but not 22Rv1, cells (Fig. 1A). In line with these findings, mibolerone, an androgen-repressed tumor suppressor gene (20), showed stronger inhibition in VCaP than 22Rv1 cells. These findings suggest that AREx3dup responds more poorly to androgens than wild-type AR.

One explanation for these findings could be that AR-regulated genes are already expressed at a high level in the absence of androgens in 22Rv1 cells. To test for ligand-independent AR activity in 22Rv1 cells, we knocked down the AR via small-interfering RNA (siRNA). Unexpectedly, we observed that siRNA targeted to AR exon 7 selectively ablated AREx3dup but had no effect on expression of the short AR isoform in these cells (Fig. 1B). Moreover, these experiments showed that the previously described short AR isoform (14) occurs as a doublet. Whereas the AR exon 7–targeted siRNA selectively ablated AREx3dup, an siRNA targeted to AR exon 1 ablated all AR isoforms (Fig. 1B). We used a mouse mammary tumor virus (MMTV)–regulated promoter reporter in these experiments to monitor AR activity. siRNA-mediated ablation of AREx3dup abolished MMTV-Luc androgen-responsiveness but had no effect on ligand-independent MMTV-Luc activity (Fig. 1B). Conversely, a significant inhibition of ligand-independent MMTV-Luc activity was observed...
Figure 1. Short AR isoforms specifically mediate ligand-independent transcriptional activation of AR target genes. A, RNA was isolated from VCaP and 22Rv1 cells grown in vehicle or 1 nmol/L mibolerone (Mib) and subjected to quantitative real-time RT-PCR using primers specific for PSA, TMPRSS2, and maspin. Expression levels are relative to vehicle treatment, which was arbitrarily set to 1. B, 22Rv1 cells were transfected with MMTV-Luc, nontargeted control (CTRL) siRNA, or siRNAs targeted to AR exon 1 or exon 7 (schematic on right; DBD*, three zinc-finger DBD). Cells were grown 24 h in serum-free medium and treated with 1 nmol/L Mib or ethanol (vehicle control) for 24 h. Luciferase activity was determined. Columns, mean from at least three independent experiments each performed in duplicate; bars, SE. MMTV promoter activity without androgens and siRNAs was arbitrarily set to 1. Lysates from vehicle-treated cells were subjected to Western blot using a polyclonal antibody targeted to the AR NTD. ERK-2 levels were measured as a control. C, 22Rv1 cells were transfected with nontargeted control (CTRL) siRNA, or siRNAs targeted to AR exon 1 (siAR-1) or 7 (siAR-7). RNA was isolated from transfected cells grown for 72 h without androgens. RT-PCR was performed using primers specific for hK2, PSA, TMPRSS2, NKX3.1, SCAP, and maspin. Expression levels are relative to vehicle treatment, which was arbitrarily set to 1.

upon ablation of the short AR isoforms. Similarly, ligand-independent expression of the endogenous AR target genes hK2 (21), PSA, TMPRSS2, and NKX3.1 (22) was strongly inhibited by siRNA targeted to AR exon 1 but not AR exon 7 (Fig. 1C). Expression of maspin and SCAP, an AR-activated gene (23), were not affected by AR-targeted siRNAs. These results suggest that AREx3dup mediates weak androgen-dependent AR activity in 22Rv1 cells, whereas the shorter AR isoforms mediate constitutive, albeit selective, ligand-independent AR activity. Differential ablation by exon 1–targeted versus exon 7–targeted siRNA shows that AREx3dup and the short AR isoforms are derived from distinct mRNA species. Therefore, contrary to previous reports (14, 15), the short AR isoforms expressed in 22Rv1 cells is not a proteolytic cleavage product of AREx3dup.

Short AR isoforms mediate androgen-independent proliferation of 22Rv1 cells. To test whether selectively knocking down AREx3dup and the short AR isoforms also selectively affected androgen-dependent versus androgen-independent cell growth, we performed growth assays on 22Rv1 cells 96 hours after transfection with AR-targeted siRNAs (Fig. 2A). In line with RT-PCR and reporter gene assays (Fig. 1), ablation of AREx3dup inhibited androgen-dependent, but not androgen-independent, 22Rv1 growth (Fig. 2B). Conversely, ablation of the short AR isoforms inhibited growth of 22Rv1 cells in the absence of androgens (Fig. 2B). Cell proliferation assays with BrdUrd showed that ablation of the short AR isoforms specifically inhibited the ability of 22Rv1 cells to proliferate in the absence of androgens (Fig. 2C) but did not affect levels of apoptosis (data not shown). These findings suggest that AREx3dup specifically mediates the growth of 22Rv1 cells in response to androgens, whereas the short AR isoforms specifically promote the androgen-independent proliferation of 22Rv1 cells.

Identification and characterization of a novel AR exon. Our data show that the short AR isoforms expressed in 22Rv1 cells mediate critical features of the androgen refractory phenotype. Our siRNA data also shows that these short AR isoforms are translated from mRNA species distinct from AREx3dup. We therefore generated cDNA from 22Rv1 RNA and performed 3′ RACE with an exon 1–anchored primer to isolate any potential AR mRNAs with novel 3′ ends (Fig. 3A). This approach yielded two discrete ~800 and ~900 bp PCR products, which were reduced by exon 1–targeted but not exon 7–targeted siRNA (Fig. 3A). Cloning and sequencing of these products revealed the presence of a novel 17-bp sequence spliced to either exon 2 or exon 3 (Fig. 3B). Using human genome sequence data, we mapped this sequence to a region of chromosome 17q12 that is not predicted to be spliced (Fig. 3C). This approach revealed the presence of a novel 17-bp sequence spliced to either exon 2 or exon 3 (Fig. 3A and B). Using human genome sequence data, we mapped this sequence, which we termed exon 2b, to a region ~37 kb downstream from the AR exon 2 (Fig. 3B). Genomic sequence immediately following this exon 2b sequence tag was followed by an A-rich segment, which suggested the oligo(dT) primer used for 3′ RACE had generated a premature 3′ cDNA end. Therefore, we designed a second 3′ RACE primer, which was targeted to a sequence downstream from a putative translation stop codon in...
exon 2b (Fig. 3C). This approach yielded a ~90-bp to 120-bp PCR product, the expression of which was selectively reduced in 22Rv1 cells treated with exon 1–targeted siRNA (Fig. 3C). Cloning and sequencing revealed that this additional sequence contained the remainder of exon 2b, which was polyadenylated at several sites 91-bp to 113-bp downstream from the beginning of the exon (Fig. 3B).

Examination of exon 2b sequence indicated that mRNAs consisting of exons 1/2/2b would generate a truncated AR consisting of the NTD, the first zinc finger in the DBD, and 11aa of exon 2b–derived sequence (Fig. 3B). An mRNA consisting of exons 1/2/3/2b would only exist in cells, such as 22Rv1, where exon 3 has been duplicated within the AR locus. Exons 2 and 3 have the same reading frame; therefore, an mRNA consisting of exons 1/2/3/2b would generate a second truncated AR consisting of the NTD, the entire two zinc finger DBD, and exon 2b–derived sequence (Fig. 3B). We confirmed the existence of contiguous mRNAs consisting of exons 1/2/2b and 1/2/3/2b in 22Rv1 cells via RT-PCR using primers anchored within exons 1 and 2b (Fig. 3B and D). To confirm that mRNAs harboring exon 2b gave rise to the short AR isoforms observed in 22Rv1 cells (hereafter called AR1/2/2b and AR1/2/3/2b), we designed two separate siRNAs targeted to AR exon 2b. As expected, these siRNAs knocked down the expression of AR1/2/2b and AR1/2/3/2b but did not affect the expression of AR

AR isoforms encoded by exons 1/2/2b and 1/2/3/2b are constitutively active. We next performed functional tests of AR1/2/2b and AR1/2/3/2b by cotransfecting AR-null DU-145 cells with expression vectors and AR-responsive reporter constructs (Fig. 4D). Expression of full-length AR (ARex3) rendered both MMTV-Luc and 4XARE-E4-Luc responsive to androgens. Expression of either AR1/2/2b or AR1/2/3/2b induced strong activation of these reporters in the absence of androgens. As expected, androgen treatment had no effect on the transcriptional activities of these short AR isoforms. These results show that both AR1/2/2b and AR1/2/3/2b can independently function as constitutively active transcription factors.

AR1/2/2b expression in PCa cell lines. Expression of the AR1/2/2b isoform should be restricted to cells with duplication of exon 3, which permits the generation of pre-mRNAs with exon 2b downstream of exon 3. However, synthesis of mRNAs encoding AR1/2/2b could be achieved in any cell type with a normal AR locus. Our finding that AR1/2/2b is a constitutively active AR isoform highlights exon 2b splicing as a simple and effective mechanism by which PCa could circumvent androgen ablation. Therefore, to assess if AR mRNAs composed of exons 1/2/2b exist outside of the 22Rv1 model system, we performed RT-PCR with oligo(dT)-primed cDNAs generated from androgen-dependent LNCaP, VCaP, and LAPC4 PCa cell lines (Fig. 5A and B). After 35 cycles of amplification, we observed a faint PCR product from VCaP cDNA, which corresponded to the product composed of exons 1/2/2b from 22Rv1 cells (data not shown). However, using a two-step RT-PCR strategy, wherein an initial 20-cycle PCR was diluted and used for a second 35-cycle PCR, we observed products of the expected size in LNCaP, VCaP, and LAPC4 cells (Fig. 5B). Cloning and sequencing confirmed these products resulted from splicing of exons 1/2/2b. In addition to this exons 1/2/2b product, we also observed a product of ~600 bp generated from LAPC4 cDNA. Interestingly, cloning and sequencing revealed that this product resulted from splicing of exon 1 to exon 2b. As a control, we also tested for generation of full-length AR mRNA using exon 1 and exon 4 PCR primers. Products of the expected size were generated with cDNAs from LNCaP, VCaP, and LAPC4 PCa cells. Together,

![Figure 2](attachment:image.png)

**Figure 2.** Short AR isoforms specifically mediate ligand-independent growth of androgen-refractory 22Rv1 cells. 22Rv1 cells were transfected with nontargeted control (CTRL) siRNA or siRNAs targeted to AR exon 1 (siAR-2) or 7 (siAR-1). A, transfected cells were cultured 72 h posttransfection, lysed, and subjected to Western blot using a polyclonal antibody targeted to the AR NTD. B, transfected cells were seeded and cultured for 24 h in medium supplemented with 5% CSS. Cells were treated with 1 nmol/L DHT or ethanol (EtOH, vehicle control). The relative number of viable cells 0 and 96 h after exposure to these compounds was determined by MTS assay. Values shown are relative to siCTRL-treated cells without androgens, which was arbitrarily set to 100%. Columns, mean from two experiments performed in quadruplicate; bars, SE. C, transfected cells were seeded and treated as described in B. The relative number of proliferating cells 24 h after treatment was determined by BrdUrd incorporation. Values are relative to siCTRL-treated cells grown without androgens, which was arbitrarily set to 100%. Columns, mean from two experiments performed in quadruplicate; bars, SE.
these results show that the cellular machinery required for exon 2b inclusion is not restricted to 22Rv1 cells.

To quantitatively compare the amounts of full-length AR mRNA and AR exon 1/2/2b mRNAs in these cell lines, we performed absolute quantitation using a standard curve of known target concentrations. For measurement of full-length AR mRNA, we used an exon 2b–anchored forward primer indicated in B. PCR products were cloned and sequenced, which revealed 3’ termini 91-113 bp downstream from the start of exon 2b. These putative sites of polyadenylation are indicated in B. RT-PCR was performed on RNA used in A and C with the exon 1–anchored forward primer used for 3’ RACE and a reverse primer indicated in B. PCR products were cloned and sequenced and confirmed to consist of spliced exons 1/2/2b or 1/2/3/2b.

E, 22Rv1 cells were transfected with nontargeted control (CTRL) siRNA or siRNAs targeted to exon 1 (siAR-2) or 2b. Transfected cells were cultured 48 h posttransfection, lysed, and subjected to Western blot using a polyclonal antibody targeted to the AR NTD.

Figure 3. Short AR isoforms are encoded by novel mRNAs containing a novel AR exon 2b. A, 22Rv1 cells were transfected with nontargeted control (siCTRL) siRNA or siRNAs targeted to AR exon 1 (siAR-2) or 7 (siAR-1) as indicated. RNA was isolated and subjected to 2-step 3’-RACE using exon 1–anchored forward primers. PCR products were cloned and sequenced. A schematic of this approach and resultant sequence data are shown (right). B, relative location of exon 2b in the AR locus and summary of 3’ RACE and RT-PCR experiments. Details are discussed in the text.

C, a second 3’ RACE reaction was performed on RNA as described in A using an exon 2b–anchored forward primer indicated in B. PCR products were cloned and sequenced, which revealed 3’ termini 91-113 bp downstream from the start of exon 2b. These putative sites of polyadenylation are indicated in B. D, RT-PCR was performed on RNA used in A and C with the exon 1–anchored forward primer used for 3’ RACE and a reverse primer indicated in B. PCR products were cloned and sequenced and confirmed to consist of spliced exons 1/2/2b or 1/2/3/2b.

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E, 22Rv1 cells were transfected with nontargeted control (CTRL) siRNA or siRNAs targeted to exon 1 (siAR-2) or 2b. Transfected cells were cultured 48 h posttransfection, lysed, and subjected to Western blot using a polyclonal antibody targeted to the AR NTD.
isolated total RNA from AD and androgen-refractory/AI versions of LuCaP tumors that had been propagated in intact and castrated mice, respectively. Similar to our observations in PCa cell lines, we detected mRNAs resulting from splicing of exons 1/2/2b in these xenografts (Fig. 6A). The absolute abundance of both full-length AR mRNA and exon 1/2/2b mRNA were increased in AI versus AD samples for both LuCaP 23.1 and 35 tumors (Fig. 6B). To further support this observation, we performed Western blotting on lysates from AD and AI LuCaP 23.1 and 35 tumors (Fig. 6C). Using two separate antibodies targeted to the AR NTD, we observed immunoreactive species with the exact same mobility as AR1/2/2b from 22Rv1 lysates (Fig. 6C). These species were not detected using an AR CTD-directed antibody, which further indicated they likely represented AR1/2/2b protein. Importantly, increased expression of this putative AR1/2/2b species was observed in AI versus AD xenografts, which was similar in magnitude to the increase observed for AR 1/2/2b mRNA. We also observed significantly more full-length AR protein in AI versus AD xenografts, which has been previously reported (24). Our data suggest that increased expression of AR1/2/2b may play a previously unrecognized role in mediating the androgen refractory phenotype of LuCaP 23.1 AI and LuCaP 35 AI xenografts.

Discussion

Nuclear receptor CTDs serve a dual function, possessing both the LBD and AF-2 coactivator binding surface. The model for activation of the AR and other steroid receptors is that ligand binding induces an AF-2 conformation that is permissive for docking with NR boxes (LxxLL and related motifs) of coactivator molecules, which are required for transcriptional activation. For the AR, this model is complicated by the fact that AR AF-2 can also bind an FxxLF motif in the AR NTD and thus mediate an interaction between the NH2 and COOH termini (9, 25). In vitro assays, the affinity of AF-2 for AR-derived FxxLF peptides is higher than for coactivator-derived LxxLL peptides (6, 26). As a result, the importance and role of AR AF-2 in transcriptional activation of target genes has been a topic of debate. Indeed, AR AF-2 functions as a very weak ligand-dependent transcriptional activation domain in isolation (9, 27, 28). However, the isolated AR NTD is stronger in reporter gene assays than the isolated AR CTD (27, 29). Our previous studies have shown that the AR NTD may play a major role in mediating PCa therapy resistance (10, 11). The data we present in this study supports this concept and has shown for the first time that naturally occurring AR isoforms, consisting of the NTD and DBD, can support the expression of many endogenous AR target genes, as well as the growth of androgen-refractory PCa cells in the absence of androgens.

Various mechanisms of resistance to androgen ablation therapy have been proposed and shown in various models of PCa progression. For example, mutations in the AR LBD broaden the ligand specificity of the AR, thus permitting activation by alternative steroids or even antiandrogens (2). AR overexpression,
which may result from AR gene amplification and/or enhanced AR transcription, has been shown to sensitize the AR to castrate levels of androgens (24, 30). Indeed, tissue androgens in androgen refractory PCa may persist at levels sufficient to elicit AR activation and may arise from aberrant intratumor androgen production (31–33). All of these mechanisms are ligand-dependent in nature and have called into question the importance of ligand-independent AR activation for the development and progression of androgen-refractory PCa. However, in this study, our data show that the androgen-refractory phenotype of 22Rv1 cells is due to novel AR isoforms that function in a completely ligand-independent fashion. Moreover, these isoforms would be completely resistant to all current androgen ablation regimens, which are targeted to the AR CTD. Our observation that other commonly used PCa cell lines possess the machinery required to synthesize the ligand-independent AR\textsuperscript{Ex1/2/2b} isoform provides a novel and simple avenue for these PCa cells to achieve androgen-independence. Indeed, in two xenograft-based models of PCa progression, we observed increased expression of AR exon 1/2/2b mRNA in AI versus AD tumors. Moreover, we observed a similar increased expression of a 75-kDa AR species in these AI versus AD tumors. Our data strongly suggest that this protein species is AR\textsuperscript{Ex1/2/2b}; however, a definite conclusion will have to await the development of antibodies specific for this AR isoform. Importantly, in these models, AR\textsuperscript{Ex1/2/2b} overexpression was accompanied by overexpression of full-length AR, which has been previously described (24). Because mRNAs encoding both full-length AR and AR\textsuperscript{Ex1/2/2b} are spliced from the same pre-mRNA, enhanced transcription of the AR gene would result in enhanced overall expression of both isoforms. It will therefore be important to assess the relative contributions of AR and AR\textsuperscript{Ex1/2/2b} overexpression to PCa progression during androgen ablation.
Two previous studies have investigated the nature of the small AR isoform(s) expressed in 22Rv1 cells and have concluded that these species arise from calpain-mediated cleavage of AR<sup>Ex3dup</sup> (14, 15). Our data oppose this conclusion and show that the AR NTD/DBD isoforms expressed in 22Rv1 cells arise from splicing of a novel AR exon 2b after either AR exon 2 or exon 3. AR exon 2b spliced after exon 2 or exon 3 gives rise to protein species possessing the entire AR NTD fused to the first zinc finger of the AR DBD (termed ARE<sup>1/2/2b</sup>) or the entire AR NTD fused to the complete AR DBD (termed ARE<sup>1/2/3/2b</sup>), respectively. Structural studies with AR DBD show that the first zinc finger, encoded by exon 2, harbors the recognition helix that directly engages with one hexameric half-site in an androgen response element (ARE; ref. 5). The second zinc finger, encoded by exon 3, mediates dimerization with an AR molecule engaged with the neighboring ARE half-site (5). Our finding that ARE<sup>1/2/2b</sup> is able to constitutively activate AR-responsive promoters shows that the first zinc finger is sufficient for the AR to engage with AREs, which agrees with this structural data. However, because the second exon 3-encoded zinc finger is responsible for AR dimerization and subsequent stabilization of ARE-bound ARs, the repertoire of genes that are activated by AR<sup>1/2/2b</sup> may be diminished compared with the repertoire of genes that are activated by full-length, liganded AR. Dissecting the differential patterns of gene activation by AR<sup>1/2/2b</sup> and full-length, liganded AR will be important for understanding the relative abilities of these isoforms to mediate PCa growth.

In summary, our data indicates that AR truncation, resulting from aberrant splicing of a novel AR exon 2b, couples androgen signaling from AR activity in 22Rv1 cells. AR mRNA and protein containing exon 2b were also identified in commonly used PCa cell lines. Our data further shows that AR mRNA species containing exon 2b were enriched in xenograft-based models of PCa progression. Therefore, inclusion of exon 2b in AR mRNAs represents an effective mechanism by which constitutively active AR proteins can be produced that are completely resistant to current CTD-directed PCa therapies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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