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PSA Regulates Androgen Receptor Expression In Prostate Cancer Cells

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Abstract

BACKGROUND—Prostate Specific Antigen (PSA) is a pivotal downstream target gene of the Androgen Receptor (AR), and a serum biomarker to monitor prostate cancer (PrCa) progression. It has been reported that PSA transactivates AR, but the mechanistic requirements of this response have not been investigated.

METHODS—We studied the localization of PSA, AR and Src in intracellular compartments of synthetic androgen (R1881)-stimulated LNCaP and C4-2B PrCa cells, using immunofluorescence and subcellular fractionation approaches. We also investigated the effect of downregulation of PSA on AR expression by immunoblotting and real time PCR using short hairpin RNA (shRNA) and small interfering RNA (siRNA). Src activity was analyzed by immunoblotting.

RESULTS—R1881 stimulation induced nuclear localization of both PSA and AR in LNCaP and C4-2B PrCa cells as well as increased phosphorylation of Src. Stable shRNA or transient siRNA knockdown of PSA resulted in reduced AR protein levels as well as AR mRNA levels in C4-2B cells. Similar to C4-2B cells, ablation of AR levels upon silencing of PSA was also confirmed in VCaP cells, another androgen-independent cell line. Silencing of PSA did not cause significant changes in Src activation; besides, Src regulation by integrins did not appear to affect AR transcriptional activity.

CONCLUSIONS—PSA localizes to nuclei of androgen-stimulated PrCa cells, and controls AR mRNA and protein levels. This regulatory loop is specific for PSA, does not involve known AR

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activators such as Src and AKT, and may contribute to AR signaling under conditions of increasing PSA levels in patients.

Keywords

Src; Integrins; AKT

Introduction

PrCa is the second leading cause of cancer death among men in the United States. Patients with advanced PrCa initially benefit from androgen-ablation therapy, which leads to a decreased level of androgens and temporary tumor remission due to apoptosis of androgen-sensitive tumor cells. However, relapse of disease due to the growth of androgen-independent (AI)/castrate-resistant tumors is frequently observed in patients, and renders the conventional hormone ablation therapy ineffective (1).

As one of the pivotal AR downstream target genes, PSA is a member of the human kallikrein (KLK) family of serine proteases (2) which are synthesized as preproenzymes (3), proteolytically processed by removal of the signal peptide prior to secretion as inactive proenzymes. Processing to obtain the enzymatically active form occurs by further proteolytic removal of a 4–9 amino acid long peptide at the N-terminus (with the exception KLK15). Most KLKs usually form complexes with anti-chymotrypsin (ACT) or α -2-macroglobulins (A2M) (4,5), and are activated by members of the same family or other proteases, such as Matrix Metalloproteinases (MMPs), and urinary Plasminogen Activator (uPA) (4,5). PSA is activated by KLK2 in vitro (6,7), and as a secreted protein functions to inhibit coagulation of the seminal fluid; its major substrates are seminogelin I and II, fibronectin, transforming growth factor β (TGF β), parathyroid hormone (PTH) related peptide, and plasminogen (8,9). PSA protein levels often increase in the serum of PrCa patients, and this has been used, although not without controversies, as an easily accessible and clinically relevant biomarker of early diagnosis as well as emergence of recurrent, castrate-resistant disease (10,11). In addition, it has also been proposed that PSA may directly participate in PrCa development, potentially by promoting AR signaling (12), but the requirements of this pathway are still largely elusive.

In this context, aberrant activation of AR by deregulated signaling pathways, including MAPK, Src/Ras/Raf, or JNK plays a pivotal role in the development and progression of PrCa in humans (13). Specifically, multiple studies support a role for Src tyrosine kinase and its family members in PrCa (13), potentially by integrating signals from growth factors and chemokines (14). Src binds to and phosphorylates AR on Tyr534 (15), inducing its activation. This oncogenic pathway can be further independently contributed by the $\alpha_v\beta_3$ integrin (16), a member of a large family of transmembrane heterodimeric receptors that promote a plethora of adhesion-dependent cellular responses, including proliferation, survival, migration and invasion (16,17).

In this study, we investigated a potential contribution of PSA in the regulation of AR. Unexpectedly, we found that PSA accumulates in nuclei of androgen-stimulated PrCa cells, co-localizing with AR, and maintaining its mRNA and protein expression levels, in a pathway independent of Src activation or integrin expression. These data suggest the existence of a novel feedback loop between AR and PSA, in which PSA can play an active role in the control of AR expression.

Materials and Methods

Reagents

Synthetic androgen R1881 was obtained from Perkin-Elmer. The following rabbit polyclonal antibodies (pAbs) were used for immunoblotting (IB): p-Src (Y416, Cell Signaling), AKT1/2/3 (H-136), p-AKT (S473), ERK1 (C16, Santa Cruz), Survivin (SV, Novus Biologicals), AR (N-20, Santa Cruz), PSA (DAKO Cytomation). The goat pAb to RCC1 (Santa Cruz) was used as a marker of the nuclear fraction (18,19) in sub-cellular fractionation experiments. The following mouse monoclonal antibodies (mAbs) were used against AR (AR441, Santa Cruz), c-Src (Cell Signaling), and α -tubulin (DM1A, Sigma) for IB. Purified non-immune mouse IgGs (mIgG) and rabbit IgGs (rIgG) (Pierce) were used as negative controls, and HRP-conjugated secondary Abs to mouse, rabbit (Cell Signaling) and goat (Santa Cruz) were used for secondary detection. Rabbit pAb to PSA (DAKO Cytomation), mAbs to c-Src (Santa Cruz) and Giantin (Abcam, 9B6), and rhodamine and fluorescein-conjugated secondary Abs (Jackson Laboratories) were used for immunofluorescence (IF). 4', 6-diamidino-2-phenylindole (DAPI) used for IF was purchased from Molecular Probes, Invitrogen. MG-132, proteasome inhibitor, was obtained from Boston Biochem.

Cells and Culture Conditions

LNCaP (17) and C4-2B cells were cultured in RPMI (GIBCO, Invitrogen) supplemented with 5% FBS (fetal bovine serum) (Gemini Bioproducts Inc.), 2 mmol/L glutamine, 100 μ g/ml streptomycin-100U/ml penicillin, 0.1 mmol/L nonessential amino acids and 1 mmol/L sodium pyruvate (GIBCO, Invitrogen). Cells were starved using RPMI supplemented with 2% CSS (charcoal stripped serum) for 24 hrs. VCaP cells (20) were cultured in DMEM (GIBCO, Invitrogen) supplemented with 10% FBS.

Sub-Cellular Fractionation

3×10^6 cells were trypsinized, and the pellet was washed once with PBS. The pellet was then resuspended in Buffer A (10 mM HEPES at pH 7.9, 10 mM KCL, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 10 mM NaF, 1 mM Na_2VO_3 , 0.1% Triton X-100, protease inhibitors, 0.2mM PMSF), vortexed and incubated for 30 mins. Cell homogenate was centrifuged at 3,300 RPM for 15 mins, and the supernatant was collected and stored as cytosolic extract. The pellet obtained (nuclear fraction) was incubated in an equivalent volume of buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.2 mM PMSF, and protease inhibitors) for 30 mins, the homogenate was subjected to two cycles of sonication (10 seconds/cycle) and centrifuged at 13,000RPM for 15mins to remove contaminants such as chromatin. The supernatant obtained from the above centrifugation was collected and stored as the "nuclear fraction". All steps were performed at 4°C.

Immunoblotting (IB)

Preparation of cell lysates, SDS-PAGE and IB experiments were performed as previously described (21,22). For experiments performed using MG-132, a proteasome inhibitor, cells were incubated for 7 hrs in serum-free medium supplemented with 10 μ M MG-132. Cells treated with vehicle (DMSO) were used as controls. After treatment, cells were lysed and analyzed by IB for detection of AR expression.

Immunofluorescence (IF)

Cells were seeded on glass coverslips coated with poly-L-lysine (1 mg/ml) in RPMI supplemented with 5% FBS. After starvation, they were stimulated with either R1881 (1 nM) or vehicle (ethanol, EtOH) and then fixed in 4% paraformaldehyde for 15 min. After

two washes with PBS, the cells were permeabilized with 0.2% Triton-X100 for 5 min, and incubated in blocking buffer (5% BSA in PBS) for 1 hr at room temperature (RT). Staining with primary Abs diluted in blocking buffer was performed for 1 hr at RT. After washing with PBS, the coverslips were incubated with goat anti-rabbit rhodamine or anti-mouse fluorescein-conjugated secondary Abs for 1 hr at RT. After two washes with PBS, the cells were counterstained with DAPI to visualize nuclei before mounting. The slides were visualized using an Olympus IX71 inverted microscope with IPLab V3.55 (Scanalytics, Inc, Rockville, MD), magnification 400X.

Small Interfering RNA Transfection

LNCaP cells were transfected with β 1A and β 1C integrin siRNA duplexes (IDT Inc.), and VCaP cells were transfected with PSA siRNA duplexes (IDT Inc.). The sequences of β 1A siRNA were: sense strand, 5'-AUGGGACACGGGUGAAAAUTT-3'; antisense strand, 5'-AUUUUCACCCGUGUCCCAUTT-3'. The sequences of PSA siRNA were: sense strand, 5'-GUGCGAGAAGCAUCCCAATT-3'; antisense strand, 5'-UUGGGAAUGCUUCUCGCACTT-3'. The sequences of (non-silencing) NS/ β 1C siRNA were: sense strand, 5'-CCUCUGACUCCAGAUUCCTT-3'; antisense strand, 5'-GGAAUCUGGAAGUCAGAGGTT-3'. siRNA duplexes at a final concentration of 100 nM (PSA siRNA) were transfected using Oligofectamine (Invitrogen). Cells were subjected to two rounds of transfection, and harvested 48 hrs later.

shRNA mediated PSA knockdown

293FT cells were transfected with PSA shRNA and non-silencing (NS) shRNA constructs using Lipofectamine 2000 (Invitrogen). The lentivirus-containing medium was collected 48 hrs later. The pLKO.1/PSA shRNA lentiviral vector was purchased from Open Biosystems (cat. RHS3979-9575622). C4-2B cells were infected with lentivirus for 24 hrs and the medium was replaced with RPMI supplemented with 5% FBS. Stable PSA knockdown cells were obtained by selection with 0.5 μ g/ml puromycin.

Quantitative real time PCR

Real time PCR analysis was carried out as described earlier (23). Δ Ct values were calculated by subtracting the Threshold Cycle (Ct) values of GAPDH (housekeeping gene) from Ct values of target genes. Further, $\Delta\Delta$ Ct values were calculated by subtracting Δ Ct value of non-silencing shRNA transfected C4-2B cells from PSA shRNA expressing samples. Relative expression was calculated by using the formula $2^{-\Delta\Delta\text{Ct}}$. The sequences of PSA oligonucleotides were: sense strand, 5'-AGGTCAGCCACAGCTTCCCA-3'; antisense strand, 5'-GGGCAGGTCCATGACCTTCA-3'. The sequences of AR oligonucleotides were: sense strand, 5'-CGACTACCGCATCATCACAG-3'; antisense strand, 5'-TCTGGAAAGCTCCTCGGTAG-3'. The sequences of GAPDH oligonucleotides were: sense strand, 5'-GGGAAGGTGAAGGTCGGAGT-3'; antisense strand: 5'-GTTCTCAGCCTTGACGGTGC-3'. The sequences of G6PD (Glucose 6-phosphate dehydrogenase) oligonucleotides were: sense strand: 5'-CCCCAGAGGAGAAGCTCAAG-3'; antisense strand: 5'-GCAAGGCCAGGTAGAAGAGG-3'.

Statistical Analysis

For IF experiments detecting PSA and Giantin, a total of 700 cells were counted for different experimental conditions. Each experiment was repeated three times. The average and standard deviation values were calculated for every experiment. For real time PCR assays, each reaction was carried in triplicate. Statistical analysis was performed pairwise using the Student's t-test. All p-values were based on two-tailed tests.

Results

PSA Localization

We began this study by investigating the subcellular localization of AR and PSA in LNCaP or C4-2B PrCa cells treated with R1881. In biochemical subcellular fractionation experiments, treatment with R1881 stimulated AR translocation from the cytoplasm to the nucleus (Fig. 1), consistent with earlier observations. Surprisingly, IB analysis of isolated subcellular fractions also revealed a specific accumulation of PSA in nuclear extracts of R1881-treated LNCaP cells, compared to vehicle-treated cultures (Fig. 1, left). These results are reminiscent of data reported for another member of the KLK protein family, KLK4 (24), which is devoid of signal peptide, and therefore expected to localize intracellularly. In C4-2B cells, PSA was detected in nuclear and cytoplasmic fractions (Fig. 1, right).

Based on these findings, we next carried out immunofluorescence analysis to visualize the localization of PSA in LNCaP or C4-2B cells in response to androgen deprivation or stimulation (Fig. 2A). In these experiments, between 80–90% of the cells analyzed exhibited nuclear localization of PSA, whereas about 10–20% of the cells only contained cytoplasmic staining. Notably, PSA reactivity was detected as aggregates in specific areas of the cytoplasm. In double labeling experiments, PSA co-localized with the cis-medial Golgi marker Giantin (100% of cells) (25,26) in both LNCaP and C4-2B cells (Fig. 2B), suggesting that in the current analysis PSA still retained the signal peptide required for maturation and secretion in the extracellular milieu.

PSA Down-regulation Affects AR Expression

To investigate a potential significance of nuclear-localized PSA, we next silenced PSA expression in PrCa cells by shRNA or siRNA, and looked at differential protein expression by IB. C4-2B cells stably transfected with control, non-targeting shRNA exhibited high levels of AR and PSA (Fig. 3A). Conversely, two independent PSA-directed shRNA sequences completely abolished PSA expression in C4-2B cells (Fig. 3A). Strikingly, shRNA silencing of PSA under these conditions was associated with nearly complete ablation of AR levels in C4-2B cells (Fig. 3A). shRNA silencing of PSA and associated loss of AR did not affect the expression of the anti-apoptotic and cell cycle regulator, Survivin, and ERK1,2 used as loading controls (Fig. 3A). To determine whether a potential PSA regulation of AR expression was specific, we next examined changes in AKT or Src activation, two critical signaling pathways for PrCa progression that have been previously implicated in AR regulation and function (15,27). At variance with this model, shRNA silencing of PSA did not significantly affect AKT phosphorylation on Ser473 or Src phosphorylation on Tyr416, compared with control transfectants (Fig. 3B). Overall protein levels of AKT or Src did not change in PrCa cells after shRNA knockdown of PSA, indistinguishably from control cells transfected with non-targeting shRNA (Fig. 3B). Similar to C4-2B cells, ablation of AR levels upon silencing of PSA was also confirmed in VCaP cells (Fig. 3C), another androgen-independent cell line (20), reported to express full-length AR and also 6 splicing variants (28). Finally, we investigated the mechanisms underlying PSA-mediated control of AR expression. Treatment of C4-2B/PSA shRNA and C4-2B/NS shRNA cells with the proteasome inhibitor MG-132 did not affect AR protein levels (data not shown). Further analysis by real time PCR performed using C4-2B/PSA shRNA and C4-2B/NS shRNA cells revealed that AR mRNA was significantly downregulated upon silencing of PSA (Fig. 3D), suggesting that PSA-mediated regulation of AR expression occurs to some extent at the mRNA level in PrCa cells.

$\alpha_v\beta_3$ Integrin-Src Pathway Does Not Contribute to AR Activation

Stimulation of LNCaP (Fig. 4A) or C4-2B (Fig. 4B) cells with R1881 resulted in strong phosphorylation of Src on Tyr416 in both isolated cytosolic and nuclear extracts (Fig. 4). In contrast, androgen stimulation under these conditions did not affect Src total protein levels (Fig. 4A, B). Therefore, we next asked whether as upstream regulators of Src (30), members of the integrin family, in particular $\alpha_v\beta_3$, affected AR activation and PSA levels in PrCa cells. In these experiments, ectopic expression of $\alpha_v\beta_3$ in LNCaP (Fig. 5A) or C4-2B cells (Fig. 5B) resulted in strong Src activation, as determined by Tyr416 phosphorylation, compared to control transfectants. Under these conditions of Src activation, ectopic expression of $\alpha_v\beta_3$ integrin had no effect on PSA levels in LNCaP or C4-2B PrCa cells.

Discussion

In this study, we have shown that PSA, a known critical downstream target gene of the AR, and important biomarker of disease onset and progression, accumulates in nuclei of androgen-stimulated PrCa cells and is required for AR mRNA and protein expression. This pathway is specific as recognized signaling pathways implicated in AR regulation, including Src and integrins, have no effect. Our data complement and extend earlier studies that PSA transactivates AR through the co-factor ARA70 (12), and suggest the existence of a novel mechanism which regulates AR mRNA and protein expression under conditions of increasing PSA levels.

Although previous studies have postulated a potential link between PSA and AR (12), a physical co-localization of these two proteins in nuclei of PrCa cells, as obtained here has not been reported before. These data are significant as they anticipate a potential role of PSA not only as a secreted protease, but also as a nuclear-compartmentalized direct regulator of AR expression. In this context, there is precedence for other members of the kallikrein protein family to exhibit multiple and differential subcellular localizations. For instance, the mature/active protease KLK4-205, which is devoid of a signal peptide encoded by exon 1, is found predominantly localized in the nucleus of PrCa cells (31). While additional work is required to elucidate the mechanism(s) of PSA import into the nuclei of PrCa cells, one possible model is that full-length PSA becomes imported in the nucleus upon extracellular activation, or, alternatively, that as yet unknown intracellular protease(s) participate in PSA cleavage, thus enabling its nuclear localization.

Functionally, there is extensive experimental evidence underscoring a pivotal role of PSA in promoting PrCa cell growth in vivo and in vitro (32). This stimulatory pathway has important implications for PrCa therapy. As an example, immunotherapy studies with PSA-encoding recombinant vaccinia vectors (rV-PSA) have shown promising safety and immunogenicity in the clinic (33,34). Similarly, phase I and II clinical trials with a PSA vaccine have shown a 44% reduction in death rate, and 8.5-month improvement in median overall survival in men with castrate-resistant PrCa (35,36). Although the clinical risk(s) associated with PSA immunotherapy need to be further elucidated, especially the risk of autoantibody production (37), these results reinforce the concept of a pivotal role of PSA signaling in PrCa maintenance and progression in humans. The pathway described here fits well with this model, and suggests a potential alternative role of nuclear-localized PSA in promoting the expression of AR, in vivo.

Given our results demonstrating that PSA regulates AR mRNA as well as protein levels, it remains to be investigated whether the changes observed at the protein level are a consequence of variations of mRNA due to multiple transcriptional modulators that synergize with PSA. Alternatively, we may speculate that AR regulation occurs also at a translational or post-translational level.

The signaling requirements of the PSA-AR feedback loop need to be further elucidated. Initial evidence presented here reinforces the specificity of this pathway, as activation of Src or AKT, or forced changes in integrin expression, which have all been previously associated with regulation of AR activity, do not affect PSA-AR modulation. In particular, Src signaling has been implicated in androgen-induced proliferation of PrCa cells (38), and may also participate in the transition from androgen-dependent tumor to AI growth and metastasis (14,39,40). Mechanistically, this pathway hinges on the ability of constitutively active Src to phosphorylate AR and increase AR-dependent gene expression (15), and this response can be further augmented via integrin signaling, resulting in ligand-dependent Src activation (16). At variance with this scenario, the data presented here suggest a direct modulation of AR by PSA, and future studies will test whether a direct or indirect association between PSA and AR in the nucleus is required to maintain constant AR expression levels, or examine a potential role of additional regulatory molecules or binding partners in this response.

In summary, we have uncovered a novel feedback regulatory loop by which PSA promotes the expression of AR in androgen-stimulated PrCa cells. This mechanism may have important implications as an additional aspect of AR function, contributing to castrate-resistant disease, and reinforce the potential relevance of PSA as a bona fide molecular target for PrCa therapy.

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Fig. 1.

AR and PSA co-fractionation. LNCaP and C4-2B cells were starved for 24 hrs using RPMI containing 2% CSS, and then stimulated with R1881 (+) or ethanol (–) for 24 hrs. Cytoplasmic (C) and nuclear (N) fractions were collected from cell lysates subjected to fractionation. The collected fractions were analyzed by IB for expression of AR and PSA. α-tubulin and RCC1 were used as a loading control for the cytoplasmic and nuclear fractions, respectively. Each experiment was repeated three times.

Fig. 2. Nuclear localization of PSA. LNCaP and C4-2B cells were seeded on poly-L-Lysine-coated coverslips for two days. **A:** Cells were starved for 24 hrs, then treated with R1881 (1 nM) or ethanol (EtOH) and finally fixed and stained for detecting the intracellular distribution of PSA. Arrows, cytoplasmic localization. Arrowheads, nuclear localization. Asterisks, cytoplasmic aggregates of PSA. **B:** LNCaP and C4-2B cells subjected to the same treatments as shown in panel A, were stained for PSA and Giantin (a Golgi marker). Nuclear counterstaining was performed using DAPI.

Fig. 3.

PSA regulates AR expression. **A:** Downregulation of PSA protein levels was obtained by infection of C4-2B cells with two different shRNA-expressing lentiviruses. Cell lysates were analyzed by IB for expression of AR and PSA. Survivin and ERK1/2, protein loading controls. **B:** C4-2B/PSA shRNA cells were analyzed by IB for levels of p-AKT, AKT, p-Src (Y416), c-Src and PSA. β_1 integrin, protein loading control. The experiment was repeated three times. **C:** PSA protein levels were downregulated in VCaP cells by transfecting specific siRNA oligonucleotides. Cells were lysed after 48 hrs and analyzed by IB for expression of AR and PSA. AKT, protein loading control. **D:** RNA isolated from C4-2B/PSA shRNA and C4-2B/NS shRNA was evaluated for transcript levels of PSA, AR and G6PD using quantitative real time PCR. Expression levels were normalized with transcript levels of GAPDH and data are presented as expression of each target gene in C4-2B/PSA shRNA relative to its expression in C4-2B/NS shRNA (set as 1.0). Each reaction was run in triplicate. *, $P=0.0148$; **, $P=0.0000987$. A non-silencing (NS) shRNA lentivirus was used as negative control in all experiments.

Fig. 4. Nuclear localization of p-Src in R1881-treated PrCa cells. **A.** LNCaP and **B.** C4-2B cells were treated as in Fig. 1, and cytoplasmic (C) and nuclear (N) fractions were analyzed by IB for expression of p-Src (Y416) and c-Src. α-tubulin and RCC1, loading controls for the cytoplasmic and nuclear fractions, respectively. Each experiment was repeated three times.

Fig. 5. $\alpha_v\beta_3$ integrin activates Src but does not affect PSA expression levels. Lysates from **A.** LNCaP/ $\alpha_v\beta_3$ versus LNCaP/Mock, and **B.** C4-2B/ $\alpha_v\beta_3$ versus C4-2B parental cells were analyzed by IB for expression levels of PSA, p-Src (Y416) and c-Src.