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Prosaposin Is an AR-Target Gene and Its Neurotrophic Domain Upregulates AR Expression and Activity in Prostate Stromal Cells

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Abstract Recent studies have introduced prosaposin (PSAP) as a pleiotrophic growth factor for prostate cancer (PCa). We have previously reported that PSAP or one of its known active molecular derivatives, saposin C functions as an androgen-agonist and androgen-regulated gene (ARG) for androgen-sensitive (AS) PCa cell lines. Due to the potential significance of androgen receptor (AR)-expressing stroma in PCa, we evaluated a possible bi-directional paracrine regulatory interactions between DHT and PSAP in AR-positive prostate stromal (PrSt) cells. We report that saposin C in a ligand-independent manner increased AR expression, its nuclear content, and tyrosine phosphorylation. DHT treatment of PrSt cells increased PSAP expression. We also demonstrated both serum- and androgen-inducibility of a previously characterized hormone-responsive element (HRE) located in the proximal region of *PSAP* promoter. In addition, conditioned-media derived from PrSt cells and bone fibroblasts (i.e., MSF) differentially increased *PSAP*-promoter activity in androgen-independent (AI) PC-3 and AS LNCaP cells. Our data for the first time demonstrate that not only saposin C or PSAP regulates AR expression/activity, but also function as an ARG in PrSt. Ligand-independent activation of AR by PSAP or saposin C in PCa and stromal cells may contribute not only to prostate carcinogenesis at an early stage, but also in AI progression of the disease in an androgen-deprived tumor microenvironment. J. Cell. Biochem. 104: 2272–2285, 2008.

Key words: PSAP; AR; stromal cells; prostate; DHT

Prostate cancer (PCa) presents as the second leading cause of cancer mortality in western men [Jemal et al., 2007]. It initiates as an androgen-dependent (AD) disease which progresses to androgen-independent (AI) state. Although PCa patients initially benefit from androgen-ablation therapy, most cases eventually fail this therapy and die from recurrent AI tumors. Androgen receptor (AR) is probably the most important receptor involved at any stage during normal and neoplastic development of the prostate gland. Using tissue recombination, it has been demonstrated that mesenchymal–epithelial interactions dictates normal prostate epithelial growth, development, and differentiation in AD-manner [Chung et al., 1991].

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Dynamic interactions between prostate epithelial or cancer cells and stromal cells play a fundamental role in disease progression and hormone responsiveness [Chung et al., 1991; Sung et al., 2007]. Lack of recognition of the functional involvement of the stromal components has been considered as a strong reason for the failure of our therapeutic interventions for PCa. It is now believed that therapies directed at co-targeting tumor and stroma may be the only alternative therapeutic approach [Cher et al., 2006; Chung et al., 2006; Vessella and Corey, 2006; Morrissey and Vessella, 2007; Sung et al., 2007]. In PCa, several mechanisms might be responsible for AI activation of AR including genomic amplification or mutation, overexpression of AR or its co-activators, or signaling cross-talk with tyrosine kinases (e.g., HER2) [Craft et al., 1999; Feldman and Feldman, 2001].

Prosaposin (PSAP) is a highly conserved glycoprotein and is the precursor of four small lysosomal proteins known as saposins A, B, C, and D, which are required for intracellular degradation of certain sphingolipids [Kishimoto et al., 1992; Huwiler et al., 2000; Sandhoff and Kolter, 2003; Koochekpour, 2006]. Extracellularly, PSAP exists as a secreted molecule known for its neurotrophic activities. Several reports have identified the saposin C domain of PSAP responsible for its in vitro and in vivo neurotrophic activity. Synthetic peptides (e.g., TX14A) derived from its N-terminal portion exert similar biological activities to saposin C or its precursor.

The first evidence demonstrating a potential developmental role for PSAP in the prostate originated in male mice with homozygous deletion of the *PSAP* gene. These mice showed distinct abnormalities in reproductive organs, with gross pathologic features including a reduction in size and weight of the testes, epididymis, seminal vesicles, and prostate gland. In spite of these abnormal findings, the testosterone level was normal or even elevated. Microscopic examination also revealed rudimentary and involuted prostate, seminal vesicles, and epididymis with undifferentiated epithelial cells [Morales et al., 2000].

We have previously reported that (1) PSAP expression is higher in metastatic AI PCa cells than in androgen-sensitive (AS) PCa, normal prostate epithelial, and stromal cells [Koochekpour et al., 2004b, 2005b]; (2) the *PSAP* gene is amplified in PCa cells, punch biopsy specimens of PCa xenografts (LuCaP), and metastatic tissues; (3) prosaposin and/or its active molecular derivatives (i.e., saposin C, TX14A synthetic peptide) stimulate growth, migration, and invasion, act as cell survival and anti-apoptotic factors, activate MAPK- and PI3K/Akt-signaling pathways, and upregulate uPA/uPAR proteolytic enzyme expression in both AI and, AD PCa cells; and (4) immunohistochemical staining of benign and malignant prostate tissues also revealed an intense granular (extracytosolic) anti-PSAP immunoreactivity in tumor cells and in stromal, endothelial, and inflammatory mononuclear cells [Koochekpour et al., 2004a; Lee et al., 2004].

Recently, we reported that PSAP and/or saposin C, in an AI-manner, upregulated AR and AR-target genes (i.e., PSA, probasin) expression and activity in AS LNCaP cells [Koochekpour et al., 2007a]. In agreement with these observations, a recent report has demonstrated that ectopic expression of neurotrophic peptide of amino-terminal domain of saposin C stimulated AS- and AI PCa cells proliferation and upregulated AR expression and transactivation in androgen-responsive PCa cell lines [Ding et al., 2007]. In addition to the saposin C effect on AR expression and activity, DHT treatment of LNCaP cells increased PSAP expression. We also demonstrated androgen-responsiveness of *PSAP* promoter and AR occupancy to a hormone-responsive element (HRE) located in its proximal region [Koochekpour et al., 2007b]. Overall, our data indicate that PSAP (or saposin C) not only functions as an androgen-agonist, but also as a novel androgen-regulated gene (ARG) in AR-expressing PCa cell lines.

Here, we examined the functional relationship between saposin C and AR in a previously characterized prostate stromal (PrSt) cell line [Koochekpour et al., 2004a]. Our data show that saposin C increases PrSt cells' and AR expression, phosphorylation state, and its nuclear content. In addition, we also show that bicalutamide (antiandrogen) cannot efficiently antagonize the androgenic induction of *PSAP* expression and promoter activity in the cells. We also found that *PSAP* promoter activity could be induced by serum and androgen in PrSt and PCa cells. Finally, conditioned media derived from PrSt cells and bone fibroblasts (MSF) was also able to increase *PSAP* promoter activity in both AI- and AS-PCa cells.

Overall, our results implicate PSAP and saposin C as potentially important paracrine mediators of PCa and stromal cells interactions. By upregulating AR expression and AR-target gene activity in tumor microenvironment, saposin C and/or PSAP could potentially play an important role in AR-dependent, but AI-progression of PCa.

MATERIALS AND METHODS

Cell Culture, Reagents, and Antibodies

PrSt cells were originally derived from a primary culture of normal PrSt cells purchased from BioWhittaker (Walkersville, MD) and initially maintained in stromal cell growth medium (SCGM; Walkersville). After several cycles of trypsinization and under in vitro culture condition, these cells were spontaneously transformed and could be cultured in DMEM, RPMI-1640, or T-medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) and antibiotics (penicillin, 100 units/ml and streptomycin, 100 μg/ml) at 37°C with 5% CO_2 in a humidified incubator. AI (PC-3 and DU-145) and AS (LNCaP) PCa cell lines, MRC-5 (Lung embryonal fibroblast), and NIH 3T3 cell lines were purchased from ATCC (Manassas, VA). PC-3, DU-145, and MRC-5 cell lines were cultured in DMEM supplemented with 10% FBS and 1% antibiotics. The LNCaP cell line was grown in RPMI-1640 supplemented with 10% FBS, 1 mM sodium pyruvate and 10 mM HEPES. MSF (bone fibroblast) was kindly provided by Dr. Martin Gleave and cultured in T-medium (Invitrogen) supplemented with 5% FBS. All tissue culture media were from Invitrogen. The source and characterization of purified recombinant human saposin C has been described before [Henseler et al., 1996; Koochekpour et al., 2004b]. Mouse monoclonal anti-human PSAP (or saposin C) has been previously characterized [Koochekpour et al., 2004b, 2005a,b]. Anti-GAPDH antibody and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Charcoal-stripped fetal bovine serum (CS-FBS), dihydrotestosterone (DHT), cycloheximide, and actinomycin D were obtained from Sigma (St. Louis, MO).

Immunoprecipitation and Immunoblotting

Cells were grown up to 65–70% confluency in their respective maintenance medium, washed with PBS, and incubated in phenol red (PR)-free RPMI supplemented with 5% CS-FBS for 24–36 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 or saposin C at the indicated concentrations.

To evaluate for tyrosine phosphorylation of AR, after androgen-deprivation, PrSt cells were incubated with PR-free basal medium in the presence or absence of DHT or Saposin C for 6 h. Whole cell lysates were immunoprecipitated with anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY; 2 μg antibody for 340 μg protein in PrSt and 3 μg antibody for 750 μg protein in LNCaP) followed by immunoblotting with anti-AR antibody [Koochekpour et al., 1999]. Total AR was determined by stripping the membrane and reprobing with AR antibody.

In order to evaluate the effect of various pharmacological inhibitors on protein and mRNA expression of AR and PSAP, 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), cycloheximide (at 1 μg/ml), or bicalutamide (10 μM) for 30 min. After this period, saposin C or DHT (1.0 or 10 nM) was added directly to the culture medium. To evaluate the potential cytotoxic effect of the reagents used, parallel tissue culture plates were also prepared and treated as mentioned above; cell viability was determined using the trypan blue dye-exclusion assay. To detect PSAP expression in culture supernatants, all tissue culture plates were incubated for 16–24 h in basal medium and at the end of the incubation period, cell-free culture supernatants were collected and concentrated up to 10 times by using a Centriprep-3 concentrator (with a 3.0 kDa molecular weight cut-off, Millipore, Billerica, MA). Whole cell lysates were also prepared from the same tissue culture plates and protein samples (for AR in LNCaP 10 μg/lane and PrSt 15 μg/lane, or 15 μg/lane for PSAP) were subjected to SDS–PAGE and immunoblotting as previously described [Koochekpour et al., 2007b]. Membranes were probed with anti-AR (at 1 μg/ml; Clone PG-21, Upstate Biotechnology, Inc.), or mouse monoclonal anti-human PSAP/saposin C (at 1:1,000 dilution) as the primary antibody and signals were detected by ECL detection system (Amersham,

Piscataway, NJ). Normalization of loading control for culture supernatants was based on the total cell number and/or protein content. To demonstrate the relative higher expression of AR in LNCaP cells compared to PrSt cells, we performed densitometric analysis (Quantity one, Bio-Rad, CA) on AR and GAPDH protein.

Preparation of Nuclear and Cytoplasmic Extract

Cells were grown up to 75% confluency and androgen-deprived as described above. After washing plates with PR- and serum-free medium RPMI, cells were incubated in this medium in the presence or absence of saposin C or DHT (1.0 nM) for 3 h. Nuclear and cytoplasmic extracts were prepared from a single plate by using the NE-PER Nuclear Extraction Kit (PIERCE, Rockford, IL) according to manufacturer's instructions and as described in previous reports [Koochekpour et al., 2007a,b].

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 AR or PSAP. Total RNA extraction and RT-PCR on *AR*, *PSAP*, and *GAPDH* were carried out as described before [Koochekpour et al., 2007a,b]. The sizes of amplified cDNA fragments, the optimized Tm, time period, and the number of cycles for PCR were: 702 bp/52°C for 60 s/35 cycles for PrSt cells for *AR*, and 898 bp/52°C for 60 s/35 cycles for *PSAP*, and 566 bp/57°C for 60 s/25 cycles for *GAPDH* (amplified either alone or in a duplex PCR). 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 *PSAP* promoter deletion constructs H-813, H-343, and H-213 in firefly luciferase reporter 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 *PSAP* 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 [Koochekpour et al., 2007a,b]. Promoterless pGL3B vector was used as control for transfection.

Transient Transfections and Reporter Gene Assay

PCa and stromal cells were seeded at a density of $1.5–2 \times 10^5$ 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 36 h in PR-free RPMI (for LNCaP and stromal cells) supplemented with 5% CS-FBS or DMEM (for PC-3 cells transfected with pGL3B-813-Luc). Transfections were carried with 1.2 ml of serum-free OPTI-MEM containing 7.5 μl/well of lipofectine reagent (Invitrogen), 0.25 μg of pSV-β-galactosidase vector (Promega), and 0.75 μg of test DNA (pGL3B-H813, -H213, or-H343, or pGL3B-HRE). 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 the removal of the transfection medium, serum- and PR-free basal media was added to the wells for an additional 12 h. Cells were treated with different concentrations of DHT, FBS, CS-FBS, or serum-free conditioned medium (=0.4 mg protein) prepared under androgen-deprived culture conditions as indicated previously. Pre-treatment or treatment of cells with various pharmacological inhibitors was completed at the concentration indicated above. A time- and dose-course study was performed to evaluate the kinetic of the effect of DHT, or other treatment reagents on individual test DNAs and cell lines under investigation. Due to variations in transfection efficiencies among experiments, after considering the basal control value in individual experiments, relative values for co-transfections assays have been calculated and normalized.

Luciferase activity was determined as described in details in previous reports [Koochekpour et al., 2007a,b]. Luciferase activity of the test DNAs was referenced to those from the promoter-less (empty) vector. The results are

presented as relative luciferase activity (RLA), and are defined as luciferase activity normalized to internal control SV40/β-galactosidase activity for transfection efficiency. All transfection experiments were conducted in triplicates (or more) and repeated 3–5 times independently. RLA is presented as the mean ± SEM.

RESULTS

PSAP and AR Expression in Prostate Stromal and Cancer Cells

The expression of AR and PSAP was determined by western analysis of cell lysates and culture supernatants, respectively. As demonstrated by densitometric analysis, AR was expressed at a higher level in LNCaP than in PrSt cells, but not in AI PC-3 and DU-145 cells (Fig. 1). The expression level of secreted PSAP in PrSt cells was less than PCa cell lines. Saposin C, as a mature soluble neurotrophic protein with an approximate molecular weight of 10 kDa, expressed at higher level in AI PCa than in AS LNCaP or PrSt cells. Since saposin C cannot be secreted, it is likely that extracellular saposin C is the result of catalytic cleavage of the secreted PSAP. This result clearly demonstrates a differential expression pattern for saposin C and its precursor (PSAP) in PrSt, AS-, and AI-PCa cells.

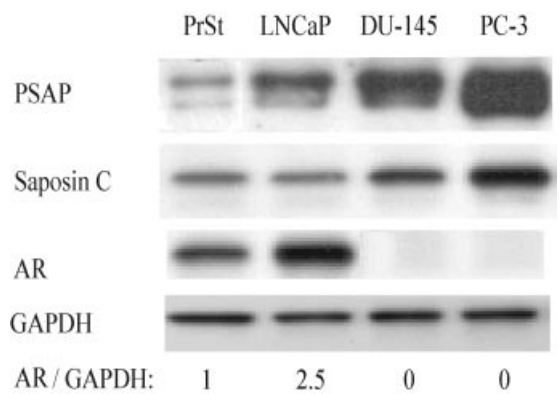

Fig. 1. PSAP and AR expression in prostate stromal and cancer cells. Cells were grown up to 70% confluency and incubated in serum-free basal medium for 24 h. Culture supernatants were collected, centrifuged, and concentrated. Fifteen micrograms protein per cell line was subjected to SDS–PAGE, and immunoblotted with a mouse monoclonal anti-human PSAP/Saposin C antibody. PrSt; normal prostate stromal cells. DU-145 and PC-3 were used as androgen-independent and LNCaP was used as androgen-sensitive prostate cancer cell lines. Anti-GAPDH antibody used as loading control for cell lysates. Loading control for culture supernatants was based on protein content and total cell number. Relative AR expression in the cells was (AR/GAPDH ratio) determined by densitometric analysis. The experiment was repeated at least twice independently.

DHT Upregulates AR and PSAP Expression in Prostate Stromal Cells

We have previously shown that DHT increases AR and PSAP expression in LNCaP cells. Here, we examined such possibility for AR-expressing PrSt cells. We found that DHT increases AR and PSAP protein and mRNA expression in the cells with a peak effect at 1.0 nM concentration (Figs. 2 and 3). Pre-treatment and treatment of PrSt cells with inhibitors of RNA transcription or protein translation substantially reduced DHT effect. Treatment of

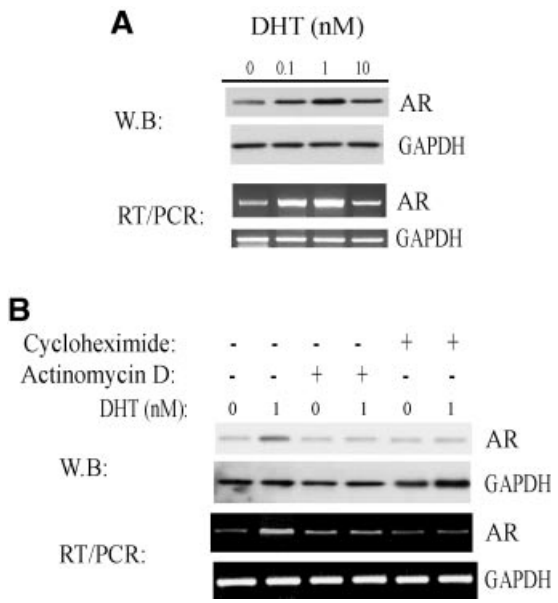

Fig. 2. DHT increases AR expression in prostate stromal cells. Cells were grown up to 70% confluency in their respective maintenance media, androgen-deprived in 5% CS-FBS/PR-free RPMI (for PrSt cells) or DMEM (for PC-3 and DU-145 cells) for 24 h, and incubated in basal PR-free medium supplemented with or without DHT at the indicated concentrations for 16 h. Parallel tissue culture plates were also pretreated and treated with actinomycin D or cycloheximide for 30 min in the presence or absence of the above effectors. **A**: Protein samples (15 μg/lane) were subjected to SDS–PAGE and immunoblotting using anti-AR antibody. **B**: Total RNA was also extracted from the parallel tissue culture plates using RNAzol B reagent. First strand cDNA was synthesized and used for PCR as described in ''Materials and Methods'' Section. GAPDH antibody and primers were also used for loading control. Results are representative of three independent experiments.

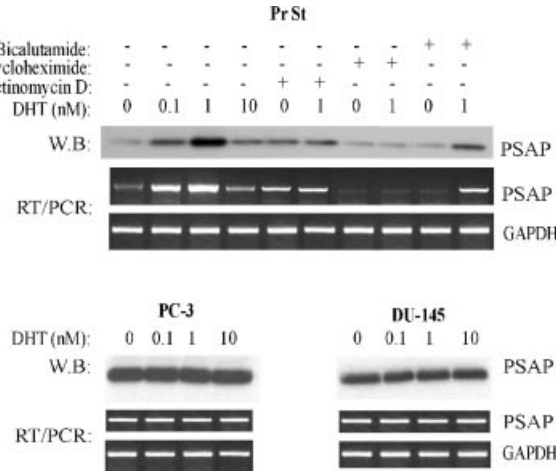

Fig. 3. DHT upregulates PSAP expression in prostate stromal cells. Cells were androgen-deprived and pretreated with actinomycin D (0.5 μg/ml for 30 min), cycloheximide (1 μg/ml for 30 min), or bicalutamide (10 μM for 30 min) in serum-free/PR-free RPMI. After washing, cells were then treated with DHT at the indicated concentrations. Plates pretreated with actinomycin D, cycloheximide, or bicalutamide were treated with DHT without changing the culture medium. All tissue culture plates were incubated for 16 h and after this period, culture supernatants (20 μg/lane) and total RNA were prepared from the same tissue culture plate. Protein and RNA samples were used for western analysis and RT/PCR. Normalization of culture supernatant was based on the total cell number as well as on protein content. GAPDH antibody and primers were also used to monitor loading control. The experiment was repeated at least twice independently.

PrSt cells with antiandrogen bicalutamide reduced androgenic induction of PSAP to the level expressed at 0.1 nM saposin C. On the contrary, DHT treatment of AI PC-3 and DU-145 cells did not increase PSAP protein or mRNA levels. Overall, these data indicate that (a) DHT upregulation of PSAP is being mediated by AR; (b) PSAP is an AR-target gene; (c) androgen-induction of *PSAP* expression is regulated both at the gene transcription and translation level; and (d) bicalutamide does not appear at least to be an efficient antiandrogen in PrSt cells.

PSAP Promoter Activity Is Induced by Serum and Androgen in Prostate Stromal and Cancer Cells

We have previously demonstrated the androgen-inducibility of PSAP promoter activity in LNCaP cells. In addition, we also showed that upon androgen stimulation, AR associates with the only HRE motif (−242/−237 bp upstream to ATG) located in the early region of PSAP promoter [Koochekpour et al., 2007b]. Next, we examined the effect of DHT on activity of proximal region of *PSAP* promoter (−813 bp). This region contains the highest frequency of transcription factors consensus sites (e.g., Sp1, SRY) within the 5-kb upstream to transcription initiation site of the *PSAP* gene.

DHT induced transcriptional activity of (−813 bp)-*PSAP* by 45–116% in PrSt cells (Fig. 4A). This result demonstrates that DHT has a paracrine stimulatory effect on *PSAP* promoter activity and this effect is mediated by AR. Next, we investigated the effect of DHT on luciferase activity on several deletion constructs (e.g., −95, −213, −343, and −493 bp 5′ to ATG) in the proximal *PSAP* promoter that were characterized previously [Sun et al., 1998]. DHT induced luciferase activity of H-213 deletion construct slightly (by 13%) in PrSt cells. The activity of H-343 construct activity (containing the HRE-motif) was also increased by 68% in PrSt cells (Fig. 4A). However, the H-95 construct was non-responsive to DHT and the H-493 deletion construct activity was considerably less than H-343 promoter fragment (data not shown). These differential responses could be due to the presence of positive or negative regulatory elements located in individual deletion constructs [Sun et al., 1998].

Among all other growth factors, serum also contains androgens, saposin C, and PSAP. Next, we decided to test the effect of serum (FBS) on transcriptional activity of *PSAP* promoter (−813 bp) in the AR-negative cells PC-3 and DU-145 and AR-positive LNCaP and PrSt cells. FBS in a dose- and time-dependent manner induced *PSAP*-luciferase activity by 100% in PC-3, by 64% in DU-145, and by 60% in LNCaP cells. Serum induced transcriptional activity of *PSAP* in PrSt cells by sixfold; this was considerably higher than the DHT effect (Fig. 4B). Together, these data indicate that *PSAP* promoter activity could be induced by the naturally existing paracrine androgenic and non-androgenic factors.

DHT Induces Transcriptional Activity of a Hormone-Response Element Located in the Proximal *PSAP* Promoter in Prostate Stromal Cells

We demonstrated that DHT can increase PSAP protein/mRNA expression in PrSt cells. This data clearly indicate that AR is involved and its binding to an ARE or some other androgen-responsive consensus site(s) in the *PSAP* promoter is a strong possibility. In addition,

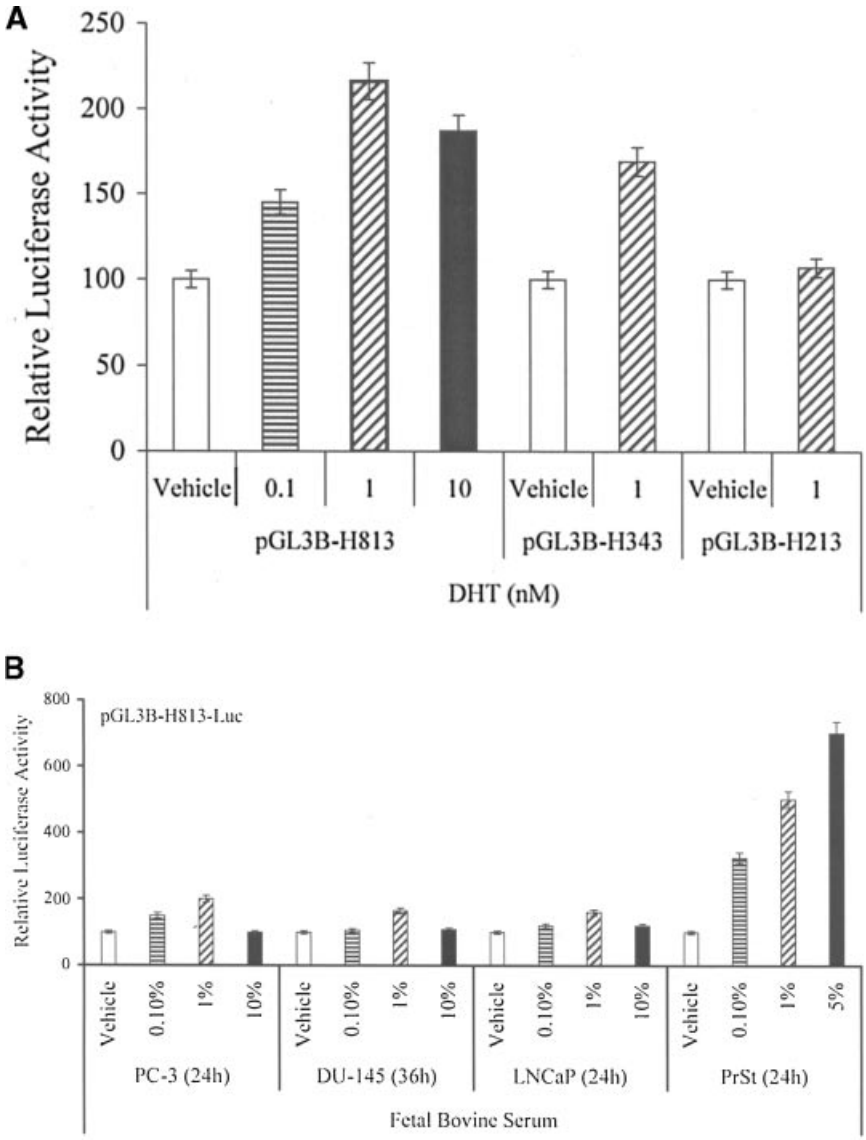

Fig. 4. Induction of transcriptional activity of proximal PSAP promoter by serum and androgen in prostate cancer and stromal cells. Transcriptional activity of *PSAP* promoter in prostate cancer cells is androgen (**A**) and serum dependent (**B**). Deletion constructs H-813, H-213, and H-343 of human early *PSAP* promoter region were subcloned in pGL3B firefly luciferase reporter vector. Cells were seeded at a density of $1.5–2 \times 10^5$ 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 PR-free basal media. Co-transfection of cells with the promoter-less pGL3B vector and pSV-β-galactosidase were used as control for transfection and luciferase activity assay (Vehicle). After 12 h, the transfection medium removed, PR-free basal media were added to the wells for an additional 12 h, and cells were incubated in the presence or absence of DHT (for LNCaP) or serum at the indicated concentrations and treatment periods. Luciferase activity assay was determined as described under ''Materials and Methods'' Section. The results are presented as relative luciferase activity (RLA) and are defined as luciferase activity normalized to internal control pSV-β-galactosidase activity for transfection efficiency. All transfection experiments were conducted in triplicates and repeated at least three times independently. 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. Statistical significance of the effect of androgen or serum 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 $P < 0.05$.

we showed that *PSAP* proximal (−813 bp) promoter is androgen-inducible and among deletion constructs investigated, the −343 and −213 bp fragments of *PSAP* promoter showed the highest and lowest reporter gene activity in response to androgen, respectively. As reported recently, in LNCaP cells, we identified a single consensus hexamer (−242/−237), 5′-GTTCTT that is equivalent to HRE and also closely resembles the 3′-half of a glucocorticoid-responsive element (GRE1/2) [Cato et al., 1987; Parker et al., 1988; Koochekpour et al., 2007b]. Following transient transfection of cells with the pGL3B-HRE-luciferase, cells were treated with DHT in the presence or absence of antiandrogen bicalutamide. DHT induced reporter gene activity in a dose-dependent manner in PrSt cells. Luciferase activity was increased by 30–103% in PrSt cells (Fig. 5A). Treatment of PrSt cells with pure antiandrogen bicalutamide decreased androgenic induction of the reporter gene activity. However this inhibition was only partial and luciferase activity was still 48% more than the control values. Together, our results indicate that bicalutamide is not efficient in blocking the induction of androgen-responsive HRE-reporter gene of *PSAP* in PrSt cells. To determine serum-responsiveness of this reporter gene, we examined the effect of both CS-FBS and FBS on luciferase activity. Although both types of serum in a dose-dependent manner were capable of inducing reporter gene activity, the effect of androgen-depleted FBS was less prominent than the normal FBS (Fig. 5B). These data indicate that not only the androgen-sensitivity of HRE-reporter gene, but also the presence of other serum factors that could synergize with androgens in transcriptional regulation of *PSAP*.

Conditioned Media Derived From Prostate Stromal Cells and Bone Fibroblasts Differentially Induce *PSAP* Promoter Activity in AI- and AS-PCa Cells

Since serum also had a synergistic effect on *PSAP* transcriptional activity, we decided to test whether stromal-derived soluble factors can play a similar role. Following androgen-deprivation and transient transfection of pGL3B-813-*PSAP* reporter gene, cells were treated with culture supernatants derived from PrSt cells, MSF (bone fibroblasts), MRC-5 (embryonal lung fibroblast), or NIH3T3 cells. PrSt cells' conditioned medium increased *PSAP*

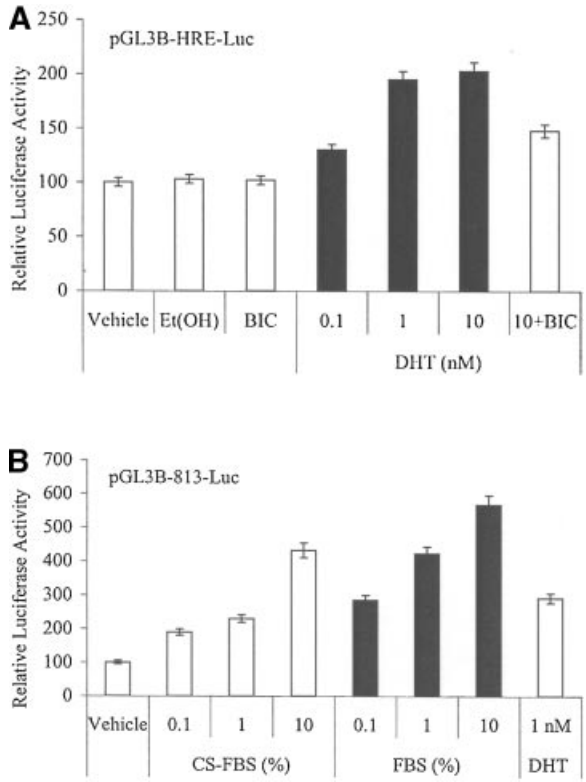

Fig. 5. Effect of androgen, charcoal stripped-FBS, or FBS on transcriptional activity of HRE-containing motif of PSAP promoter in prostate stromal cells. PrSt cells were seeded at a density of $1.5–2 \times 10^5$ cells per well in 6-well plates in their maintenance media and grown up to 70–75% confluency. Cells were incubated in PR-free medium containing 5% CS-FBS for 24 h. Cells were co-transfected with pGL3B-HRE-Luciferase constrcut (**A**) or a deletion construct of early *PSAP* promoter region (**B**) containing HRE sequence (0.75 μg) and pSV-β-galactosidase (0.25 μg). After 12 h, the transfection medium removed, PR-free basal media was added to the wells for an additional 12 h, and cells were incubated in the presence or absence of DHT, CS-FBS, or FBS for 24 h. Co-transfection of cells with the pGL3B vector and pSV-β-galactosidase was used as control for transfection and luciferase activity assay (Vehicle). To evaluate the effect of bicalutamide, cells were pre-treated with bicalutamide (BIC; 10 μM for 30 min) before the addition of DHT. Luciferase activity assay was determined as described under ''Materials and Methods'' Section. The results are presented as relative luciferase activity (RLA) and are defined as luciferase activity normalized to internal control pSV-β-galactosidase activity for transfection efficiency. All transfection experiments were conducted in triplicates and repeated at least three times independently. 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. Statistical significance of the effect of DHT or serum on 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 $P < 0.05$.

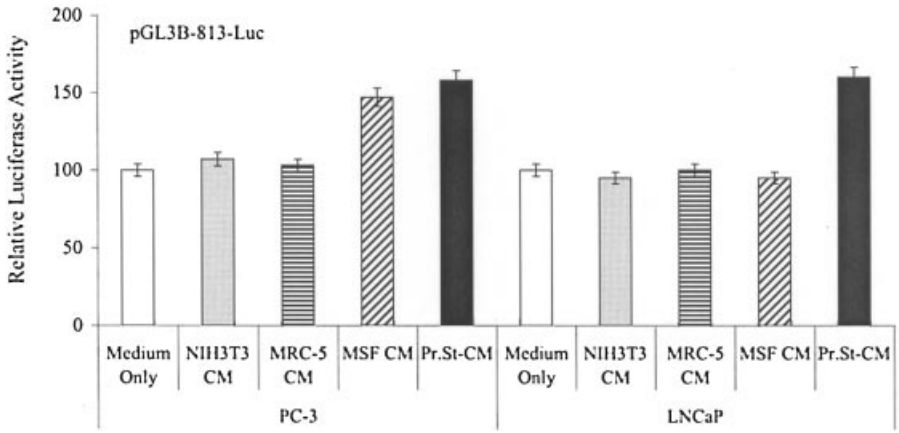

Fig. 6. Androgen-independent induction of transcriptional activity of proximal *PSAP* promoter by tissue-specific stromal cells' conditioned medium in prostate cancer cells. Conditioned media derived from various stromal cells were prepared by androgen-deprivation and incubation of the cells in PR-free basal medium for 24 h. Cell-free culture supernatants were then concentrated and 0.4 mg protein per 1.2 ml was used for a 12 h treatment of the cells. For transfection, PC-3 and androgen-deprived PrSt cells, were grown up to 75% confluency and co-transfected with *PSAP*-H813-luciferase (0.75 μg) and pSV-β-galactosidase (0.25 μg) vectors for 12 h before addition of individual conditioned medium. Cells co-transfected with the pGL3B and pSV-β-galactosidase vectors and treated with the conditioned medium were used as controls. Luciferase activity assay was determined as described under ''Materials and Methods'' Section. The results are presented as relative luciferase activity (RLA) and are defined as luciferase activity 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. Statistical significance for the effect of tissue-specific stromal conditioned medium on *PSAP* promoter activity was evaluated by one-way ANOVA test with Bonferroni adjustment. For each cell line, differences between medium-only and other individual experimental group were evaluated by Student's *t*-test and statistical significance was set at $P < 0.05$.

(−813 bp)-luciferase activity by 57% in PC-3 and by 60% in LNCaP cells (Fig. 6). However, MSF conditioned medium increased PSAP promoter activity only in PC-3 cells. These data indicate the presence of tissue-specific stromal-derived soluble factor(s) capable of inducing *PSAP* transcription in both AI- and AS-PCa cells.

Saposin C Acts as a Novel Androgen-Independent Regulator of AR in Prostate Stromal Cells

Recently, we reported PSAP or its biologically active molecular derivatives (i.e., saposin C, TX14A synthetic peptide), increased AR expression in LNCaP cells. Here, we examined the effect of saposin C on AR expression in PrSt cells under androgen-deprivation culture conditions. We found that saposin C at concentrations as low as 0.1 nM was able to increase AR mRNA and protein levels in PrSt cells (Fig. 7A). To determine whether saposin C induction of AR expression is regulated at the level of gene translation or transcription, we treated cells with the (low) non-toxic dosage of inhibitors of protein translation (cycloheximide; 1 μg/ml) or RNA transcription (actinomycin D; 0.5 μg/ml). Using western analysis and RT/PCR, we found that actinomycin D treatment downregulated AR mRNA and protein expression in PrSt cells (Fig. 7B). This observation indicates a transcriptional regulatory function for saposin C induction of AR. In addition, cycloheximide also suppressed DHT-induced AR expression in PrSt cells, both at the protein and mRNA level. This result indicates that in addition to the direct translational regulatory mechanism(s), DHT modulate AR mRNA expression via an indirect mechanism that requires de novo protein synthesis.

Saposin C Tyrosine Phosphorylates AR and Increases Its Nuclear Content in LNCaP Cells

Although AR upregulation increase androgen-sensitivity of PrSt cells, by itself it is not an indication for AR activation. Therefore, as a sign of steroid hormone receptor activity [O'Malley et al., 1991; Culig et al., 2000], we examined both AR-nuclear translocation and phosphorylation status in the cells. While the total AR level was not affected, treatment of cells with saposin C for 3 h increased nuclear-

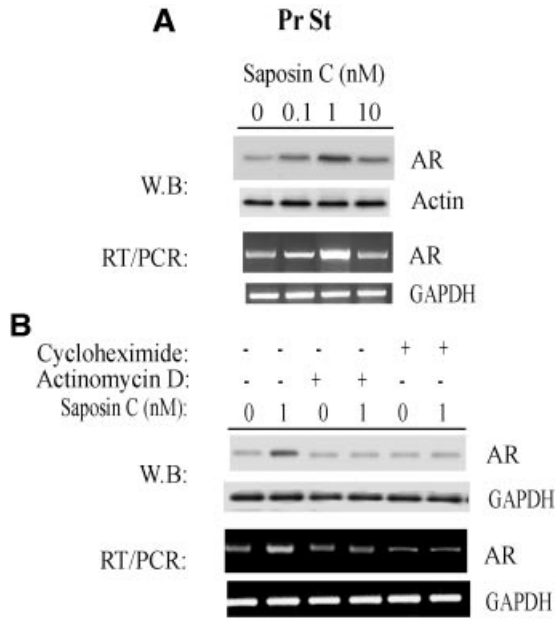

Fig. 7. Saposin C upregulates AR expression in an androgen-independent manner in prostate stromal cells. PrSt cells were grown up to 70% confluency in their respective maintenance media, androgen-deprived in 5% CS-FBS/PR-free RPMI for 24 h, and incubated in serum- and PR-free RPMI supplemented with or without saposin C at the indicated concentrations for 16 h. Parallel tissue culture plates were also prepared for pre-treatment and treatment with actinomycin D or cycloheximide. Protein and total RNA were extracted and samples were subjected to immunoblotting (**A**) or RT/PCR (**B**) as described in Figure 2 and ''Materials and Methods'' Section. GAPDH antibody and primers were also used as controls.

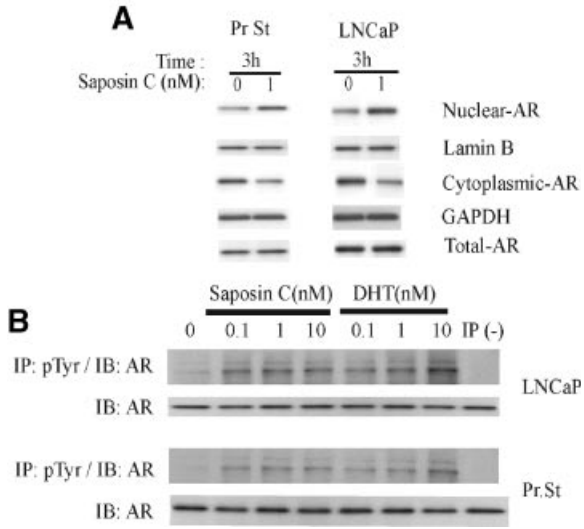

Fig. 8. Saposin C phosphorylates AR at tyrosine residues and increases its nuclear content in an androgen-independent manner in prostate stromal and LNCaP cells. Cells were grown up to 70% confluency in their maintenance medium, androgen-deprived in 5% CS-FBS and PR-free medium for 36 h, and then treated in PR-free basal medium in the presence or absence of saposin C or DHT. **A**: Saposin C increases nuclear AR level in androgen-sensitive PrSt and LNCaP cells. Nuclear and cytosolic extracts were prepared after 3 h treatment of androgen-deprived cells with saposin C or DHT, followed by SDS–PAGE and immunoblotting with anti-AR antibody. Whole cell lysate prepared from parallel tissue culture plates was used for loading control with anti-AR antibody. Results are representative of three independent experiments. Loading control for nuclear and cytoplasmic protein extracts was provided by reprobing the membrane with anti-Lamin B and -GAPDH antibodies, respectively. **B**: Saposin C induction of AR-tyrosine phosphorylation. Cell lysates were immunoprecipitated using anti-phosphotyrosine antibody (4G10) followed by immunoblotting with anti-AR antibody. Total AR was determined by stripping the membrane and reprobing with AR antibody.

AR content up to onefold as determined by densitometry (Fig. 8A). In parallel with this observation, there was a reduction in the level of cytoplasmic AR. As a positive control, DHT treatment of cells for 24 h led to an increase in both nuclear and total AR level (data not shown). Loading control for nuclear and cytoplasmic protein extracts was provided by reprobing the membrane with anti-Lamin B and -GAPDH antibodies, respectively. To further show AR-activation status, we examined serine- and tyrosine-phosphorylation of AR in the presence of DHT or saposin C. Under our experimental condition, we did not detect increased phosphoserine activity. However, we found higher levels of anti-pTyr activity in DHT or saposin C treated cells (Fig. 8B). The rapidity of nuclear AR-translocation and its phosphorylation indicates that saposin C transactivates AR and this effect is not dependent on upregulation of the total AR level.

DISCUSSION

Dynamic and reciprocal interactions between stroma and tumor cells both in primary and metastatic PCa have a fundamental role in the progression of disease [Chung et al., 1991]. These interactions are mediated by steroid hormones, soluble and insoluble factors, and extracellular matrix (ECM) components of tissue microenvironment. Growth promoting effect of androgen on prostatic epithelium is dependent on the presence of functional AR in stroma [Chung et al., 1991; Sung et al., 2007]. A number of studies have raised the possibility for AR signaling in prostate stroma as a contributing factor to tumor recurrence following androgen-ablation therapy [Kurita et al., 2001; Johansson et al., 2005; Ohlson et al., 2007]. It

has been shown that involution of prostate gland caused by castration was secondary to changes in the prostatic stroma [Kurita et al., 2001; Ohlson et al., 2007]. Recently, it has been demonstrated that castration decreased nuclear AR presence in both PCa and stromal cells and local tumor recurrence following surgical castration was associated with the reappearance of nuclear AR in prostate epithelial and stromal cells [Wikstrom et al., 2007]. These studies indicate a more active role for AR expression/signaling in prostate stroma.

Recently we reported that saposin C or its precursor PSAP not only upregulates AR and PSA expression and activity, but also presents as an androgen-regulated gene in LNCaP and/or other AR-expressing PCa cell lines [Koochekpour et al., 2007b]. These observations prompted us to explore the possibility for a functional relationship between saposin C and AR in AS PrSt cells.

Our data showed that saposin C, also in an AI-manner, increased AR and PSAP expression in the cells. As a natural consequence, this effect could potentially provide a growth advantage to AD tumor cells in primary tumor foci. In addition, by conferring androgenic effects to both stromal and/or cancer cells in a post-treatment androgen-deprived environment, it may compensate the iatrogenic androgen-deficiency in favor of AI progression of PCa. In our previous report, we observed that the antiandrogen bicalutamide not only failed to inhibit DHT-induction of PSAP expression, but also acted as a partial androgen-agonist in LNCaP cells [Koochekpour et al., 2007a]. On the contrary, bicalutamide reduced DHT upregulation of PSAP expression in PrSt cells. This cell type-specific response to bicalutamide could be due to the mutated AR in LNCaP cells as compared to wild-type AR in PrSt cells.

PSAP upregulation by DHT and its inhibition by bicalutamide indicates that AR is involved in transcriptional regulation of *PSAP*. In LNCaP cells, we have previously reported that proximal *PSAP* promoter (−813 bp) is androgen-inducible [Koochekpour et al., 2007b]. In addition, in this region, we identified a single consensus hexamer (−242/−237; 5′-GTTCTT-3′) that resembles closely to the 3′-half of consensus glucocorticoid-responsive elements (GRE1/2) and androgen-responsive elements (ARE-1 and ARE-2). This motif collectively represents HREs and could also be regulated by steroid hormones such as glucocorticoids, progestins, and androgens among others [Cato et al., 1987; Parker et al., 1988; Simon et al., 1995; Yeung et al., 2000].

Using a set of deletion constructs, we first demonstrated that DHT in a dose-dependent manner increased the *PSAP* (−813 bp) luciferase activity in PrSt. Among all deletion constructs examined, DHT differentially induced luciferase activity of H-813, H-343, and H-493 deletion constructs (containing the hexanucleotide sequence of interest) in PrSt cells. In our preliminary data, we also found that deletion of HRE-motif from pGL3B-H813 and pGL3B-H343 constructs reduced luciferase activity in PrSt cell by 15–20% (data not shown). This effect could be due to the presence of more than one transcription factor site in deletion constructs and a potential for overlapping effects (additive, diminutive, or counteracting) among different consensus sites for transcription factors binding and might also involve a variety of signal transduction pathways and AR co-regulatory factors. In addition, serum also in dose-dependent manner was able to induce *PSAP* (−813 bp) reporter gene activity in AI PCa cell lines (PC-3 and DU-145) and AS LNCaP cells. It is noteworthy that PrSt cells response was considerably higher than any other cell types. This could be due to the presence of natural androgens and/or serum factors. To further relate the androgen-responsiveness to the specific 5′-GTTCTT-3′sequence, we constructed the pGL3B-HRE vector and tested its response to DHT. In agreement with bicalutamide inhibition of DHT induction of PSAP expression, the activity of reporter gene containing the HRE in the presence of bicalutamide was reduced significantly in PrSt cells. The exact mechanism(s) for this relative inefficiency of bicalutamide as an anti-androgen in PrSt cells is not known. However, it could be potentially due to (a) increased AR-synthesis; (b) increased availability of AR (following bicalutamide inhibition of AR binding to some other AR-target genes promoter); (c) relative abundance and contribution of specific AR-co-activators (that due to bicalutamide inhibition, were not consumed by engagement and transcriptional activity of AR on promoters of some other AR-targets) to the assembly of the HRE-transcription machinery complex of *PSAP* promoter; (d) DHT-induced production of paracrine stimulatory factors that could stimulate an AR-independent *PSAP* tran-

scriptional activity; or a combination of these. Whether or not AR expression and activity in stromal components of tumor microenvironment might have a role in the development of anti-androgen withdrawal syndrome in PCa remains to be understood. Several reports have demonstrated that the 5′-GTTCT-3′ sequence of HREs confers regulation by glucocorticoids, progestins, and androgens [Strahle et al., 1987; Ham et al., 1988]. In vitro binding studies have demonstrated that the receptors for androgens, glucocorticoid, and progesterone have conserved recognition sequences that encompass the hexanucleotide motif or its closely related variants such as the 3′-right half GRE [Cato et al., 1987; Morin et al., 2000]. Therefore, AR can function as a trans-acting transcription factor for genes containing this DNA motif.

To evaluate whether serum-induction of HRE-containing motif of *PSAP*-promoter is due to the presence of androgenic or non-androgenic factors that exist in the serum, we examined the effect of normal FBS and CS-FBS. Luciferase activity in the cells treated with CS-FBS was approximately 30–46% less than normal FBS. This indicates that non-androgenic serum factors might also synergize with androgens and stimulate transcriptional activity of *PSAP* gene. In addition, our data showed that only conditioned medium derived from the prostate or bone fibroblast (MSF) was able to induce *PSAP*-reporter gene activity in AI PC-3 or AS LNCaP cells as compared to the lung embryonal (MRC-5) or NIH3T3 fibroblasts. In addition, MSF-conditioned medium increased reporter gene activity only in PC-3 cell line. While both cell types were originated from metastatic bone tumors, unlike LNCaP cells, PC-3 cells are poorly differentiated and highly tumorigenic in in vivo tumorigenesis assay. Taken together, these data indicate the androgen-responsiveness of *PSAP* promoter and are suggestive of the presence of potential paracrine positive regulatory factors (e.g., growth factors) present in the serum or secreted as soluble factor(s) by tissue-specific stromal cells, and provides a model system to study the signal transduction pathways involved in regulation of *PSAP* gene activity in PrSt, AS-, and AI-PCa cells.

As a member of the steroid receptor superfamily and nuclear transcription factor, AR-activation or -transactivation requires nuclear AR translocation and an elevated phosphorylation level. Here, we show that as in LNCaP cells, saposin C in an AI-manner increases nuclear AR content. Using two-dimensional phosphopeptide mapping, Edman degradation, tandem mass spectrophotometry studies, AR phosphorylation at serine residues have been previously reported [Kuiper and Brinkmann, 1995; Gioeli et al., 2002]. In our study, we also reported AR-phosphorylation by saposin C and DHT at Serine 81 in LNCaP cells [Koochekpour et al., 2007a]. Here, we demonstrated that saposin C in an AI-manner increased nuclear AR content in PrSt cells. However, using immunoprecipitation study and a pan-phosphoserine antibody, we were not able to detect phosphoserine activity in the cells (data not shown). In addition to serine-phosphorylation on AR, the NetPhos approach predicted several tyrosine phosphorylation sites on AR [Blom et al., 1999]. Recent studies also confirmed this prediction and have provided evidence for AR tyrosine phosphorylation in LNCaP cells in response to androgens, EGF, FGF, and IL-6 [Kraus et al., 2006]. In our study, we showed that both saposin C and DHT are equally potent in phosphorylating AR at tyrosine residues in LNCaP and PrSt cells.

We conclude that (a) our data for the first time provide evidence for the presence of a cross-regulation between PSAP (or saposin C) and DHT/AR in androgen-responsive PrSt; (b) ligand-independent activation of AR by PSAP/saposin C in PCa and stromal cells in tumor microenvironment may contribute to prostate carcinogenesis at an early androgen-dependent or in the development and maintenance of metastatic and/or AI-phenotype; (c) both androgen-activated AR or transactivated-AR can function as a trans-acting transcription factor and via binding to the universal HRE of genes with pleiotrophic activities (e.g., PSAP) may contribute to antiandrogen withdrawal syndrome.

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