The role of protein kinase A pathway and cAMP responsive element-binding protein in androgen receptor-mediated transcription at the prostate-specific antigen locus

J Kim¹, L Jia², M R Stallcup³ and G A Coetzee^{1,2}

Departments of ¹Molecular Microbiology and Immunology, ²Urology and Preventive Medicine, and ³Pathology, Biochemistry and Molecular Biology, USC/Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089, USA

(Requests for offprints should be addressed to G A Coetzee; Email: coetzee@usc.edu)

Abstract

Androgen-independent prostate cancer is a lethal form of the disease that is marked by metastasis and rapid proliferation in its final stages. As no effective therapy for this aggressive tumor currently exists, it is imperative to elucidate and target the mechanisms involved in the progression to androgen independence. Accumulating evidence indicates that aberrant activation of androgen receptor (AR) via signal transduction pathways, AR gene mutation and/or amplification, and/or coregulator alterations may contribute to the progression of prostate cancer. In the present study, the effects of protein kinase A (PKA) signaling and its downstream factors on AR activity at the prostate-specific antigen (PSA) gene were tested. Activation of PKA by forskolin resulted in enhanced androgen-induced expression of the PSA gene, an effect that was blocked by the AR antagonist, bicalutamide. Interestingly, when either p300 or CBP was overexpressed, PKA activation was sufficient to stimulate PSA promoter-driven transcription in the absence of androgen, which was not inhibited by bicalutamide. PKA activation did not significantly alter AR protein levels but significantly increased the phosphorylated form of its downstream effector, cAMP responsive element-binding protein (CREB) in the presence of androgen. Furthermore, chromatin immunoprecipitation showed that the combination of androgen and forskolin increased phosphorylated CREB occupancy, which was accompanied by histone acetylation, at the putative cAMP responsive element located in the 5' upstream regulatory region of the PSA gene. Remarkably, mammalian two-hybrid assay indicated that p300/CBP may bridge the interaction between AR and CREB, suggesting a novel enhanceosomal cooperation. These results demonstrate an intriguing interplay between a signal transduction pathway, coactivator overexpression and AR signaling as a possible combined mechanism of progression to androgen-independent prostate cancer.

Journal of Molecular Endocrinology (2005) **34**, 107–118

Introduction

Untreated prostate cancer progresses from androgen-dependent tumors that respond favorably to clinical therapies to recurrent, metastatic, androgen-independent tumors that are invariably fatal. Even at the latter, hormone-refractory stage, the tumor growth is dependent on a functional androgen receptor (AR) signaling pathway, as most androgen-independent prostate tumors continue to express AR and AR-regulated genes, such as prostate-specific antigen (PSA) (Buchanan *et al.* 2001). This evidence suggests that a critical process in tumor recurrence and progression may involve some compensatory regulation of AR activity in the low-androgen environment. Although the exact molecular basis for the progression to androgen-independent prostate cancer has yet to be defined, it is becoming increasingly clear that more than one specific mechanism may be involved. The proposed means of subverting growth control include AR gene mutation

and amplification, coregulator alterations and androgen-independent activation of AR via various signal transduction pathways (Feldman & Feldman 2001). These mechanisms are not mutually exclusive but may in fact constitute compensatory measures that cooperate to establish androgen independence (Weber & Gioeli 2004).

Recent attention has focused on the hypothesis that the aberrant activation of AR through various signal transduction pathways plays an important role in tumor progression. Numerous studies indicate that AR signaling is affected by a diverse array of cytokines and growth factors, such as insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF), epidermal growth factor (EGF), interleukin 6 (IL-6) and forskolin, which act through a web of complex signal cascades, such as mitogen-activated protein kinase (MAPK), janus-activated kinase (JAK), signal transducers and activators of transcription (STAT), and protein kinase A (PKA) (Culig *et al.* 1994, Nazareth & Weigel 1996,

Reinikainen *et al.* 1996, Sadar *et al.* 1999, Ueda *et al.* 2002, Jia *et al.* 2004). Of these pathways, PKA, as activated by forskolin, is particularly interesting because of its possible ability to phosphorylate AR *in vivo* and stimulate the expression of the AR-regulated gene, PSA, in the absence of androgen (Sadar *et al.* 1999, Gioeli *et al.* 2002). Taken together, these observations suggest that PKA may modulate AR function, in part, by activating AR through phosphorylation in the absence of androgen. However, site-directed mutagenesis of AR phosphorylation sites has shown that the transcriptional activity of AR may not be affected by the post-translational modification (Gioeli *et al.* 2002). Thus, it is plausible that other mechanisms downstream of the PKA pathway are involved in the activation of AR-regulated genes.

One such mechanism that is downstream of the PKA pathway is the phosphorylation of cAMP responsive element-binding protein (CREB). CREB is a conceivable candidate for mediating PKA effects on AR-regulated transcription due to the presence of a putative cAMP responsive site (CRE) at the 5' upstream regulatory region of the PSA gene (Sadar *et al.* 1999). Although no reports have yet confirmed this phenomenon, it is a striking association in view of the PKA-induced transcription of PSA and the mechanism of CREB activity. PKA stimulation by cAMP, which is synthesized by forskolin-activated adenylyl cyclase, leads to the phosphorylation of CREB at serine 133 (Ser 133), located within a transcriptionally critical region called kinase-inducible domain (KID) (Mayr & Montminy 2001). Phosphorylation of Ser 133 promotes binding of CREB to CRE and recruitment of the coactivator histone acetylase (HAT) paralogs CREB-binding protein (CBP) and p300 (Shaywitz & Greenberg 1999). The recruitment of CBP/p300 may specifically enhance the transcriptional potential of CREB by acetylating histones around the CRE and/or bridging the interaction between CREB and components of the basal transcription machinery (Nakajima *et al.* 1996, Korzus *et al.* 1998).

Interestingly, the physical bridging function of CBP/p300 and their HAT activities are both fundamentally important features that are also shared in the transcription activation modulated by nuclear hormone receptors, including the AR. Compelling studies suggest that, in the specific case of PSA gene transcription, CBP/p300 may not only bridge the interaction between AR and the basal transcription apparatus but also mediate the contacts between AR/coactivator complexes bound to the promoter and enhancer regions, thus forming 'enhanceosomes' (Shang *et al.* 2002, Louie *et al.* 2003). This theory of communication between 5' regulatory elements is especially intriguing because of its implication that CBP/p300 could possibly mediate interactions between different transcription factors, such

as AR and CREB, that share coactivators in common. Furthermore, another inference of the theory is that coactivators may play a crucial role in coordinating the increased transcriptional activity of AR in low-androgen environments by assembling stable, higher-order complexes. Indeed, transcriptional synergy among nuclear receptor coactivators via ternary complex formation has been documented (Lee *et al.* 2002). This also suggests that increased expression of coactivators may be an important mechanism that could compensate for the androgen-depleted state of advanced prostate cancer.

In the present study, the effects of PKA signaling and its mechanism of action on AR-regulated transcription at the PSA gene were investigated. The experiments confirmed that PSA gene expression was enhanced by PKA activation and demonstrated a novel mechanism of CREB and AR cooperation through CBP/p300. The evidence illustrated here presents a possibility of a unique cross-talk mechanism between AR and PKA signaling pathways.

Materials and methods

Cell culture and reagents

The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in RPMI 1640 (Invitrogen, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA, USA); it was used between passages 19 to 24. Cos 7 cells were also obtained from the ATCC and grown in OPTI-MEM (Invitrogen) supplemented with 5% (v/v) heat-inactivated FBS; they were used between passages 12 to 16. 5 α -Dihydrotestosterone (DHT) was purchased from Sigma-Aldrich (St Louis, MO, USA). H-89 was purchased from Calbiochem (San Diego, CA, USA). Bicalutamide was obtained from Astra Zeneca. Forskolin was purchased from Sigma-Aldrich.

Transient transfection and luciferase detection

LNCaP cells (5×10^4 cells/well) in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS (Gemini) were plated in 96-well plates for 3 days. Cells were then transfected with reporter PSA-luc (100 ng/well; pGL3_PSA540), pCMV-300 or pCMV-CBP (50 ng/well; Dr T-P Yao, Duke University, Durham, NC, USA), and/or pCAT-basic (50 ng/well; Promega), a negative control to balance out the total amount of DNA, using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. pGL3-PSA540-enhancer

(PSA-luc) is a mammalian expression vector that contains firefly luciferase linked to the androgen-responsive 548 bp PSA promoter region (–541 to +7) preceded by 1450 bp of the PSA enhancer region (–5322 to –3873) provided by Bristol-Myers Squibb (Princeton, NJ, USA). After transfection, cells were grown in phenol red-free RPMI 1640 containing 0.5% charcoal/dextran-stripped FBS with DHT, forskolin, bicalutamide and/or H-89 as indicated. After additional 30-h incubation, the cells were lysed with the passive lysis buffer (Promega). The extracts were assayed for luciferase activity with the Promega kit, according to the manufacturer's protocol, and measured on a Dynex MLX Microtiter Plate Luminometer (Chantilly, VA, USA). Relative luciferase units (RLU) are shown as the means $\pm$ S.D. of quadruplicate wells. Total protein concentrations of the extracts were assayed with the Bio-Rad Protein Assay Kit (Hercules, CA, USA), according to the manufacturer's protocol, and 600 nm absorbance was measured on a Molecular Devices EMax Microplate Reader (Sunnyvale, CA, USA). No significant differences in total protein concentrations were observed among the different wells within a given experiment.

Chromatin immunoprecipitation (ChIP) assays

LNcap cells (5×10^6 cells/150 mm dish) were cultured in phenol red-free RPMI 1640 supplemented with 5% charcoal/dextran-stripped FBS (Gemini) for 3 days. Cells were treated with DHT and/or forskolin for 2 h, cross-linked by adding formaldehyde (1%) directly to the culture medium, and incubated at room temperature for 10 min. The cells were washed twice with ice-cold PBS and harvested by scraping and centrifugation at 3000 g for 5 min. The cell pellets were resuspended in 0.5 ml lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0, with $1 \times$ complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA)) and incubated for 20 min on ice. The cell lysates were sonicated at setting 4 on a Branson Sonifier Cell Disruptor 185 for 10 s (Fisher Scientific, Los Angeles, CA, USA). The sonication was repeated five times (with 1-min incubations on ice between sonications), and insoluble materials were removed by centrifugation at 15 500 g for 10 min. For each immunoprecipitation, 100 μ l supernatant containing soluble chromatin was diluted 10-fold in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.0), 167 mM NaCl and $1 \times$ protease inhibitor cocktail). After preclearing with 75 μ l of protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at 4 °C for 1 h, the supernatant was immunoprecipitated by incubating at 4 °C overnight with 25 μ l anti-AR (N20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 10 μ l anti-CREB, 10 μ l anti-phospho CREB, 5 μ l anti-dimethyl H3-K4 and 5 μ l anti-AcH3 (Upstate

Biotechnology, Lake Placid, NY, USA). Immune complexes were obtained by incubating with 50 μ l protein G-Sepharose at 4 °C for 1 h. Immunoprecipitates were sequentially washed for 5 min each in low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0) and 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% IGEPAL CA 630, 1% deoxycholate, 1 mM EDTA and 10 mM Tris-HCl (pH 8.0)) and TE buffer (twice). Washed beads were extracted with 250 μ l elution buffer (1% SDS and 100 mM NaHCO₃) twice, the elution was combined, and the protein-DNA cross-linking was reversed by incubation at 65 °C overnight. Each sample was treated with 20 μ g proteinase K (Gibco BRL, Grand Island, NY, USA) in proteinase K buffer (50 mM Tris-HCl (pH 6.5) and 10 mM EDTA) at 45 °C for 1 h. DNA was purified by phenol/chloroform extraction, precipitated by ethanol with glycogen, and resuspended in 100 μ l H₂O. One percent of total soluble chromatin was processed in parallel without immunoprecipitation, and values obtained from this DNA were used as denominators to calculate immunoprecipitated DNA as percentage of input.

Real-time PCR of ChIP DNA

Immunoprecipitated DNA was analyzed by quantitative real-time PCR, as described previously (Jia *et al.* 2003). Briefly, triplicate PCRs for each sample were mixed with AmpliTaq Gold PCR master mix (Applied Biosystems, Branchburg, NJ, USA), forward and reverse primers and probe to be analyzed by Bio-Rad iCycler optical system. The primers and probes were as follows: enhancer forward, 5'-GCCTGGATCTGAGAGAGATATCATC-3'; reverse, 5'-ACACCTTTTTTTTCTGATTGTTG-3'; CRE forward, 5'-CTGGGCAAGCACAATCTGA-3'; reverse, 5'-GTGTGATGCTGGATGATGAGTG-3'; enhancer probe, 5'-6-FAM-TGC AAGGATGCCTGCTTTACAAACATCC-BHQ-1-3'; and CRE probe, 5'-6-FAM-CATGAAAGATGCCCCAGAGGCCCTTG-BHQ-1-3' (Biosearch Technologies, Novato, CA, USA). This assay provides a precise quantitation of target DNA and is based on the principle of fluorophore release from a self-quenching probe; the instrument measures the number of cycles (C_t) required for fluorescence to exceed a set threshold. A standard curve of known target DNA is constructed in parallel, from which the relative amount of target DNA in the sample is calculated. Values are presented as percentage input, which is analyzed at the same time. The precise quantitative nature of this analysis is superior to analyses commonly used by others that rely on semiquantitative end-point assessments of PCR bands on agarose gels.

Western blot analysis

LNCaP cells (6×10^5 cells/60 mm dish) were plated and grown in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS for 3 days. After treatment with DHT, forskolin and/or H-89 as indicated, cells were harvested in 100 μ l RIPA buffer (10 mM sodium phosphate, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1% SDS, 1% IGEPAL CA-630, 1% sodium deoxycholate and 0.2 mM Na_3VO_4 (pH 7.2)) that contained a cocktail of mammalian protease inhibitors. Total protein concentration of the protein extract was determined by the Bio-Rad Protein Assay Kit, according to the manufacturer's protocol, and 600 nm absorbance was measured on a Molecular Devices Emax Microplate Reader (Sunnyvale, CA, USA). Equal amounts of each protein extract were analyzed by SDS-PAGE, transferred to Hybond-P membrane (Amersham Pharmacia Biotech), and probed with polyclonal anti-AR (AR-N20), anti-actin (Santa Cruz Biotechnology) and anti-CREB/anti-phospho-CREB (Upstate Biotechnology). HRP-conjugated anti-IgG (Santa Cruz Biotechnology) was used as the secondary antibody. Detection was performed by the enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech), according to the manufacturer's protocol. Images are representative of three independent immunoblots and were analyzed by the Fluor-S Max MultiImager Quantification System (Bio-Rad).

Real-time RT-PCR of RNA

PSA mRNA was analyzed, as described previously, by quantitative RT-PCR. After treatment of the cells with DHT and/or forskolin, total cellular RNA was prepared and treated with RNase-free DNase I by the SV Total RNA Isolation System (Promega). A two-step RT-PCR method was employed, using the TaqMan Gold RT-PCR Kit (Applied Biosystems). In these analyses, the same probe and reverse primer located in exon 4 of the PSA gene were used for both the pre- and mature mRNA determinations (see below). The forward primer is located in intron 3 for pre-mRNA and in exon 3 for mature mRNA determinations. mRNA quantitation was performed by real-time RT-PCR. Thus, the primers and probes were as follows: PSA mRNA forward, 5'-GGCAGCATTGAACCAGAGGAG-3'; PSA reverse, 5'-GCATGAACCTTGGTCACCTTCTG-3'; PSA probe, 5'-6-FAM-ATGACGTGTGTGCGCAAGTTCACCC-BHQ-1-3'; GAPDH forward, 5'-GTC-ATGGGTGTGAACCATGAGA-3'; GAPDH reverse, 5'-GGTCATGAGTCCTTCCACGATAC-3'; and GAPDH probe, 5'-6-FAM-CAGCCTCAAGATCATCAGCAATGCCTC-BHQ-1-3' (Biosearch Technologies). TriPLICATE PCR reactions were conducted. GAPDH mRNA expression was analyzed in each sample in parallel wells.

The results are represented as PSA/GAPDH mRNA ratios. Due to extensive DNase treatment of the RNA preparations (total RNA Isolation System; Promega), no significant genomic DNA contamination was apparent: 1. negative controls lacking reverse transcriptase were normally less than 1% of the experimental values (the value of each sample was adjusted by subtracting these negative values); 2. PCR analyses of promoter sequences gave negligible values.

Mammalian two-hybrid assay

We used reagents from the Clontech Mammalian Matchmaker Two-Hybrid Assay Kit to clone full-length CREB (pCF-CREB kindly provided by M R Montminy of the Salk Institute, La Jolla, CA, USA) into pM vector, and AR NTD and AR LBD into VP16 vector. Cos 7 cells (2×10^4 cells/well) in phenol red-free OPTI-MEM, containing 5% charcoal/dextran-stripped FBS (CSS), were plated in 96-well plates for 24 h. Cells were then transfected with reporter PGK1 (100 ng/well), pM CREB (20 ng/well), VP16 T Ag or VP16 AR NTD (20 ng/well), and GRIP1, p300 or CBP (50 ng/well), using Lipofect AMINE 2000 (Invitrogen), according to the manufacturer's protocol. After 4-h transfection, the lipofection mixture was removed and replaced with 5% CSS media with either ethanol (vehicle), DHT or forskolin as indicated. After 24-h incubation at 37 °C, the medium was removed, and cells were lysed with the passive lysis buffer (Promega). The extracts were assayed for luciferase activity as described above.

Results

Forskolin induces CBP/p300-mediated transcription of PSA-driven reporter

Previously, we reported that forskolin enhanced androgen-induced transcription of a PSA-driven reporter gene (Jia *et al.* 2003). In the present study, we sought to explore plausible mechanisms responsible for the observed effect, and considered downstream consequences of PKA activation. Our initial hypothesis was that the forskolin-activated PKA pathway may phosphorylate proteins involved in the AR signaling pathway, including AR and its coactivators. However, a recent study has demonstrated that the phosphorylation of AR does not affect its transcriptional activity (Gioeli *et al.* 2002). Thus, we examined whether coactivators that can be phosphorylated, such as CBP and p300 (Kitabayashi *et al.* 1995, Ait-Si-Ali *et al.* 1998), mediate the increased transcription through PKA signaling. We cotransfected LNCaP cells, which express a functional AR, with a luciferase reporter driven by PSA promoter and enhancer along with, either control, CBP or p300 plasmid (Fig. 1A). As previously described (Jia *et al.* 2003), transcription of

Figure 1 Forskolin enhances DHT-mediated and induces CBP/p300-mediated PSA transcription. (A) Transient transfection of LNCaP cells with AR-responsive reporter, PSA-luciferase, along with control (pCAT-basic), CBP or p300 expression plasmid. The transfected cells were treated with various combinations of ethanol (vehicle), 10 nM 5 α -dihydrotestosterone (DHT), 50 μ M forskolin (FSK) and/or 10 μ M bicalutamide (BIC) as indicated. The values are represented as fold activation over the vehicle control (set as value, 1) in each transfection. (B) Transient transfection of LNCaP cells with PSA-luciferase with either pCAT-basic (as a control) or p300 expression plasmid. The transfected cells were treated with various combinations of vehicle, 10 nM DHT, 50 μ M FSK and/or 10 μ M H-89 (specific PKA inhibitor). The values are represented as fold activation over the vehicle control in each transfection. All values are means $\pm$ S.E.M. of quadruplicate determinations, and the data are representative of three independent experiments.

PSA reporter induced by the AR ligand, DHT, was significantly enhanced by forskolin treatment. This effect was inhibited by the addition of the AR antagonist, bicalutamide, suggesting that AR signaling is necessary. Contrary to the earlier report by Sadar *et al.* (1999), we did not detect androgen-independent stimulation of PSA reporter transcription by forskolin treatment alone. Inter-

estingly, however, when we overexpressed CBP or p300, we did observe forskolin-induced transactivation. Moreover, this induction was not inhibited by bicalutamide, indicating that AR does not mediate the androgen-independent stimulation. Moreover, when AR was blocked by bicalutamide, transcription stimulated by DHT and forskolin was reduced to the level of forskolin treatment alone. These results suggested that when CBP or p300 is overexpressed, a mechanism other than AR signaling is stimulated by the PKA pathway, and when combined with AR activity, it produces at least an additive, if not a synergistic, effect on PSA transcription. Next, we tested the effects of PKA pathway suppression with a specific inhibitor, H-89 (Fig. 1B). Surprisingly, when PKA signaling was blocked, both DHT- and DHT/forskolin-induced activities were abolished, indicating that the PKA pathway may be a crucial component of AR signaling in PSA gene transactivation. When p300 was overexpressed, forskolin stimulation was also inhibited by H-89, confirming that PKA signaling is responsible for the CBP/p300-mediated stimulation.

PKA activation enhances endogenous PSA gene expression and CREB phosphorylation

Due to the limitations of transient transfection assays, we were cautious in interpreting the transient transfection results; therefore, we sought to investigate the PKA effects *in vivo*. A quantitative analysis of PSA mRNA expression in LNCaP cells yielded similar results to the transient assays (Fig. 2A). Again, in contrast to the findings by Sadar *et al.* (1999), we did not observe an androgen-independent production of PSA mRNA by forskolin treatment alone. PKA activation by forskolin, however, further stimulated DHT-induced PSA mRNA expression. This increased stimulation was significantly reduced but not completely abolished by bicalutamide (Fig. 2B), suggesting that inhibition of AR signaling does not eliminate the PKA component of the stimulation.

Next, we examined whether AR protein levels are affected by PKA activation (Fig. 3A). Addition of forskolin alone or in combination with DHT did not enhance AR expression. Taken together, these data strongly indicate that the enhanced expression of PSA by forskolin is not due to an increased expression or stabilization of AR by PKA signaling. Therefore, considering the results thus far and also the fact that CBP/p300 do not bind to DNA elements directly, we postulated that there may be another transcription factor that mediates both androgen-dependent enhancement and androgen-independent induction of PSA transcription. An immediately apparent candidate was the CREB protein because of its ability to be activated by PKA signaling and its association with CBP/p300. We measured the endogenous expression of CREB in LNCaP cells and determined that the protein level did

Figure 2 PKA activation enhances endogenous PSA gene expression. (A) Quantitative RT-PCR of PSA mRNA isolated from LNCaP cells after treatment of vehicle, DHT (ethanol), 10 nM DHT and/or 1 μ M forskolin (FSK) for 16 h. The values are represented as a ratio of PSA mRNA to GAPDH. (B) PSA mRNA expression induced by DHT and FSK is inhibited by bicalutamide (BIC). LNCaP cells were treated with vehicle, DHT (ethanol), 10 nM DHT and 1 μ M FSK, and/or 10 μ M BIC for 16 h. The values are represented as means of ratios of PSA mRNA to GAPDH $\pm$ S.E.M. of triplicates, and are representative of five independent experiments.

not vary with DHT and/or forskolin exposure (Fig. 3B). However, the level of the phosphorylated form of CREB was significantly elevated by the DHT and forskolin combination. This evidence suggested the interesting possibility of a cross-talk between AR and PKA signaling that synergistically induces CREB activation, which in turn enhances androgen-dependent activation of PSA gene transcription.

CREB binds to CRE at the PSA 5' regulatory region concomitant with histone acetylation

An additional rationale for CREB involvement in AR/PKA signaling at the PSA locus is provided by the fact that a putative CREB-binding site, CRE, is present

Figure 3 PKA activation does not affect AR protein expression but significantly increases CREB phosphorylation when combined with AR signaling. (A) LNCaP cells were treated with either 10 nM DHT or 1 μ M forskolin (FSK), or both for 16 h and immunoblotted with anti-AR (N20) antibody. (B) For anti-CREB and antiphosphorylated CREB (pCREB) Western blot, LNCaP cells were treated with 10 nM DHT and/or 1 μ M or 50 μ M FSK for 10 min and immunoblotted with anti-CREB and anti-pCREB antibodies. Protein extracts in each case were loaded in equal amounts, as apparent by actin blotting. The number below each lane of protein expression indicates the relative intensity of each band normalized to corresponding actin expression level. The CREB and pCREB analyses are representative of four independent experiments.

at the 5' upstream regulatory region of the PSA gene (Sadar *et al.* 1999). Although no characterization of this specific CRE exists in the literature, it is known to be a perfect 8 bp palindromic site (TGACGTCA) located at -3196/-3189 of the PSA gene (Fig. 4A). PSA CRE is apart from the AR binding sites located at the enhancer (-4148/-4134) and promoter (-170/-156). To investigate the role of CREB in AR-mediated transcription of the PSA gene, we chromatin immunoprecipitated (ChIP) for CREB binding and histone modifications at PSA CRE. Remarkably, quantitative analysis of CREB and phosphorylated CREB occupancy revealed that the

Figure 4 Phosphorylated CREB binds to CRE at the PSA 5' regulatory region, which is concomitantly histone acetylated. (A) Real-time PCR primers and probes were designed around PSA enhancer ARE site (–4148/–4134) and CRE (–3196/–3189) for chromatin immunoprecipitation (ChIP) assay. Chromatin was sonicated to around a 500 bp fragment. (B) CREB and phosphorylated CREB occupancies at the PSA CRE site after treatment with vehicle, 10 nM DHT and/or 1 μ M FSK. ChIP assay values are given as percentage of whole cell lysate (input). (C) Acetylation of histone H3 (Ac H3) and dimethylation of histone H3 at lysine 4 (dime H3-K4) at the CRE site measured by ChIP assay. The same conditions were used as above. (D) AR occupancy, acetylation of histone H3 and dimethylation of histone H3 at lysine 4 at the PSA enhancer ARE measured by ChIP assay. The same conditions were used as above. Values are means $\pm$ S.E.M. of triplicate determinations and are representative of three independent experiments.

activated form of CREB was bound to the CRE only after DHT and forskolin exposure (Fig. 4B). Although the recruitment of activated CREB was low, the results were reproducible and in parallel with the low occupancy level of AR at the androgen response element

(ARE) sites located in the promoter (Jia *et al.* 2003, Louie *et al.* 2003). We also examined acetylation of histone H3 (Fig. 4C) and H4 (data not shown) at the PSA CRE region and found that both were acetylated by DHT stimulation, suggesting that transcriptional potential is

increased at the DNA element. Although the combination of DHT and forskolin treatment also yielded histone acetylation, the absence of any significant change compared with the DHT treatment alone, taken together with the absence of acetylation with the forskolin exposure alone, may imply that acetylation of histones is a DHT-specific effect. Since we did not observe phosphorylated CREB occupancy with DHT treatment, these results raised the possibility that the immunoprecipitated CRE chromatin fragment contained the nearby ARE of the enhancer (~1 kb upstream of CRE) due to inefficient sonication. However, comparison of the dimethylation pattern of histone H3 at lysine 4 at the enhancer ARE (Fig. 4D) with that at the CRE (Fig. 4C) demonstrated that the two immunoprecipitated chromatin fragments were distinct. This is consistent with our previous finding that dimethylation pattern dynamically varies with transcription activation at the PSA AREs, but not at other regions (Kim *et al.* 2003). In addition, AR occupancy was not detected at the CRE, and CREB binding was not apparent at the enhancer ARE (data not shown), a finding which further supported the improbability of immunoprecipitating the same chromatin fragment. Therefore, it was apparent that an interesting cross-talk between AR and PKA signaling occurred at the CRE.

CBP/p300 mediates interaction between AR and CREB

Subsequently, we investigated whether a physical interaction between AR and CREB was responsible for the observed cross-talk. To test a possible AR and CREB interaction, we utilized the mammalian two-hybrid system in Cos 7 cells, which lack an endogenously expressed AR. While both AR and CREB can bind to CBP/p300 (Chrivia *et al.* 1993, Lundblad *et al.* 1995, Aarnisalo *et al.* 1998, Debes *et al.* 2002), no evidence of direct interaction between AR and CREB exists. Thus, we also examined the possibility that CBP/p300 physically bridges the interaction between AR and CREB. Full-length CREB was cloned into the GAL4 DNA-binding domain plasmid (pM-CREB), and N-terminal domain of AR was cloned into VP16 activation domain plasmid (VP16-AR NTD) (Fig. 5A). When CREB and a control vector (VP16 T Ag) were cotransfected in a mammalian two-hybrid assay (Fig. 5B), intrinsic activation by the forskolin treatment was observed, probably due to the transcriptional activation domain present in the CREB protein. As this activity does not reflect an interaction between CREB and the control protein, we determined this level to be the background, with which other interactions would be compared. Cotransfection of coactivators GRIP1 or p300 did not indicate bridging of interaction between CREB and the control protein. When CREB was

cotransfected with AR NTD, no direct interaction was observed. Moreover, GRIP1 did not bridge the interaction between CREB and AR. However, cotransfection of p300 along with CREB and AR yielded a twofold increase in activity, suggesting that p300 can bridge the interaction between CREB and AR. This result is not due to p300's enhancing the transcriptional readout of GAL4 DNA-bound CREB, since p300 cotransfected with CREB and control protein did not produce a higher activity. Similar results were obtained when CBP was cotransfected (Fig. 5C), indicating that both CBP and p300 are able to bridge the interaction between CREB and AR. Notably, when CREB and ligand-binding domain of AR (AR-LBD) were coexpressed, CBP and p300 could not bridge the interaction (data not shown). This is consistent with the previously reported finding that CBP/p300 interacts with only the N-terminal domain of AR (Fronsdal *et al.* 1998). Therefore, the data presented suggest a possible indirect AR/CREB interaction mediated by CBP/p300.

Discussion

The AR plays a central role in the biology of prostate cancer, and androgen ablation therapy remains, after more than 50 years, the most effective treatment for advanced prostate cancer. However, prostate tumors treated with androgen ablation invariably progress to the therapy-resistant form that cannot be further treated with success. Although the tumors are androgen-independent at this final stage, a functional AR signaling pathway is maintained. Various molecular bases for the progression from androgen-dependent prostate cancer to the advanced disease have been proposed. Here, we illustrated a novel mechanism of cross-talk between AR and PKA signaling pathways, which cooperate to activate androgen-responsive gene transcription. Our data confirmed that PKA activation enhances androgen-dependent transcription from both transiently transfected and chromatin-integrated genes. A proposed consequence of PKA activation is thought to be the phosphorylation of AR, which in turn could render the protein more transcriptionally active. However, in addition to the conflicting result, indicating that PKA activation may actually dephosphorylate AR (Blok *et al.* 1998), a recent study showed that mutation of the phosphorylation sites does not have an effect on AR transcriptional activity (Gioeli *et al.* 2002). Moreover, the *in vivo* phosphorylation sites may regulate nuclear transport, and not transcription (Black *et al.* 2004). Our evidence suggests that PKA enhancement of androgen-mediated transcription is not due to increased AR expression or stabilization.

Contrary to the finding by Sadar *et al.* (1999), forskolin did not stimulate androgen-independent transcription in

Figure 5 CBP/p300 mediates interaction between AR and CREB. (A) Mammalian two-hybrid assay scheme. Full-length CREB was inserted into GAL4 DNA binding pM plasmid, and N-terminal domain of AR (AR-NTD, aa 1–538) was cloned into VP16 activation domain plasmid. Luciferase reporter driven by GAL4 (PGK1), pM-CREB, VP16-AR NTD, GRIP1, CBP and/or p300 plasmids was cotransfected into Cos 7 cells lacking an endogenous AR. (B) p300, but not GRIP1, mediates the interaction between AR and CREB. Cos 7 cells were transfected with pM-CREB, VP16 T Ag (control) or VP16-AR NTD, GRIP1, and/or p300. Cos 7 cells were treated with vehicle or 10 μ M forskolin (FSK). The values are represented as relative light units (RLU). (C) CBP mediates the interaction between AR and CREB. The same conditions as above were used in the experiment with CBP. Values are means $\pm$ S.E.M. of quadruplicate determinations, and the data are representative of three experiments.

Figure 6 Proposed model of cross-talk between AR and PKA signaling pathway at the 5' transcriptional control regions of the PSA gene. In this model based on the experiments illustrated in this study, PKA signaling phosphorylates and activates CREB, which binds to the PSA CRE site after DHT-induced AR signaling recruits CBP/p300, which mediates the interaction between AR and CREB. The recruitment of CBP/p300 acetylates the histones around the AREs and CRE. This higher-ordered enhanceosome assembly may stabilize the interaction with general transcription factors (GTFs) and RNA polymerase II (RNAP II), and thus increase the transcriptional potential at the PSA promoter.

our experiments. A possible explanation is that we tested early-passage (passages 19–24) LNCaP cells while the Sadar group used later-passage cells (passages 44–55). Taken together with the observation that CBP/p300 overexpression is sufficient to induce PKA-mediated transcription that is independent of DHT, our results suggest the interesting possibility that LNCaP cells may progressively express more CBP/p300 over time to compensate for the low-androgen environment. Activation of PKA signaling and increased expression of p300/CBP may be sufficient for progression of LNCaP cells toward androgen independence with respect to growth, gene expression and tumorigenicity. CBP and p300 are transcriptional coactivators that are fundamentally important in various signal-regulated transcription events. CBP was first identified as a protein that interacts with CREB, while p300 was isolated as a crucial cellular

protein targeted by the adenovirus E1A oncoprotein (Whyte *et al.* 1989, Chrivia *et al.* 1993). Yet, CBP and p300 are highly related in structure and interact with a similar set of proteins, including p160 coactivators, general transcriptional apparatus, and nuclear hormone receptors (Giordano & Avantaggiati 1999). Since CBP/p300 does not bind to DNA elements directly, we examined whether another transcription factor was mediating the PKA effect. An attractive candidate was the CREB protein because of its association with CBP/p300 and its activation via the PKA pathway. Western blot data indicated that CREB phosphorylation is significantly increased by DHT and forskolin, corresponding to the observed enhancement of PSA transcription.

Moreover, ChIP assays demonstrated a novel occupancy of CREB at the PSA CRE site, which was

accompanied by histone acetylation. Interestingly, only the combination of DHT and forskolin treatment recruited activated CREB to CRE, further supporting a cross-talk between AR and PKA signaling. This phenomenon may reflect the significantly increased level of phosphorylated CREB induced by DHT and forskolin treatment of LNCaP cells, as evidenced by the Western blot data. Although the K_d for CREB binding to palindromic CREs is low (1 nM) compared with the nuclear concentration of the protein (100 nM), the CRE is generally less active when moved further upstream of the promoter (Shaywitz & Greenberg 1999). Thus, the increased availability of phosphorylated CREB may be a crucial event mediated by AR and PKA cross-talk. In addition, the mammalian two-hybrid data that suggest CBP/p300 is able to mediate the interaction between AR and CREB, taken together with our ChIP results, may indicate that AR and CREB form an enhanceosome at the 5' regulatory regions of the PSA gene (Fig. 6). In this model, stimulation of androgen and the PKA pathway activates both AR and CREB, which recruit CBP/p300, forming a more stable tertiary interaction in an enhanceosome. As the specific recruitment of a complex with HAT activity to a promoter may play a critical role in overcoming the repressive effects of chromatin structure on transcription (Struhl *et al.* 1998), a higher-ordered assembly that recruits histone acetylation to the regulatory elements could increase the transcription potential. CREB may also function cooperatively with CBP/p300 to regulate AR activity, in part by enhancing the recruitment of TAF_{II} 130 to the promoter (Nakajima *et al.* 1997, Felinski & Quinn 1999).

As androgen and the AR play important roles in prostate cancer progression, understanding the factors involved in the regulation of androgen/AR action may provide molecular targets for prostate cancer treatment. Aberrant activation of the AR through the PKA signaling pathway may be responsible for the progression of prostate tumors to the rapidly proliferating, androgen-independent state. Further investigation of the PKA signaling effect on AR activity may yield valuable information potentially leading to viable therapies in the future.

Acknowledgements

Grant support included NIH/NCI (R01CA84890) (to G A C), US Department of Defense (DAMD17-00-1-0102; W81 XWH-04-1-0049; W81 XWH-04-1-0823) (to G A C) and the Prostate Cancer Foundation (to G A C). L J is supported by NIH training grant T32CA009320. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Aarnisalo P, Palvimo JJ & Janne OA 1998 CREB-binding protein in androgen receptor-mediated signaling. *PNAS* **95** 2122–2127.
- Ait-Si-Ali S, Ramirez S, Barre FX, Dkhissi F, Magnaghi-Jaulin L, Girault JA, Robin P, Knibiehler M, Pritchard LL, Ducommun B, Trouche D & Harel-Bellan A 1998 Histone acetyltransferase activity of CBP is controlled by cycle-dependent kinases and oncoprotein E1A. *Nature* **396** 184–186.
- Black BE, Vitto MJ, Gioeli D, Spencer A, Afshar N, Conaway MR, Weber MJ & Paschal BM 2004 Transient, ligand-dependent arrest of the androgen receptor in subnuclear foci alters phosphorylation and coactivator interactions. *Molecular Endocrinology* **18** 834–850.
- Blok LJ, de Ruiter PE & Brinkmann AO 1998 Forskolin-induced dephosphorylation of the androgen receptor impairs ligand binding. *Biochemistry* **37** 3850–3857.
- Buchanan G, Irvine RA, Coetzee GA & Tilley WD 2001 Contribution of the androgen receptor to prostate cancer predisposition and progression. *Cancer Metastasis Reviews* **20** 207–223.
- Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR & Goodman RH 1993 Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365** 855–859.
- Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, Bartsch G & Klocker H 1994 Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Research* **54** 5474–5478.
- Debes JD, Schmidt LJ, Huang H & Tindall DJ 2002 p300 mediates androgen-independent transactivation of the androgen receptor by interleukin 6. *Cancer Research* **62** 5632–5636.
- Feldman BJ & Feldman D 2001 The development of androgen-independent prostate cancer. *Nature Reviews Cancer* **1** 34–45.
- Felinski EA & Quinn PG 1999 The CREB constitutive activation domain interacts with TATA-binding protein-associated factor 110 (TAF110) through specific hydrophobic residues in one of the three subdomains required for both activation and TAF110 binding. *Journal of Biological Chemistry* **274** 11672–11678.
- Fronsdal K, Engedal N, Slagsvold T & Saatcioglu F 1998 CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. *Journal of Biological Chemistry* **273** 31853–31859.
- Gioeli D, Ficarro SB, Kwick JJ, Aaronson D, Hancock M, Catling AD, White FM, Christian RE, Settlege RE, Shabanowitz J, Hunt DF & Weber MJ 2002 Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. *Journal of Biological Chemistry* **277** 29304–29314.
- Giordano A & Avantaggiati ML 1999 p300 and CBP: partners for life and death. *Journal of Cellular Physiology* **181** 218–230.
- Jia L, Kim J, Shen H, Clark PE, Tilley WD & Coetzee GA 2003 Androgen receptor activity at the prostate specific antigen locus: steroidal and non-steroidal mechanisms. *Molecular Cancer Research* **1** 385–392.
- Jia L, Choong CS, Ricciardelli C, Kim J, Tilley WD & Coetzee GA 2004 Androgen receptor signaling: mechanism of interleukin-6 inhibition. *Cancer Research* **64** 2619–2626.
- Kim J, Jia L, Tilley WD & Coetzee GA 2003 Dynamic methylation of histone H3 at lysine 4 in transcriptional regulation by the androgen receptor. *Nucleic Acids Research* **31** 6741–6747.
- Kitabayashi I, Eckner R, Arany Z, Chiu R, Gachelin G, Livingston DM & Yokoyama KK 1995 Phosphorylation of the adenovirus E1A-associated 300 kDa protein in response to retinoic acid and E1A during the differentiation of F9 cells. *Embo Journal* **14** 3496–3509.
- Korzus E, Torchia J, Rose DW, Xu L, Kurokawa R, McInerney EM, Mullen TM, Glass CK & Rosenfeld MG 1998 Transcription

- factor-specific requirements for coactivators and their acetyltransferase functions. *Science* **279** 703–707.
- Lee YH, Koh SS, Zhang X, Cheng X & Stallcup MR 2002 Synergy among nuclear receptor coactivators: selective requirement for protein methyltransferase and acetyltransferase activities. *Molecular and Cellular Biology* **22** 3621–3632.
- Louie MC, Yang HQ, Ma AH, Xu W, Zou JX, Kung HJ & Chen HW 2003 Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. *PNAS* **100** 2226–2230.
- Lundblad JR, Kwok RP, Lurance ME, Harter ML & Goodman RH 1995 Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. *Nature* **374** 85–88.
- Mayr B & Montminy M 2001 Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nature Reviews. Molecular Cell Biology* **2** 599–609.
- Nakajima T, Fukamizu A, Takahashi J, Gage FH, Fisher T, Blenis J & Montminy MR 1996 The signal-dependent coactivator CBP is a nuclear target for pp90 RSK. *Cell* **86** 465–474.
- Nakajima T, Uchida C, Anderson SF, Parvin JD & Montminy M 1997 Analysis of a cAMP-responsive activator reveals a two-component mechanism for transcriptional induction via signal-dependent factors. *Genes and Development* **11** 738–747.
- Nazareth LV & Weigel NL 1996 Activation of the human androgen receptor through a protein kinase A signaling pathway. *Journal of Biological Chemistry* **271** 19900–19907.
- Reinikainen P, Palvimo JJ & Janne OA 1996 Effects of mitogens on androgen receptor-mediated transactivation. *Endocrinology* **137** 4351–4357.
- Sadar MD, Hussain M & Bruchovsky N 1999 Prostate cancer: molecular biology of early progression to androgen independence. *Endocrine-Related Cancer* **6** 487–502.
- Shang Y, Myers M & Brown M 2002 Formation of the androgen receptor transcription complex. *Molecular Cell* **9** 601–610.
- Shaywitz AJ & Greenberg ME 1999 CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annual Review of Biochemistry* **68** 821–861.
- Struhl K, Kadosh D, Keaveney M, Kuras L & Moqtaderi Z 1998 Activation and repression mechanisms in yeast. *Cold Spring Harbor Symposium on Quantitative Biology* **63** 413–421.
- Ueda T, Mawji NR, Bruchovsky N & Sadar MD 2002 Ligand-independent activation of the androgen receptor by IL-6 and the role of the coactivator SRC-1 in prostate cancer cells. *Journal of Biological Chemistry* **277** 38087–38094.
- Weber MJ & Gioeli D 2004 Ras signaling in prostate cancer progression. *Journal of Cellular Biochemistry* **91** 13–25.
- Whyte P, Williamson NM & Harlow E 1989 Cellular targets for transformation by the adenovirus E1A proteins. *Cell* **56** 67–75.

Received 17 November 2004

Accepted 27 November 2004

Made available online as an Accepted Preprint
6 December 2004