Identification of a negative regulatory *cis*-element in the enhancer core region of the prostate-specific antigen promoter: implications for intersection of androgen receptor and nuclear factor- κ B signalling in prostate cancer cells

Bekir CINAR^{*1}, Fan YEUNG^{*}, Hiroyuki KONAKA[†], Marty W. MAYO^{*}, Michael R. FREEMAN[‡], Haiyen E. ZHAU^{†2} and Leland W. K. CHUNG^{†§2}

^{*}Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, Charlottesville, VA 22908, U.S.A., [†]Department of Urology, Molecular Urology and Therapeutics Program and Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA 30322, U.S.A., [‡]Departments of Urology and Surgery, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115, U.S.A., and [§]Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

The NF- κ B (nuclear factor- κ B) transcription factors mediate activation of a large number of gene promoters containing diverse κ B-site sequences. Here, PSA (prostate-specific antigen) was used as an AR (androgen receptor)-responsive gene to examine the underlying mechanism by which the NF- κ B p65 transcription factor down-regulates the transcriptional activity of AR in cells. We observed that activation of NF- κ B by TNF α (tumour necrosis factor α) inhibited both basal and androgen-stimulated PSA expression, and that this down-regulation occurred at the promoter level, as confirmed by the super-repressor I κ B α (S32A/S36A), a dominant negative inhibitor of NF- κ B. Using a linker-scanning mutagenesis approach, we identified a *cis*-element, designated XBE (X-factor-binding element), in the AREc (androgen response element enhancer core) of the PSA promoter, which negatively regulated several AR-responsive promoters, including that of PSA. When three copies of XBE in tandem were juxtaposed to GRE4 (glucocorticoid response element 4), a 4–6-fold reduction

of inducible GRE4 activity was detected in three different cell lines, LNCaP, ARCaP-AR and PC3-AR. Bioinformatics and molecular biochemical studies indicated that XBE is a κ B-like element that binds specifically to the NF- κ B p65 subunit; consistent with these observations, only NF- κ B p65, but not the NF- κ B p50 subunit, was capable of inhibiting AR-mediated PSA promoter transactivation in LNCaP cells. In addition, our data also showed that AR binds to XBE, as well as to the κ B consensus site, and that the transfection of AR inhibits the κ B-responsive promoter in transient co-transfection assays. Collectively, these data indicate that cross-modulation between AR and NF- κ B p65 transcription factors may occur by a novel mechanism involving binding to a common *cis*-DNA element.

Key words: androgen receptor, nuclear factor- κ B (NF- κ B), prostate cancer cell, prostate-specific antigen (PSA) expression, transcription factor.

INTRODUCTION

The transcriptional regulation of eukaryotic genes is a complex process that requires a cohort of basal transcription factors, which are needed for transcriptional initiation, and promoter-specific regulatory protein(s) (activators or repressors) that either enhance or repress target gene expression depending on the nature of signalling stimuli [1,2]. The AR (androgen receptor), a key transcription factor, activates target gene expression by interacting with its cognate DNA elements [AREs (androgen response elements)] and with other transcriptional co-regulators [3,4]. AR belongs to the nuclear steroid receptor family, and has distinct structural and functional domains: (a) an N-terminal region with a transactivation function, (b) a zinc finger region that mediates DNA binding, and (c) a C-terminal ligand-binding moiety [4,5]. Upon androgen binding, the AR undergoes conformational changes and translocates to the nucleus from the cytosol [6].

PSA (prostate-specific antigen) is a well known AR-regulated gene in the human prostate gland, and is expressed principally

by both normal prostate epithelium and hormone-dependent PCa (prostate cancer) cells [7]. Currently, serum PSA is widely used as a marker for the diagnosis of prostate cancer and disease progression [8]. Expression of the PSA gene is tightly regulated by androgen through the activation of AR and interaction of AR with AREs within the 6 kb PSA promoter [9,10]. Cleutjens et al. [11,12] identified three AREs in the PSA gene promoter. ARE-I and -II are located within the proximal region of the promoter, whereas ARE-III is contained within the 500 bp AREc (ARE enhancer core), located at approx. –4.2/–3.8 kb from the transcription start site. Additional non-consensus AREs in the AREc were identified and shown to transactivate an AR-responsive reporter gene co-operatively [13]. Studies from other laboratories, including our own, have shown that the AREc region of the PSA promoter is crucial for PSA gene regulation [14]. Therefore much emphasis has been placed on delineating the molecular signalling pathways that regulate PSA expression as a way to explore the transcriptional activities of AR in PCa cells. A study from our laboratory has shown that, despite the overexpression

Abbreviations used: AR, androgen receptor; ARE, androgen response element; AREc, androgen response element enhancer core; CMV, cytomegalovirus; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; β -gal, β -galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRE, glucocorticoid response element; I κ B, inhibitor of κ B; IL-1, interleukin-1; NF- κ B, nuclear factor- κ B; PCa cells, prostate cancer cells; PSA, prostate-specific antigen; RLU, relative luciferase units; SR-I κ B α , super-repressor of NF- κ B; TNF α , tumour necrosis factor α ; XBE, X-factor-binding element.

¹ Present address: Departments of Urology and Surgery, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115, U.S.A.

² To whom correspondence should be addressed: Emory University Winship Cancer Institute, 1365B Clifton Rd, N.E., Suite B4100, Atlanta, GA 30322, U.S.A. (e-mail lwchung@emory.edu or hzhau@emory.edu).

of AR, androgen was not able to induce activation of the PSA promoter in aggressive PCa cells [15]. Unlike the up-regulation of PSA in androgen-dependent PCa cells, the mechanism of down-regulation of PSA gene expression in advanced PCa remains to be elucidated.

NF- κ B (nuclear factor- κ B) is a dimeric transcription factor that belongs to the Rel homology protein family [16]. Currently, there are five known NF- κ B family members in mammalian cells: p65 (RelA), RelB, c-Rel, p50 (NF- κ B1) and p52 (NF- κ B2) [17]. The proteins in this family share a highly evolutionarily conserved Rel homology domain that is responsible for DNA binding, protein dimerization and nuclear translocation [18,19]. In addition to the Rel homology domain, the C-terminus of p65, RelB and c-Rel consists of a potent transactivation domain that is important for NF- κ B-mediated gene transactivation [20]. The family members p50 and p52, which are derived from the inactive precursors p105 and p100 respectively, possess DNA-binding and dimerization properties, but do not have a strong transactivation domain [18,19]. The differential expression of these proteins, their ability to heterodimerize with different family members, and their interactions with different components of the transcriptional apparatus have diverse effects on NF- κ B-dependent transcription [18,19]. The most common and well-studied form of NF- κ B is the p65/p50 heterodimer [19,21].

NF- κ B regulates genes that are involved in multiple cellular processes, such as immune and inflammatory responses, cellular proliferation, development and apoptosis [18,22]. In most unstimulated cells, NF- κ B subunits remain inactive in the cytoplasm by associating with I κ B inhibitory proteins, which prevents them from entering the nucleus by masking their nuclear localization sequence [19,23]. NF- κ B responds to various stimuli, such as TNF α (tumour necrosis factor α) or IL-1 β (interleukin-1 β) [19]. Upon stimulation, the IKK (I κ B kinase) complex (IKK1 and IKK2) phosphorylates I κ B α on serine residues (32 and 36), causing I κ B α to be degraded [24,25]. This event liberates NF- κ B, allowing the transcription factor to translocate into the nucleus and initiate target gene transcription.

NF- κ B also communicates with a broad range of transcriptional regulatory proteins, including the steroid hormone receptor family [26–28]. Cross-talk between signalling pathways may link processes occurring in different cellular compartments, increase regulatory diversity, and provide opportunities for cell- and tissue-specific responses. The interaction between the NF- κ B p65 subunit and steroid receptors is biologically inhibitory. Although several mechanisms have been proposed, including (i) a weak protein–protein interaction [26,27], (ii) increased I κ B α expression [29], and (iii) competition for a limited amount of cofactor [30], the molecular mechanism underlying functional antagonism between AR and NF- κ B is still unclear.

The objective of the present study is to investigate the mechanism by which NF- κ B modulates the transcriptional activity of AR by using the PSA promoter as a model. Although a recent report has shown that NF- κ B is potentially capable of up-regulating PSA promoter activity [37], here we identify a novel κ B-like *cis*-element present in the PSA core enhancer promoter, named XBE (X-factor-binding element), that inhibits AR-responsive promoters and selectively recruits the p65 subunit of NF- κ B. Our results indicate that NF- κ B p65 is capable of down-regulating AR-mediated gene expression by interacting with this negative regulatory element. These findings are consistent with reports from other laboratories that NF- κ B p65 can antagonize gene expression mediated by steroid receptors [26,27,29,30,38]. They also provide a potential mechanism capable of explaining why advanced PCa cells that express wild-type AR and constitutive NF- κ B activity frequently no longer express PSA.

EXPERIMENTAL

Cell cultures, chemicals and antibodies

PCa cell lines (LNCaP, PC3-AR and ARCaP-AR) and a Green Monkey kidney cell line (CV-1) were routinely cultured in T-medium supplemented with 5% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA, U.S.A.) [15]. Phenol Red-free RPMI 1640 medium (Invitrogen) supplemented with 5% (v/v) serum (treated with dextran-coated charcoal) was used in transfection assays. R1881, a synthetic androgen, was purchased from New England Nuclear (Boston, MA, U.S.A.). DOTAP or FuGENE 6 transfection reagent was obtained from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). Human TNF α and IL-1 β were purchased from B&D Systems, Inc. (Minneapolis, MN, U.S.A.). The sources of PG21 and CW2 anti-AR antibodies were as described in [15,31]. Anti-p50 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-p65 antibody was obtained from Rockland (Gilbertsville, PA, U.S.A.).

Plasmid construction

The construction of reporter plasmids p61PSA-Luc, p61-2-Luc, psPSA-Luc, pAREc-TATA-Luc, pCR2.1-AREc and pGRE4-TATA-Luc was described previously [31]. To construct the pAREc/GRE4-TATA-Luc chimaeric promoter, an *Eco*RI-cut AREc fragment from the pCR2.1-AREc vector was subjected to blunt-end reaction and inserted upstream of GRE4 (glucocorticoid response element 4) in the pGRE4-TATA-Luc vector, which was cut by *Nhe*I and blunt-ended in a blunt-end ligation. All blunt-end reactions were carried out using T4 DNA polymerase (NEB, Beverly, MA, U.S.A.) for 20 min at 12 °C. For the construction of the pGRE4/AREc-TATA-Luc promoter–reporter, the GRE4 fragment from the pGRE4-TATA-Luc plasmid was amplified by PCR using 5'-phosphorylated primers and Bio-x-Act *Taq* DNA polymerase (Bioline, Springfield, NJ, U.S.A.). The primers for the PCR were 5'-GATCCCTCGAGCAGCTGAGC-3' (forward) and 5'-CCTCTAGAGTCGACCTGCAG-3' (reverse). The PCR product was purified from 1.5% (w/v) agarose gels using an agarose gel DNA extraction system (Roche Molecular Biochemical) and inserted upstream of AREc in the pAREc-TATA-Luc vector, which was cut by *Nhe*I and blunt-ended prior to blunt-end ligation. The orientation of AREc or GRE4 within pAREc/GRE4-TATA-Luc or pGRE4/AREc-TATA-Luc was determined by *Cla*I restriction enzyme digestion or verified by DNA sequencing by using RV primer within a pGL3 vector backbone (Promega).

To obtain p61-7-Luc, the p61-2-Luc vector was digested by *Sac*I/*Bst*EII and ligated after the blunt-end reaction (the *Bst*EII site remained). To construct p61-7/GaL4-Luc, a *Bst*EII- and *Pst*I-cut AREc fragment in this vector was replaced with a *Bst*EII- and *Pst*I-cut AREc/GaL4 fragment from pAREc/GaL4-TATA-Luc. The construct psPSA/GaL4-Luc was obtained by digesting p61-7/GaL4-Luc with *Pst*I/*Nhe*I and performing blunt-ended ligation. To generate the pGRE4/3XBE-TATA-Luc promoter–reporter, the 5'-phosphorylated GRE4 fragment described above was re-ligated into the *Mlu*I site of the pGL3-TATA vector. Secondly, three tandem copies of a PAGE-purified 5'-phosphorylated XBE oligo (Sigma-Genosys, Woodlands, TX, U.S.A.) were annealed by heating up to 95 °C, and slowly cooled down to room temperature. The double-stranded 3XBE was then ligated downstream of GRE4 in *Sma*I-cut pGRE4-TATA-Luc vector by blunt-end reaction. The orientation of XBE in pGRE4/3XBE-TATA-Luc was determined by DNA sequencing. To obtain the p3XBE-TATA-Luc reporter vector, the GRE4 fragment from the pGRE4/3XBE-TATA-Luc vector was removed by *Kpn*I/*Nhe*I in a double digestion

reaction. The cohesive enzyme sites were filled, and the vector was re-ligated in a blunt-end reaction. All ligation reactions were performed at room temperature using a rapid ligation system (Roche Molecular Biochemical).

The pcDNA3-hAR (CMV-hAR) expression plasmid was described previously (CMV is cytomegalovirus) [15]. Expression plasmids pCMV-Flag-p50 (p50), pCMV-Flag-p65 (CMV-p65), pCMV-Flag-p65mt (CMV-p65mt) and pCMV-Flag-SR-I κ B α (CMV-SR-I κ B α , where SR-I κ B α is a super-repressor of NF- κ B) and the p3X- κ B-Luc promoter-reporter vector were provided by Dr Marty Mayo (University of Virginia, Charlottesville, VA, U.S.A.).

Cell transfections and luciferase assays

Unless stated otherwise, all transfection assays were performed according to the DOTAP liposome transient transfection protocol (Roche Molecular Biochemical). Briefly, 2×10^5 LNCaP, ARCaP-AR [15,31] or CV-1 cells per well were seeded in 12-well plates overnight. After removal of the medium, cells were washed with serum-free Phenol Red-free RPMI 1640 medium before transfections. Cells were then exposed to a plasmid DNA/lipid mixture, for which 1.5–2 μ g of plasmid DNA was allowed to form a complex with transfection reagents for 10–15 min at room temperature prior to their addition to each well containing 0.5 ml of serum- and antibiotic-free RPMI 1640 medium. A CMV-directed β -gal (β -galactosidase) reporter plasmid was co-transfected as a transfection control in the luciferase reporter assays. The cells were incubated with the complexes for 6–8 h in 5 % CO₂ at 37 °C. The DNA/lipid medium was then removed. Cells were washed with RPMI 1640 and incubated in RPMI 1640 containing 5 % dextran-coated charcoal and R1881 (1 or 10 nM) or TNF α (10 ng/ml), or respective vehicles (ethanol or BSA) for 24–36 h.

For luciferase assays, cells were washed with ice-cold PBS and lysed in luciferase lysis buffer [25 mM Tris/phosphate (pH 7.8), 2 mM DTT (dithiothreitol), 2 mM 1,2-diaminocyclohexane-*N,N',N',N'*-tetra-acetic acid, 10 % (v/v) glycerol and 1 % Triton X-100; Promega, Madison, WI, U.S.A.] at room temperature with constant rocking for 15 min. Cell lysates were collected in pre-cooled tubes, vortexed briefly, and centrifuged at 13 000 *g* for 3 min at 4 °C. To measure reporter luciferase activity, 20 μ l of supernatant was mixed with 100 μ l of luciferase substrate (Promega) and measured using a luminometer (Monolight 2010 or 3010; Analytical Luminescence Laboratory, Ann Arbor, MI, U.S.A.). For protein assays, 10 μ l of cell extract was mixed with 200 μ l of Coomassie Blue plus protein reagent (Pierce, Rockford, IL, U.S.A.) and measured at A₅₉₀; BSA was used as a standard. β -Gal activity was assessed according to the manufacturer's instructions (Promega). Briefly, equal amounts of cleared cell lysates and β -gal substrate were mixed and incubated at 37 °C for 30 min. The relative intensity of colour representing β -gal activity was measured at A₄₅₀. Either β -gal activity or total protein was used to normalize RLU (relative luciferase units), and the data were expressed as luciferase activity. Studies from our laboratory [15,31] and others [32] have indicated that no differences are observed using these two methodologies for the normalization of RLU.

Linker-scanning mutagenesis

The pAREc-Gal4-TATA-Luc construct was generated by replacing the respective DNA sequence of AREc with the Gal4 sequence (5'-CGGAGTACTGTCTCCG-3'). Briefly, two 5'-phosphoryl-

ated primers (top sequence, 5'-GTCCTCCGAACGCTGTTTCAG-CCAGAGGC-3'; bottom sequence, 5'-AGTACTCCGGGTTCT-GTCACGTATCTGTG-3'), each with half of the Gal4 site, were designed to run in PCR with the uncut pAREc-TATA-Luc plasmid. Platinum *Pfx* enzyme (Invitrogen) was used in the experiments to generate blunt-ended PCR products. The PCR products were purified as described above from a 0.8 % (w/v) agarose gel and ligated at room temperature with a rapid ligation system (Roche Molecular Biochemical). Clones were screened by PCR using a Gal4 primer (5'-CGGAGTACTGTCTCCG-3') and GLprimer2 (5'-CTTTATGTTTTTGGCGTCTTCC-3') of the pGL3-basic vector. All clones were confirmed by DNA sequencing.

Western blots

NOVEX (Invitrogen) was used to perform the immunoblotting experiments, as described previously [15], with some modifications. Briefly, proteins were separated on a 4–12 % (w/v) polyacrylamide Tris/glycine PAGE gel and transferred on to 0.45 μ m pore size nitrocellulose membrane (Nitro Pure; Osmonics, Westborough, MA, U.S.A.). Non-specific binding of the antibody was blocked with TBST blocking buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05 % Tween-20 and 5 % skimmed milk) for 1 h at room temperature. An anti-PSA primary antibody was used at 2 μ g/ml. To detect the AR protein, horseradish peroxidase-conjugated anti-rabbit IgG (Amersham-Pharmacia Biotech) was used at 1:2000 dilution. For the detection of PSA protein, horseradish peroxidase-conjugated anti-goat IgG (Amersham-Pharmacia Biotech) was used at 1:5000 dilution. Antibodies were prepared in TBST blocking buffer and incubated at room temperature for 1 h. The membrane was subjected to four 5 min washes with TBST containing 1 % Tween-20. The ECL Plus system (Amersham-Pharmacia Biotech) was used to detect the signals.

PSA immunoassay

The secreted PSA proteins were detected by an immunoassay method as described previously [33]. Briefly, conditioned media were collected and diluted in PSA diluent buffer (Abbott Laboratory, Abbott Park, IL, U.S.A.) at a ratio of 1:5 or 1:10 (v/v) in a total volume of 175 μ l. An IMX reader (Abbott Laboratory) was used to quantify the amount of secreted PSA in comparison with PSA standards.

RNA isolation and Northern blots

Total RNA from LNCaP cells was isolated as described [15]. Briefly, cells were plated in a 10 cm culture plate overnight. The culture medium was replaced by Phenol Red-free RPMI 1640 medium and incubated for an additional 24 h. Serum-depleted cells were treated with vehicle controls (BSA or ethanol), TNF α (10 ng/ml) or R1881 (1 nM). By this time, cell confluence was less than 65–75 %. Total RNA was extracted using RNeasy-B reagent (Tel-Test, Inc., Friendswood, TX, U.S.A.) according to the manufacturer's protocol, with modifications [15]. Northern blots were performed as described by Sambrook et al. [34]. Briefly, 5–10 μ g of total RNA was dissolved in 10 μ l of diethyl pyrocarbonate-treated water, mixed with an equal volume of RNA denaturing buffer, heated at 60 °C for 60 min, and then immediately chilled on ice for 5 min. After spin-down, the samples were mixed with 10 $\times$ RNA sample buffer and resolved on a 1 % (w/v) agarose-phosphate RNA gel at 100 V for 2–3 h. The RNA fragment was transferred to a nylon membrane (Zeta Probe; Bio-Rad) by an electrophoretic transfer apparatus at 0.5 A

for 2 h. The RNA was cross-linked to the membrane by using a UV cross-linker (FB-UVXL-1000; Fisher Scientific, Pittsburgh, PA, U.S.A.). The [α - 32 P]dCTP-labelled PSA cDNA probe prepared using the Random Prime Labeling System (*rediprime* II; Amersham-Pharmacia Biotech) was hybridized to pretreated membrane at 60 °C overnight. The hybridization and washing procedures were performed using the Rapid-Hyb-Buffer System (Amersham-Pharmacia Biotech). The membrane was exposed to X-ray film at -80 °C overnight, followed by autoradiography. To detect the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcript, the stripped membrane was reprobed with [α - 32 P]dCTP-labelled GAPDH cDNA probe.

Nuclear extract preparations

Nuclear extracts were prepared from PCa cell lines as described in [35] with modifications. Unless otherwise stated, all protein extractions were conducted at 4 °C. Cells at 70–80 % confluence grown in T-medium (Invitrogen) containing 5 % (v/v) fetal bovine serum were collected by trypsinization, washed three times with ice-cold PBS, and pelleted by centrifugation at 800 *g* for 5 min. The pellet was swelled in a low-salt buffer [10 mM Hepes, pH 7.9, 10 % (v/v) glycerol, 1 mM EDTA, 1 mM DTT and protease inhibitors: 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin A and 1 mM PMSF]. Swollen cells were homogenized using a Dounce homogenizer until cell membranes were disrupted. Cell nuclei that remained intact during homogenization were pelleted by centrifugation at 5000 *g* for 10 min. After decanting the supernatant, the pellet was re-centrifuged at 30 000 *g* for 10 min to remove the remaining cytosol. Then the pellet was resuspended in a high-salt buffer [20 mM Hepes, pH 7.9, 20 % (v/v) glycerol, 0.44 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 2 mM DTT and protease inhibitors as above], and the nuclear proteins were extracted for 30 min with constant rotation. After extraction, the nuclei and insoluble debris were removed by centrifugation at 30 000 *g* for 20 min. The resulting supernatant was dialysed in a dialysis buffer [20 mM Hepes, pH 7.9, 20 % (v/v) glycerol, 100 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 2 mM DTT and 1 mM PMSF] for 4 h with two changes. After dialysis, protein precipitates were removed by a second centrifugation at 30 000 *g* for 15 min. Protein concentration was determined by the Bradford method (Pierce).

EMSA (electrophoretic mobility shift assay)

PAGE-purified oligonucleotides (Sigma-Genosys) were annealed as described above. The forward sequences were 5'-ATGGAG-AATTGCCTCCC-3' for XBE and 5'-AGTTGAGGGGACTTT-CCCAGGC-3' for κ B (Santa Cruz). Double-stranded probes were end-labelled with [γ - 32 P]ATP (Amersham-Pharmacia Biotech) by T4 polynucleotide kinase (NEB) in a 20 μ l reaction volume containing 50 ng of oligo probe, 1 $\times$ T4 PNK (polynucleotide kinase) buffer, 20 μ Ci of [γ - 32 P]ATP and 20 units of T4 PNK at 37 °C for 1 h. The free nucleotides were removed using a QIA Quick Nucleotide Removal Kit (Qiagen, Valencia, CA, U.S.A.). Approx. 30 000–40 000 c.p.m. of labelled probe and 6–10 μ g of nuclear extract were incubated in a binding buffer containing 10 mM Tris/HCl, pH 7.6, 50 mM NaCl, 10 % glycerol, 1 mM DTT, 0.5 mM EDTA, 1 mM KCl and 1 μ g of poly(dI-dC) (Amersham-Pharmacia Biotech). The samples were subjected to electrophoresis at room temperature on a 4 or 5 % non-denaturing polyacrylamide gel in 1 $\times$ TGE buffer (25 mM Tris base, 188 mM glycine and 1 mM EDTA) for 1.5–2 h. For competition experiments, an excess of unlabelled oligonucleotides was incubated with nuclear extract for 10–15 min

on ice prior to the addition of the probe. For antibody supershift experiments, 2 μ l of anti-AR antibody (CW2) or 2 μ g of antibody against the p50, p52 (provided by Dr M. Mayo, University of Virginia, Charlottesville, VA, U.S.A.) or p65 subunits of NF- κ B was added to the reaction mixture and incubated for 30 min at room temperature prior to the addition of probe. The full-length AR in pcDNA3-hAR was transcribed and translated *in vitro* as a non-radioactive protein using the TNT Quick coupled transcription/translation system according to the manufacturer's protocol (Promega). Portions of 1–2 μ l of *in vitro*-transcribed AR protein were also subjected to gel-shift assays as described above. All protein–DNA interactions were performed in a 20 μ l reaction volume for 10 min at room temperature.

RESULTS

Activation of NF- κ B down-regulates PSA gene expression

It has been reported previously that AR and NF- κ B are mutually inhibitory [27]. Because most PCa cells still express AR, the molecular mechanisms by which late-stage PCa cells can lose their ability to express AR-regulated genes, such as that encoding PSA, are not currently understood. Since aggressive PCa cells typically constitutively express active NF- κ B [36], we sought to determine if there was a correlation between loss of AR-mediated gene transcription and NF- κ B activity. LNCaP cells were employed as an experimental system to assess whether activation of NF- κ B down-regulates PSA expression in PCa cells, because they naturally express PSA and AR proteins [10]. We have demonstrated previously that, unlike in LNCaP cells, expression of AR in ARCaP or PC3 PCa cells fails to activate an AR-mediated PSA promoter-reporter construct [15], and studies based on the NF- κ B-responsive promoter-reporter assay have also revealed that NF- κ B is constitutively active in PC3-AR cells and moderately active in ARCaP-AR cells (B. Cinar, unpublished work). These observations suggest that activation of NF- κ B may down-regulate AR-driven PSA promoter activity. Since TNF α is a well-known activator of NF- κ B [24], we asked whether the administration of TNF α down-regulates PSA expression or secretion. Serum-depleted cells were incubated under various conditions for 48 h. Cells were counted, and medium was collected for immunoassay of the PSA protein. The results demonstrated that TNF α antagonized androgen-dependent and -independent PSA secretion in LNCaP cells (Figure 1A).

We next examined whether the down-regulation of PSA secretion occurs at the transcriptional (mRNA) or protein level. Cells were incubated under the above conditions, and PSA mRNA and protein levels were analysed. We found that the reduction in PSA secretion correlated with reduced PSA mRNA levels, as well as with a decrease in cell-associated PSA protein (Figures 1B and 1C). Furthermore, administration of TNF α also inhibited androgen-induced PSA promoter-reporter activity in a transient transfection assay (Figure 1D). A similar result (i.e. reduced PSA protein or PSA promoter-reporter activity) was also observed when LNCaP cells were treated with IL-1 β , another activator of NF- κ B (results not shown).

Overexpression of SR-I κ B α (S32A/S36A), a super-repressor of NF- κ B, was shown to completely abolish NF- κ B transactivation [25]. Therefore we wanted to test whether NF- κ B is directly involved in the down-regulation of AR-driven PSA promoter activation. An assay of PSA promoter-reporter activity in LNCaP or PC3-AR cells in the presence (CMV-SR-I κ B α) and absence (CMV-VO) of SR-I κ B α in transient transfection assays showed that the blockade of NF- κ B function by SR-I κ B α further enhanced androgen induction of PSA promoter activity in the

Figure 1 Activation of NF- κ B by TNF α inhibits PSA secretion, expression and promoter activity in PCa cells

(A) PSA secretion (ng/ml); (B) PSA mRNA expression; (C) cellular PSA protein. Equal numbers of cells were treated with BSA, TNF α (10 ng/ml), ethanol (EtOH), R1881 (1 nM), BSA/ethanol or TNF α /R1881. Samples of 15–20 μ g of total protein were used in PSA protein analysis, and samples of 10 μ g of total RNA were used in PSA mRNA analysis. GAPDH was present as a control in Northern blots. (D) PSA promoter activity in the presence and absence of TNF α and/or R1881. LNCaP cells were transfected with p61PSA-Luc in a 12-well plate. (E) PSA promoter activity in LNCaP and PC3-AR cells. Cells were co-transfected with p61PSA-Luc and CMV-SR-I κ B α or control vector (CMV-VO), followed by incubation with R1881 or ethanol. At 24 h, cells were harvested, and lysates were assayed for luciferase activity and protein content. Luciferase activity is expressed as fold induction with respect to control vector (pGL3-basic). Means $\pm$ S.D. of determinations from three individual experiments are shown.

LNCaP and PC3-AR cell lines (Figure 1E). Together, these observations suggest that a loss of PSA promoter activity may be associated with the constitutive activation of NF- κ B in PCa cells.

The PSA promoter contains a negative regulatory element

The above results indicate that the activation of NF- κ B negatively regulates AR-mediated PSA expression by targeting negative regulatory elements within the PSA promoter. To examine whether the PSA promoter contains *cis*-elements through which NF- κ B negatively regulates AR-driven gene expression, we focused our attention on the PSA core enhancer promoter, AREc, because: (i) AREc has been demonstrated to be a major regulatory region of the PSA gene [12,31], and (ii) androgen/AR is unable to activate AREc when linked to a simple TATA-Luc construct, but activates the pGRE4-TATA-Luc promoter construct, another AR-responsive promoter (Figure 2B, and see [15]). This differential activity of two AR-responsive promoters in PCa cells provides an opportunity to examine if the PSA promoter can be regulated by a negative regulatory element(s) within the AREc. To examine this possibility, we constructed the chimaeric promoter-reporters pAREc/GRE4-TATA-Luc and pGRE4/AREc-TATA-Luc, and analysed their androgen responsiveness in transient transfection assays in cells that express either endogenous or stably transfected AR (Figure 2A). The AREc, when placed either up- or downstream of GRE4, reduced overall androgen-induced promoter-reporter activity in ARCaP-AR and PC3-AR cells compared with the effect of pGRE4-TATA-Luc alone (Figure 2B).

In order to identify a crucial *cis*-element, we first conducted linker-scanning mutagenesis analysis [31]: 17 bp DNA segments in the AREc sequence (Figure 3A) were replaced systematically with a Gal4 binding site, which resulted in a panel of 24 mutant constructs, and the androgen responsiveness of each individual construct was tested in a transient transfection assay (results not shown, but are available upon request). Among the linker-

Figure 2 AREc consists of a negative regulatory element that down-regulates AR-mediated promoter-reporter activity

(A) Western blot analysis of AR in engineered ARCaP-AR and PC3-AR cells [15]. Portions of 15–20 μ g of total protein were immunoblotted with an antibody against AR (PG21), and the signals were detected by the ECL system. The location of the 110 kDa AR is labelled (arrows). The AR in LNCaP cells was used as a positive control. (B) Effects of AREc on pAREc/GRE4-TATA-Luc and pGRE4/AREc-TATA-Luc chimaera in ARCaP-AR and PC3-AR cells. Cells were transfected with pGRE4-TATA-Luc, pAREc/GRE4-TATA-Luc or pGRE4/AREc-TATA-Luc and treated with R1881 or ethanol (EtOH). The luciferase activity was determined in lysates 24 h after transfection. Luciferase activity is expressed as fold induction with respect to a control vector (pGL3-TATA). Data are presented as means $\pm$ S.D. of determinations from three individual experiments.

scanning constructs, pAREc-Gal4-TATA-Luc, where the XBE sequence in the AREc was replaced by a Gal4 binding site (see Figure 3A for the AREc sequence and location of XBE with respect to ARE-III), showed a 4-fold increase in androgen-induced activity compared with that of pAREc-TATA-Luc (Figure 3B). In contrast, replacement of ARE-III by 17 bp Gal4 sequences in the ARE core completely abrogated androgen-induced pAREc-TATA-Luc promoter-reporter activity [31], suggesting that XBE is a negative regulatory element in the PSA promoter.

Figure 3 Linker-scanning mutagenesis identifies a 17 bp *cis*-DNA as a negative regulatory element for AR-responsive promoters

(A) Sequence of AREc used in linker-scanning mutagenesis analysis. Replacement of the XBE sequence with Gal4 DNA in pAREc-TATA-Luc generated pAREc-Gal4-TATA-Luc. The location of XBE with respect to ARE III [12] is emboldened and underlined. The location of AREc relative to the transcriptional start site in the PSA gene is indicated with numbers (5' to 3'). (B) Comparison of pAREc-Gal4-TATA-Luc and pAREc-TATA-Luc promoter-reporter activity in LNCaP and ARCaP-AR PCa cells. (C) Effect of XBE on AR-driven pGRE4-TATA-Luc activity in the chimaeric promoter pGRE4/3XBE-TATA-Luc in LNCaP, ARCaP-AR and PC3-AR cells. Cells were transfected with each promoter-reporter construct and treated with R1881 or ethanol (EtOH). Luciferase activity was measured 24 h after transfection. Normalized luciferase data are presented as fold induction with respect to the empty vector (pGL3-TATA). Data represent means $\pm$ S.D. of determinations from three individual experiments.

Figure 4 XBE is involved in the down-regulation of the PSA promoter

(A) p61-7-Luc and p61-7-Gal4-Luc and (B) psPSA-Luc and psPSA-Gal4-Luc promoter-reporter activity. LNCaP cells were transfected with reporter construct driven by p61-7-Luc, p61-7-Gal4-Luc, psPSA-Luc or psPSA-Gal4-Luc. Luciferase activity was measured after a 24 h incubation with R1881 or ethanol (EtOH). Luciferase activity is presented as fold induction with respect to vector backbone (pGL3-basic). Data represent means $\pm$ S.D. of determinations from two individual experiments.

To assess whether XBE alone possesses inhibitory activity, we generated a pGRE4/3XBE-TATA-Luc chimaeric promoter-reporter construct, where three tandem copies of XBE were placed between the GRE4 and simple TATA box sequences. Transient transfection of pGRE4/3XBE-TATA-Luc revealed that XBE by itself was capable of blocking liganded AR-mediated pGRE4/3XBE-TATA-Luc activity in ARCaP-AR, PC3-AR or LNCaP cell lines when compared with transfection of pGRE4-TATA-Luc (Figure 3C). To characterize further whether mutation of XBE in the context of more complex PSA promoter constructs induces AR-driven PSA promoter activity, we exchanged XBE with the 17 bp Gal4 DNA in psPSA-Luc or p61-7-Luc promoter-reporters. psPSA-Luc contains the AREc and proximal PSA promoter, and it was demonstrated previously to be more active

than full-length PSA promoter activity [31]; p61-7-Luc contains only the AREc plus 150 bp of proximal PSA sequence, including the TATA box. Transient transfection analysis of these mutants further enhanced androgen-induced p61-7-Luc (Figure 4A) or psPSA-Luc (Figure 4B) activity, confirming XBE as a negative regulatory *cis*-element in the PSA promoter.

XBE is an authentic NF- κ B site

To test whether XBE is a potential *cis*-element for NF- κ B, we employed bioinformatics, biochemical and molecular-biological approaches. First, we undertook a transcription factor database search (http://motif.genome.ad.jp/motif-bin/motif_markseq-transfac) to identify potential *trans*-acting factor(s). The search

Figure 5 XBE is a κ B-like element bound by both p65 and AR

(A) Sequence comparison of XBE with κ B consensus DNA. The NF- κ B protein-binding site is underlined. The sequence differences between the XBE and κ B elements are labelled with numbers above the underlined sequence. The first G (guanine) from the left within the underlined sequence is numbered 1. Half-sites A and B are divided by a vertical broken line. (B) Comparison of XBE with previously identified κ B sites from the PSA promoter (Chen and Sawyers [37]). The numbers (5' to 3') on the sites show the location of κ B sites relative to the transcriptional initiation site in the PSA gene. (C) XBE or κ B element DNA-protein complexes in LNCaP cell nuclear extracts. Nuclear extracts (5–10 μ g) were mixed with a 32 P-labelled XBE or consensus oligo, or 3 μ l of *in vitro*-transcribed/translated AR protein was mixed with 32 P-labelled XBE oligo in EMSA reactions. A 100- or 250-fold excess of unlabelled (cold) κ B or XBE oligo respectively was added in competition assays. Locations of the bound probe were labelled as complexes A, B and C from the bottom. (D) Antibody supershift assays with an ARCaP-AR cell nuclear extract (left panel) or with *in vitro*-transcribed/translated AR protein (right panel). Samples of 2 μ l of anti-AR or anti-p65 and 2 μ g of anti-p50 or anti-p52 antibodies were used in antibody supershift assays. Protein-DNA complexes were resolved in a 4% native gel. The locations of bound, supershifted and free probes are indicated (arrows). The radiolabelled 17 bp XBE sequence used in EMSA is presented below the panel. Corresponding lanes are numbered and labelled. NE, nuclear extract; SS, supershift; the asterisk denotes p65 supershift. Data represent at least five individual experiments.

results repeatedly indicated that the subunits of NF- κ B transcription factors are potential candidates for XBE binding (results not shown). The database consensus site search indicated that XBE shares high identity with the κ B consensus site (Figure 5A), where XBE differs only by 2 bp: adenine (A) at position 2 located in half-site A, and guanine (G) at position 8 located in half-site B, when compared with the κ B consensus DNA. In addition, after the present study was completed, Chen and Sawyers [37] reported multiple κ B elements in the PSA promoter. XBE, when compared with these κ B elements, is distinct from them [37] (Figure 5B).

We next compared the mobility shift patterns of protein-XBE complexes with those of the κ B consensus oligo probe in the presence or absence of specific competitor DNA. The profiles of the 32 P-labelled XBE oligo probe-protein complexes (Figure 5C, lane 2, complexes A and B, arrows) were similar to those of the 32 P-labelled κ B oligo probe-protein complexes (Figure 5C, lane 6, complexes A and B, arrows) in LNCaP cell nuclear extracts. An excess of unlabelled XBE oligo diminished the formation of both XBE (Figure 5C, lane 3) and κ B (Figure 5C, lane 8) oligo-protein complexes. Likewise, an excess of unlabelled κ B oligo also abolished both κ B (Figure 5C, lane 4) and XBE (Figure 5C, lane 7) oligo-protein complexes in competition experiments. The competition with an excess amount of unlabelled oligo in both experiments was dose-dependent (from 0 to 250-fold excess; results not shown). Thus the EMSA confirms the computer prediction that XBE serves as a binding site for the subunits of NF- κ B. Although we observed similar XBE or κ B oligo-protein complexes in PC3-AR and ARCaP-AR nuclear extracts (results not shown), LNCaP nuclear extracts repeatedly demonstrated a slower-migrating band (Figure 5C, complex C), which did not appear with PC3-AR or ARCaP-AR cells. To investigate what other factors might be able to compete for XBE, nuclear extracts were prepared from LNCaP cells grown in serum-free medium containing either R1881 (1 nM) or ethanol (vehicle) and examined for XBE oligo-protein complex formation. We found that XBE probe-protein complex formation was enhanced in the nuclear

extract treated with R1881 when compared with that of the XBE control nuclear extract (results not shown). Recognizing that androgens are an inducer of AR, this observation suggests that AR may also bind to XBE.

To characterize the molecular composition of XBE-binding complexes, we employed antibodies specific for NF- κ B subunits or for AR protein in gel mobility supershift analysis of nuclear extracts from ARCaP-AR cells that express stable ARs [15]. The results showed that antibodies against NF- κ B p65 (Figure 5D, left panel, lane 4) or AR (Figure 5D, left panel, lane 5), but not those against NF- κ B p50 or p52 (Figure 5D, left panel, lanes 2 and 3 respectively), supershifted XBE oligo-protein complexes *in vitro* in nuclear extracts from ARCaP-AR cells. In a separate experiment, antibodies against NF- κ B p50 or p52 were capable of supershifting κ B consensus oligo probe-protein complexes, and also the anti-AR antibody supershifted XBE or κ B consensus oligo probe-protein complexes in LNCaP cell nuclear extracts, where AR seemed to be present in two different locations, similar to the patterns observed in Figure 5(C), complex A or C (results not shown). Moreover, the specificity of AR for XBE was also demonstrated using an *in vitro*-transcribed/translated AR protein in an AR-specific antibody supershift assay (Figure 5D, right panel, lane 3). Taken together, these results suggest that AR and NF- κ B p65 can bind specifically to the XBE *cis*-element in the PSA promoter.

XBE is a biologically active κ B element

To examine further whether XBE is able to drive reporter gene activation, we generated a p3XBE-TATA-Luc reporter construct by linking three tandem copies of XBE to a simple TATA box. Transient transfection analysis of p3XBE-TATA-Luc demonstrated that XBE was able to respond to induction by TNF α , as well as stimulation by IL-1 β (results not shown). These results are comparable with those with the known κ B-responsive promoter-reporter (p3X- κ B-Luc) in AR-negative CV-1 cells (Figure 6A) or LNCaP cells (Figure 6B), and both reporter

Figure 6 XBE is a functional κ B element

(A) CV-1 or (B) LNCaP cells were transfected with p3X- κ B-Luc or p3XBE-TATA-Luc reporter construct. Luciferase activity was measured after a 24 h incubation with TNF α (10 ng/ml) or BSA. Luciferase activity is presented as fold induction with respect to vector (pGL3-TATA for p3XBE-TATA-Luc, or pGL3-basic for p3X- κ B-Luc). Data represent means $\pm$ S.D. of determinations from three individual experiments.

activities could be blocked by SR-I κ B α (results not shown). The data suggest that XBE is a biologically active κ B-like element. Moreover, co-transfection of NF- κ B p50 or p65 and p61PSA-Luc into LNCaP cells revealed that only NF- κ B p65, but not NF- κ B p50, blocked the liganded-AR activation of a PSA promoter-reporter construct (Figure 7A). Conversely, co-transfection of AR and p3X- κ B-Luc reporter genes showed that expression of AR also blocked the κ B-responsive promoter activity (Figures 7B and 7C respectively). Collectively, these results are consistent with a published study [27] demonstrating AR and NF- κ B p65 are mutually inhibitory, and that the functional antagonism between NF- κ B p65 and AR may occur due to binding to a common *cis*-element.

DISCUSSION

In the present study we demonstrate that activation of NF- κ B p65 inhibits AR-mediated gene expression in PCa cells by a mechanism involving binding of NF- κ B to a novel κ B-like element, XBE, located in the AREc region of the PSA promoter. We provide evidence that (1) replacement of XBE with the Gal4 binding site in the androgen-responsive promoter-reporters pAREc-TATA-Luc, p61-7-Luc and pPSA-Luc resulted in all three promoters acquiring enhanced responsiveness to androgen; (2) XBE alone, when linked to the androgen-responsive promoter-reporter pGRE4-TATA-Luc, repressed promoter activity in an androgen-dependent manner; (3) NF- κ B p65 interacted physically with XBE; (4) activation of NF- κ B by TNF α or IL-1 β inhibited PSA gene expression, protein level and protein secretion; (5) blockade of NF- κ B by SR-I κ B α (S32A/S36A) further enhanced androgen-induced AR-driven PSA promoter activation; (6) TNF α or IL-1 β was able to activate a minimal promoter in an XBE-dependent manner; and (7) AR competed with XBE, as well as with a κ B consensus oligo, in EMSA and inhibited the κ B-responsive promoter. Our results are the first physical evidence for a DNA-dependent functional antagonism between AR and NF- κ B at endogenous promoter regions, and they support the existence of a class of AR-regulated genes that is susceptible to NF- κ B-mediated repression.

NF- κ B hetero- or homo-dimers communicate with a broad range of transcriptional regulatory proteins. Previous studies have shown that interaction between the NF- κ B p65 subunit and steroid hormone receptors, including the glucocorticoid receptor, the progesterone receptor and the AR, results in the repression of steroid hormone-mediated gene expression. Several mechanisms have been described that may account for the inhibitory cross-talk between the steroid hormone and NF- κ B pathways, including: (1) weak protein-protein interactions [26,27], (2) increased I κ B α expression [29], or (3) competition for a limited amount of cofactor [30,38]. Two previous groups have reported on the intersection of the androgen and NF- κ B pathways. Functional antagonism between AR and NF- κ B signalling was observed by Palvimo et al. [27] and was attributed to a physical association

Figure 7 Cross-communication between p65 and AR is transcriptionally inhibitory

(A) PSA promoter activity in LNCaP cells. Cells were transfected with control vector (CMV-VO), CMV-p50 or CMV-p65 (250 ng), together with the reporter construct driven by p61PSA-Luc. Normalized luciferase data are presented as fold induction with respect to the vector backbone (pGL3-basic). Data represent means $\pm$ S.D. of determinations from two individual experiments. (B) Transient expression of human AR (hAR) in CV-1 cells. Portions of 7.5 μ g of total protein from mock (CMV-VO)- or CMV-hAR-transfected cell lysates were separated and transferred on to membranes. The AR signal was detected by PG21 antibody against AR by using the ECL system. (C) p3X- κ B-Luc reporter activity. CV-1 cells were co-transfected with 2.5 μ g of p3X- κ B-Luc promoter-reporter and 0.5 μ g of control vector (CMV-VO) or CMV-hAR expression vector in six-well plates. Luciferase activity was determined 24 h following transfections. Luciferase activity is presented as fold induction with respect to the vector backbone (pGL3-basic). Data represent means $\pm$ S.D. of determinations from three individual experiments.

between AR and NF- κ B p65. Chen and Sawyers [37] identified four NF- κ B-binding sites in the PSA promoter and demonstrated that these sites are capable of mediating promoter activation in the absence of androgen upon cell stimulation with TNF α . In the present study, we have identified a fifth NF- κ B site at positions –3946/–3938 relative to transcriptional start site, which, in the context of the PSA promoter, is capable of repressing AR-dependent gene activation, but in the context of a minimal promoter is capable of mediating transcriptional activation by TNF α or IL-1 β . A computer-aided search (MOTIF) identified XBE as a potential binding site for NF- κ B, and we confirmed by EMSA that the NF- κ B p65 subunit binds to this site. Our results further suggest that the NF- κ B p50 subunit cannot bind to XBE. Consistent with these observations, we found that p65 inhibited androgen-mediated activation of the PSA promoter, whereas p50 enhanced the same promoter in the presence of androgen. Activation of this promoter by p50 is consistent with the previous demonstration that binding of p50 to other NF- κ B sites located at positions –3996/–3987, –4245/–4236, –4299/–4290 and –4355/–4346 with respect to the transcriptional initiation site in the PSA promoter resulted in transcriptional activation in response to TNF α in the absence of androgen [37]. These observations suggest different roles for NF- κ B subunits in the AR-mediated regulation of the PSA promoter in PCa cells. Collectively, these results indicate that the NF- κ B and androgen-mediated pathways are likely to converge in complex ways that may depend on the hormonal milieu, the oncogenic state of the cell, and the protein complexes associated with a specific androgen-responsive gene.

Although a computer search of the transcription factor database did not predict XBE as a binding site for AR, the AR protein also interacts physically with XBE. Similarly, we observed that AR interacted with a κ B consensus DNA, as demonstrated by antibody supershift and by DNA-affinity precipitation assays; consistent with this finding, when overexpressed, AR trans-repressed the NF- κ B-driven promoter-reporter, demonstrating that AR-protein- κ B-element interactions are biologically relevant. Altogether, the data suggest that AR and κ B transcription factors may regulate gene expression in PCa cells by interacting with a common *cis*-DNA element, and this interaction may be the molecular basis of the trans-repression of AR and NF- κ B target genes.

The C-terminal domain of NF- κ B p65 contains an acidic activation domain that has been demonstrated to interact with elements of the general transcriptional machinery, such as TFIIB [39] or TBP (TATA box-binding protein) [39]. We have shown that the transfection of full-length NF- κ B p65 repressed AR-driven PSA promoter-reporter activation, whereas the introduction of mutant p65 which lacked the C-terminal transactivation domain but maintained DNA-binding capability was less repressive (B. Cinar, unpublished work). These results can be interpreted as indicating that NF- κ B p65 binds to DNA and may interact with the components of the basal transcriptional machinery or with cofactor proteins for which AR may also compete [40]. Alternatively, NF- κ B p65, upon binding to XBE, may introduce a conformational change at the PSA promoter or recruit cofactors so that AR-induced PSA expression is inhibited. These assumptions are consistent with a more recent observation that endogenous promoter activity was not only encoded by the κ B-site sequence itself [41]. Thus further studies are needed to confirm whether NF- κ B p65 competes with AR for binding to general transcription factors or cofactors in regulating AR-mediated gene transactivation.

Based on our findings, we present a model (Figure 8) in which AR and p65 compete for XBE (model 1) depending on the nature

Figure 8 Proposed models of inhibition by p65 of PSA expression in PCa cells

Model 1: p65 and AR compete for XBE. Model 2: p65 binds to XBE and inhibits PSA promoter activity by communicating with the transcriptional machinery in a negative manner. T-Complex, transcriptional complex.

of signals (e.g. androgen and TNF α respectively). An activated NF- κ B p65 may first interact with XBE and secondly trans-repress an AR-driven promoter-reporter by competing with AR for the same general transcription factors or co-regulatory proteins (model 2). This inhibition may exhibit less repression in androgen-independent than in androgen-independent cells. However, in the presence of reactive AR, NF- κ B p65 may have a minor negative regulatory effect on AR function in androgen-dependent cells, because AR competes for XBE in response to androgen action. Overall, our data suggest that DNA binding, possibly to common *cis*-DNA elements, seems to be necessary for NF- κ B p65 to antagonize AR function. Likewise, AR may trans-repress NF- κ B function by a similar mechanism. Previous reports support the idea that the glucocorticoid receptor can disrupt the interaction of NF- κ B p65 with the basal transcription machinery by direct interaction either with NF- κ B p65 or with general transcription factors [42]. Therefore our results provide a novel finding that protein–DNA interactions may be another key to the mechanism of functional antagonism between AR and NF- κ B.

NF- κ B is inducible in most cells, but is constitutively active in the majority of metastatic cancers [43,44]. We have demonstrated that the blockade of NF- κ B by SR-I κ B α (S32A/S36A) further enhanced androgen-induced AR-driven PSA promoter activity in PCa cells, suggesting that activation of NF- κ B may limit or abrogate AR-mediated PSA expression. This limitation may be enhanced by androgen independence and metastatic progression, implying that loss of PSA expression or the metastatic potential of prostate cancer may be correlated with the presence of constitutively active NF- κ B. It was reported that the inactivation of NF- κ B p65 by antisense [45,46] or dominant negative mutant I κ B α [36,47] reduced the angiogenic and tumorigenic potential of some cancer cells.

In summary, we have presented the first evidence that NF- κ B and AR are mutually antagonistic at the promoter level by competing for binding at a common DNA element. These findings may provide a mechanistic explanation for the mutual transcriptional inhibition between NF- κ B p65 and other members of the steroid

receptor family. Further investigations into the interaction between NF- κ B or steroid receptors and members of the general transcriptional machinery are needed in order to explore the mechanisms of gene repression. In addition, with regard to the role of NF- κ B in promoting human prostate cancer progression and metastasis, it is worth investigating whether the blockade of NF- κ B, along with restoration of AR 'homeostasis' as we reported [15], might reduce the tumorigenicity and metastatic potential of human PCa cells in animal models.

We thank Dr Tucker Collins and Dr Keith Solomon (Children's Hospital Boston, Harvard Medical School, Boston, MA, U.S.A.) for critical reading of the manuscript and for helpful suggestions. We also thank Mr Gary Mawyer for editorial assistance before submission. This study was supported by National Cancer Institute grants CA-76620 to L. W. K. C. and CA-82739 to H. E. Z., by National Cancer Institute grants CA78595 and CA75080 and a Paul Mellon Prostate Cancer Research Institute award to M. W. M., and by NIH grant R37 DK47556 to M. R. F.; B. C. was supported by a scholarship from the Higher Education Council, Ankara, Turkey, and from the School of Medicine, University of Virginia. These studies were presented in preliminary form as an abstract (no. 596) at the 93rd Annual Meeting of the American Association for Cancer Research (AACR), held in San Francisco, on 6–10 April 2002.

REFERENCES

- Lillie, J. W. and Green, M. R. (1989) Gene transcription: activator's target in sight. *Nature (London)* **341**, 279–280.
- Mitchell, P. J. and Tjian, R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**, 371–378.
- Wong, C. I., Zhou, Z. X., Sar, M. and Wilson, E. M. (1993) Steroid requirement for androgen receptor dimerization and DNA binding. Modulation by intramolecular interactions between the NH2-terminal and steroid-binding domains. *J. Biol. Chem.* **268**, 19004–19012.
- Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, M. J. and O'Malley, B. W. (1997) Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. *Recent Prog. Horm. Res.* **52**, 141–164.
- Brinkmann, A. O., Blok, L. J., de Ruiter, P. E., Doesburg, P., Stekete, K., Berrevoets, C. A. and Trapman, J. (1999) Mechanisms of androgen receptor activation and function. *J. Steroid Biochem. Mol. Biol.* **69**, 307–313.
- Tyagi, R. K., Lavrovsky, Y., Ahn, S. C., Song, C. S., Chatterjee, B. and Roy, A. K. (2000) Dynamics of intracellular movement and nucleocytoplasmic recycling of the ligand-activated androgen receptor in living cells. *Mol. Endocrinol.* **14**, 1162–1174.
- Young, C. Y., Andrews, P. E. and Tindall, D. J. (1995) Expression and androgenic regulation of human prostate-specific kallikreins. *J. Androl.* **16**, 97–99.
- Catalona, W. J., Smith, D. S., Ratliff, T. L., Dodds, K. M., Coplen, D. E., Yuan, J. J., Petros, J. A. and Andriole, G. L. (1991) Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. *N. Engl. J. Med.* **324**, 1156–1161.
- Zhang, J., Zhang, S., Murtha, P. E., Zhu, W., Hou, S. S. and Young, C. Y. (1997) Identification of two novel cis-elements in the promoter of the prostate-specific antigen gene that are required to enhance androgen receptor-mediated transactivation. *Nucleic Acids Res.* **25**, 3143–3150.
- Trapman, J. and Cleutjens, K. B. (1997) Androgen-regulated gene expression in prostate cancer. *Semin. Cancer Biol.* **8**, 29–36.
- Cleutjens, K. B., van Eekelen, C. C., van der Korput, H. A., Brinkmann, A. O. and Trapman, J. (1996) Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. *J. Biol. Chem.* **271**, 6379–6388.
- Cleutjens, K. B., van der Korput, H. A., van Eekelen, C. C., van Rooij, H. C., Faber, P. W. and Trapman, J. (1997) An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. *Mol. Endocrinol.* **11**, 148–161.
- Huang, W., Shostak, Y., Tarr, P., Sawyers, C. and Carey, M. (1999) Cooperative assembly of androgen receptor into a nucleoprotein complex that regulates the prostate-specific antigen enhancer. *J. Biol. Chem.* **274**, 25756–25768.
- Schuur, E. R., Henderson, G. A., Kmetec, L. A., Miller, J. D., Lamparski, H. G. and Henderson, D. R. (1996) Prostate-specific antigen expression is regulated by an upstream enhancer. *J. Biol. Chem.* **271**, 7043–7051.
- Cinar, B., Koenenman, K. S., Edlund, M., Prins, G. S., Zhau, H. E. and Chung, L. W. (2001) Androgen receptor mediates the reduced tumor growth, enhanced androgen responsiveness, and selected target gene transactivation in a human prostate cancer cell line. *Cancer Res.* **61**, 7310–7317.
- Blank, V., Kourilsky, P. and Israel, A. (1992) NF- κ B and related proteins: Rel/dorsal homologies meet ankyrin-like repeats. *Trends Biochem. Sci.* **17**, 135–140.
- McKay, L. I. and Cidlowski, J. A. (1999) Molecular control of immune/inflammatory responses: interactions between nuclear factor- κ B and steroid receptor-signaling pathways. *Endocr. Rev.* **20**, 435–459.
- Baeuerle, P. A. and Henkel, T. (1994) Function and activation of NF- κ B in the immune system. *Annu. Rev. Immunol.* **12**, 141–179.
- Ghosh, S., May, M. J. and Kopp, E. B. (1998) NF- κ B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16**, 225–260.
- Schmitz, M. L., dos Santos Silva, M. A., Altmann, H., Czisch, M., Holak, T. A. and Baeuerle, P. A. (1994) Structural and functional analysis of the NF- κ B p65 C terminus. An acidic and modular transactivation domain with the potential to adopt an alpha-helical conformation. *J. Biol. Chem.* **269**, 25613–25620.
- Urban, M. B., Schreck, R. and Baeuerle, P. A. (1991) NF- κ B contacts DNA by a heterodimer of the p50 and p65 subunit. *EMBO J.* **10**, 1817–1825.
- Schmidt-Ullrich, R., Memet, S., Lilienbaum, A., Feuillard, J., Raphael, M. and Israel, A. (1996) NF- κ B activity in transgenic mice: developmental regulation and tissue specificity. *Development* **122**, 2117–2128.
- Baeuerle, P. A. and Baltimore, D. (1988) Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF- κ B transcription factor. *Cell* **53**, 211–217.
- Beg, A. A. and Baldwin, Jr, A. S. (1993) The I kappa B proteins: multifunctional regulators of Rel/NF- κ B transcription factors. *Genes Dev.* **7**, 2064–2070.
- Brown, K., Gerstberger, S., Carlson, L., Franzoso, G. and Siebenlist, U. (1995) Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. *Science* **267**, 1485–1488.
- Kalkhoven, E., Wissink, S., van der Saag, P. T. and van der Burg, B. (1996) Negative interaction between the RelA(p65) subunit of NF- κ B and the progesterone receptor. *J. Biol. Chem.* **271**, 6217–6224.
- Palvimo, J. J., Reinikainen, P., Ikonen, T., Kallio, P. J., Moilanen, A. and Janne, O. A. (1996) Mutual transcriptional interference between RelA and androgen receptor. *J. Biol. Chem.* **271**, 24151–24156.
- McKay, L. I. and Cidlowski, J. A. (1998) Cross-talk between nuclear factor- κ B and the steroid hormone receptors: mechanisms of mutual antagonism. *Mol. Endocrinol.* **12**, 45–56.
- Auphan, N., DiDonato, J. A., Rosette, C., Helmsberg, A. and Karin, M. (1995) Immunosuppression by glucocorticoids: inhibition of NF- κ B activity through induction of I kappa B synthesis. *Science* **270**, 286–290.
- Sheppard, K. A., Phelps, K. M., Williams, A. J., Thanos, D., Glass, C. K., Rosenfeld, M. G., Gerritsen, M. E. and Collins, T. (1998) Nuclear integration of glucocorticoid receptor and nuclear factor- κ B signaling by CREB-binding protein and steroid receptor coactivator-1. *J. Biol. Chem.* **273**, 29291–29294.
- Yeung, F., Li, X., Ellett, J., Trapman, J., Kao, C. and Chung, L. W. (2000) Regions of prostate-specific antigen (PSA) promoter confer androgen-independent expression of PSA in prostate cancer cells. *J. Biol. Chem.* **275**, 40846–40855.
- Lu, S., Tsai, S. Y. and Tsai, M. J. (1997) Regulation of androgen-dependent prostatic cancer cell growth: androgen regulation of CDK2, CDK4, and CK1 p16 genes. *Cancer Res.* **57**, 4511–4516.
- Zhou, H. E., Goodwin, T. J., Chang, S. M., Baker, T. L. and Chung, L. W. (1997) Establishment of a three-dimensional human prostate organoid coculture under microgravity-simulated conditions: evaluation of androgen-induced growth and PSA expression. *In Vitro Cell Dev. Biol. Anim.* **33**, 375–380.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn, vol. 1, pp. 7.37–7.52. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Snoek, R., Rennie, P. S., Kasper, S., Matusik, R. J. and Bruchovsky, N. (1996) Induction of cell-free, in vitro transcription by recombinant androgen receptor peptides. *J. Steroid Biochem. Mol. Biol.* **59**, 243–250.
- Huang, S., Robinson, J. B., Deguzman, A., Bucana, C. D. and Fidler, I. J. (2000) Blockade of nuclear factor- κ B signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. *Cancer Res.* **60**, 5334–5339.
- Chen, C. D. and Sawyers, C. L. (2002) NF- κ B activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. *Mol. Cell. Biol.* **22**, 2862–2870.
- McKay, L. I. and Cidlowski, J. A. (2000) CBP (CREB binding protein) integrates NF- κ B (nuclear factor- κ B) and glucocorticoid receptor physical interactions and antagonism. *Mol. Endocrinol.* **14**, 1222–1234.
- Schmitz, M. L., Stelzer, G., Altmann, H., Meisterernst, M. and Baeuerle, P. A. (1995) Interaction of the COOH-terminal transactivation domain of p65 NF- κ B with TATA-binding protein, transcription factor IIB, and coactivators. *J. Biol. Chem.* **270**, 7219–7226.

-
- 40 McEwan, I. J. and Gustafsson, J. (1997) Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8485–8490
- 41 Hoffmann, A., Leung, T. H. and Baltimore, D. (2003) Genetic analysis of NF- κ B/Rel transcription factors defines functional specificities. *EMBO J.* **22**, 5530–5539
- 42 De Bosscher, K., Vanden Berghe, W., Vermeulen, L., Plaisance, S., Boone, E. and Haegeman, G. (2000) Glucocorticoids repress NF- κ B-driven genes by disturbing the interaction of p65 with the basal transcription machinery, irrespective of coactivator levels in the cell. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 3919–3924
- 43 Gilmore, T. D., Koedood, M., Piffat, K. A. and White, D. W. (1996) Rel/NF- κ B/I κ B proteins and cancer. *Oncogene* **13**, 1367–1378
- 44 Yamamoto, Y. and Gaynor, R. B. (2001) Therapeutic potential of inhibition of the NF- κ B pathway in the treatment of inflammation and cancer. *J. Clin. Invest.* **107**, 135–142
- 45 Higgins, K. A., Perez, J. R., Coleman, T. A., Dorshkind, K., McComas, W. A., Sarmiento, U. M., Rosen, C. A. and Narayanan, R. (1993) Antisense inhibition of the p65 subunit of NF- κ B blocks tumorigenicity and causes tumor regression. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9901–9905
- 46 Sharma, H. W., Perez, J. R., Higgins-Sochaski, K., Hsiao, R. and Narayanan, R. (1996) Transcription factor decoy approach to decipher the role of NF- κ B in oncogenesis. *Anticancer Res.* **16**, 61–69
- 47 Wang, C. Y., Mayo, M. W. and Baldwin, Jr, A. S. (1996) TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF- κ B. *Science* **274**, 784–787

Received 30 October 2003/7 January 2004; accepted 9 January 2004

Published as BJ Immediate Publication 9 January 2004, DOI 10.1042/BJ20031661