Androgenic Induction of Prostate-specific Antigen Gene Is Repressed by Protein-Protein Interaction between the Androgen Receptor and AP-1/c-Jun in the Human Prostate Cancer Cell Line LNCaP*

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In exploring the possible mechanisms of androgen independence of prostate-specific antigen (PSA) gene expression, we investigated the effect of elevating AP-1 by both 12-O-tetradecanoylphorbol 13-acetate (TPA) treatment and transfection of the c-Jun expression vector in LNCaP cells. Transcription of PSA is initiated when ligand-activated androgen receptor (AR) binds to a region in the PSA promoter that contains an androgen-responsive element (ARE). It was found that TPA inhibited androgen-induced PSA gene expression by a mechanism that did not alter nuclear levels of AR protein. Overexpression of AP-1 (Jun and fos proteins) also inhibited androgen-induced PSA promoter activity. These observations were apparently related to the disruption of AR-ARE complexes as demonstrated by the results of electrophoretic mobility shift assays. Specifically, c-Jun inhibited the formation of AR-ARE complexes and conversely that AR-glutathione S-transferase proteins inhibited the formation of c-Jun-TPA-responsive element (TRE) complexes. Consistent with the inhibitory effect of both proteins, anti-c-Jun antibody blocked the inhibition of AR-ARE complex formation by c-Jun. A similar, but less marked, effect was obtained when anti-AR antibody was used to prevent AR inhibition of c-Jun-TRE complex formation. These findings together with results obtained from co-immunoprecipitation experiments strongly suggest that mutual repression of DNA binding activity is due to direct interaction between the two proteins and that the degree of repression may be determined by the ratio of AR to c-Jun. The mechanism of repression studied in mutant analysis experiments yielded evidence of an interaction between the DNA and ligand-binding domains of AR and the leucine zipper region of c-Jun. Thus, the AR is similar to other nuclear receptors in its ability to interact with AP-1. This association provides a link between AP-1 and AR signal transduction pathways and may play a role in the regulation of the androgen-responsive PSA gene.

Prostate-specific antigen (PSA)1 belongs to the family of kallikrein-like serine proteases (for a review, see Ref. 1). In males, expression of PSA occurs exclusively in the prostate with serum levels of PSA glycoprotein being an important marker in the diagnosis and progression of prostate cancer (2, 3). PSA is an androgen-induced gene that contains androgen response elements (AREs) to which the androgen receptor (AR) binds (4, 5).

The AR belongs to the superfamily of nuclear receptors that mediate the responses of lipophilic ligands, including steroids, retinoids, vitamin D₃, and thyroid hormones (6). These receptors contain a highly conserved DNA-binding domain comprised of zinc finger-like motifs responsible for sequence-specific DNA binding, as well as protein-protein interactions. There is evidence to suggest that the DNA-binding domain of a nuclear receptor may interact with the leucine zipper of AP-1 to result in mutual transrepression (7–9). However, this interaction between nuclear receptors and AP-1 may be cell-specific, gene-specific, and may involve various mechanisms, including protein-protein interaction and/or adjacent or overlapping binding sites (10).

AP-1 is a transcriptional factor whose components are nuclear proteins encoded by c-fos and c-jun proto-oncogenes induced by 12-O-tetradecanoylphorbol 13-acetate (TPA). AP-1 has been implicated in cell growth, differentiation, and development with its activity modulated by growth factors, cytokines, oncogenes, and tumor promoters activating protein kinase C (PKC) (10). AP-1 induces transcriptional activation through interaction with the TPA-responsive element (TRE or AP-1 DNA-binding site) (11, 12). TREs are recognized by Jun homodimers and Jun/Fos heterodimers that are formed through the leucine zipper domain of both proteins (11). The basic region adjacent to the leucine zipper on Jun and Fos proteins mediates AP-1 DNA binding activity (13–15).

Since the zinc finger motifs of the AR share a high degree of homology with the same regions of other nuclear receptors and in the face of mounting evidence of AP-1 interaction with such motifs, the question arose whether AR function could also be affected by interaction with AP-1. Accordingly, in this study we examined the direct interaction of AP-1 with AR protein and

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1 The abbreviations used are: PSA, prostate-specific antigen; AR, androgen receptor; ARE, androgen-responsive element; TPA, 12-O-tetradecanoylphorbol 13-acetate; PKC, protein kinase C; TRE, TPA-responsive element; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay; GR, glucocorticoid receptor.
the effects of elevated AP-1 on androgen-stimulated PSA gene expression.

MATERIALS AND METHODS

Cell Culture and Northern Blot Analysis—All chemicals were purchased from Sigma, unless stated otherwise. PCS and CV-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% FBS (Life Technologies, Inc., Burlington, Ontario, Canada). LNCaP cells were maintained in RPMI 1640 supplemented with 5% FBS. Cells between the 37th and 49th generation were used in the these experiments. For Northern blot analyses, LNCaP cells were down-shifted to RPMI 1640 containing 2% FBS plus 2% TCMM (serum replacement obtained from Celox Corp., Hopkins, MN), for 10–14 days and 5 x 10^6 or 3 x 10^6 cells were initially plated on 6-cm dishes or 6-well plates, respectively. Culture medium was changed to RPMI 1640 with 2% TCMM (serum-free), with or without 10 ng R1881, when cells were 60–70% confluent with the cells. Total RNA was extracted with acid guanidium thiocyanate/phenol/chloroform (16) and fractionated by electrophoresis prior to blotting onto Hybond-N filters (Amersham, Oakville, Ontario, Canada). The 1.4-kb EcoRI fragments of the PSA cDNA (17), 0.7-kb EcoRI/HindIII fragments of human AR cDNA (18) and 1.9-kb PstI fragments of chicken β-actin (19) were labeled with [γ-32P]ATP by using the Random Primer DNA labeling kit (Life Technologies, Inc.). The 40-base oligonucleotides for either c-Jun or c-Fos (Ciderlane, Toronto, Ontario, Canada) were end-labeled by T4 polynucleotide kinase with [γ-32P]ATP. Hybridization was performed as reported previously (20). Filters with PSA and β-actin were washed in 0.1 × SSC, 0.1% SDS for 30 min at 65 °C, while filters with AR, c-Jun and c-Fos were in 0.5 × SSC, 0.2% SDS for 30 min at 55°C. Deniosometric analyses of mRNA bands were performed using NIH image (National Institutes of Health) from scanned x-ray films.

Immunoblots—LNCaP cells were incubated in RPMI 1640 containing 2% TCMM for 24 h prior to the addition of vehicle (0.1% ethanol), 10 ng R1881, or 1 nM TPA. After incubation with compounds, cytosolic and nuclear extracts were prepared as described by Antras et al. (21). Western blots were performed with approximately 40 μg of protein in each lane. Cytosolic extracts were blocked and washed 3 × in 5% dextran-coated stripped serum, 10 nM R1881 for 24 h, and 10% RPMI 1640 and Dulbecco's modified Eagle's medium, respectively, containing 5% FBS (Life Technologies, Inc.). Western blots were performed with 1 nM R1881, when wells were incubated in mild Nonidet P-40 buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 5 μg/ml leupeptin 5 μg/ml aprotinin, 5 μg/ml trypsin inhibitor, 5 μg/ml bacitracin, and 1 μm phenylmethylsulfonyl fluoride) for 15 min at 4 °C. The nuclear extracts were precleared with protein A-Sepharose for 30 min and incubated with anti-AR antibody PG-21 (22) or 1507A1 (Pharmingen, San Diego, CA) for 1 h at 4 °C. The antigen antibody complexes were collected by the addition of protein A-Sepharose. Immune complexes were washed once with mild Nonidet P-40 buffer prior to separating on a 10% SDS-PAGE. Western blots analyses were carried out with anti-c-Fos antibody and anti-c-Jun antibody as described above.

PSA Promoter Plasmid Constructs and Luciferase Assay—PSA 5'-flanking DNA was obtained by PCR-mediated amplification of human genomic DNA using oligonucleotide primers corresponding to the PSA gene. The sequences for primers were 5'-CATTTTTGCTGCAATTTG-3' and 5'-TCCCGGGTCAGGTTAGAATCTTTG-3'. The PCR fragments were purified by gel electrophoresis, blunted-ended, and ligated with EcoRV-digested pBluescript (pBS SK+) (Strategene, La Jolla, CA). pBS containing the PSA 5'-flanking DNA was designated as pBS-PSA-630 (23). The fragment was amplified and purified from transformed Escherichia coli DH-5α and sequenced by dyeodeoxynucleotide chain termination method using double-stranded DNA cycle sequencing kit system (Life Technologies, Inc.). DNA fragments corresponding to −630/+12 of the PSA 5'-flanking region were excised from pBS-PSA-630 with HindIII and inserted into the HindIII site of promoterless plasmid, pG-L2-basic (Promega, Madison, WI), which contains firefly luciferase as a reporter gene. This pG-L2 basic containing the −630/+12 fragment of the PSA 5'-flanking DNA was referred to as pPSA-630. LNCaP cells (2–2.5 x 10^4) were plated on 6-well plates and incubated in RPMI 1640 with 5% FBS for 3 days, resulting in 50–60% confluence. Plasmid DNA was mixed with 5 μl of Lipofectin agent (Life Technologies, Inc.) and incubated at room temperature for an min at room temperature. The total amount of plasmid DNA used was normalized to 3 μg/well by the addition of empty plasmid. Medium was replaced after 24 h by RPMI 1640 with 2% TCMM with or without R1881 (i.e. serum-free media). Cells were collected after 48-h incubation using cell lysis buffer (100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100, and 1 mM dithiothreitol). Luciferase activities were assayed using a commercial kit from Promega according to the manufacturer’s protocol, and activities were normalized by either protein concentration determined by the method of Bradford (23) or β-galactosidase activities measured with Galacto-Light (Tropix Inc.). Luciferase activities are expressed as relative luminescent units/mg of protein/min. All transfection experiments were carried out in triplicate wells and repeated two to eight times using at least two sets of plasmids prepared separately.

Other Plasmids—Full-length rat AR cDNA (amino acid 1–902) was cloned into pcDNA3 whose transcription is driven by cytomegalovirus promoter and this plasmid was referred to as pAR6. In our previous report, transcriptional efficacy of pAR6 was determined by expression of mRNA and binding assay in AR-negative PC3 cells when stably transfected with pAR6 (24). Several mutant AR expression plasmids were constructed in our laboratory. Regions of amino acids encoded by these mutant AR expression plasmids are as follows: pAR4 (232–649), pAR5 (390–649), pAR7 (232–902), and pAR8 (232–902). Several mutant c-Jun/c-Fos expression plasmids were constructed in our laboratory. Regions of amino acids encoded by these mutant AR expression plasmids are as follows: pAR4 (232–649), pAR5 (390–649), and pAR7 (232–902). pRSV-c-Jun and pRSV-c-Fos were wild type c-Jun and c-Fos expression plasmids, respectively. pRSV-c-Jun-D1 lacks a small portion of the N-terminal activator domain, c-Jun-D3 lacks the N-terminal domain, and c-Jun-L2 lacks the leucine zipper. pRSV-Jun-B,-Jun D, -Fra 1, and -Fra 2 are Jun B, Jun D, Fra 1, and Fra 2 expression plasmids, respectively. pRSV-0 is the empty plasmid for pRSV series of plasmids. ARR-tk-luciferase reporter construct consists of three congruent rat probasin AREs (−244 to −96) ligated in tandem into the HindIII site of the pBS1 luciferase vector (ATCC, Rockville, MD) as described by us previously (25). pCH110 (Amersham), a β-galactosidase expression plasmid, was co-transfected as an internal marker for normalizing efficacy of transient transfection.

Preparation of Recombinant c-Jun Protein and AR-GST Fusion Proteins—The prokaryotic expression vector pET-8c-c-Jun coding for the full-length c-Jun was transformed into E. coli BL21(DE3)pLYSs. The recombinant protein was induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside and extracted from inclusion bodies according to the protocol of Lin and Cheng (26) with slight modifications. Briefly, the inclusion bodies were isolated and subjected to several rounds of sonication in the appropriate buffers. The insoluble pellet was solubilized in 5 M guanidinium HCl, followed by stepwise dialysis against buffers containing 2 M, 1 M, and 0.5 M guanidinium HCl. Yields were typically 14 mg of c-Jun protein per 200 ml of bacterial culture with 90% purity. AR1 and AR2 were expressed in E. coli as isopropyl-β-d-thiogalactopyranoside-induced fusion proteins with glutathione S-transferase (GST), purified through glutathione affinity chromatography, and calculated to have greater than 90% purity when assayed by Coomassie Blue staining of polyacrylamide gels (25). AR1 encodes amino acids 524–902 (rat AR) encompassing the DNA-binding domain, hinge region, and ligand-binding domain. AR2 encodes amino acids 524–649 (rat AR) encompassing the DNA-binding domain and hinge region.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from LNCaP cells or purified proteins were used for EMSA studies. Nuclear extracts were prepared from cells treated with vehicle (0.1% ethanol), 10 ng R1881, or TPA (1 and 10 nM) for 6 h before harvesting. DNA binding reactions were carried out in a total volume of 25 μl, containing DNA binding buffer (20 mM HEPS, pH 7.9, 20% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 1 mM diethiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 500 ng of poly(dI-dC) (Pharmacia Biotech Inc.). Approximately 1.5 fmol of double-stranded 32P-labeled TRE oligonucleotide (5'-GGCTGTAGATGCAGCGGAAG-3'), PSA ARE oligonucleotide (5'-TTGCAAGACACGAAGCTGTCCTC-3'), PSA mutant ARE (5'-TTGGCAGAATGCTGGCTGCT-3'), pRSV ARE oligonucleotide (5'-ATTCGCTGGTGCCGGGGGAG-3') and the TRE oligonucleotide were obtained from Promega, purified through several rounds of purification by correction for the amount of buffer and total protein. For competition experiments, excess unlabeled oligonucleotide was used. Competition DNA-protein complexes were separated under non-denaturing conditions in a 8% polyacrylamide gel (29:1) containing 2.5% glycerol and run in 0.5 × TBE (1 × 389 mM Tris borate, 89 mM boric acid, and 2 mM EDTA, pH 8.3) at 200 V. Bands from dried EMSA
TPA concentrations of 1 nM and less did not alter morphology nor reduce cell viability (data not shown). Therefore, treatment and levels continued to decline for the duration of the experiment at 24 h. β-Actin mRNA levels remained consistent, and androgen-induced levels of PSA mRNA remained elevated in the absence of TPA for the duration of the study.

TPA has been shown to induce c-jun and c-fos mRNA levels in LNCaP cells (27). In agreement, TPA (1 nM) caused a transient increase in both c-jun and c-fos mRNA levels (Fig. 1D), regardless of the presence of androgen (R1881). This suggests that androgen stimulation and the subsequent activation of AR does not interfere with the signal transduction pathway leading from PKC to c-jun and c-fos induction in LNCaP cells.

A time course study of TPA induction of c-Jun and C-Fos proteins in LNCaP cells showed that maximum levels were achieved after 4.5 h of exposure (Fig. 2). At this time point, c-Jun levels were 6.6-fold higher and c-Fos levels 49-fold higher than levels in untreated cells. c-Jun levels remained 3–4-fold higher in TPA-treated cells, as compared with levels in untreated cells, for the duration of the experiment (32 h).

TPA Effects on Nuclear and Cytosolic Levels of Androgen Receptor—Androgen induction of PSA mRNA has been shown to be mediated by the AR which binds to AREs on the PSA promoter (4, 5). To determine whether the TPA-associated decreases in androgen-induced PSA mRNA demonstrated in Fig. 1A were due to reduced expression of AR, we examined levels of AR protein. AR protein was detected as a band at approximately 110 kDa in both nuclear and cytosolic extracts (Fig. 3). Nuclear extracts prepared from cells exposed to R1881 had increased levels of nuclear AR, as compared with cells not exposed to androgen. Cytosolic and nuclear levels of AR were not altered by treatment with TPA (1 nM) for 24 h. Therefore, TPA does not appear to decrease PSA gene expression by a mechanism that involves decreasing the nuclear levels of AR protein.

Inhibition of Androgen-induced PSA Promoter Activity by TPA and Overexpression of c-Jun and c-Fos—PSA promoter activity was examined by transient transfection of LNCaP cells with a PSA promoter-luciferase reporter plasmid (pPSA-630).
LNCaP cells express endogenous AR, and the addition of 10 nM R1881 to cells resulted in a 5-fold increase of PSA promoter activity (data not shown). Co-transfection of cells with the rat wild-type AR (pAR6) expression plasmid (0.5 μg/well) resulted in a 34-fold increase in androgen-induced PSA promoter activity in the presence of 10 nM R1881 (Fig. 4A). Therefore, all subsequent studies measuring androgen-induced PSA promoter activities were performed with cells transiently transfected with pAR6.

In Fig. 1, A and C, it was demonstrated that TPA decreased androgen-induced PSA mRNA. In agreement with these data, androgen-induced PSA promoter activity was also inhibited by 39% in LNCaP cells exposed to 1 nM TPA for 24 h (data not shown).

Fig. 2. Time course study of TPA induction of c-Jun (A) and c-Fos (B) protein in LNCaP cells. LNCaP cells were maintained in serum-free media in the absence of R1881 for 24 h prior to the addition of 1 nM TPA. Cells were incubated with TPA for various time points before harvesting and Western blots performed. Forty μg of protein was loaded in each lane and separated by SDS-PAGE, blotted, and analyzed for c-Jun (A) and c-Fos (B) protein using antibodies (sc-045 and sc-052, respectively).

Fig. 3. TPA effects on nuclear and cytosolic levels of AR protein. LNCaP cells were incubated in serum-free media for 24 h prior to the addition of R1881 (10 nM) and TPA (1 nM) for an additional 24 h before cytosolic (lanes 1–4) and nuclear (lanes 5–8) fractions were prepared. Forty-six μg of protein was loaded in each lane and separated by SDS-PAGE, blotted, and analyzed for AR protein using the AR antibody (PG-21).

Numerous nuclear receptors have been shown to interact with TPA-inducible proteins, c-Jun and/or c-Fos (10). Therefore, to determine whether c-Jun and/or c-Fos are involved in TPA repression of androgen-induced PSA promoter activity, LNCaP cells were transfected with pPSA-630 (1 μg/well), pAR6 (0.5 μg/well) with or without c-Jun, c-Jun plus c-Fos, or c-Fos expression plasmids. Subsequent to transfection, cells were incubated for 48 h in serum-free RPMI 1640 containing 2% TCM™ in the presence of 10 nM R1881. The percent inhibition of relative luciferase activities is expressed as the mean ± S.D. of three independent experiments with triplicate determinations, using controls (with RSV-0) set at 0%. *, **, and ***, significantly different from the control value.
AP-1 Inhibition of Androgen-induced PSA

PSA promoter activity by transfection of the respective expression vectors into LNCaP cells. All members of the jun family examined significantly attenuated androgen-induced PSA promoter activity relative to RSV-0 values (Fig. 5). However, for members of the fos family investigated, only c-Fos significantly inhibited androgen-induced PSA promoter activity in the absence of jun members. Maximum inhibition of androgen-induced PSA promoter activity was generally observed in cells co-transfected with expression plasmids from both jun and fos families (lanes 8–16).

Mutual Interaction of DNA Binding Activity by Co-incubation of AR and c-Jun—EMSA with radiolabeled synthetic oligonucleotides containing a TRE consensus site and nuclear extracts prepared from LNCaP cells exposed to TPA for 6 h showed comparable DNA binding activities in vehicle-treated (0.1% ethanol) and 10 nM R1881-treated cells (Fig. 6A). Cells treated with 1 nM TPA (lane 3) showed a 3.7-fold increase in DNA binding activity (compared with vehicle-treated levels, lane 1), which was similar to levels obtained with nuclear extracts from cells treated with 1 nM TPA and R1881 (lane 4). These data are comparable with the 4-fold increase in cellular levels of TPA-induced c-Jun protein for this time point as shown in Fig. 2. In Fig. 6A, TPA (10 nM) was the more potent of the two concentrations of TPA examined and resulted in an 11.7-fold increase in DNA binding activity of nuclear extracts to the TRE oligonucleotide (lane 5). The addition of R1881 to 10 nM TPA-treated cells consistently resulted in approximately a 33% reduction in AP-1 DNA-binding activity (lane 6). Specificity of DNA binding activity was shown by competition experiments using 100-fold excess unlabeled SP-1 oligonucleotide (nonspecific competitor) (lane 7) and TRE oligonucleotide (specific competitor) (lane 8).

Thus, nuclear extracts from TPA-treated LNCaP cells are characterized by enhanced AP-1 DNA-binding activity which is reduced by R1881.

The work of Kallio et al. (32) indicated that AR inhibits c-Jun DNA binding activity, while c-Jun does not affect AR DNA binding to the C3 ARE. Similarly, the c-Jun DNA binding activity seen in our experiments (Fig. 6B, lane 3) was also inhibited by peptide fragments of the AR. AR1-GST, containing both the ligand- and DNA-binding domains, was more potent in the inhibition of c-Jun DNA binding activity (lanes 4–8) than the AR2-GST which contains only the DNA-binding domain (lanes 10–13). A 5-fold increase in molar ratio of AR1-GST to c-Jun resulted in a 100% decrease in c-Jun DNA binding (lane 6); by comparison, at the same ratio AR2-GST seemed to be less effective, inhibiting c-Jun binding by 94% (lane 11). At equimolar concentrations of AR1 and AR2 to c-Jun, there was an 82 and 75% inhibition of c-Jun DNA binding activity, respectively, which is comparable with that reported by Kallio et al. (32). AR inhibition of c-Jun DNA binding activity could be partially preincubated with the AR with an antibody to the AR DNA-binding domain before incubation with c-Jun (compare Fig. 6B, lanes 11 and 12, and inset, lanes 6 and 7). Specificity of c-Jun binding to the TRE oligonucleotide was shown by a supershift of the c-Jun-c-Jun-TRE complex with an antibody to c-Jun (lane 2). AR-GST proteins did not bind to the TRE oligonucleotide (lanes 8 and 13).

To examine whether the presence of c-Jun could alter AR DNA binding activity to the PSA ARE, we investigated several different molar ratios of c-Jun to AR. c-Jun at a molar concentration 10-fold higher than AR1-GST protein levels, caused a 44% decrease in AR1-GST binding to the PSA ARE (Fig. 6C, compare lanes 2 and 7). c-Jun inhibition of AR DNA binding activity could be completely blocked by preincubation of c-Jun with an antibody to c-Jun prior to incubation with the AR (compare lanes 7 and 9). Specificity of AR-GST protein binding to the PSA ARE was confirmed using three different approaches. These included supershift of the AR:ARE complex with an antibody to the AR DNA binding domain (lane 11), lack of AR binding to the ARE oligonucleotide containing a mutated AR half-site (lane 12), and by competition experiments using 100-fold excess unlabeled SP-1 oligonucleotide (nonspecific competitor) (lane 13) and ARE oligonucleotide (specific competitor) (lane 14).

Co-immunoprecipitation of AP-1 with AR—The above results from transfection experiments and EMSA suggest that mutual interference between AR and AP-1 may be due to direct protein-protein interaction. To investigate this possibility, co-immunoprecipitation studies were performed using LNCaP and PC3 cells. The PC3 cell line is a poorly differentiated prostate cancer cell line that does not express PSA or AR (33) and was included as a control. In Fig. 7A (lane 5), c-Jun is immunoprecipitated with the AR from LNCaP cells when using an antibody to the first 21 N-terminal amino acids of the AR (PG-21). As expected, c-Jun could not be immunoprecipitated from PC3 cells when using the PG-21 antibody (lane 4), because this cell line lacks AR. Since the DNA-binding domains of other steroid receptors have been suggested to be involved in protein-protein interaction with c-Jun, we used another antibody that binds to the AR DNA binding domain (15071A). c-Jun could not be immunoprecipitated with the AR when using this antibody (PC3 cells, lane 2; LNCaP cells, lane 3). This suggests that the AR DNA-binding domain is involved in protein-protein interaction between the AR and c-Jun. Similar results were obtained with c-Fos (Fig. 7B).

c-Jun Mutant Analyses—To determine the region(s) of c-Jun that may be involved in protein-protein interaction with the
AR, we transfected expression vectors for various c-Jun mutants into LNCaP cells and examined their effects upon androgen-induced PSA promoter activity. Androgen-induced PSA promoter activity observed in cells transfected with pAR6 was inhibited 60% by transfection with wild type c-Jun (Fig. 8). Deletion of a small region of the N-terminal activator domain (c-Jun-D1) did not alter the ability of c-Jun to repress androgen-induced PSA promoter activity. c-Jun inhibition was impaired when the N terminus region of c-Jun was deleted (c-Jun-D3). The most pronounced effect was observed upon deletion of the leucine zipper (c-Jun-ΔLZ), which completely abolished the protein’s ability to inhibit androgen-induced PSA promoter activity. Therefore the leucine zipper motif of c-Jun may interact with the AR.

AR Mutant Analyses—To define possible AR domains involved in AP-1 repression, the expression plasmid for wild type c-Jun was co-transfected with expression plasmids for several AR mutants. Consistent with Fig. 4, transfection of the wild type AR (pAR6) resulted in over 30-fold induction of PSA promoter activity by R1881 (Fig. 9, lane 2). This androgenic induction was inhibited by 60% when cells were co-transfected with the c-Jun expression plasmid (lane 3). Maximum induction (>50-fold) of PSA promoter activity was observed in cells transfected with the pAR7, which lacks part of the N terminus region (amino acids 1–231) (lane 5). Androgenic induction of pAR7 was inhibited by approximately 65% when cells were co-transfected with c-Jun (lane 6). Cells transfected with pAR4, which lacks the ligand-binding domain, resulted in constitutively high PSA promoter activity (lane 7) regardless of R1881 addition (lane 8). Levels of PSA promoter activities in pAR4-transfected cells were not significantly affected by co-transfection with c-Jun (lane 9). Transfection of cells with pAR5 re-

FIG. 6. Mutual inhibition of AR and c-Jun DNA-binding activity. EMSA were performed using the radiolabeled TRE oligonucleotide (A and B) or the PSA ARE (C). A, AP-1 DNA binding activity in nuclear extracts isolated from LNCaP cells exposed to TPA for 6 h. Lane 1, control; lanes 2, 4, and 6–8, 10 nM R1881; lanes 3, 4, 7, and 8, 1 nM TPA; lanes 5 and 6, 10 nM TPA. Specificity of binding is indicated by competition experiments with unlabeled SP-1 (lane 7) and TRE (lane 8) oligonucleotides. B, inhibition of c-Jun binding to the consensus TRE. Purified c-Jun protein (lanes 2–7 and 9–12, 250 ng) was incubated with increasing amounts of either AR1-GST (lane 4, 444 ng; lane 5, 888 ng; lanes 6–8, 2220 ng) or AR2-GST (lane 9, 160 ng; lane 10, 320 ng; lanes 11–13, 800 ng). Antibody studies were performed by preincubation of the AR with PG-21 antibody 30 min before the addition of c-Jun. Numbers in the figure represent the molar ratios. Lane 2 shows c-Jun–TRE complex supershifted by incubation with anti-c-Jun antibody. The inset shows a longer exposure time of lanes 5–7. C, inhibition of AR binding to the PSA ARE. Purified AR1-GST (lanes 2–7 and 9–14, 240 ng) was incubated with increasing amounts of c-Jun (lane 3, 75 ng; lane 4, 150 ng; lane 5, 300 ng; lane 6, 750 ng; lanes 7–9, 1500 ng). Antibody studies were performed by preincubation of the c-Jun with anti-c-Jun antibody for 30 min before the addition of AR1-GST. Numbers in the figure represent the molar ratios. Lanes 10–14 show AR–ARE specificity. Lane 11 shows AR–ARE complex supershifted by incubation with PG-21 antibody. Lane 12, radiolabeled PSA ARE is substituted with a radiolabeled mutant PSA ARE. Competition experiments with nonlabeled SP-1 oligonucleotide (lane 13) and nonlabeled PSA ARE (lane 14).
sulted in slight androgen induction of PSA promoter activity (lane 11) that was not affected by co-transfection with c-Jun (lane 12). Thus, the ligand-binding domain of the AR may be required for inhibition by c-Jun.

Promoter and Cell Specificity—A previous report examining nuclear receptors and AP-1 interaction showed different results depending on the promoter and cell line examined (34). Therefore, we investigated whether AP-1 could inhibit another androgen-induced promoter in LNCaP cells. These cells were transfected with the ARR3-tk-luciferase reporter construct, that contains three repeats of the rat probasin ARE1 and ARE2 region ligated in tandem in a luciferase reporter (25). ARR3-tk-luciferase activity was induced 72-fold in cells treated for 48 h with 10 nM R1881, as compared with cells exposed to vehicle only. LNCaP cells transfected with 0.5 µg of c-Jun expression plasmid showed 43% inhibition of androgen-induced ARR3 promoter activity (Fig. 10A, lane 2). Cells transfected with 0.5 µg of c-Fos expression plasmid had 71% inhibition of androgen-induced ARR3 promoter activity (lane 3). Co-transfection of both c-Jun and c-Fos expression plasmids resulted in an 89% inhibition of androgen-induced ARR3 promoter activity (lane 4). Hence, there was a difference between the PSA and ARR3 promoter in AP-1 inhibition of androgen-induced activity in LNCaP cells. c-Jun was more potent than c-Fos in the inhibition of androgen-induced PSA promoter activity (Fig. 4B), while c-Fos was more potent than c-Jun in the inhibition of androgen-induced ARR3 activity (Fig. 10A).

The cellular specificity of AP-1 inhibition of PSA promoter activity was examined using CV-1 cells (kidney, African green monkey) that are considered to be glucocorticoid receptor (GR)-deficient. Co-transfection of pAR6 was required for androgen induction of PSA promoter activity in CV-1 cells. PSA promoter activity was induced 32-fold in cells transfected with 0.5 µg pAR6 and exposed to 10 nM R1881 (data not shown). CV-1 cells co-transfected with 0.5 µg of both pAR6 and c-Jun resulted in 38% inhibition of androgen-induced PSA promoter activity (Fig. 10B, lane 2). These levels were comparable with those in cells co-transfected with 0.5 µg of both pAR6 and c-Fos (43% inhibition of androgen-induced PSA promoter activity) (lane 3). Co-transfection of both c-Jun and c-Fos resulted in a 68% inhibition of androgen-induced PSA promoter activity (lane 4) that was similar to the percentage inhibition seen in LNCaP under the same conditions (Fig. 4B). These results infer that AP-1 inhibition of androgen-induced PSA promoter activity is cell-specific.

Fig. 8. c-Jun mutant analyses. pPSA-630 (1 µg/well) was co-transfected with pAR6 (0.5 µg/well) and a wild type (wt) or a mutant c-Jun expression plasmids (c-Jun-Δ1, -Δ3, and -ΔLZ) at 0.5 mg/well into LNCaP cells and incubated for 48 h with 10 nM R1881. Relative luciferase activities are expressed as the mean ± S.D. of five independent experiments with triplicate determinations, setting the control (RSV-0) as 100%. *, significantly different from RSV-0; (column 1 versus column 2, p < 0.00001; column 1 versus column 3, p < 0.0001; column 1 versus column 4, p < 0.01; column 1 versus column 5, not significant). Schema of structure of c-Jun: NA, the N-terminal activator domain; BR, the basic region (the DNA-binding domain); and LZ, the leucine zipper, which is essential sequences for dimerization with Jun or Fos.

Luciferase Activity (RLU x 10⁶ / min / mg protein)

Fig. 9. AR mutant analyses. pPSA-630 (1 µg/well) was co-transfected with a wild type (pAR6), mutant rat AR expression plasmids, or pRc-RSV (empty plasmid) at 0.5 µg/well into LNCaP cells and incubated for 48 h in the absence (−) or presence (+) of 10 nM R1881. Some cells were further co-transfected with c-Jun expression plasmids. Luciferase activities are expressed as the mean ± S.D. of three independent experiments with triplicate determinations. Schema of structure of the AR: N, the N-terminal domain; D, the DNA-binding domain; H, the hinge region; and A, the androgen-binding domain. RLU, relative luminescent units(s).
Inhibition of androgen-induced PSA promoter activity by overexpression of c-Jun and c-Fos suggests that TPA-associated decreases in PSA gene expression may be the result of increased AP-1 and interaction between the AR and these proteins. The order of potency of inhibition from co-transfection experiments observed in LNCaP cells was: c-Jun and c-Fos > c-Jun alone > c-Fos alone (Fig. 4B). These results would be consistent with cooperativity between c-Jun and c-Fos. Alternatively, they are compatible with a requirement for AP-1 dimerization, since Jun homodimers are considerably less stable than the heterodimeric Jun/Fos complex (49, 50). However, dimer formation would theoretically mask the leucine zipper of c-Jun, a domain that our results (Fig. 8) suggest may be involved in AR/AP-1 interaction. Another study examining AP-1 modulation of androgen induction found that an increase in the intracellular levels of c-Jun stimulated, while c-Fos inhibited the transcriptional activation induced by androgen in numerous cell lines (34). However, these responses appeared to be cell-specific, which we also see when comparing PSA promoter activity in LNCaP cells (Fig. 4) and CV-1 cells (Fig. 10B), and gene-specific as shown by differences in AP-1 inhibition of PSA promoter activity (Fig. 4) and ARR3 activity (Fig. 10A) in LNCaP cells.

GR studies show mutual inhibition of DNA binding as the result of protein-protein interaction between the GR and AP-1 and do not show GR binding to TREs or vice versa (7, 8). Similarly our EMSA results with the AR and AP-1 do not show any evidence for c-Jun binding to the PSA ARE (Fig. 6C) nor was there evidence for AR binding to the TRE (Fig. 6B). In agreement with the lack of AP-1 binding to the PSA ARE, the sequence of this region bears no homology to the TRE consensus site. Despite the lack of DNA sequence homology between the consensus TRE and the PSA ARE, incubation of the AR with c-Jun leads to loss of mutual DNA binding activities. DNA binding activity could be Partially to fully recovered by preincubation of either protein with specific antibodies. Collectively, these data strongly suggest that the mutual inhibition of DNA binding activities may result from the formation of abortive heterodimers or complexes between the two proteins. This hypothesis was supported by the demonstration that c-Jun and c-Fos are co-immunoprecipitated with the AR (Fig. 7).

The zinc finger motifs of GR are thought to interact with the DNA binding and/or leucine zipper domains of c-Jun (7, 8). The homology between the GR and AR protein is 77% in the zinc finger regions. It is thus not surprising that results from EMSA and mutant analyses with both AR (Fig. 9) and c-Jun (Fig. 8) imply that the same structural components of AR and c-Jun may interact. In addition, the results of co-immunoprecipitation studies indicate that c-Jun and c-Fos are co-precipitated with the AR by an antibody to the AR N terminus region (Fig. 7). In contrast, neither c-Jun nor c-Fos could be co-precipitated with AR by an antibody to the AR DNA-binding domain (Fig. 7). These observations draw attention to the importance of the AR DNA-binding domain for AR and AP-1 interaction. Furthermore, the AR ligand-binding domain also appears to be in-
involved in the interaction between the AR and c-Jun (Figs. 6 and 9). EMSA data supporting the view that the AR DNA- and ligand-binding domains may interact with c-Jun is depicted in Fig. 6B. In these studies, two different AR-GST proteins were employed and the AR protein consisting of both the DNA- and ligand-binding domains (AR1) was more potent in repression of androgen-induced PSA promoter activity (Fig. 5). The DNA-binding domain of AR is high, there would be less AR available for binding to any ARE or TRE or both. It follows that such a mechanism might depend on the ratio of AR to c-Jun not excluding other AP-1 proteins or co-regulators. The possible importance of the ratio of AR to AP-1 protein is especially relevant in prostate cells in which the promoter region of the PSA gene may contain several TREs (4, 54). In a situation where the ratio of c-Jun to AR is high, there would be less AR available for binding to any ARE to initiate transcription. In contrast, there would be excess c-Jun available for binding to PSA TREs, a condition that conceivably might result in androgen-independent stimulation of PSA gene expression. In this regard, it is of interest that the PC3 cell line, which has progressed to an advanced stage of androgen-independence, is characterized by a 7-fold greater intracellular concentration of c-Jun relative to that in the more differentiated LNCaP cell line.2 The possible role of c-Jun and its related family of proteins in contributing to the androgen-independent regulation of the PSA gene appears worthy of further investigation.

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