Androgen Receptor Expression in Androgen-independent Prostate Cancer Is Associated with Increased Expression of Androgen-regulated Genes

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ABSTRACT

The human prostate cancer (CaP) xenograft, CWR22, mimics human CaP. CWR22 grows in testosterone-stimulated nude mice, regresses after castration, and recurs after 5–6 months in the absence of testicular androgen. Like human CaP that recurs during androgen deprivation therapy, the recurrent CWR22 expresses high levels of androgen receptor (AR). Immunohistochemical, Western, and Northern blot analyses demonstrated that AR expression in the androgen-independent CWR22 is similar to AR expression in the androgen-dependent CWR22 prior to castration. Expression of prostate-specific antigen and human kallikrein-2 mRNAs, two well-characterized androgen-regulated genes in human CaP, was androgen dependent in CWR22. Despite the absence of testicular androgen, prostate-specific antigen and human kallikrein-2 mRNA levels in recurrent CWR22 were higher than the levels in regressing CWR22 tumors from 12-day castrate mice and similar to those in the androgen-stimulated CWR22. Other AR-regulated genes followed a similar pattern of expression. Differential expression screening identified androgen regulation of α-enolase and α-tubulin as well as other unknown mRNAs. Insulin-like growth factor binding protein-5, the homeobox gene Nkx3.1, the AR coactivator ARA-70, and cell cycle genes Cdk1 and Cdk2 were androgen regulated in CWR22. In recurrent CWR22, the steady-state levels of all these AR-dependent mRNAs were similar to those in the androgen-stimulated CWR22, despite the absence of testicular androgen. Expression of AR and AR-regulated genes in the androgen-deprived recurrent CWR22 at levels similar to the androgen-stimulated CWR22 suggests that AR is transcriptionally active in recurrent CWR22. Induction of these AR-regulated genes may enhance cellular proliferation in relative androgen absence but through an AR-dependent mechanism. Alternatively, in androgen-independent tumors, induced expression of the AR-regulated gene network might result from a non-AR transcription control mechanism common to these genes.

INTRODUCTION

AR is a member of the subfamily of steroid receptors and the larger family of nuclear receptors that function as transcription factors. AR is activated by androgen, binds specific nucleotide sequences known as androgen response elements, and interacts with other factors to control transcription of androgen-regulated genes, thereby stimulating development and function of the male reproductive tract (1–9). CaP is androgen dependent (10), and its growth is mediated by a network of AR-regulated genes that remains to be fully characterized.

MATERIALS AND METHODS

Transplantation of CWR22 Tumors

Nude mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). CWR22 tumors were transplanted into nude mice containing s.c. testosterone pellets (12.5 mg for sustained release of ~10 μg/day; Innovative Research of America, Sarasota, FL) as described previously (17). Testosterone pellets eliminated the wide variation in serum testosterone of male nude mice and maintained a serum concentration of ~4.0 ng/ml. Intact mice bearing androgen-stimulated tumors and castrated mice (testes and testosterone pellets eliminated the wide variation in serum testosterone of male nude mice and maintained a serum concentration of ~4.0 ng/ml. Intact mice bearing androgen-stimulated tumors and castrated mice (testes and testosterone pellets were removed) carrying regressed or recurrent CWR22 tumors were exposed to methoxyflurane and sacrificed by cervical dislocation. Tumors were harvested rapidly and frozen in liquid nitrogen or fixed in 10% buffered formalin for paraffin embedding. All procedures used were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

RNA Isolation and Northern Hybridization

Total RNA was isolated from CWR22 tumors as described (19). RNA resuspended in sterile H2O was glyoxylated and fractionated through 1.0% agarose gel. The abbreviations used are: AR, androgen receptor; CaP, prostate cancer; hK-2, human kallikrein-2; IGFBP, insulin-like growth factor binding protein; Cdk, cyclin-dependent kinase; CK, castrate; PSA, prostate-specific antigen; TP, testosterone propionate; ARA-70, AR-associated protein-70.

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agaro-gels and transferred to Biotrans nylon membrane (ICN Biomedicals, Inc., Aurora, OH). cDNA probes were labeled with [32P]dCTP (Amerham Corp., Arlington Heights, IL) using the Prime-a-Gene System (Promega Corp., Madison, WI). Membranes were hybridized in aqueous solution (5X SSC, 5X Denhardt’s solution, 1% SDS, and 100 µg/ml salmon sperm DNA) overnight at 68°C. After washing at 50°C for 1 h in 0.1X SSC, 0.1% SDS, the membranes were exposed to X-ray film (Eastman Kodak Co., Rochester, NY) with an intensifying screen at -80°C. hK-2 cDNA was kindly provided by Dr. Charles Y-F. Young (Mayo Clinic, Rochester, MN). 1×EcoRI cDNA was from Dr. Albert S. Baldwin (University of North Carolina at Chapel Hill). IGFBP cDNAs were provided by Dr. Shunichi Shimasaki (Scripps Research Institute, La Jolla, CA).

**Western Immunoblot Analysis of AR**

Lysates were prepared from frozen CWR22 tumors. Tumor tissue (100 mg) was pulverized in liquid nitrogen, allowed to thaw on ice, and mixed with 1.0 ml of RIPA buffer with protease inhibitors (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM pepstatin, 4 µM aprotinin, 80 µM leupeptin, and 5 mM benzamidine). Tissue was homogenized on ice for 30 s using a Biohomogenizer (Biospec Products, Inc., Bartlesville, OK). Two µl of 0.2 m phenylmethylsulfonyl fluoride were added and the homogenates incubated 30 min on ice. Homogenates were centrifuged at 10,000 × g for 20 min; supernatants were collected and centrifuged again to prepare the final lysate. Supernatant protein (100 µg) from each sample was electrophoresed in 12% SDS-polyacrylamide gels, followed by electroblotting to Immobilon-P membrane (Millipore Corp., Bedford, MA) and immunodetection. Anti-human AR monoclonal antibody F39.4.1 (Biogenex, San Ramon, CA) was used at a 1:10,000 dilution. LCNP cell lysate was used as a positive control on immunoblots. Secondary antibody (goat-anti-mouse 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 and Ki-67**

Formalin-fixed, paraffin-embedded sections of tumor tissue were processed for AR immunostaining using anti-human AR monoclonal antibody F39.4.1. After deparaffinization and rehydration, tissue sections were heated at 100°C for 30 min in a vegetable steamer in the presence of antigen retrieval solution (CITRA, pH 6.0; Biogenex). F39.4.1 antibody was used at a concentration of 0.13 µg/ml and dianinobenzidine was used to detect immunoperoxidase antigen-antibody reaction products.

Ki-67 immunostaining for cellular proliferation was performed with the MIB-1 monoclonal antibody (Oncogene, Cambridge, MA) at an IgG concentration of 0.5 µg/ml (1:50). All other steps were as described for AR immunostaining. Automated digital image analysis to determine the percentage of tumor cells expressing Ki-67 was performed as described (20).

**cDNA Library Construction and Screening**

**Library Construction.** Five µg of poly(A)+ mRNA was used to construct directional cDNA libraries in lambda ZAP II (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The primary library from intact, testosterone-stimulated CWR22 represented 0.95 × 10⁶ unique clones (>99% recombinant). The primary library from the recurrent CWR22 represented 1.8 × 10⁶ unique clones (91% recombinant). Both libraries were amplified once. The average insert size for the libraries was 1.5 kb.

**Library Screening.** Lambda phage plaques from the primary unamplified CWR22 library were plated at low density. Two or three individual plaques were placed into each 1.5-ml Eppendord tube containing 500 µl of suspension media plus chloroform. Ten µl of phage stock were PCR amplified in 50 mM KCI, 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 0.001% gelatin, 50 µm primers (standard M13 forward and reverse primers), 0.1 mM deoxyxynucleotide triphosphates, 1.5 units of Taq (Perkin-Elmer, Foster City, CA), and 0.25 unit cloned Pfu (Stratagene) in a 60-µl reaction. PCR conditions were 95°C for 3 min, 55°C for 3 min, 68°C for 15 min for 1 cycle, then 95°C for 30 s, 55°C for 1 min, and 72°C for 3 min (36 cycles), followed by a 10-min incubation at 72°C. PCR products were run on duplicate 1.5% Tris-borate-EDTA agarose gels. After electrophoresis, the gels were photographed and treated for 20 min in 0.4 m NaOH. DNA was transferred to a positively charged nylon membrane (Biotrans, ICN, Costa Mesa, CA) in 0.4 m NaOH by overnight capillary transfer. After transfer, membranes were washed for 10–15 min in 2× SSC followed by 5 min in double-distilled H₂O. Membranes were air-dried and stored at 4°C until hybridization.

Radiolabeled first-strand cDNA was prepared from poly(A)+ mRNA as described previously (21) and used to screen the CWR22 intact library by Southern hybridization. Probes from CWR22 intact and 2-day CX mice were hybridized with duplicate Southern blots (30 lanes, each lane containing a pool of 2–3 pfu). Prehybridization of the duplicate Southern was done in 5× SSC, 5× Denhardt’s, 1% SDS, and 100 µg/ml single-strand DNA at 68°C for 2–3 h. Hybridization was performed overnight at 68°C using either CWR22 intact or CWR22 2-day CX cDNA probes at 10⁶ cpn/ml. After hybridization, the blots were washed briefly in 2× SSC-0.1% SDS, followed by 1 h at 50°C in 0.1× SSC-0.1% SDS. Autoradiographs of the duplicate Southern blots hybridized to intact and 2-day CX cDNA probes were analyzed to identify bands with differential hybridization to the two probes. Phage plagues corresponding to bands tentatively identified as being androgen-regulated were plaque purified and reanalyzed by Southern hybridization to confirm androgen regulation. Confirmation of androgen regulation of the mRNA was done using Northern blot analysis. Blots containing total RNA from CWR22 intact, 2-day CX, 6-day CX, 6-day CX + 24 h of TP, and recurrent CWR22 tumors were hybridized to radiolabeled probe prepared from plaque-purified phage.

**RNase Protection Assay**

The RiboQuant Multiprobe RNase Protection Assay System (PharMingen, San Diego, CA) was used to quantitate mRNA transcripts for Cdk1–4 and p16. Ten µg of total RNA from each of the CWR22 tumors were hybridized with the radiolabeled hCC-1 multiprobe template set (PharMingen) according to the manufacturer’s instructions. After RNase treatment, protected probes were resolved on 5% polyacrylamide sequencing gels, followed by exposure of the dried gel to BioMax MR film (Kodak) overnight at -70°C.

**RESULTS**

AR protein expression in CWR22 human CaP xenografts was determined using immunohistochemical and Western blot analyses. AR staining in nuclei of epithelial cells was abundant in CWR22 tumors from androgen-stimulated intact mice (Fig. 1A) and decreased 1–4 days after castration (Fig. 1B) during the period of most rapid apoptosis. At 6 days after castration (Fig. 1C), AR protein was increased and remained so at 12 days (not shown). In the CWR22 tumor that recurred 5 months after androgen withdrawal (Fig. 1D), the intensity of AR staining and strong nuclear localization was similar to that observed in CWR22 tumors from intact androgen-stimulated mice. Western blotting yielded similar results (Fig. 2). AR in the intact androgen-stimulated CWR22 decreased within 1 day after castration, increased 6 and 12 days after castration, but was less than in the recurrent tumor. In the recurrent tumor, AR protein levels were similar to those in CWR22 from androgen-stimulated intact mice. AR mRNA expression was determined by Northern analysis. A normalized transcript of 9.6 kb was present, and bands of similar intensity were obtained with CWR22 tumors from intact androgen-stimulated mice and recurrent CWR22 tumors from CX mice (Fig. 3).

Differential expression screening of cDNA libraries constructed from CWR22 tumors was performed to identify androgen-regulated genes in human CaP. A total of 1652 plaques were screened initially by this method (936 from intact CWR22 library and 696 from the recurrent CWR22 library). A second screening to eliminate false positives resulted in 48 regulated clones. Northern blot analysis confirmed androgen regulation of 24 clones. PSA was identified twice, whereas seven clones had no homology to DNA sequences in GenBank (National Center for Biotechnology Information, Bethesda, MD). The remaining 15 clones had sequence homology to transcripts identified previously. Both α-1-antitrypsin and α-tubulin showed strong
up-regulation by androgen, whereas testosterone-repressed prostate
message-2 was down-regulated. Vascular endothelial growth factor
and mitochondrial hinge protein mRNAs were up-regulated by an-
drogen but <2-fold (data not shown). Several ribosomal protein
mRNAs and other unknown transcripts were regulated minimally
either in an up or downward direction.

The effects of castration and androgen replacement on mRNAs for
a prostate-specific homeobox gene, Nkx3.1, and the human kallikrein
gene, hK-2, both of which are androgen-regulated in human prostate
(22–24), were demonstrated using Northern hybridization (Fig. 4).
IGFBP-5 was androgen up-regulated, whereas other IGFBP mRNAs
(IGFBP-2, IGFBP-3, and IGFBP-6) were unchanged (data not shown). We also found that the AR coactivator ARA-70 was androgen
up-regulated. As shown in Fig. 4, the levels of all these mRNAs were
decreased 6 and 12 days after castration when compared with the
levels in CWR22 tumors from androgen-stimulated intact mice. Treat-
ment of the 6- and 12-day CX mice with TP (0.1 mg/animal) for 24,
48, or 72 h increased the level of each of these mRNA transcripts,
implicating AR in the control of their expression. In the CWR22
tumor that recurred in the absence of testicular androgen, levels of all
these androgen-regulated mRNAs increased compared with castrates
and were similar to those in CWR22 tumors from androgen-stimu-
lated intact mice (Fig. 4).

Androgen regulation of genes controlling cell cycle progression in
CWR22 tumors was investigated using RNase protection assays to
compare the levels of mRNA transcripts encoding the Cdk5 (Cdk 1–4)
and the Cdk inhibitor p16 (Fig. 5). Androgen withdrawal from
CWR22 tumors for 6 or 12 days resulted in down-regulation of Cdk1
and Cdk2 and up-regulation of p16. Testosterone treatment of 6- or
12-day CX animals resulted in up-regulation within 24 h of Cdk1 and
Cdk2 and down-regulation of p16. In recurrent CWR22, Cdk1 and
Cdk2 were expressed at levels similar to those of the androgen-
stimulated CWR22 from intact mice. However, levels of p16 were
variable in 10 different recurrent CWR22 tumors examined. Cdk4
levels did not change appreciably after castration or with testosterone
replacement. Ki-67 immunostaining, which correlates well with bro-
modeoxyuridine incorporation and thymidine labeling (25), was used
to estimate cellular proliferation in CWR22 tumors (Fig. 6). The
percentage of Ki-67-positive cells decreased from 79.4 ± 3.6 in
tumors from intact mice to 0.8 ± 0.5 at 6 days after castration.
Injection of TP (0.1 mg) increased Ki-67 immunostaining to
4.5 ± 0.42 within 24 h and to 31.0 ± 6.7% positive within 48 h.
CWR22 tumors that recurred in the absence of testicular androgen
demonstrated a high percentage of Ki-67-positive cells (67.1 ± 3.3)
similar to that of CWR22 tumors from intact mice.
Several factors are known to control AR gene transcription in mammalian cell lines (27–38); however, the specific factors that induce AR expression in CaP remain unknown. Increased AR expression in a significant number of androgen-independent CaPs may result from amplification of the AR gene locus. AR gene amplification was present in ~30% of CaPs that relapsed after androgen deprivation but in none of the original primary tumors (39). In a series of 54 locally recurrent CaPs from patients who survived for 1 year or more on androgen deprivation therapy, comparative genomic hybridization and interphase fluorescence in situ hybridization demonstrated amplification in 15 tumors (40). Amplification was associated with increased expression of AR mRNA and may contribute to the AR expression in recurrent CWR22; however, this remains to be determined.

AR and androgens are essential for development, growth, and function of the prostate. In human and other mammalian species, castration results in apoptosis and loss of androgen-dependent prostate cells. In normal prostate cells that survive androgen deprivation, AR does not become reactivated in the absence of testicular androgen (41). However, in CaP cells, genetic alterations occurring during the months after androgen deprivation might turn on signaling mechanisms and reactivate AR-induced transcription independent of androgen or at a lower concentration of androgen or other steroids (42). AR activation might result from direct effects on AR that alter its structure or phosphorylation state or indirect effects involving protein-protein interactions with AR coactivators or repressors. Modulators of protein kinases A and C might activate AR in the absence of ligand by altering AR phosphorylation (43–46). Other AR-activating pathways might be mediated by transmembrane receptors for growth factors. Studies on CaP cell lines suggested that keratinocyte growth factor, epidermal growth factor, and IGF-I signaling pathways activate AR in recurrent CWR22 based on decreased steady-state levels after castration and increased levels after testosterone replacement. In the recurrent tumors, the levels of all androgen-regulated mRNAs were higher than in CWR22 tumors after castration and similar to levels in androgen-stimulated tumors from intact mice. This novel observation is consistent with AR induction of gene transcription in androgen-independent CaP. It suggests also that growth of androgen-independent tumor cells is stimulated by a network of AR-regulated genes.

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the absence of androgen (47). With respect to IGF-I, our results suggest a mechanism whereby its signaling may be enhanced in the recurrent tumor. Because IGFBP-5 is up-regulated by AR and is a positive modulator of IGF-I in some tissues (48), IGFBP-5 may amplify IGF-I activation of AR in the recurrent tumor thereby creating a self-perpetuating autocrine feedback loop at the level of the IGF-I receptor. Moreover, PSA and hK-2 are AR-regulated members of the human kallikrein family of serine proteases that degrade the IGF-I inhibitor, IGFBP-3 (48). Thus, the increased expression of these proteases in the recurrent tumor could further enhance the activity of IGF-I.

PSA and hK-2 genes are transcriptionally up-regulated by androgen and contain androgen response elements in their 5′-flanking regions (49–53). Additional upstream PSA gene enhancer elements have been identified that amplify androgen responsiveness in a cell type-specific manner, suggesting that other DNA-binding proteins cooperate with AR in amplifying the transcriptional response (49). Studies on several AR-regulated genes indicated that DNA-binding proteins, some of which are androgen-regulated, cooperate with AR in the induction of gene transcription (3–6, 54–57). However, these interacting factors remain to be characterized, and as yet no factor has been shown to induce transcription of these genes in the absence of AR. This leads to the question: What accounts for the increased expression of PSA and hK-2 in CaP that recur after androgen deprivation? The increase in serum PSA associated with tumor recurrence during androgen deprivation treatment has often been explained on the basis of increased tumor volume; however, our studies on CWR22 demonstrate that PSA mRNA is increased per tumor weight. In a recent clinical study of 46 patients undergoing treatment for CaP, de Vere White et al. (11) obtained results on AR and PSA similar to our findings in CWR22. In patients who underwent a relatively short period of “combined androgen blockade” for downsizing prior to radical prostatectomy, most residual CaP expressed AR but low to undetectable PSA, whereas CaP that relapsed after a longer period of androgen blockade expressed both AR and PSA, indicating a link between AR and PSA expression in androgen-independent CaP. Factors that modulate PSA expression in androgen-responsive cells may act by enhancing the action of AR. PSA-stimulating activity was identified by Hsieh et al. (58) in conditioned medium of an LNCaP cell subline derived from a s.c. xenograft that exhibited relapsed growth after castration of the host male nude mouse. The conditioned medium was active in the absence of testosterone; however, the LNCaP cells expressing PSA were androgen responsive, suggesting the possibility that AR could have mediated the increase in PSA expression. Similarly, vitamin D (1,25-dihydroxyvitamin D₃) increased PSA secretion and inhibited growth of cultured LNCaP cells through what was believed to be an effect on cell differentiation (59). Moreover, Gleave et al. (60) demonstrated an increase in LNCaP PSA mRNA expression by treatment with transforming growth factor β that was independent of cellular proliferation.

The finding that several AR-controlled genes are up-regulated in androgen-independent CWR22 strengthens the concept that AR has a role in mediating androgen-independent growth and emphasizes further the importance of identifying AR-regulated, growth-promoting genes. ARA-70 was first identified in a thyroid carcinoma as a RET fusion gene in which 790 5′-nucleotides of the ARA-70 gene were fused to the tyrosine kinase domain of the RET oncogene. The full significance of this rearrangement remains to be established; however, the RET gene is mutated frequently in papillary thyroid carcinomas (61). ARA-70 is reported to be an AR coactivator and potentiates the transcriptional activity of AR in DU-145 cells cotransfected with a reporter gene (62). Its up-regulation in recurrent CWR22 could amplify effects of other ligand-independent AR activators.

The Nkx3.1 gene encodes a 234-amino acid homeodomain protein and is a homologue of the Drosophila Nk-3 gene (63). Human and mouse proteins are identical within the homeodomain region. In mouse prostate and seminal vesicle, Nkx3.1 mRNA increases in parallel with androgen stimulation of sexual development, and castration reduces mRNA levels in sexually mature mice (64). Nkx3.1 mRNA was demonstrated in prostate and to a lesser extent in testis in adult human males. It is also expressed in LNCaP cells and up-regulated by androgen. Expression was not detected in the PC-3 or DU-145 androgen-independent cell lines that lack AR (23). The Nkx3.1 gene maps to chromosome 8p2.1, a region commonly mutated in prostate cancer. However, in 50 CaP tissue specimens removed at radical prostatectomy, only one deletion was found, and no sequence-altering mutation was detected in the Nkx3.1 coding region (65). Chromosome 8 alleles are present in both CWR22 and recurrent CWR22 (16), and their Nkx3.1 mRNAs are of equal size. Homeodomain proteins have the ability to route extracellular signals to specific target genes through protein-protein interactions (66–68). Thus, Nkx3.1 may integrate a number of growth-promoting signals in both the androgen-dependent and androgen-independent CWR22.

α-Enolase is the glycolytic enzyme that converts 2-phosphoglycerate to phosphoenolpyruvate, the precursor of pyruvate. Glycolysis is believed to have an important role in DNA synthesis (69). Oxidative decarboxylation of pyruvate to form acetyl-CoA links glycolysis with the citric acid cycle. Human and rat prostate secrete large amounts of citric acid formed by the reaction of acetyl-CoA with oxaloacetate and H₂O. Citric acid production is androgen dependent. In the rat, androgens increase the expression of pyruvate dehydrogenase-E1 that catalyzes the conversion of pyruvate to acetyl-CoA and mitochondrial aspartate aminotransferase that converts aspartate to oxaloacetate. The latter was regulated by a combined increase in transcription rate and mRNA stabilization (70–72).

α-Tubulin and β-tubulin subunits, together with their associated proteins, make up the mitotic apparatus, a microtubule machine for separating chromosomes. α-Tubulin has been considered a constitutive housekeeping gene; however, our data indicate its expression is regulated either directly or indirectly by androgen in CWR22. The relatively slow increase in α-tubulin mRNA in response to androgen

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Fig. 6. Quantitative analysis of Ki-67 immunostaining in CWR22 tumors. Cellular proliferation decreased 80-fold 6 days after castration of CWR22-bearing mice. TP (0.1 mg/animal) administration to 6-day CX mice caused a ~5-fold increase at 24 h and a 30-fold increase after 48 h. Recurrent CWR22 tumors showed a percentage of Ki-67-positive cells similar to that seen in androgen-stimulated CWR22 tumors from intact mice. Bars, SE.
suggests it is a secondary response gene perhaps controlled by multiple gene products, some of which are androgen-regulated. Androgen regulation of α-tubulin may link AR action to cell division in CaP. Cdk5 together with cyclins control the normal progression of cells through the cell cycle. Abrupt activation of Cdk5 may potentiate the growth of certain cancers (73), including CaP. Cdk2 and Cdk4 were positively regulated by androgen in cultured LNCaP cells in association with increased cellular proliferation (74). Cdk1 and Cdk2 mRNAs in androgen-dependent CWR22 decreased after castration and were up-regulated by androgen replacement. In the recurrent CWR22, Cdk1 and Cdk2 mRNAs were expressed at levels similar to those in androgen-dependent CWR22 tumors from intact mice. Cdk4 mRNA remained unchanged after castration or androgen replacement. The up-regulation of Cdk1 and Cdk2 mRNAs after testosterone replacement in 6-day CX mice coincided with an increase in cellular proliferation (as assessed by Ki-67 immunohistochemistry). Whereas protein synthesis and subsequent phosphorylation or dephosphorylation is required for Cdk complex formation with cyclins, our data suggest that AR induction of Cdk1 and Cdk2 mRNA expression is an important step in reactivating the cell cycle. Cdk1, which appeared to be strongly androgen-regulated, complexes with cyclin B and is activated by dephosphorylation just prior to mitosis (75). Negative regulation of the Cdk inhibitor p16 in CWR22 would diminish the inhibitory effect of p16, further enhancing the initiation of cellular proliferation. However, p16 mRNA levels were variable in recurrent CWR22 tumors. Chi et al. (76) found similar variability in p16 mRNA expression in 116 human prostate tissue specimens, with 43% of untreated primary CaP showing reduced expression.

Our results indicate that a network of androgen-regulated genes has a role in driving the growth of androgen-independent CaP. Furthermore, they provide a conceptual framework within which to investigate further the role of AR and to identify additional gene products important in the recurrence of CaP after androgen deprivation. The expression of AR and androgen-regulated genes in androgen-independent CaP suggests AR is active in the absence of testicular androgen. Alternatively, a compensatory transcriptional or mRNA-stabilizing mechanism common to androgen-regulated genes might become activated in the absence of androgen stimulation. Pursuit of these possibilities should lead to a better understanding of the transition of CaP from androgen-dependent to androgen-independent growth.

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