Androgen Receptor Activity at the Prostate Specific Antigen Locus: Steroidal and Non-Steroidal Mechanisms

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Abstract

Ligand-activated androgen receptors (ARs) occupy target genes and recruit histone modifiers that influence transcriptional competency. In LNCaP prostate cancer cells, the natural ligand 5α-dihydrotestosterone (DHT) activates transiently transfected AR-responsive promoter constructs; concurrent treatment with the protein kinase A activator forskolin enhanced AR stimulation induced by DHT. Additional treatment with the cytokine IL-6, purportedly an AR activator, markedly inhibited receptor activity. To assess AR activity on natural chromatin-integrated promoters/enhancers, we determined AR occupancy of the endogenous prostate specific antigen (PSA) promoter/enhancer as well as PSA expression in LNCaP cells treated with DHT; AR occupancy of the PSA enhancer was rapid (within 1 h of stimulation), robust (10-fold over background), and sustained (8–16 h). In contrast, AR occupancy of the PSA promoter was only increased by 2-fold. Histone H3 acetylation at both the enhancer and promoter was evident 1–2 h after DHT treatment. Detectable pre- and mature PSA mRNA levels appeared after 1 and 6 h treatment, respectively. Substantial qualitative and quantitative differences in PSA expression and AR occupancy of the PSA enhancer were observed when DHT-induced and ligand-independent activations of the AR were compared; forskolin stimulated PSA mRNA and protein expression, whereas IL-6 inhibited both DHT- and forskolin-stimulated expression. IL-6 did not diminish DHT-dependent AR occupancy of the PSA enhancer but inhibited CBP/p300 recruitment, histone H3 acetylation, and cell proliferation. These findings provide a contextual framework for interpreting the contribution of non-steroidal activation of the AR to signaling in vivo, and have implications for prostate cancer cell growth.

Introduction

Androgen receptor (AR) activity is implicated in all phases of prostate cancer (1–3) including the final stages of the disease that ensue following failure of androgen ablation therapy which frequently is termed androgen independent. Recent evidence suggests that prostate cancer cells surviving after androgen ablation therapy are not necessarily resistant to subsequent alternative hormone manipulations that depend on a functional AR. Continued signaling of the AR in a castrated hormone environment could result from overexpression of the receptor, gain-of-function AR gene somatic mutations, AR coactivator overexpression, and ligand-independent activation of the AR (1, 3). It was recently proposed that stimulation of the Janus kinase–signal transducers and activators of transcription (JAK-STAT) and/or mitogen-activated protein kinase (MAPK) pathways by interleukin-6 (IL-6) and forskolin activated the AR in the absence of ligand in LNCaP prostate cancer cells (4, 5). Mechanisms for this possibly include phosphorylation of the steroid receptor coactivator-1 (6) and/or recruitment of the coactivator p300 (7). While these mechanisms theoretically may account for the continued activity of the androgen-signaling axis following androgen ablation, their contribution to AR signaling and prostate cell growth in vivo is not known.

The newly synthesized AR resides in the cell cytoplasm associated with a multi-protein chaperone complex essential for receptor maturation and the acquisition of ligand binding competence (8). Following binding of the native ligand, 5α-dihydrotestosterone (DHT), the multiprotein receptor complex, dissociates and the receptor is rapidly translocated into the nucleus (9) where it dimerizes and binds in the major groove of the DNA double helix to specific DNA sequences called androgen response elements (AREs). Subsequent transactivation or repression of target genes depends on the relative abundance of specific cofactors (coactivators and corepressors) in a given cell type; coactivators recruit histone acetyl transferases and certain methyl transferases, whereas corepressors recruit histone deacetylases to the transcription complex resulting in chromatin decondensation (activation) and condensation (inhibition), respectively (reviewed in Ref. 10). In this model, AR-induced activation versus repression of the transcription of a particular target gene is a function of the relative amount and type of cofactors recruited to the ARE receptor complex.

The expression of prostate specific antigen (PSA) is dependent on androgen signaling in prostate epithelial cells and has been used extensively as a marker of prostate cancer growth. The binding of transcription factors to two upstream cis regions, a proximal promoter and a distal enhancer, of the PSA gene results in transcriptional regulation. Two AREs are located...
in the promoter and six are located in the enhancer region some 4.2 kb upstream from the transcription start site (11–13). Recently, chromatin immunoprecipitation (ChIP) experiments have revealed that AR complexes with both the PSA promoter and enhancer to mediate PSA expression via a ligand-dependent mechanism (14). Because most of the work assessing androgen-independent activation of the AR thus far has relied on transiently transfected reporter genes, we developed a model system for androgen-dependent and -independent activation of the AR by targeting the endogenous chromatin-integrated PSA gene and measuring its mRNA expression by reverse transcription (RT)-PCR and promoter/enhancer occupancy by ChIP analyses in LNCaP cells. Using this model system, we examined AR-mediated PSA expression and promoter/enhancer occupancy after AR activation with the natural ligand DHT, the cytokine IL-6, and the adenylate cyclase activator forskolin, and unexpectedly found that IL-6 opposed the effects of DHT and forskolin on PSA expression.

Results

AR Transactivation Activity on Transiently Transfected AR Responsive Promoter-Luciferase Constructs

Neither IL-6 nor forskolin treatment was able to stimulate the activity of any of three different androgen responsive luciferase reporter plasmids containing either a PSA promoter/enhancer (PSA-luc), a probasin promoter fragment (Probasin-luc), or an MMTV-LTR (MMTV-luc) in transiently transfected LNCaP cells (Fig. 1A). Forskolin treatment in the presence of DHT, however, resulted in a dramatic increase in activity from all three promoters, and this activity was significantly inhibited by the inclusion of IL-6 (Fig. 1B). IL-6 inhibited the DHT-mediated activity of PSA-luc, had no effect on the DHT-mediated activity of Probasin-luc, and stimulated the DHT-mediated activity of MMTV-luc (Fig. 1B).

Establishment of an Endogenous AR Reporter System

AR-mediated endogenous PSA promoter activity can be synchronized and monitored using ChIP and RT-PCR assays. This is achieved by starving the cells of steroids in growth medium containing charcoal-stripped serum, followed by the addition of the natural ligand, DHT, or alternative AR activators. The AR can bind to cis elements (AREs) found in the promoter and enhancer regions upstream of the transcription start site of the PSA gene (diagrammatically represented in Fig. 2A). Treatment of LNCaP cells with 10 nM DHT resulted in rapid occupancy of the PSA enhancer by the AR with maximum levels (on average some 10-fold higher than the minus antibody or minus DHT controls) being attained during the first 4 h (Fig. 2B). Acetylated histone H3 (AcH3) immunoprecipitation increased in a quantitatively similar fashion. The AcH3 time course is delayed, however, by about 1–2 h relative to AR occupancy, which is consistent with a mechanism by which the AR recruits histone acetyl transferases to the complex to allow histone H3 modification. The situation on the PSA promoter, by contrast, is different (Fig. 2B). AR occupancy is barely raised to levels 2-fold over the no antibody or minus DHT controls, and this level was maintained for at least 16 h. The reason for the observed difference between AR occupancy on the PSA promoter and enhancer is unclear but might be related to multiple putative AREs found in the enhancer. Three different anti-AR antibody preparations raised against different epitopes gave similar results in ChIP assays (data not shown) making it unlikely that specific AR epitope masking occurred on the promoter. PCR analysis of a negative control (3′-irrelevant DNA about 6kb downstream) yielded negligible values (Fig. 2B).

PSA Expression

PSA pre-mRNA was detectable by real-time RT-PCR immediately after AR enhancer occupancy, the level increasing steadily over the following 16 h (Fig. 3A). The mature mRNA (i.e., spliced) time course lagged behind that of PSA pre-mRNA with processed PSA mRNA only being detectable after about 6 h at which time it increased linearly (Fig. 3A). IL-6 treatment of LNCaP cells inhibited DHT-stimulated (Fig. 3B) and forskolin-stimulated (Fig. 3C) PSA mRNA synthesis.

FIGURE 1. Effects of DHT, IL-6, and forskolin on transiently transfected PSA-luc, Probasin-luc, and MMTV-luc activity in LNCaP cells. LNCaP cells were transiently transfected with PSA-luc, Probasin-luc, or MMTV-luc (100 ng/well) and incubated with DHT (10 nM) and/or IL-6 (50 ng/ml) and/or forskolin (FSK; 5 μM) for 30 h. A and B are two independent experiments. Columns, means of determinations from quadruplicate wells; bars, SD. RLU, relative luciferase units.
FIGURE 2. The recruitment of AR to the enhancer and promoter sequences of the PSA gene by DHT. A. A schematic representation is depicted of the PSA promoter and enhancer regions. Vertical dark bars, approximate locations of AREs. Horizontal dark bars, real-time PCR targeted regions. Arrow, transcription start site. B. LNCaP cells (7 × 10⁶ cells/150 mm dish) were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS for 5 days and then treated with DHT (10 nM) for various times as indicated. The occupancy of AR and AcH3 on PSA gene enhancer, promoter, and 3'-irrelevant region were examined. Values are presented as percentage input. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed.

FIGURE 3. IL-6 inhibits PSA expression in LNCaP cells. LNCaP cells (2 × 10⁶) were seeded in 100-mm dishes in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS and grown for 4 days. A. Cells were treated with DHT (10 nM) for various times as indicated. Real-time RT-PCR was conducted to measure the levels of pre-mRNA (open symbols) and mature mRNA (closed symbols). B. In a separate experiment, cells were treated with DHT (10 nM) (dotted lines) or DHT (10 nM) + IL-6 (10 ng/ml) (solid lines), and analyzed as in A above. C. In a separate experiment, cells were treated with forskolin (1 μM) (dotted lines) or forskolin (1 μM) + IL-6 (10 ng/ml) (solid lines), and analyzed as in A above. D. Western analyses of endogenous PSA protein levels were conducted in LNCaP cells exposed to DHT (10 nM) and/or IL-6 (10 ng/ml) and/or forskolin (FSK, 1 μM) for 12 h. PSA expression values are shown as percentage of untreated controls and normalized to actin expression on the same gels. ** Columns, means of three independent immunoblots; bars, SD. Two-tailed P-values were calculated using the Student t test.
Immunoblot analysis of expressed PSA protein after 12 h of similar treatments (Fig. 3D) revealed that IL-6 significantly inhibited both the DHT- and forskolin-stimulated PSA protein expression consistent with the mRNA data.

**IL-6 Does Not Inhibit AR Occupancy of the PSA Enhancer**

In an attempt to understand the mechanism of IL-6 inhibition of PSA expression, we analyzed AR occupancy and histone H3 acetylation of the PSA enhancer (Fig. 4). IL-6 had little effect on DHT-mediated AR occupancy but failed to stimulate histone H3 acetylation (Fig. 4A), a result consistent with the inhibitory effects of IL-6 on PSA expression. In the absence of DHT, no significant AR localization on the PSA enhancer was observed after either IL-6 or forskolin treatment (data not shown). Because CBP/p300 has intrinsic histone acetyl transferase activity and is often recruited to the transcription complex by steroid receptors, we analyzed for its presence on the PSA enhancer/promoter by ChIP. DHT-mediated recruitment of CBP/p300 was inhibited by IL-6 on both the PSA promoter and enhancer (Fig. 4B).

**IL-6 Inhibits DHT- and Forskolin-Stimulated Cell Proliferation**

Consistent with the AR activity as measured on the PSA promoter/enhancer (see above), IL-6 significantly inhibited both the DHT- and forskolin-stimulated increases in cell number after 3 days of treatment (Fig. 5).

**Discussion**

Novel findings of this study include the significantly higher DHT-induced AR occupancy of the PSA enhancer compared to the PSA promoter, and the opposing activities of the non-steroidal activators, forskolin and IL-6, at the same locus. These findings were made possible by the development of an in vivo system to determine ligand-dependent and -independent activity of the AR. It employs endogenous, chromatin-integrated PSA gene expression as a reporter in LNCaP cells, coupled with quantitative assessments of DNA occupancy by proteins using ChIP assays. Since most of the previous studies on AR activation have relied on functional and interaction assays performed in transient transfection systems, a more physiological system was sought. The ChIP assay provides a method to interrogate the molecular composition of complexes on natural promoters in vivo. In the case of steroid receptors, promoter occupancy can be synchronized by the addition of ligand to the cultures. As a result, occupancy can be assessed as a function of both time (i.e., kinetically) and composition by using appropriate antibodies. Multiple protein-protein interactions are likely to be required to recruit AR and its associated proteins in the appropriate conformation to initiate transcription via the basal transcription machinery. In addition, ligand-independent activation of the AR may differ from ligand-dependent activation in both promoter and cell contexts. For example, ligand-independent activation may cause the AR to adopt a different conformation than that assumed by the ligand-bound AR, which in turn could result in exposure of different protein-protein interaction surfaces in the AR leading to the recruitment of different coregulator proteins and altered activation of AR regulated genes.

In the present study, we observed quantitatively and qualitatively different results with the natural ligand, DHT, and the putative ligand-independent activators of the AR, forskolin and IL-6, depending on the promoter context and whether we measured endogenous or transiently transected gene expression. Forskolin treatment increased endogenous PSA expression, but not the expression from transiently transfected reporters in the absence of DHT. IL-6, on the other hand, increased transiently expressed reporters in the presence of DHT from some but not all promoters and inhibited both DHT- and forskolin-mediated expression from endogenous and some transiently expressed reporters. These results might be explained by the differential recruitment of coactivators and corepressors (10) to cis-regulatory regions in the transiently transfected plasmids and chromatin-integrated genes. AR-mediated coactivator recruitment has been extensively studied and includes p160 coactivator interactions with both the AR ligand-binding domain and the carboxy-terminal portion of the AR amino-terminal domain (15, 16). The p160 coactivators recruit secondary coactivators via two activation domains,
AD1 and AD2. AD1 binds CBP/p300, which possesses intrinsic histone acetyl transferase activity, whereas AD2 recruits the coactivator-associated arginine methyltransferase (17). AR-mediated corepressor recruitment has recently been observed with SMRT interacting with the AR amino-terminal domain (18) and NCoR with the AR ligand-binding domain (19).

Using quantitative real-time PCR assessments of the amount of DNA recovered after immunoprecipitation of cross-linked chromatin, we examined the kinetics of AR occupancy on the PSA enhancer and promoter. AR occupancy of the PSA enhancer was rapid (within 1 h of stimulation), robust (10-fold over background), and sustained (8–16 h). In contrast, AR occupancy of the PSA promoter was only 2-fold over background. No evidence of significant cycling of the AR on and off the PSA promoter and enhancer, as previously reported for the ER (20) and AR (14, 21), was observed. This may reflect the quantitative nature of the current ChIP analysis. Previous studies used non-quantitative end-point assessments of PCR bands on agarose gels, which may have exaggerated small differences in amounts of target DNA and thus not reflected the true situation in vivo.

Forskolin, which has previously been shown to activate the AR through a PKA-stimulated pathway involving the amino terminus of the receptor (reviewed in Ref. 22), activated PSA promoter activity independently from DHT in our endogenous system, and only in the presence of DHT in the transiently transfected system. In LNCanP cells, forskolin most likely increases MAPK activity via PKA stimulation (23). Inhibitors of MAPK or a mutation of the AR (S513A), which is located in a consensus MAPK phosphorylation site in the AR-NTD, abrogate AR transactivation activity in transient transfection assays in the presence of low concentrations of androgen (24, 25). Taken together, MAPK-mediated activity most likely contributes to ligand-independent AR activation and might be a major signaling mechanism in hormone refractory prostate cancer.

IL-6, a $M_t$ 21,000–28,000 cytokine, has been proposed as a contributor to prostate cancer progression (reviewed by Smith et al. 26). Interestingly, IL-6 had opposite growth effects on prostate cancer cells depending on whether they are androgen dependent or refractory. Chung et al. (27) compared a series of hormone-dependent and -refractory cell lines with respect to IL-6 secretion and growth and concluded that IL-6 might undergo a transition from a paracrine growth inhibitor to an autocrine growth stimulator during the progression of prostate cancer to become hormone refractory. Similarly, Hobisch et al. (28) found that long-term treatment of LNCanP cells with IL-6 led to abolishment of its inhibitory growth response. In an earlier study, IL-6 inhibited the stimulatory action of androgens on apolipoprotein D and gross cystic disease fluid protein 15 (both known AR target genes) expression in human breast cancer cells (29). Taken together with the results in the present study, where we have demonstrated unequivocally that IL-6 inhibited DHT- and forskolin-stimulated gene expression and cell proliferation, they suggest that long-term exposure of hormone-dependent prostate epithelial cells to IL-6 will shift its effect on the AR from inhibitory to stimulatory depending on the balance of coactivators/corepressors recruited to the AR. If this indeed is the case, inflammatory responses in the prostate mediated by IL-6 secretion might have initial benefits, but later deleterious effects with respect to prostate cancer growth and progression.

Whereas IL-6 and forskolin potentially can activate the MAPK signaling pathway, it appears that their effects on PSA expression in LNCanP cells occur via different mechanisms. In our system, IL-6 and forskolin had opposite effects on endogenous PSA expression. It is possible that the inhibitory effects of IL-6 observed in our study are mediated via non-MAPK mechanisms such as the JAK-STAT pathway. The MAPK and JAK-STAT pathways might therefore affect AR and/or coactivator and/or corepressor phosphorylation in different ways to elicit the opposite effects on AR-mediated gene expression. The challenge remains to disentangle such mechanisms to understand maintenance of AR activity in the prostate in a castrate or androgen ablated state. The endogenous PSA promoter/enhancer system described here provides a model system for such an elucidation.

**Materials and Methods**

**Cell Culture and Materials**

Human prostate cancer LNCanP cells obtained from the American Type Culture Collection (Manassas, VA) (ATCC CRL-1740) were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) and were used between passages 25–70 at a split ratio of 1:4 at each passage. DHT and forskolin were purchased from Sigma Chemical Co. (St. Louis,
MO). Recombinant human IL-6 was obtained from R&D Systems, Inc. (Minneapolis, MN). Antibodies were anti-AR (N20) and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PSA (DAKO Corp., Carpinteria, CA) and anti-ACh3, and anti-CBP/p300 (Upstate Biotechnology, Inc., Lake Placid, NY).

Transient Transfection and Luciferase Detection

LNCaP cells (5 × 10³ cells/well) were plated in 96-well plates and grown in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS (Gemini, Woodland, CA) for 3 days. Cells were then transfected with reporter PSA-luc (pGL3_PSA540), Probasin-luc (ARR3-tk-luc/tk81-PB3), or mouse mammary tumor virus-luciferase (MMTV-luc) plasmid DNA (100 ng/well) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. pGL3-PSA540-enhancer (PSA-luc) is a mammalian expression vector that contains firefly luciferase linked to the androgen responsive 548-bp PSA promoter region (−541 to +7) preceded by 1450 bp of the PSA enhancer region (−5322 to −3873) provided by Bristol-Myers Squibb (Princeton, NJ). ARR3-tk-luc/tk81-PB3 (Probasin-luc) is a mammalian expression vector that contains firefly luciferase linked to three repeats (LTR) with four hormone responsive elements (HRE) of the PSA promoter (−244 to −96) ligated in tandem to the thymidine kinase (tk) enhancer element (30, 31) provided by Dr. R.J. Matusik (The Vanderbilt Prostate Cancer Center, Nashville TN). MMTV-luc is a mammalian expression vector that contains firefly luciferase linked to MMTV long terminal repeats (LTR) with four hormone responsive elements provided by Dr. R.M. Evans (The Salk Institute, La Jolla, CA). After transfection, cells were grown in phenol red-free RPMI 1640 containing 0.5% charcoal/dextran-stripped FBS with DHT and/or IL-6 and/or forskolin as indicated. After additional 30 h incubation, the cells were lysed with the passive lysis buffer (Promega, Madison, WI). The extracts were assayed for luciferase activity using the Promega assay kit according to the manufacturer’s protocol and measured on a Dynex MLX Microtiter Plate Luminometer (Chantilly, VA). Relative luciferase units (RLU) are shown as the means ± SD of quadruplicate wells. Total protein concentrations of the extracts were assayed using Bio-Rad Protein Assay Kit according to the manufacturer’s protocol and measured on a Molecular Devices EMax Microplate Reader (Sunnyvale, CA). No significant differences in total protein concentrations were observed among the different wells within a given experiment.

ChIP Assays

LNCaP cells (7 × 10⁶ cells/150 mm dish) were plated and grown in phenol red-free RPMI 1640 supplemented with 5% charcoal/dextran-stripped FBS for 5 days. Cells were treated with DHT and/or IL-6 and/or forskolin for various times as indicated, cross-linked by adding formaldehyde (1%) directly to the culture medium, and incubated at room temperature for 10 min. The dishes were rinsed twice with ice-cold PBS, and the cells were scraped into PBS containing protease inhibitors (Sigma) and centrifuged for 3 min at 2000 rpm. The cell pellets were resuspended in 0.35 ml lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 2 mM protease inhibitors] and incubated for 10 min on ice. The resulting cell lysates were sonicated in 1.5 ml microfuge tubes for 5 s, followed by a 1 min incubation on ice for a total of four cycles using setting 10 of a Fisher Sonic Dismembrator Model 60 (Tustin, CA). Cell debris was removed by centrifugation for 10 min at 14,000 rpm in a microfuge. The soluble chromatin (100 µl) per immunoprecipitation of the supernatant was transferred to a new microfuge tube and diluted 10-fold in dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.0), 167 mM NaCl, protease inhibitors]. The diluted suspension (1 ml) was precleared by incubating with 2 µl sheared salmon sperm DNA (1 µg/µl) (Life Technologies) and 45 µl protein G-Sepharose bead suspension (Amersham Pharmacia Biotech, Piscataway, NJ) [50% slurry in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA] for 1 h at 4°C with rotation. The supernatant was immunoprecipitated by incubating overnight at 4°C with the indicated specific antibodies. The next morning, 2 µl sheared salmon sperm DNA and 45 µl protein G-Sepharose were added for 1 h at 4°C with rotation. The Sepharose-bound immune complexes were sequentially washed for 10 min each in a low salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl], a high salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl], and a LiCl wash buffer [0.25 M LiCl, 1% IGEPAL CA-630, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0)] with rotation. The Sepharose-bound immune complexes were further washed twice with TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)]. The immune complexes were recovered from the beads with an elution buffer (1% SDS and 0.1 M NaHCO₃). Eluates were heated at 65°C for 4 h to reverse the formaldehyde cross-links. DNA was purified by phenol/chloroform extraction and ethanol precipitation. The DNA pellets were finally resuspended in 100 µl H₂O ready for real-time PCR analysis (see below). Ten percent input samples were treated in the same way except that no immunoprecipitation steps were performed.

Real-Time PCR of DNA Obtained From ChIP Assays

DNA samples from ChIP preparations were analyzed by real-time PCR using an iCycler optical system (Bio-Rad) and AmpliTaq Gold PCR master mix (Applied Biosystems, Branchburg, NJ). The primers and probes were: enhancer forward, 5′-GGGAGGGAGAGCTAGCACTTG-3′; reverse, 5′-ACACCTTTTTTTTTCTGGATTGTTG-3′; promoter forward, 5′-CTCTAGATGAAAGTCTCCATGAGCTACA-3′; reverse, 5′-GGGAGGGAGAGCTAGCTGGAGC-3′; 3′-irrelevant locus forward, 5′-TCACTCATGAAATCGCACCCTGTAAGC-3′; reverse, 5′-GCCCAAAGTGCTTGGTAGTATACC-3′; enhancer probe, 5′-6-FAM-TGACAGAGTGTCTCAGATG-3′; promoter probe, 5′-6-FAM-CAATTACTAGATCACCCTG-3′; and 3′-irrelevant locus probe, 5′-6-FAM-TGACTAGATGCTCTGACAGCGC-3′ (Biosearch Technologies, Novato, CA). Triplicate PCR reactions for each sample (5 µl) were conducted. The results are given as percentage of input and represent mean values ± SD of triplicate determinations.
Real-Time RT-PCR of RNA

After treatment of the cells with DHT or forskolin and/or IL-6, total cellular RNA was prepared and treated with RNase-free DNase I using the SV Total RNA Isolation System (Promega). A two-step RT-PCR method was employed using the TaqMan Gold RT-PCR Kit (Applied Biosystems). In these analyses, the same probe and reverse primer located in exon 4 of PSA gene were used for both the pre- and mature mRNA determinations (see below). The forward primer is located in intron 3 for pre-mRNA and in exon 3 for mature mRNA determinations. The size of RT-PCR product was checked by agarose gel electrophoresis (data not shown), whereas mRNA quantitation was performed by real-time RT-PCR. Thus, the primers and probes were: PSA pre-mRNA forward, 5′-GTTTTTTGCTGGCCCCGTAG-3′; mature mRNA forward, 5′-GGCGAGCATTTGAACCAAGGAG-3′; PSA reverse, 5′-GCATGAACTTTGTCACCTTCTG-3′; PSA probe, 5′-6-FAM-ATGACGTGTGTGCGCAAGTTCACC-BHQ-1-3′; GAPDH forward, 5′-GGTTTTTGGTGAACCATGAGA-3′; GAPDH reverse, 5′-GGTCCATGCTCTTCCACGATC-3′; and GAPDH probe, 5′-6-FAM-CAGCCTCAAGATCATCAGCAATTGCTC-BHQ-1-3′ (Biosearch Technologies). Triplicate PCR reactions were conducted. GAPDH mRNA expression was analyzed in each sample in parallel wells. The results are represented as PSA/GAPDH mRNA ratios. Due to extensive DNase treatment of the RNA preparations (total RNA Isolation System; Promega), no significant genomic DNA contamination was apparent; (a) negative controls lacking reverse transcriptase were normally less than 1% of the experimental values (the value of each sample was adjusted by subtracting these negative values), and (b) PCR analyses of promoter sequences gave negligible values.

Immunoblotting

LNCaP cells (6 × 10^6 cells/60 mm dish) were plated and grown in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS for 3 days. After treatment with DHT and/or IL-6 and/or forskolin as indicated, cells were harvested in 100 μl RIPA buffer [10 mM sodium phosphate, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1% SDS, 1% IGEPSAL CA-630, 1% sodium deoxycholate, 0.2 mM Na3VO4 (pH 7.2)] that contained a cocktail of mammalian protease inhibitors. Equal amounts of each extract were analyzed by SDS-PAGE. Proteins were transferred to Hybond-P membrane (Amersham Pharmacia Biotech) and probed with rabbit polyclonal anti-PSA or anti-actin antibody. HRP-conjugated anti-rabbit IgG (Santa Cruz Biotech) and probed with rabbit polyclonal anti-PSA or anti-transcript blotting system (Amersham Pharmacia Biotech) was used as the secondary antibody. Detection was performed using the enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech) according to the manufacturer’s protocol. Autoradiograms from three independent Western gels were analyzed by scanning densitometry using a Model GS-710 Imaging Densitometer (Bio-Rad).

References


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