Androgen Receptor–Dependent PSA Expression in Androgen-Independent Prostate Cancer Cells Does Not Involve Androgen Receptor Occupancy of the PSA Locus

Li Jia and Gerhard A. Coetzee

Department of Urology and Preventive Medicine, Norris Cancer Center, USC Keck School of Medicine, Los Angeles, California

Abstract
It is widely suspected that androgen-independent prostate cancer growth depends on androgen receptor signaling via ill-defined mechanisms. Prostate-specific antigen (PSA) expression is often used to measure androgen receptor activity in cells and prostate cancer progression in patients. In the present study, we have compared androgen receptor activity using PSA and human male germ cell–associated kinase (hMAK), as read-outs in androgen-dependent LNCaP and androgen-independent C4-2B cells. As expected, very little PSA and hMAK expression were detected in LNCaP cells in the absence of androgens, whereas substantial expression of PSA was observed only in C4-2B cells under the same conditions. The addition of dihydrotestosterone to the culture medium increased the expression of both genes in both cell types. Comprehensive chromatin immunoprecipitation analysis of the entire PSA locus and an androgen-response element in hMAK unexpectedly revealed that androgen receptor was not occupying any site in the absence of dihydrotestosterone in either cell type. In line with the expression data, and in the absence of dihydrotestosterone, histone acetylation and RNA polymerase II occupancy was substantial at the PSA locus in C4-2B but not in LNCaP cells. In the presence of dihydrotestosterone, androgen receptor was found to occupy mainly the enhancer region of PSA in both cell types, accompanied with increases in histone acetylation and RNA polymerase II occupancy. Although the androgen receptor was not directly involved in the androgen-independent expression of PSA in C4-2B cells, small interfering RNA knock-down of androgen receptor significantly reduced PSA expression in both the presence and absence of dihydrotestosterone. In contrast, hMAK expression was decreased only in the presence of dihydrotestosterone after androgen receptor knock-down. We conclude that androgen-independent expression of PSA in C4-2B cells does not rely on the direct occupancy of the androgen receptor at the PSA locus, but is nevertheless affected indirectly via unknown androgen receptor–dependent mechanism(s) that influence the expression from some but not all androgen receptor target genes. (Cancer Res 2005; 65(17): 8003-8)

Introduction
Androgen ablation therapy of prostate cancer, although initially efficacious, nearly always leads to treatment resistance and progression of the disease (1). Although the disease is then referred to as androgen-independent, it simply reflects resistance to the original therapy protocol. Mechanisms underlying androgen independence are thought to involve an adaptation of androgen receptor signaling to function under low or absent androgen levels (2). Prostate-specific antigen (PSA) is an androgen-responsive gene and its expression is often used as a measure of androgen receptor signaling and as a monitor of prostate cancer progression (3). Human male germ cell–associated kinase (hMAK) is a newly identified target gene of the androgen receptor that may participate in prostate cancer cell physiology (4). Because the role of the androgen receptor in androgen-independent prostate cancer is not clearly defined, we have embarked on a detailed analysis of androgen receptor signaling measuring PSA and hMAK expression in LNCaP and C4-2B cells. LNCaP cells were originally obtained from a needle aspiration biopsy of the left supravclavicular lymph node of a 50-year-old Caucasian male with confirmed diagnosis of metastatic prostate cancer (5). The cells are responsive to dihydrotestosterone in terms of growth and PSA expression. Although the androgen receptor in LNCaP cells is mutated (6), it is generally normal with respect to transactivation activities (7). C4-2B is a subline of LNCaP obtained by passage and growth in castrated athymic mice (8). The cells are no longer responsive to hormone manipulation in animals (although they still express a functional androgen receptor; ref. 9) and metastasize readily to lymph nodes and bone in athymic mice. Our results reveal the striking finding that androgen receptor–dependent PSA expression in C4-2B cells does not involve androgen receptor occupancy at the PSA locus.

Materials and Methods
Cell culture and materials. Human prostate cancer LNCaP cells, obtained from the American Type Culture Collection (Manassas, VA) and C4-2B, obtained from ViroMed Laboratories (Minneapolis, MN), were both maintained in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum. 5α-Dihydrotestosterone was purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies were anti–androgen receptor NH2-terminal (N20), anti–androgen receptor COOH-terminal (C19) anti-Pol II (N20), anti–actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-AcH3 (Upstate Biotechnology, Inc., Lake Placid, NY) and anti-PSA (DAKO Corp., Carpinteria, CA). Immunoblotting was done as described previously (10), with the indicated antibodies.

Real-time reverse transcription-PCR. After the indicated treatments of LNCaP and C4-2B cells, total cellular RNA was prepared using SV Total RNA Isolation System (Promega, Madison, WI). A two-step reverse transcription-PCR method was employed using the TaqMan Gold RT-PCR kit (Applied Biosystems, Branchburg, NJ) and primers and probes for PSA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression were as previously described (10). Primers and probe for hMAK expression were: 5′-GGTCAACAGCCACCATACCTGTA-3′ (forward); 5′-ACTTCCAACACGCCTCAAACATCAGTGA-3′ (reverse); 5′-FAM-CCAGATGTTAATGCCTGGAGATTT-BHQ1-3′ (probe). Triplicate PCR reactions were done. GAPDH mRNA expression was analyzed for each sample in parallel. The results are represented as PSA or hMAK/GAPDH mRNA ratios.
Transient transfection and luciferase assays. LNCaP (6 × 10⁵ cells/well) and C4-2B (3 × 10⁶ cells/well) were plated in six-well plates and grown in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum (CSS) for 2 days. Cells were then transfected with an androgen receptor–responsive reporter, pGL3-PSA5.85 (2 µg/well) using LipofectAMINE 2000 (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s protocol. pGL3-PSA5.85 (a gift from Dr. Hong-Wu Cheng) was constructed by inserting the entire 5.85 kb PSA upstream sequence into pGL3 basic vector (11). After transfection, cells were grown in phenol red-free RPMI 1640 containing 5% CSS with dihydrotestosterone or vehicle control for 24 hours. Luciferase activity determinations in cell lysates were conducted as previously described (10).

Chromatin immunoprecipitation assays. LNCaP (6 × 10⁵ cells/150 mm dish) and C4-2B (3 × 10⁶ cells/150 mm dish) were cultured in phenol red-free RPMI 1640 supplemented with 5% CSS for 3 days. Cells were treated with dihydrotestosterone or vehicle control for 4 hours, and chromatin immunoprecipitation (ChIP) assays were conducted as described previously (10). DNA samples from ChIP preparations were analyzed by real-time PCR using an iCycler optical system (Bio-Rad, Hercules, CA) and AmpliTaq Gold PCR master mix (Applied Biosystems). The primers and probes (synthesized by Biosearch Technologies, Novata, CA) were (as they relate to the positions at the PSA locus; see Fig. 2):

A 5'-GCTTGGATCTGAGAGATACTAC-3' (forward)
5'-CAACTTTTTTTTTTCTGGAGTGTG-3' (reverse)
5'-6-FAM-TGCAAGGTGCTGCTCTTACAACATC-BHQ-1-3' (probe)

B 5'-CTGGGGAGCACAATCAGA-3' (forward)
5'-GTGTGATGCTGATGATGTTG-3' (reverse)
5'-6-FAM-CATGAAAGATGCCCCAGAGGCCTTG-BHQ-1-3' (probe)

C 5'-CAGTGGGCAAGCACAATCTGA-3' (forward)
5'-AACATCTCCAAGTCTTACATACA-3' (reverse)
5'-6-FAM-CCCAAC-GCAATTAACCTAACAAG-BHQ-1-3' (probe)

D 5'-TTCAGAGGTAGTGATCATTAG-3' (forward)
5'-GGAGACGGAGCAGGAGATC-3' (reverse)
5'-6-FAM-CAGCTCAGTTAGCTGAGTCCTGGC-BHQ-1-3' (probe)

E 5'-CCTAGATGAGGCTCTAGATAGA-3' (forward)
5'-GGAAGGAGGA-GCTAGACTTGG-3' (reverse)
5'-6-FAM-CAATTACTAGATACCCCTCTTTGATGACAGCAG-GH-BHQ-1-3' (probe)

F 5'-CACCCCAGCTCTAGATGAGG-3' (forward)
5'-GAGCCAGGGACTGCTCTGTA-3' (reverse)
5'-6-FAM-CTCCAGCAGACATTAGCTGCTG-BHQ-1-3' (probe)

G 5'-ATCTAGATGACCTGCTGTCAG-3' (forward)
5'-GCCAAGGTGCTTTGTATACC-3' (reverse)
5'-6-FAM-TCATTCTACACTTGATGACGCAGCAGC-3' (probe)

The primers and probes (synthesized by Biosearch Technologies) were (as they relate to the indicated hMAK locus; see Fig. 4):

- 5'-AGAAGTGGAGAGCAGCTGAA-3' (forward)
- 5'-CAACTAAGAATCAGGGATGAGTA-3' (reverse)
- 5'-6-FAM-CCAAACACATCAGCAGCTTAATGTGG-TAGBHQ-1-3' (probe)

Duplicate immunoprecipitations for each antibody and triplicate PCR reactions for each immunoprecipitation sample were done. The results are given as a percentage of input.

Small interfering RNA transfection. C4-2B cells (1.5 × 10⁶ cells/well) were plated in six-well plates and grown in phenol red-free RPMI 1640 containing 5% CSS for 2 days. Cells were transfected with one of two androgen receptor small interfering RNA (siRNA) duplexes. The first was sense, 5'-UGAUCACUCGAGUGUT-3', and antisense, 5'-AGUCUGAUCGAGUGUT-3', directed against the coding region of androgen receptor mRNA. The second was sense, 5'-ACGCUUACGUUATT-3' and antisense, 5'-GCAUAUAAGACCGGGUUU-3', directed against the 3'- untranslated region of androgen receptor mRNA. A nonspecific siRNA duplex, 5'-AGACUUGCCUGGGGUATT-3' and 5'-UCCGAGAUGAGCGAGUUGU-3' was used as a control. All siRNA duplexes were at a final concentration of 100 nmol/L and transfected using OligoectAMINE reagent (Invitrogen) according to the manufacturer’s instructions. After transfection, cells were grown in phenol red-free RPMI 1640 containing 5% CSS for 48 hours and then treated with dihydrotestosterone or vehicle control for 18 hours. Total RNA and protein extractions were conducted for reverse transcription-PCR and immunoblot analyses, respectively, as described above.

Results

PSA and hMAK expression in LNCaP and C4-2B cells. In LNCaP cells, virtually no detectable PSA expression was observed in the absence of androgens as measured by immunoblot of the PSA protein (Fig. 1A) or expression of PSA mRNA (Fig. 1B and C). On the other hand, PSA expression was substantial in C4-2B cells in the absence of androgens (Fig. 1A-C). In contrast, the low level of hMAK expression in the absence of dihydrotestosterone stimulation was not higher in C4-2B cells compared with LNCaP (Fig. 1D). Addition of dihydrotestosterone increased the expression of PSA and hMAK in both cell lines; expression values of PSA in C4-2B cells in the absence of dihydrotestosterone were similar or even higher than values in LNCaP cells in the presence of androgens. Even at low concentrations of dihydrotestosterone, C4-2B expressed more than an order of magnitude higher PSA mRNA than did LNCaP cells at any given dihydrotestosterone concentration (Fig. 1C). However, when a transiently-transfected PSA-driven luciferase reporter was used, virtually no detectable luciferase expression was measured in the absence of androgens in either LNCaP cells or C4-2B cells (Fig. 1E). Therefore, the observed ligand-independent expression of endogenous PSA in C4-2B cells is somehow related to the chromatin-integrated nature of the native PSA gene (see below).

Chromatin immunoprecipitation analysis of the PSA and hMAK loci. In previous work, we began to define transcriptional activity at the PSA locus in terms of androgen receptor and cofactor occupancies and histone modifications (7, 10, 12). We have now extended the analysis to interrogate seven sites at the locus spanning the enhancer, promoter, an exon and 3'-untranslated region of the gene (Fig. 2A). Androgen receptor recruitment, histone H3 acetylation and RNA polymerase II occupancy were analyzed in LNCaP and C4-2B cells in the absence or presence of dihydrotestosterone stimulation (Fig. 2B). The IgG-negative controls gave very low signals throughout in both cell lines. No androgen receptor occupancy was detected in either cell line in the absence of dihydrotestosterone using an NH₂-terminal androgen receptor antibody (N20; Fig. 2B) or a COOH-terminal antibody (C19; Fig. 3), whereas significant occupancy was observed at the enhancer (A) and to a lesser extent at the promoter (E) in both cell lines in the presence of dihydrotestosterone (Figs. 2B and 3). This is an interesting finding considering the high expression levels of PSA in C4-2B cells in the absence and presence of dihydrotestosterone (orders of magnitude higher than in LNCaP cells), yet no androgen receptor was found at the PSA locus in the absence of dihydrotestosterone, and in the presence of dihydrotestosterone, androgen receptor occupancy was lower in C4-2B than in LNCaP cells. The fact that this result was obtained using androgen receptor antibodies directed to two different epitopes indicated that it is unlikely that epitope masking...
is the reason for the lack of androgen receptor detection at these loci in the absence of dihydrotestosterone treatment. The results, therefore, indicate that a significant proportion of PSA expression occurs either via non–androgen receptor mechanisms or androgen receptor–dependent mechanisms not related to androgen receptor occupancy at the PSA locus in C4-2B cells. Histone H3 acetylation and RNA polymerase II occupancy was substantially higher in C4-2B than LNCaP cells in the absence of dihydrotestosterone (at A, B, E, and F sites) in line with dihydrotestosterone-independent expression of PSA in these cells. This indicates that these regions, especially E and F, are in readily accessible chromatin, which likely facilitate the recruitment of general basal transcription factors and RNA polymerase II to mediate the observed transcription of PSA mRNA.

Dihydrotestosterone addition resulted in a further increase of these variables in C4-2B cells, which was now also detected in LNCaP cells. Overall, these results indicate that PSA expression in the absence of dihydrotestosterone occurs in C4-2B cells in the absence of androgen receptor occupancy at this locus.

The same ChIP DNA obtained for the experiment depicted in Fig. 2 was assessed for an androgen-response element located about 3.5 kb upstream from the transcription start site of the hMAK gene (Fig. 4).

The androgen-response element (GGAACATGATGGCCT) was identified by scanning the 5'-upstream region of the hMAK gene using ConSite (web-based prediction of regulatory elements; ref. 13). In the absence of dihydrotestosterone, very low to negligible androgen receptor occupancy, histone acetylation or polymerase II occupancy were detected at this site in both cell lines consistent with the very low expression levels of this gene under these conditions. Note that this result is in contrast with the observations at the PSA locus in C4-2B cells. The addition of dihydrotestosterone to the medium resulted in increases in all the parameters, especially in C4-2B cells, again consistent with expression values obtained for this gene under these conditions. The only inconsistency noted between ChIP results and expression levels of the two genes is the lack of androgen receptor occupancy at the PSA enhancer in the absence of dihydrotestosterone in C4-2B cells, whereas substantial expression of PSA occurred under these conditions in the cells. We therefore tested whether the androgen receptor is even involved under these conditions.

Androgen receptor involvement in PSA and hMAK expression levels in the absence of dihydrotestosterone in C42B cells. To determine whether the androgen receptor was involved, even indirectly, in PSA expression in the absence of dihydrotestosterone in C4-2B cells, we knocked down the androgen receptor using a siRNA approach and measured PSA mRNA expression (Fig. 5). As a negative control, we also assayed the expression of hMAK using the same cDNA preparation, and as a positive control, the expression of these genes when the cells were stimulated by dihydrotestosterone. In the experiment depicted, we used the siRNA directed against the coding region of androgen receptor mRNA (see
Materials and Methods). Androgen receptor was knocked-down successfully as revealed by immunoblot analysis (Fig. 5A). This maneuver caused a substantial inhibition of both androgen-dependent and -independent PSA expression roughly to the same extent as the androgen receptor protein was knocked down (Fig. 5B). Similar results were obtained using a siRNA directed against the androgen receptor mRNA 3'-untranslated region (data not shown), indicating that the results were not due to off-target effects.

Figure 2. ChIP analysis at PSA locus. A, schematic representation of the PSA gene. ARE, androgen response element; CRE, cyclic AMP-responsive element; black box, real-time PCR targeted regions; arrow, transcription start site. B, LNCaP and C4-2B cells were incubated in phenol red–free RPMI 1640 containing 5% CSS for 3 days and then treated with or without dihydrotestosterone (10 nmol/L) for 4 hours. Androgen receptor occupancy (using N20 antibody), histone H3 acetylation and RNA Pol II recruitment were examined by ChIP analyses. The value of each immunoprecipitation sample was obtained from the average of triplicate real-time PCR assays from duplicate immunoprecipitations; columns, mean; bars, ± SD. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were done.

Figure 3. Androgen receptor ChIP with COOH-terminal androgen receptor antibody. LNCaP and C4-2B cells were incubated in phenol red–free RPMI 1640 containing 5% CSS for 3 days and then treated with or without dihydrotestosterone (10 nmol/L) for 4 hours. Androgen receptor occupancies on PSA enhancer and promoter were examined by ChIP analyses using the antibody against androgen receptor COOH terminus. The value of each immunoprecipitation sample was obtained from the average of triplicate real-time PCR assays from duplicate immunoprecipitations, and are presented as percentage input and represent mean values ± SD. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were done.
of the siRNA. Using the same cDNA, no equivalent decrease in the low but measurable level of hMAK expression was observed (Fig. 5C). The decrease in dihydrotestosterone-stimulated activity observed with the nonspecific siRNA treatment might be related to a general siRNA effect or possibly due to stimulation of the IFN system elicited by double-stranded RNA (14). This was not seen in the PSA expression activity in the absence of dihydrotestosterone. The fact that androgen receptor knock-down caused PSA expression inhibition (but not hMAK expression inhibition) in the absence of dihydrotestosterone, indicates that the androgen receptor is somehow involved in PSA expression specifically. The involvement, however, seems to be indirect because no androgen receptor occupancy at the androgen receptor enhancer was apparent in our ChIP analyses in the absence of dihydrotestosterone treatment in C4-2B cells (Figs. 2 and 3).

Discussion

The androgen receptor signaling axis is involved in all phases of prostate cancer, from genetic predisposition through disease progression, including the development of resistance to androgen
ablation therapies. Resistance to androgen ablation is not due to a loss of androgen responsiveness, but in most cases, is associated with altered androgen receptor activity (reviewed in refs. 2, 15, 16). Such aberrant androgen receptor signaling is thought to include androgen receptor hypersensitivity to androgens, androgen receptor insensitivity to antagonists, increased androgen receptor expression levels, or androgen receptor activity modulation by nonsteroidal signaling pathways. Although the details of these molecular processes are largely unknown, they may be linked, operating together in a given tumor. It was recently shown that increased androgen receptor expression levels are necessary and sufficient to convert prostate cancer to an ablation-resistant state (17).

The main finding of the present work, which adds another dimension to the notions referred to above, is that the androgen receptor is involved in PSA expression in the absence of androgen in the androgen-independent cell line, C4-2B, without occupying regulatory sites in the PSA upstream control region. What are the possible mechanisms to explain this? This question is not trivial because as stated above, androgen-independent receptor activity may be one of the main mechanisms governing growth in androgen-independent, advanced prostate cancer.

One possibility to explain our findings is that the androgen receptor may affect the transcription of other transcription factors that in turn activate PSA expression. Candidates for such indirect effects are general transcription factors like SP1 or transcription factors affected by non-steroid hormone signals like cyclic AMP–responsive element binding protein, for which response elements exist at the PSA locus. We recently analyzed the involvement of cyclic AMP–responsive element binding protein at the PSA locus, and although we could define an enhancer with cooperation between the androgen receptor and cyclic AMP–responsive element binding protein via p300/CBP, its contribution to overall transcription was relatively small and could only be observed by protein kinase A stimulation with forskolin (18). This does not exclude the involvement of other transcriptional factors in the androgen-independent activation of PSA expression with which the androgen receptor may cooperate in an indirect fashion.

Another and even more intriguing mechanism of androgen receptor–dependent, but androgen-independent PSA expression, may be related to the nongenomic actions of the androgen receptor as proposed to be active in many mammalian cell types (19). Such nonclassical androgen receptor mechanisms may work via classic nonsteroidal signaling pathways affecting any number of other transcription factors. For example, it was reported that androgen receptor forms a triple complex containing androgen receptor/p85α/Src affecting phosphoinositide-3-kinase/Akt activation and thus mediating androgen-induced cell growth and survival (20). More recently, it was reported that the Src-HEK-1-2-ERK-1/2-cyclic AMP–responsive element binding protein pathway, associated with the transition of LNCaP cells to androgen-independence, correlates with the nongenotropic actions of androgen receptor where nuclear translocation and the ability of the androgen receptor to bind to DNA were not prerequisites for androgen receptor activity (21). The challenge therefore will be to identify and understand the actions of such factors and signaling mechanisms not only to better understand the progression of androgen-independent prostate cancer, but also to devise schemes of better therapies for this life-threatening disease.

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References

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