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Prosaposin Is a Novel Androgen-Regulated Gene in Prostate Cancer Cell Line LNCaP

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Abstract Androgen-regulated genes (ARG) are implicated in normal and neoplastic growth of the prostate. Recently, we reported genomic amplification and/or overexpression of a previously known neurotrophic factor, prosaposin, in androgen-independent (AI) or metastatic prostate cancer (PCa) cells and tissues. Prosaposin and/or its known active molecular derivatives (e.g., saposin C) function as a pluripotent growth factor with diverse biological activities that favor malignant phenotypes in PCa cells. In addition, prosaposin or saposin C upregulates androgen receptor (AR) and AR-target genes (i.e., prostate-specific antigen, Probasin) expression and activity in LNCaP cells. Here, we examined prosaposin as an ARG. We report that DHT treatment of LNCaP cells increases prosaposin expression. In addition, we demonstrate androgen-responsiveness of prosaposin promoter and AR occupancy to a hormone-responsive element located in the proximal region of the prosaposin promoter. Our data for the first time identify prosaposin as an ARG. This observation, together with the pleiotropic growth factor activity of prosaposin, might suggest a role for this molecule in AR-dependent progression of prostate cancer at its early or late AI-state. J. Cell. Biochem. 101: 631–641, 2007.

Key words: prosaposin promoter; AR; HRE; PSA; prostate

Prostate cancer (PCa) is one of the leading causes of morbidity and mortality among men in the United States [Jemal et al., 2005]. Androgenic hormones are not only critical determinants in normal growth and development of PCa, but also play a significant role in prostate carcinogenesis and progression. Their actions are mediated by the androgen receptor (AR), as a transcription factor member of the nuclear steroid receptor superfamily. After androgen binding, AR dissociates from heat shock proteins and forms homodimer and binds to androgen-response elements (AREs). AR also in association with other effectors, (e.g., coactivators) activates the transcription of

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androgen-responsive or—regulated genes [Jenster, 1999; Feldman and Feldman, 2001].

Almost all patients with PCa are initially androgen-dependent (AD) and respond to anti-androgen therapy. However, a significant percentage of patients will eventually progress to the clinically incurable androgen-independent (AI) stage [Jenster, 1999; Arnold and Isaacs, 2002]. Identification and characterization of androgen-regulated genes (ARGs) that are differentially expressed between AD and AI PCa and function as multipotential modulators of diverse biological activities in favor of malignancy may be important not only to understand downstream signaling events in AR pathway and the mechanisms underlying the development of hormone-refractory PCa, but also to develop potential diagnostic and/or therapeutic targets for PCa patients.

Prosaposin is a dual function protein. As an intracellular lysosomal protein, it is the precursor of sphingolipid activator proteins (saposin A-D) involved in hydrolysis of sphingolipids [Kishimoto et al., 1992; Koochekpour, 2006a]. In soluble form, prosaposin and at least one of its mature active domains (i.e., saposin C) are well-known neurotrophic factors [O'Brien et al., 1994]. Homozygous inactivation of prosaposin gene in mice led to the development of a number of abnormalities in male reproductive organs including involution and atrophy of the prostate gland [Morales et al., 2000]. This finding indicates a developmental role for prosaposin in the prostate. Our results to date show: (1) prosaposin expression is higher in metastatic AI than in AD PCa cells and tissues, (2) prosaposin gene is amplified in a number of AI PCa cells and punch biopsy specimens of prostate cancer xenografts and metastatic lymph nodes, (3) prosaposin expression in C4-2B (a bone metastatic-subline of the LNCaP PCa progression model derived under in vivo androgen-deprived conditions) is higher than its parental androgen-sensitive (AS) cell line, and (4) prosaposin stimulates growth-, migration-, and invasion-promoting activities, activates multiple core-signal transduction pathways (e.g., MAPK, PI3/Akt), and acts as a cell survival and anti-apoptotic factor of both AS- and AI-PCa cells [Lee et al., 2004; Koochekpour et al., 2004a,b, 2005a,b, 2006a,b]. Interestingly, we have recently demonstrated that prosaposin or its active molecular derivatives (saposin C, TX14A synthetic peptide) in an AI-manner and via upregulation of AR expression and activity, induced AR-target gene activity in LNCaP cells [Koochekpour et al., 2006b]. Overall, these observations suggested that together with its pleiotrophic regulatory functions, prosaposin may contribute to prostate carcinogenesis at its early AD- or hormone-refractory state.

In this study, we examined whether or not prosaposin (PSAP) expression could be regulated by androgen. Our data presented show that DHT increased prosaposin expression in LNCaP cells. In addition, we have demonstrated that in response to DHT, AR associates with an androgen-responsive hexanucleotide motif located in the proximal prosaposin promoter region that matches to the sequence of hormone-response element (HRE) or the right half of the glucocorticoid-response element (GRE1/2). Our data also show that bicalutamide was not only inefficient to block DHT induction of prosaposin expression, but also demonstrated an agonistic effect and increased the transcriptional activity of the proximal prosaposin promoter (with the HRE motif) in LNCaP cells. These data for the first time demonstrate that prosaposin is an ARG. Overall these data led us to hypothesize that prosaposin as a pluripotent growth factor, a regulator of AR expression and activity, and as an ARG, could play an important role in AR-dependent and AI-progression of PCa.

MATERIALS AND METHODS

Cell Culture, Reagents, and Antibodies

LNCaP cell line was obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI-1640 supplemented with 10% FBS, 1 mM sodium pyruvate, and 10 mM HEPES. All tissue culture media were from Invitrogen (Carlsbad, CA). Mouse monoclonal anti-human saposin C was generated through a contract with the antibody production unit at AnaSpec (San Jose, CA) and has been previously characterized [Koochekpour et al., 2004a, 2005b]. Anti-human PSA (sc-7638), actin, GAPDH, and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human interleukin-6 (IL-6) and rabbit anti-human AR (PG-21) were purchased from R&D systems (Minneapolis, MN) and Upstate Biotechnology (Lake Placid, NY), respectively. Recombinant

human insulin-like growth factor-I (IGF-I), charcoal stripped-fetal bovine serum (CS-FBS), dihydrotestosterone (DHT), cycloheximide, and actinomycin D were obtained from Sigma (St. Louis, MO).

Western Analysis

LNCaP cells were grown to 65%–70% confluency in their respective complete culture media. Cells were washed with PBS and incubated in phenol red (PR)-free RPMI supplemented with 5% CS-FBS for 24 h. After washing the cells twice with PR- and serum-free RPMI, they were incubated overnight (16 h) in this medium in the presence or absence of DHT at the indicated concentrations.

In order to evaluate the effect of various pharmacological inhibitors on protein and/or mRNA expression of PSA or prosaposin, after the initial 24 h androgen deprivation, cells were washed with serum- and PR-free RPMI and pretreated in this medium with actinomycin D (0.5 μg/ml) or cycloheximide (at 1 μg/ml) or bicalutamide (10 μM) for 30 min. Cells were then treated with DHT (at 1.0 nM). To evaluate the potential cytotoxic effect of the reagents used, parallel tissue culture plates were also prepared and treated as mentioned above and cell viability was determined using the trypan blue dye-exclusion assay. All tissue culture plates were incubated for 16–24 h, and at the end of the incubation period, cell-free culture supernatants were collected and concentrated up to 20 times by using a Centriprep-3 concentrator (with a 3.0 kDa molecular weight cut-off, Millipore, Billerica, MA). Protein samples (20 μg/lane for PSA, 15 μg/lane for prosaposin, or 10 μg/lane for AR) were subjected to SDS–PAGE and immunoblotting as previously described (10, 12). Normalization of culture supernatants was based on the total cell number or protein content. Membranes were probed with anti-PSA (at 1:100 dilution), anti-AR (at 1:500), mouse monoclonal anti-human saposin C (at 1:250 dilution), anti-actin, or anti-GAPDH (at 1:2,000) as the primary antibody and signals were detected by ECL detection system (Amersham, Piscaraway, NJ).

RNA Extraction and RT-PCR

In order to study mRNA expression, we used tissue culture plates prepared in parallel with those used for protein expression analysis of PSA. Total RNA was extracted and used for first-strand synthesis of cDNA and RT-PCR as described before [Koochekpour et al., 2004b]. The oligonucleotides used (according to the human gene sequences with accession numbers: X07730 for *PSA*, BC007612 for prosaposin, and M33197 for *GAPDH*, as deposited at the NCBI/genome data bank) were as follows: *PSA* sense, 5′-TACCCACTGCATCAGGAACA-3′ and *PSA* antisense, 5′-CCTTGAAGCACACCATTACA-3′; prosaposin sense, 5′-AAGGAGAAATGAGCCGTCCT-3′ and prosaposin antisense, 5′-CGATCCAAATAACCCACCAG-3′, and *GAPDH* sense, 5′-GCAGGGGGGAGCCAAAAGGG-3′ and *GAPDH* antisense, 5′-TGCCAGCCCCAGCGTCAAAG-3′. PCR was carried out using a T-gradient model (Biometra, Horsham, PA) under the following conditions: 29 cycles of 95°C for 90 s, Tm (melting temperature) as indicated below, 72°C for 1.5 min with a final 10 min extension cycle at 72°C. The sizes of amplified cDNA fragments, the optimized Tm, time period, and the number of cycles for PCR were: 486 bp/62.5°C for 90 s/19 cycles for *PSA*, 898 bp/52°C for 60 s/35 cycles for prosaposin, and 566bp/64°C for 90 s/25 cycles for *GAPDH* [Jung et al., 2003; Koochekpour et al., 2004b]. The PCR product was confirmed as a single band using 1.2% agarose gel electrophoresis and loading was also normalized with *GAPDH*. A non-template control was included in each PCR experiment, each sample was analyzed twice, and PCR experiments were repeated three times independently.

Plasmid Constructs

Human prosaposin promoter deletion constructs H-813, H-343, H-213, and H-95 in firefly luciferase reporter-gene vector pGL2B [Sun et al., 1998] were restriction digested with *Kpn*1 and *Hind*III and subcloned into the similar sites of the pGL3-Basic luciferase reporter vector (Promega, Madison, WI). pGL3B-HRE was constructed with primers 5′-CGGGGTACCTCTCCAAGTTCTTCCTCACTAAGCTTCCC and 5′- GGGAAGCTTAGTGAGGAAGAACTTGGAGAGGTACCCCG containing the 5′ flanking HRE sequence from −242 to −237 of prosaposin promoter. After annealing the sense and antisense primers, the dsDNA oligo was cut with *Kpn*I and *Hind*III, electro-eluted from the agarose gel, and ligated into similarly cut pGL3B vector. All plasmid constructs were

sequenced in both directions to ensure correct sequence. The 5.8-kb PSA promoter upstream to luciferase reporter gene in pGL3B vector has been characterized before [Yeung et al., 2000].

Transient Transfections and Reporter Gene Activity

Cells were seeded at a density of 1.5 to 2×10^5 cells per well in 6-well plates in their maintenance media and grown up to 70%–75% confluency. Culture dishes were washed twice with their respective basal medium, and incubated for 24 h in PR-free RPMI (for LNCaP) supplemented with 5% CS-FBS. Transfections were carried with 1.2 ml of serum-free OPTI-MEM containing 7.5 μl/well of lipofectine reagent (Invitrogen, Carlsbad, CA), 0.25 μg of pSV-β-galactosidase vector (Promega, Madison, WI), and 0.75 μg of test DNA. Test DNA-lipid were mixed and incubated for 45 min at room temperature before addition to each well. Cells were incubated in transfection medium for 12 h. After removal of transfection medium, serum- and PR-free basal medium was added to the wells for an additional 12 h. Cells were then treated with DHT at different concentrations and time-periods indicated. A time- and dose-course study was performed to evaluate the kinetic effect of DHT, or other treatment reagents on individual test DNAs under investigation. Luciferase activity was determined with luciferase and β-galactosidase assay systems as instructed by the manufacturer (Promega, Madison, MI). To equilibrate for potential variation in well to well transfection effciciency, in addition to synchronoizing the transfections among comparable reporter genes in the cells, luciferase activity for each transfection was normalized to SV40/β-galactosidase activity. All transfection experiments were conducted in triplicate (or more) and repeated three to five times independently. Data are presented as the mean (±SEM) luciferase activity of the test DNAs relative to basal activity (promoter-specific vehicle) which was set as 100%.

Chromatin Immunoprecipitation Assay

Cells were androgen-deprived in 5% CS-FBS and PR-free RPMI-1640 for 24 h, and treated with 1 nM DHT for 3 h. ChIP assay was carried out using the Active Motif ChIP-IT kit (Carlsbad, CA) according to the protocol provided by manufacturer. After cross-linking, washing, and harvesting the cells, chromatin pellets were resuspended in shearing buffer and sonicated. Clarified supernatants containing the soluble chromatin were immunoprecipitated using 2 μg of anti-AR antibody (Mouse monoclonal antibody, clone H441; Santa Cruz Biotech, CA) and the salmon-sperm DNA and BSA blocked protein G beads by overnight incubation at 4°C with rotation. The kit's negative control IgG and positive control antibody (TFIIB) were used at 4 μg per ChIP reaction. Mouse IgG (2 μg) was also used as a control antibody for AR. The protein G agarose-antibody-AR complex was pelleted and the immune complexes were sequentially washed with wash buffers provided by the manufacturer. After recovering the immune complexes from the beads with the elution buffer, the eluates were heated at 65°C for 4 h to reverse the formaldehyde cross-links. The immunoprecipitated DNA fragments were then treated with proteinase-K, incubated at 45°C for 2 h, and the eluted DNA was purified using DNA purification mini-column. PCR was performed on DNA templates isolated through TFIIB ChIP, DNA isolated through the negative control IgG ChIP, or the input DNA ChIP (with anti-AR antibody). The purified DNA were subjected to PCR using a pair of primers for prosaposin promoter (−260/−39) sense, 5′-TCCCTTCCCTTTCTCCAAGT-3′ and antisense, 5′-CTGATCCCCCGCAGATATAA-3′, which were designed to flank the DNA site of interest (sequence from −242 to −237 containing HRE sequence). To demonstrate the specificity of the potential DNA-binding motif, additional primer sets were also designed to amplify DNA sequence both immediate upstream and downstream to HRE sequence of prosaposin promoter.

These primer sequences were prosaposin promoter (−567/−243) sense, 5′-ACCCTGTTACACGACTACTTATTCTAT-3′ and antisense, 5′-TTGGAGAAAGGGAAGGGAAA-3′ and prosaposin promoter (−220/+5) sense, 5′ CCCACTATAGCCCTTCCTTTC-3′ and antisense 5′-TACATAGCGCCGTCTGACTC-3′. Positive control PCR primers (sense, 5′-TACTAGCGGTTTTACGGGCG-3′ and antisense, 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′) flanking the TFIIB site of the constitutively active *GAPDH* promoter and positive control antibody against the basal transcription factor TFIIB was provided by the manufacturer. Triplicate PCR reactions were conducted for prosaposin as

follows, 95°C for 5 min, then 40 cycles of 96°C for 90 s, 56°C for 60 s, and 72°C for 60 s with a final extension for 6 min at 72°C. Negative control PCR primers flanked a segment of genomic DNA between *GAPDH* and *CNAP1* genes. PCR conditions for control genes were set as suggested by the manufacturer. Equal volumes of each PCR products were subjected on 2% agarose gels, stained with ethidium bromide, and photographed under UV illumination.

RESULTS

DHT Upregulates Prosaposin Expression in LNCaP Cells

We have recently demonstrated that prosaposin or one of its active domains, in an AI-manner, upregulated *AR* and *PSA* mRNA and protein expression in AS LNCaP cells [Koochekpour et al., 2006b]. Here, we examined the effect of DHT on prosaposin expression in the cells. We found a dose-dependent increase up to two-fold in prosaposin protein and mRNA expression in LNCaP cells (Fig. 1A). Pre-treatment and treatment of the cells with inhibitors of RNA transcription or protein translation substantially reduced DHT-induction of prosaposin expression. As it was expected, DHT increased PSA expression in a dose-dependent manner and this effect was inhibited in the presence of bicalutamide (Fig. 1B). Surprisingly, bicalutamide treatment in LNCaP cells did not inhibit DHT-induction of prosaposin. This data indicate that prosaposin is an ARG and insensitive to bicalutamide inhibition.

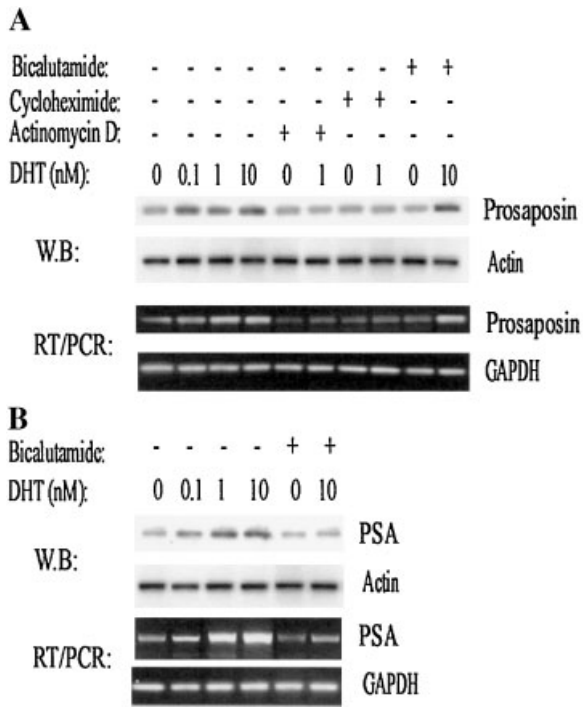

Fig. 1. DHT uprergulates prosaposin expression in androgen-responsive LNCaP cells. Cells were androgen-deprived for 24 h and then incubated overnight in serum- and PR-free RPMI in the presence or absence of DHT for 16 h. Parallel tissue culture plates were also pretreated first for 0.5 h with bicalutamide (10 μM), cycloheximide (1 μg/ml), or actinomycin D (0.5 μg/ml), and then DHT was added to the cells. Concentrated culture supernatants were subjected to immunoblotting by using antibodies against prosaposin (**A**) or PSA (**B**). Normalization of culture supernatant was based on the protein content. Total RNA was extracted from the same tissue culture plates using RNAzol B reagent. First strand cDNA was synthesized and RT/PCR was carried out as described in details in the text. GAPDH primers and actin expression were used to monitor loading control in RT-PCR and western blot analyses, respectively. The experiment was repeated at least twice independently.

Prosaposin Promoter Activity in LNCaP Cells Is Androgen-Inducible

We have demonstrated that DHT increased staeady-state mRNA expression of prosaposin in LNCaP cells. This data clearly indicate that AR is involved. To examine the role of AR as a transcription factor, we examined the effect of DHT on activity of proximal region of prosaposin promoter (−813 bp) containing the highest frequency of transcription factor binding sites within 5 kb upstream to transcription start site ATG (1+) [Sun et al., 1998]. DHT induced transcriptional activity of (−813 bp)-prosaposin promoter by 79% in LNCaP cells. This result demonstrates that DHT has a stimulatory effect on prosaposin promoter activity in androgen-responsive prostate cancer cells and this effect is mediated by AR. To determine the approximate location of androgen-responsive fragment, we investigated the effect of DHT on luciferase activity of a set of deletion constructs (−95, −213, −343, and −493 bp 5′ to ATG) that were characterized before [Sun et al., 1998]. The H-95 and H-213 constructs were non-responsive to DHT and the response of the H-493 construct was minimal (data not shown). However, DHT increased H-343 promoter activity by 53% (Fig. 2). These data indicate that the H-343 deletion construct might contain the androgen-responsive sequence within the proximal region of prosaposin promoter.

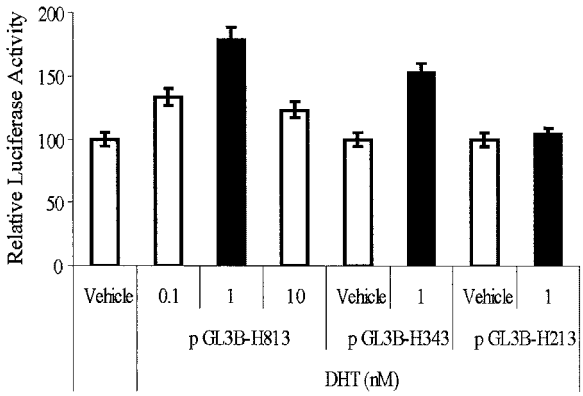

Fig. 2. Induction of transcriptional activity of proximal prosaposin promoter by androgen in androgen-responsive LNCaP cells. Deletion constructs H-813, H-213, and H-343 of human proximal prosaposin promoter region were subloned in firefly luciferase reporter-gene vector pGL3B. LNCaP cells were seeded at a density of 1.5 to 2×10^5 cells per well in 6-well plates in their maintenance media and grown up to 70–75% confluency. Cells were incubated in PR-free medium containing 5% CS-FBS for 24 h and were co-transfected with individual deletion constructs (0.75 μg) and pSV-β-galactosidase vector (0.25 μg) in serum- and PR-free basal media. Co-transfection of cells with the individual constructs and pSV-β-galactosidase was used as control for transfection and luciferase activity assay (Vehicle). After 12 h, the transfection medium removed, serum- and PR-free basal media was added to the wells for an additional 12 h, and cells were incubated in the presence of DHT for 18 h. Luciferase activity assay was determined as described under ''Materials and Methods''. The results are presented as relative luciferase activity (RLA) and are normalized to internal control pSV/β-galactosidase activity for transfection efficiency. Data are presented as the mean (±SEM) luciferase activity relative to the basal activity level, which was set as 100%, from 3 to 5 independent experiments. All transfection experiments were conducted in triplicate. Statistical significance of the effect of saposin C on individual reporter gene activity was evaluated by one-way ANOVA test with Bonferroni adjustment. Differences between vehicle and any other single experimental group of interest were evaluated by Student's *t*-test and statistical significance was set at $P < 0.05$.

DHT Induces Transcriptional Activity of the Hexanucleotide 5′-GTTCTT-3′ of Proximal Prosaposin Promoter in LNCaP Cells

In our search to find a potential DNA sequence that could potentially interact with steroid hormone receptor, we identified a single consensus hexamer (−242/−237), 5′-GTTCTT, which closely resembles the 3′-half of consensus glucocorticoid-responsive element (GRE1/2) or androgen-responsive elements (ARE-1 and ARE-2) [Cato et al., 1987; Parker et al., 1988; Simon et al., 1995]. It is noteworthy that the TGTTCT sequence in hormone-responsive elements (HREs) confers regulation by glucocorticoids, progestins, and androgens. Following transient transfection of cells with the pGL3B-HRE-luciferase, cells were treated with DHT in the presence or absence of antiandrogen bicalutamide. DHT, in a dose-dependent manner, induced reporter gene activity by 67% in LNCaP cells. Interestingly, pre-treatment and treatment of LNCaP cells with bicalutamide not only did not antagonize androgen effect, but also showed agonistic effect and further increased reporter gene activity by 36% as compared to DHT (at 10 nM) (Fig. 3A). In addition, bicalutamide treatment in LNCaP cells transfected with the early prosaposin promoter (pGL3B-H813-Luc) in the presence of DHT also increased the reporter gene activity by 21% (Fig. 3B). Control experiment showed the bicalutamide inhibition of PSA-luciferase activity in the cells (Fig. 3C).

DHT Induces AR Association With the Hormone-Response Element of The Proximal Prosaposin Promoter Region in LNCaP Cells

Our data collectively provided evidence for prosaposin as an ARG in LNCaP cells. In light of the fact that receptors for glucocoticoid, progesterone, and androgens share recognition sequences that encompass the hexanucleotide motif 5′-TGTTCT-3′ or closely related variants of this sequence [Strahle et al., 1987; Ham et al., 1988; Parker et al., 1988; Denison et al., 1989; Simon et al., 1995; Morin et al., 2000]), by using ChIP assays in androgen-responsive LNCaP cells and a mouse monoclonal anti-AR antibody (H-441), we decided to test whether AR may also have the potential to associate physically with 5′-GTTCTT-3′ sequence of the proximal prosaposin promoter as a transcriptional regulatory mechanism. As shown in Figure 4, in the absence of DHT, a small amount of AR was detected at the HRE sequence of interest [Prosaposin (−260/−39) top panel]. However, after 3 h exposure of the cells to DHT, we observed a two-fold increase in AR association to the HRE motif of prosaposin promoter. However, we did not detect any signal for control mouse IgG-immunopreciptates. The AR occupancy in the absence of androgen could be because of the promiscuity of the mutant AR in LNCaP cells allowing low level (basal) activity and engagement with this specific DNA sequence or binding of trace amounts of androgens and/or other steroids under our

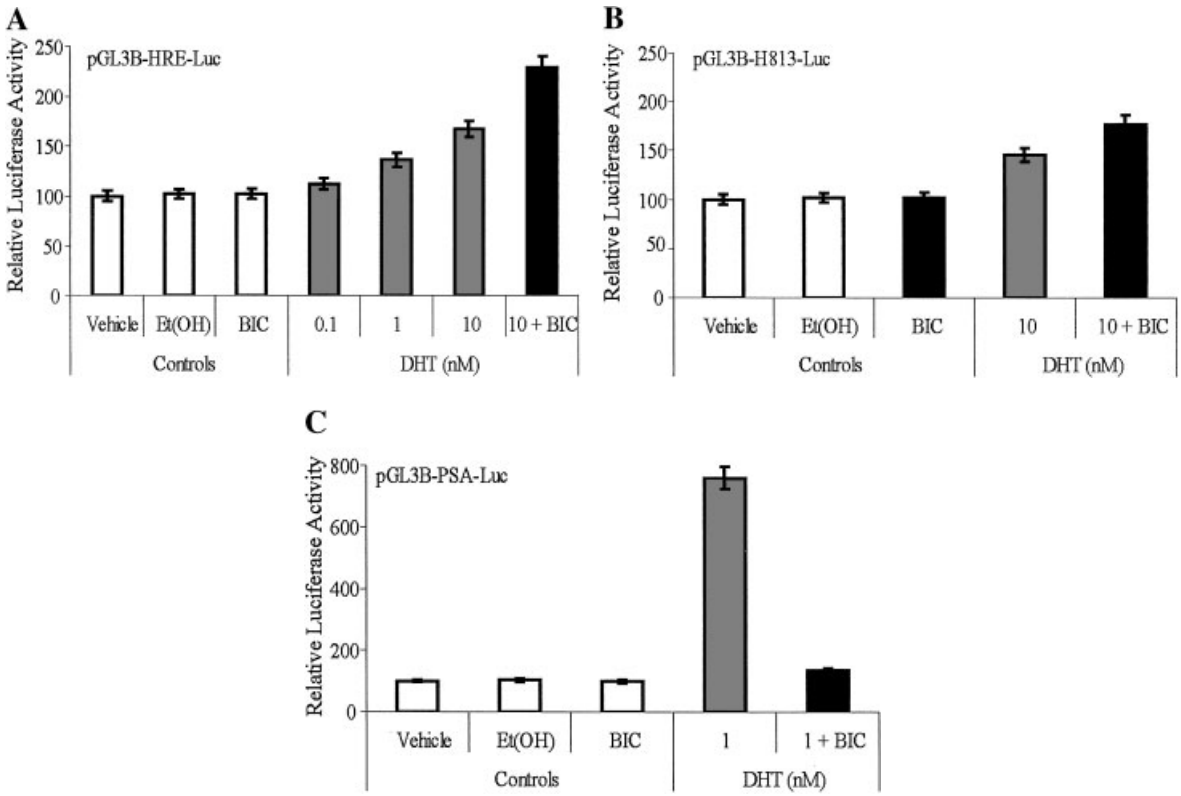

Fig. 3. Effect of androgen on transcriptional activity of HRE-containing sequence of prosaposin promoter in LNCaP cells. Cell culture, androgen-deprivation, co-transfection, and reporter gene activity assay was performed as described in Figure 2 and in ''Materials and Methods''. **A**: DHT effect on pGL3B-HRE-luciferase activity. **B**: DHT effect on early prosaposin promoter (pGL3B-H813) activity. **C**: DHT effect on pGL3B-PSA-luciferase activity. To evaluate the effect of bicalutamide, cells were pre-treated with bicalutamide (BIC; 10 μM for 30 min) before the addition of DHT. The results are presented as relative luciferase activity (RLA) and are normalized to internal control pSV/β-galactosidase activity for transfection efficiency. Data are presented as the mean (±SEM) luciferase activity relative to the basal activity level, which was set as 100%, from 3 to 5 independent experiments. All transfection experiments were conducted in triplicate. Statistical significance of the effect of saposin C on individual reporter gene activity was evaluated by one-way ANOVA test with Bonferroni adjustment. Differences between vehicle and any other single experimental group of interest were evaluated by Student's *t*-test and statistical significance was set at $P < 0.05$.

experimental conditions. Specificity of AR association with HRE sequence was demonstrated by lack of any AR localization for the amplified DNA sequences immediately upstream [Prosaposin (−567/−243)] or downstream [Prosaposin (−220/+5)] to the hexanucleotide sequence of interest. Positive control antibody against the basal transcription factor TFIIB showed the localization of TFIIB on *GAPDH* promoter with no changes in the presence or absence of androgen. In contrast to TFIIB binding, antibody against AR did not show any AR occupancy on *GAPDH* promoter (Fig. 4, bottomn panel).

IL-6 and IGF-I Increase Prosaposin Expression in LNCaP Cells in the Absence of Androgen

In addition to androgens, other growth modulators, such as IL-6 and IGF-I, could upregulate the expression and activity of AR and ARGs (e.g., PSA). Therefore we determined the effect of different concentrations of IGF-I or IL-6 on prosaposin expression under androgen-deprived culture conditions. We found that IGF-I, at 10 ng/ml, increased prosaposin expression (Fig. 5). However, IL-6 demonstrated a dose-dependent increase with a peak effect at 25 ng/ml. Overall, IL-6 was found to be more effective than IGF-I. We observed a similar effect for PSA expression. In parallel experiments, as it was expected, both IGF-I and IL-6 were able to upregulate AR expression in the cells (Fig. 5). These data collectively indicate that in addition to androgen, other growth modulators capable of AR-transactivation are equally potent in upregulating prosaposin expression.

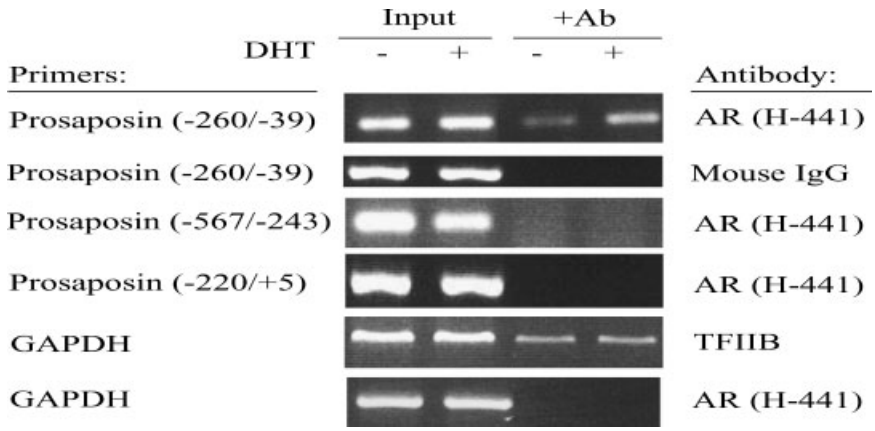

Fig. 4. AR association with the prosaposin promoter is induced in an androgen-dependent manner in LNCaP cells. LNCaP cells were grown up to 70% confluency in their maintenance medium, androgen-deprived in 5% CS-FBS and PR-free RPMI 1640 for 24 h, and then treated in basal PR-free medium with 1 nM DHT for 3 h. ChIP assay was performed to investigate AR occupancy with the early prosaposin promoter region (−260/−39) containing an HRE site in the presence or absence of androgen (DHT). Clarified supernatants containing the soluble chromatin were immunoprecipitated using 2 μg of mouse monoclonal anti-AR antibody followed by PCR analyses incorporating primers specific for DNA sequence of interest. Non-immune mouse IgG was used as a negative control in conjunction with anti-AR antibody immunoprecipitation. To demonstrate the specificity of the potential DNA-binding motif, additional primer sets were also designed to amplify DNA sequence both immediate upstream (−567/−243) and downstream (−220/+5) to HRE sequence of prosaposin promoter. For positive control ChIP, antibody against TFIIB transcription factor and primers flanking its consensus site on the constitutively active *GAPDH* promoter was included. PCR reactions were performed in triplicate and the experiment repeated twice independently. Equal volumes of each PCR product were subjected on 2% agarose gels, stained with ethidium bromide, and photographed under UV illumination. Input samples refer to crude chromatin extract prior to immunoprecipitation. The experiment was repeated twice independently.

DISCUSSION

AR expression and activity is required at any stage of normal or neoplastic growth of the prostate. In spite of maximum androgen ablation therapy, AR and AR-target genes are constitutively expressed or overexpressed in the majority of proliferative hormone refractory prostate cancer (HRPCa) cells [Jenster, 1999; Feldman and Feldman, 2001; Arnold and Isaacs, 2002].

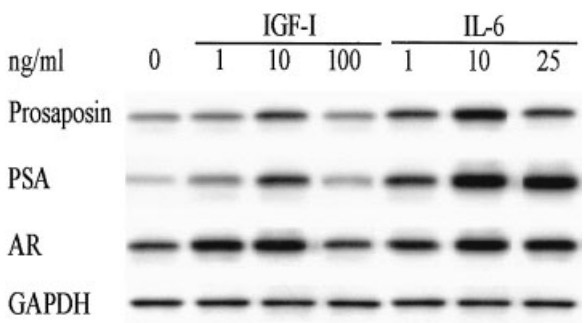

Fig. 5. IL-6 and IGF-I uprergulate prosaposin expression in an androgen-independent manner in LNCaP cells. Cells were androgen-deprived for 24 h and then incubated overnight in serum- and PR-free RPMI in the presence or absence of IL-6 or IGF-I for 16 h. Whole cell lysates and concentrated culture supernatants were subjected to immunoblotting using antibodies against prosaposin, PSA, AR, or GAPDH as described in ''Materials and Methods''. Normalization of culture supernatant was based on the protein content. The experiment was repeated at least twice independently.

We have recently reported cloning of prosaposin from poorly differentiated PCa cell line, PC-3. Our data demonstrated that prosaposin is genomically amplified and/or overexpressed in AI PCa cells, xenograts, and metastatic lymph nodes [Koochekpour et al., 2005b]. In addition, prosaposin or its active molecular derivatives (e.g., saposin C, TX14A peptide): stimulates proliferation, migration, and invasion, activates multiple interacting signal transduction pathways (e.g., MAPK, PI3K/Akt), and functions as a cell-survival and anti-apoptotic factor for both AD and AI PCa cells [Lee et al., 2004; Koochekpour et al., 2004a, 2005a,b]. Interestingly, we also found that like some other growth factors (e.g., EGF, IGF-I, KGF), prosaposin or saposin C, in an AI-manner upregulated AR and AR-target genes expression and activity in AS LNCaP cells [Koochekpour et al., 2006b].

In this study, we demonstrated upregulation of prosaposin expression by DHT. Cycloheximide and actinomycin D inhibition of DHT effect indicate that protein and RNA synthesis

are both required for androgenic induction of prosaposin. On the other hand, the fact that cycloheximide blocks prosaposin mRNA induction implies that protein synthesis is also required for the prosaposin transcription. These findings prompted us to analyze prosaposin promoter activity in AS LNCaP cells. We showed that DHT, in a dose-dependent manner, increased the prosaposin (−813 bp)-luciferase activity in these cells. These data indicate androgen-responsiveness of prosaposin promoter. In addition, within this early promoter region, we located a single consensus hexamer (−242/−237), 5′-GTTCTT-3′, equivalent to the HRE and with close resemblance to 3′-half of GRE (25). In our study, DHT induced luciferase activity of H-343 deletion construct (containing the hexanucleotide sequence of interest) in LNCaP cells. This response was slightly less than H-813 construct. The differences in the androgen-responsiveness among the three deletion constructs H-813, H-343, and H-493 could be due to the presence of other transcription factors that bind to potential positive or negative regulatory elements of the early promoter region [Sun et al., 1998]. It is noteworthy that the 5′-GTTCT-3′ sequence in HREs confers regulation by glucocorticoids, progestins, and androgens [Strahle et al., 1987; Ham et al., 1988]. In vitro binding studies have also demonstrated that the receptors for androgens, glucocorticoid, and progesterone share recognition sequences that encompass the hexanucleotide motif or its closely related variants such as the 3′-right half GRE [Cato et al., 1987]. By using ChIP assay and two different AR-antibodies, we also demonstrated both the specificity and occupancy of AR to the 5′-GTTCTT-3′ sequence. In addition, we have demonstrated that saposin C treatment of the cells led to a punctuate nuclear distribution pattern of AR, further indicating its association with the nuclear matrix and possibly the engagement and/or recruitment in the assembly of basal transcription machinery of androgen-regulated genes [Koochekpour et al., 2006b]. The GTTCTT sequence has also been identified in ARE of a number of other AR-target genes [Rennie et al., 1993] such as rat PB gene, seminal vesicle secretory (SVS) protein family related mouse gene (i.e., MSVSP99), or rat SVS VI gene [Cato et al., 1987; Parker et al., 1988; Simon et al., 1995]. The inclusion of the AREs in HRE cateogory is based largly on the observation that AR can induce transcription through GREs of mouse mammary tumor virus (MMTV) DNA and the tyrosine aminotransferase (TAT) gene [Ham et al., 1988; Denison et al., 1989]). Therefore, AR may function as a trans-acting transcription factor for genes containing this DNA motif.

Antiandrogen therapy in the form of monotherapy or combined androgen blockade (surgical or chemical castration plus antiandrogen) remains as the main treatment strategy for advanced prostate cancer [Kelly et al., 1997]. Almost all antiandrogens (e.g., bicalutamide, flutamide, magestrol) during the course of treatment show some degrees of androgen-agonistic activity known as antiandrogen withdrawal syndrome and could be recognized with the increase in serum PSA, clinical deterioration of the patient, or disease regression following cessation of bicalutamide or antiandrogen therapy [Suzuki et al., 1996; Kelly et al., 1997; Sella et al., 2004]. In addition to this, the antagonist-agonistic conversion of bicalutamide in LNCaP cells has been demonstrated under long-term androgen-deprived condition in vitro [Culig et al., 1999].

In our previous study, we demonstrated the inhibition of saposin C-induced PSA expression and its reporter gene activity by bicalutamide [Koochekpour et al., 2006b]. However, here our data indicate that bicalutamide did not efficiently inhibit or block the DHT-induced prosaposin expression. In addition, bicalutamide not only failed to block the effect of DHT on pGL3B-HRE luciferase activity, but also acted as an androgen agonist and further stimulated the reporter gene activity in LNCaP cells. We obtained relatively similar finding with the effect of bicalutamide on DHT-induction of the early prosaposin promoter activity (pGL3B-H813-Luc).

Although the exact molecular mechanisms underlying the antiandrogen withdrawal syndrome are not completely known, several studies provide evidence in support of the influence of AR coactivators (e.g., ARA70), mutation in the ligand binding domain (LBD) of AR, or the development of mutant AR during bicalutamide treatment [Suzuki et al., 1996; Culig et al., 1999, 2001; Hara et al., 2003; Bohl et al., 2005]. Investigation on X-ray crystal structure of R-bicalutamide bound to W741L mutant AR has provided a well-developed structural basis for the acquisition of agonistic function for

bicalutamide [Bohl et al., 2005]. In the present study, failure of bicalutamide in blocking the effect of DHT could be potentially due to (a) increased availability of AR (following inhibition of AR binding to PSA promoter); (b) bicalutamide-binding to AR may not interfere with physical association of AR with HRE of the prosaposin promoter; (c) the availability of AR coactivators (that were not consumed by engagement and transcriptional activity of AR on PSA promoter) and their contribution to the assembly of the HRE-transcription machinery complex of the prosaposin promoter; (d) DHT-induced production of paracrine stimulatory factors that could stimulate prosaposin transcriptional activity; or a combination of these. Interestingly, we have also located the presence of HRE sequence (5′-GTTCTT-3′) in the promoters of human EGF (position −583/−588, accession number AF023155) and mouse insulin-like growth factor binding proteins-1 and -3 (IGFB-1/-3; position −2221/−2226, accession number AL6071241) and the right half-GRE sequence (5′-GTTCT-3′) in promoters of human VEGF (position −1455/−1459, accession number AF095785) and human IGF-1 (two positions −1111/−1115 and −1125/−1129, accession number M12659). Among these, EGF and IGF-I can not only transactivate AR, but also are AR-target genes [Culig et al., 1994; Martin and Pattison, 2000], while the other two are regulated by DHT/AR [Sordello et al., 1998; Martin and Pattison, 2000]. Whether or not the presence of the HRE or half-GRE sequence in these genes and their functional relationship with DHT/AR and/or bicalutamide could support a general mechanistic explanation for agonistic conversion of antiandrogens remains an interesting subject for future investigations. Activated AR can function as a trans-acting transcription factor and, via binding to the universal HRE or half-GRE sequence of genes (with pleiotrophic avtivities) such as prosaposin, may contribute to antiandrogen withdrawal syndrome.

Additionally, we were also concerned about nonsteroidal AR-activation by cytokines and polypeptide growth factors as paracrine regulatory stimulants for prosaposin as an ARG. Among polypeptide growth modulators, IL-6 and IGF-I are the two prominent examples. Here we provided preliminary evidence indicating that in addition to androgens, both IL-6 and IGF-I, in an AI-manner, were able to upregulate prosaposin expression in the cells. These observations provide strong supportive evidence that persistent AR-signaling pathways by native ligands (androgens) and/or nonsteroidal AR-transactivators (e.g., EGF, IL-6, IGF-I) may maintain an elevated level of prosaposin both at AD- and AI-stages of the disease which ultimately favors malignant phenotypes of hormone-naïve and/or hormone refractory PCa cells. Dissection of molecular mechanisms underlying nonsteroidal activation and maintenance of AR-signaling pathways might lead to a better understanding of prosaposin-mediated control of PCa cells growth.

While prosaposin and some other ARGs hold promise as potential markers or targets in the diagnosis and treatment of PCa, taking into consideration that as a pleiotrophic growth factor overexpressed in AI PCa cells and regulates AR expression and activity and as a novel ARG, it might be involved in AR-dependent progression of PCa.

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