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Androgen receptor-associated protein complex binds upstream of the androgen-responsive elements in the promoters of human prostate-specific antigen and kallikrein 2 genes

Zijie Sun*, Jing Pan and Steven P. Balk

Cancer Biology Program, Division of Hematology–Oncology, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215, USA

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ABSTRACT

An increasing number of proteins which bind to hormone-dependent nuclear receptors and mediate their effects on gene expression are being identified. The human prostate-specific antigen (PSA) and kallikrein 2 (KLK2) genes are regulated by the androgen receptor (AR). Using electrophoresis mobility shift assays (EMSA), a common nuclear protein(s) which binds upstream of the androgen-responsive elements (AREs) in the PSA and KLK2 promoters was identified. Binding occurred between bp –539 and –399 and bp –349 and –224 in the PSA and KLK2 promoters respectively, which were shown previously to be necessary for AR-mediated transactivation. Glutathione S-transferase (GST)–AR fusion proteins were constructed to determine whether the AR interacted directly with this protein or protein complex. Specific interactions were observed with AR fusion proteins containing the DNA binding domain. EMSA supershift experiments and GST–AR pull-down experiments followed by Western blotting identified a Fos-related protein(s) of ~40 kDa as part of this complex. Competition experiments with a double-stranded oligonucleotide containing an AP-1 binding site demonstrated that DNA binding was not mediated by AP-1. These results indicate that a Fos-containing protein complex distinct from AP-1 binds upstream of the AREs in the PSA and KLK2 promoters, interacts with the AR and may participate in regulation of these two androgen-responsive genes.

INTRODUCTION

The androgen receptor (AR), which is a member of the superfamily of hormone-dependent nuclear receptors, plays a central role in development of the normal prostate and prostate cancer (1–4). Hormone-dependent nuclear receptors contain three domains, a C-terminal hormone binding domain (HBD), an N-terminal transactivation domain (TAD) and a DNA binding domain (DBD) located between these regions. The AR regulates transcription upon binding to cognate AR-responsive elements (AREs) located in the vicinity of target genes. The activity of the AR is regulated by androgens, primarily dihydrotestosterone, binding to the HBD. Androgen binding disrupts an inhibitory association between the AR and a heat shock protein complex and probably facilitates dimerization and binding of additional proteins (4). It has become clear recently that transcriptional regulation by hormone-dependent nuclear receptors involves protein–protein interactions with a number of additional proteins which may function to either activate (co-activators) (5–7) or repress transcription (8–10). With respect to the AR specifically, associated proteins which have been identified and shown to modulate transcriptional activation are ARA70 (11) and GRIP (12).

Human prostate-specific antigen (PSA) and kallikrein 2 (KLK2) are closely related and linked genes expressed specifically by normal prostate epithelial cells and most prostate cancers. Expression of these genes is regulated at the transcriptional level by the AR (13–15) and AREs have been identified immediately upstream of the TATA box in both the PSA and KLK2 genes (16,17). Previous reports have shown that in addition to these AREs, DNA sequences located immediately upstream of the AREs in both the PSA and KLK2 promoters are also necessary for AR-mediated transcriptional activation (14,15). These studies suggest that other transcription factors binding upstream of the AREs cooperate directly or indirectly with the AR to regulate expression of these genes.

To identify transcription factors which contribute to regulation of androgen-dependent genes, this study focused on the promoters of the PSA and KLK2 genes. The regions upstream of the AREs in both genes were analyzed for DNA–protein interaction by electrophoresis mobility shift assays (EMSA). A common protein complex which bound to the promoters of both genes was identified in nuclear extracts from prostate- and non-prostate-derived cell lines. A series of glutathione S-transferase (GST)–AR fusion proteins was constructed to determine whether

* To whom correspondence should be addressed at: Division of Hematology–Oncology, HIM 1047, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215, USA. Tel: +1 617 667 0719; Fax: +1 617 667 0610; Email: zsun@bih.harvard.edu

this protein complex could regulate expression of the PSA and KLK2 genes through direct interaction with the AR. The results from absorption experiments demonstrated that the involved protein complex specifically interacted with the DBD of the AR. Competition experiments with an AP-1 binding site oligonucleotide indicated that DNA binding was not AP-1 mediated (18), but c-Fos family proteins of ~40 kDa were identified in this protein complex by gel mobility supershift experiments and Western blotting. Further characterization of this AR-associated DNA binding protein complex is described.

MATERIALS AND METHODS

Cell culture and preparation of nuclear extracts

Two prostate cancer cell lines, LNCaP and PC3, were used and maintained as described previously (19,20). AR expression by LNCaP and the lack of AR expression by PC3 were confirmed by Western blotting (data not shown). In addition, two other cell lines utilized in these studies were the monocytic U937 line and the human cervical carcinoma line HeLa. Nuclear extracts were prepared from LNCaP, PC3, U937 and HeLa cell lines essentially according to the method of Dignam *et al.* (21).

Electrophoresis mobility shift assay (EMSA)

DNA fragments from the PSA and KLK2 promoters were generated by PCR. Prior to PCR amplification for probe generation, one of the primers was ^{32}P-labeled with T4 polynucleotide kinase. The PCR fragments were then purified by gel electrophoresis. The EMSAs were performed essentially as described by Singh *et al.* (22). Each reaction contained 2 µg crude nuclear extract with 0.5 µg poly(dI·dC) (Pharmacia) and 10^4 c.p.m. ^{32}P-labeled DNA fragment to be analyzed in 20 µl buffer containing 20 mM HEPES, pH 7.9, 50 mM KCl, 5 mM EDTA, 1 mM DTT, 3 mM $MgCl_2$ and 5% glycerol. After a 20 min incubation on ice, the binding reaction was analyzed by electrophoresis on a native 4% polyacrylamide gel (19:1 bis acrylamide) in 0.25× TBE buffer at 5 V/cm for 4–6 h at 4°C. In the experiments to detect AP-1 and Oct-1 complexes electrophoresis was performed for 2–3 h. Gels were dried and autoradiographed. Competition experiments to demonstrate binding specificity were performed by preincubating the nuclear extracts and a 50- or 100-fold molar excess of unlabeled DNA fragments at 4°C for 10 min.

A c-Fos family polyclonal rabbit antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA; catalog no. sc-253x) and used in the supershift experiments. This antibody was generated and affinity purified with a common peptide, KVEQLSPEEEEKRRIRRIRNKMAAA, in a highly conserved region of c-Fos family members, including c-Fos, FosB, Fra1 and Fra2. Antibody was incubated with PC3 crude nuclear extracts for 10 min either before or after adding the ^{32}P-labeled DNA fragments. A rabbit polyclonal antibody to human CBP (Santa Cruz Biotechnology; catalog no. sc-369x) served as a control. An AP-1 double-stranded oligonucleotide, 5′-CGCTTGATGACTCAGCCGGAA-3′, was labeled and used as a probe in EMSA (18). Both cold AP-1 and ARE (5′-GTCTGGTACAGGGTGTTCTTTTG-3′) double-stranded oligonucleotides were used as competitors in EMSA (23).

Construction and expression of GST–AR fusion proteins

Portions of the AR gene were amplified by PCR with specific primers to generate the appropriate fragments with in-frame restriction enzyme sites. After cleavage, the fragments containing different portions of the AR were cloned downstream of GST in the pGEX-2TK vector (Pharmacia) (Fig. 4A). All constructs were sequenced from two directions to confirm that there were no mutations introduced by the PCR. The plasmids were transformed into DH5α (Gibco BRL) or BP12 (Novogen). The GST fusion proteins were prepared as described before (24). After final washing, the GST beads were resuspended in 200 µl NETN (0.5% NP-40, 1 mM EDTA, 20 mM Tris–HCl, pH 8.0, 100 mM NaCl) with 10% glycerol and 2–5 µl GST beads were analyzed on 10% SDS–PAGE.

Depletion experiments with GST–AR fusion proteins

To deplete possible AR binding proteins, 50 µl crude nuclear extract from PC3 cells (1 µg/µl) were incubated with equal amounts of GST–AR fusion proteins or GST on agarose beads for 12–14 h at 4°C with gentle shaking. The beads with absorbed PC3 nuclear proteins were removed by centrifugation and the unbound fractions were then carefully transferred to clean tubes for further analysis. In order to detect remaining DNA binding activity, different amounts of the unbound PC3 nuclear extracts were analyzed by EMSA with the labeled KC fragment from the KLK2 promoter as described above.

Western blotting

One hundred microliters of PC3 crude nuclear extracts (1 µg/µl) were incubated with several different GST–AR fusion proteins at 4°C overnight as indicated in the figure legends. The GST beads were washed four times and resuspended in 100 µl protein storage buffer (NETN with 10% glycerol and 5 mM DTT). Samples of 10 µl were analyzed by 10% SDS–PAGE and transferred to a nitrocellulose membrane. A 1:1000 dilution of a polyclonal antibody for c-Fos family proteins (Santa Cruz Biotechnology; catalog no. sc-253x) was used as the first antibody and binding was detected by ECL (Amersham).

RESULTS

Identification of a common nuclear protein(s) which binds upstream of the AREs in the PSA and KLK2 genes

Previous functional studies using CAT reporter gene constructs determined that *cis*-acting elements immediately upstream of the AREs in the PSA and KLK2 genes were required, in conjunction with the AR and an ARE, for androgen-induced transcriptional activation (14,15). By deletion analysis, these elements were mapped to a region between –539 and –320 in the PSA promoter (16) and –411 and –171 in the KLK2 promoter (17) (Fig. 1). To identify *trans*-acting factors binding to these sites, the sequences upstream of the AREs in each gene were analyzed by EMSA. The 223 bp fragment upstream of the ARE in the PSA gene (–539 to –317, PF in Fig. 1) was labeled and used as a probe with nuclear extracts from several cell types. A single major DNA–protein complex was observed using extracts from LNCaP, a prostate cancer cell line which expresses the AR (Fig. 2A). This complex was also observed using extracts from HeLa, U937 (Fig. 2A) and PC3 (Fig. 3A) cells. An additional faster migrating band observed

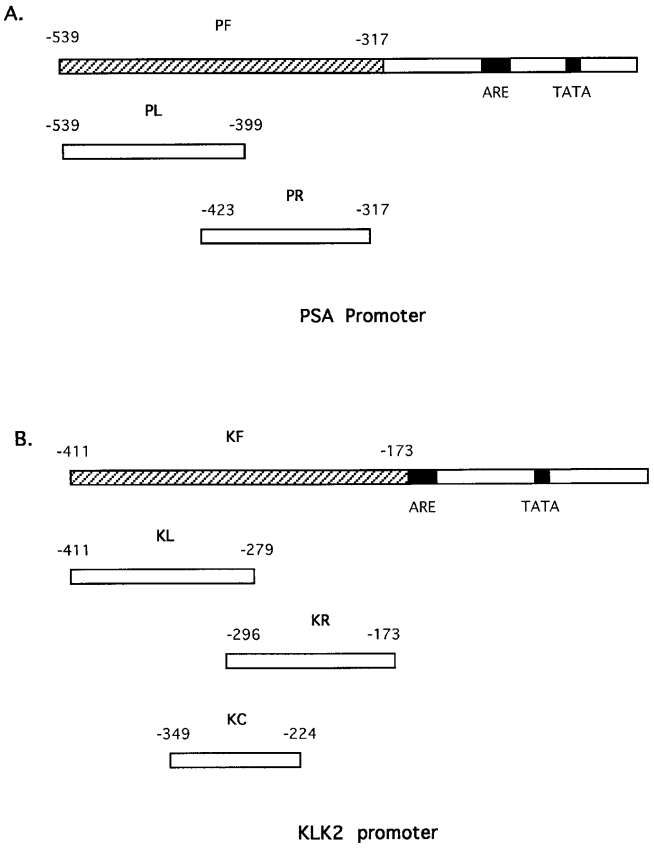

Figure 1. Structures of the PSA (**A**) and KLK2 (**B**) promoters. The AREs and TATA boxes are labeled as the dark boxes in the PSA and KLK2 promoters. The PF (–539 to –399 bp) and KF (–411 to –279 bp) fragments which contain the functional regions required for androgen induction in previous studies (14,15) and are used in the EMSA are shown. Subsequently, smaller PCR fragments from both promoters were generated as indicated in the figure.

in nuclear extracts from U937 cells was further confirmed due to proteolysis. The specificity of these gel shifts was shown by competition with the unlabeled PSA probe (PF). This complex formation could also be inhibited by a 239 bp fragment from upstream of the ARE in the KLK2 promoter (–411 to –173, KF), which contained *cis*-acting elements shown previously to be required for androgen inducibility (14).

Similar results were obtained when the KLK2 fragment KF was used directly as probe. Complexes were formed with nuclear extracts from each of the cell lines and these could be competed by both the unlabeled KLK2 and PSA fragments (KF and PF respectively, Fig. 2B). Although the PSA fragment was less effective, suggestive of a relatively lower affinity, there was no competition with a series of control DNA fragments (Fig. 2C). These binding and cross-competition experiments indicated that a common protein or protein complex expressed in several cell types could bind to sites upstream of the ARE in both the PSA and KLK2 promoters.

Further mapping of the binding sites in the PSA and KLK2 promoters

Additional overlapping fragments from the PSA promoter were generated to map the binding site by EMSA (Fig. 1A). The binding site was mapped to the PL fragment based upon its ability to fully compete, although partial competition was also observed using the PR fragment (Fig. 3A). EMSA using the labeled PL fragment directly also yielded a specific band (not shown). The site in the PL fragment could not be mapped further by EMSA as a series of smaller fragments, such as PL-L and PL-R, could only partially compete for binding to the PF and PL probes (data not shown).

Similar results were obtained upon mapping the binding site in the KF fragment from the KLK2 promoter. The KL and KR fragments, which overlapped by 18 bp, were able to partially compete for binding to the KF probe (not shown). Another smaller fragment from the central portion of the KF probe, KC (Fig. 1B), was able to completely inhibit binding to the KF probe (not shown). The activity of the KC fragment was further assessed by using it directly as a probe with PC3 nuclear extracts. Like the full-length KF fragment, the KC fragment was able to form a DNA–protein complex which could be competed with the unlabeled probe itself and the KF and PL fragments (Fig. 3B). However, smaller fragments generated from the KC probe, including an oligonucleotide corresponding to the overlap between the KL and KR fragments, were not able to inhibit binding to the KC or KF probes (data not shown).

The KC fragment from the KLK2 promoter and the PL fragment from PSA were also able to cross-compete with each other for binding (Fig. 3B and C). These results, in conjunction with the data above, indicate that a common factor was binding to *cis*-acting elements located in the PL and KC fragments of the PSA and KLK2 genes respectively. Moreover, they suggest that the binding region may be comprised of multiple weak binding sites, as it appeared to require a relatively large DNA fragment. Further efforts to map a discrete binding site using the methylation interference and DNase I footprinting methods have similarly failed to identify strong binding sites in the regions which cover the PL and KC fragments (data not shown).

Interaction between the protein complex identified by DNA binding and the AR

One mechanism through which the protein or protein complex which binds upstream of the AREs in the PSA and KLK2 promoters could contribute to androgen inducibility would be by direct interaction with the AR. Therefore, a series of GST–AR fusion proteins were generated to assess possible interactions with the AR (Fig. 4A). The GST–AR fusion proteins were isolated and initially analyzed by SDS–PAGE. EMSA experiments using an ARE probe were then carried out and showed that the DBD of these bacterially synthesized proteins was functional (data not shown).

To detect potentially weak interactions with the AR, a sensitive depletion assay was developed. PC3 nuclear extracts were incubated with a large excess of GST–AR fusion protein bound to glutathione–agarose beads overnight at 4°C. Proteins which bound to the GST–AR fusion protein were then removed by centrifugation and activity which remained in the supernatant was determined by EMSA using a KC probe. To quantitate binding activity, serial dilutions of the supernatants were examined.

The factor(s) which binds to the KC probe was not diminished by incubation with beads containing GST alone (GST), an irrelevant GST fusion protein (FAS or CD95) or fusion proteins derived from the HBD of the AR (AR/676–844, AR/676–919 and

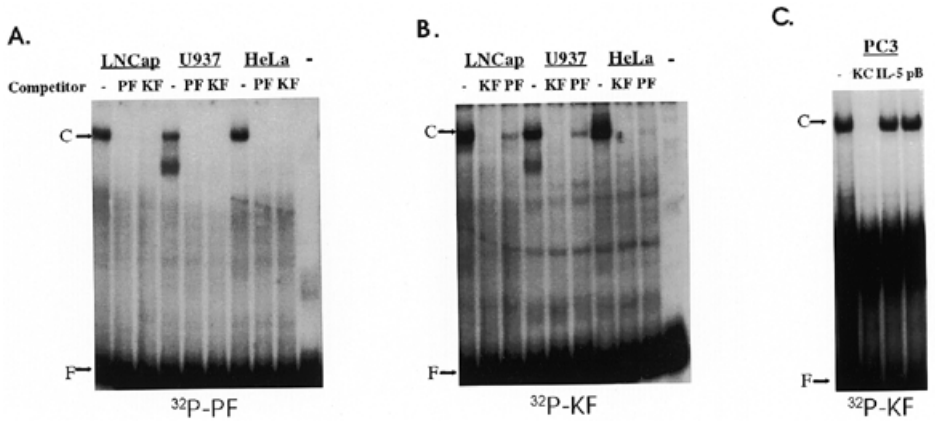

Figure 2. Gel mobility shift analysis of the PSA (**A**) and KLK2 (**B**) promoters. (A) A 224 bp PCR fragment from the PSA promoter indicated as PF (see Fig. 1) was labeled with T4 polynucleotide kinase. Two micrograms of crude nuclear extracts from human prostate cancer cells (LNCaP), monocytic cells (U937) and human cervical carcinoma cells (HeLa) were mixed with 0.5 μg poly(dI·dC) and 1×10^4 c.p.m. labeled DNA fragments and incubated for 20 min on ice. They were then analyzed by electrophoresis on native 4% polyacrylamide gels at 5 V/cm for 4–6 h at 4°C. A DNA–protein complex is indicated as C and free DNA probe is indicated as F. A 50-fold molar excess of unlabeled PCR fragments was used for competition. The last lane contains probe only with no nuclear extract. (B) A 239 bp fragment of the KLK2 promoter was analyzed as described in (A). (**C**) In this control experiment, PC3 nuclear extracts were analyzed with a labeled KF fragment. A 50-fold molar excess of cold DNA fragments was used as competitor. They included a KC fragment, a 184 bp fragment (between bp –561 and –377) from the human IL-5α receptor promoter, labeled IL-5 (42), and a 107 bp fragment from the pBluescript vector, labeled pB (Stratagene).

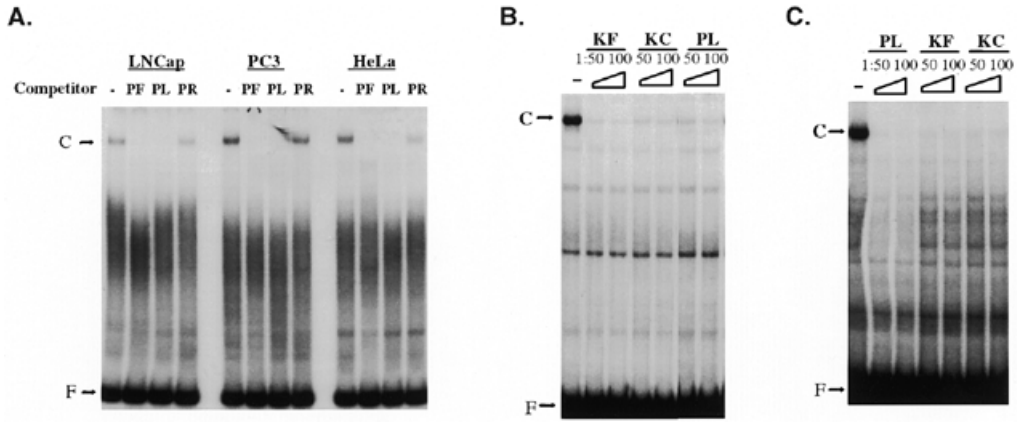

Figure 3. Further analysis of the PSA and KLK2 promoters using EMSA. (**A**) A 224 bp PF fragment from the PSA promoter was labeled and used as a probe in EMSA. The specific competition experiments were performed using a 50-fold molar excess of unlabeled PF, PL and PR fragments (outlined in Fig. 1). (**B**) A 126 bp fragment from the KLK2 promoter marked KC was labeled with T4 polynucleotide kinase and used as a probe with PC3 crude nuclear extracts. Several unlabeled DNA fragments were used as competitors in 50- and 100-fold molar concentrations. (**C**) A ^{32}P-labeled PL fragment was used as probe in the EMSA and the indicated PCR fragments from the PSA and KLK2 promoters were added as specific competitors.

AR/875–919). However, this factor(s) was depleted by incubation with GST–AR/505–919, a GST–AR fusion protein incorporating both the DBD and HBD (Fig. 4B). Depletion was also observed using the AR/505–676 construct, indicating that the factor was binding to a region of the AR in or near the DBD (Fig. 4C).

Additional GST–AR fusion proteins were generated to determine whether the binding site was in the DBD or in the hinge regions flanking the DBD. Fusion proteins incorporating the N- or C-terminal hinge regions (GST–AR/505–559 and GST–AR/624–676) respectively did not deplete the KC binding factor (Fig. 4D). In contrast, the construct containing precisely the DBD (GST–AR/559–624) fully depleted the KC binding factor(s). These results indicate that the factor(s) detected by EMSA using the KC fragment binds to a site in the DBD of the AR.

In further control experiments the depleted nuclear extracts were analyzed in EMSA with a labeled double-stranded oligonucleotide containing an Oct binding site (ATGCAAAT) (22). An Oct-1 DNA–protein complex was detected and its identity was further confirmed by competition experiments using excess cold Oct oligonucleotide and by supershifting with a specific anti-Oct-1 antibody (Fig. 5A). Incubation with the GST–AR fusion proteins had no effect on Oct-1 binding (Fig. 5A). Specific binding to a Sp1 oligonucleotide was also unaffected by preincubation with the GST–AR DBD fusion proteins (data not shown). In contrast, Figure 5B again shows specific depletion of the KC binding factor by the GST–AR DBD fusion protein. These results indicate that depletion by the AR–DBD fusion protein was specific to the protein(s) binding to the KC fragment.

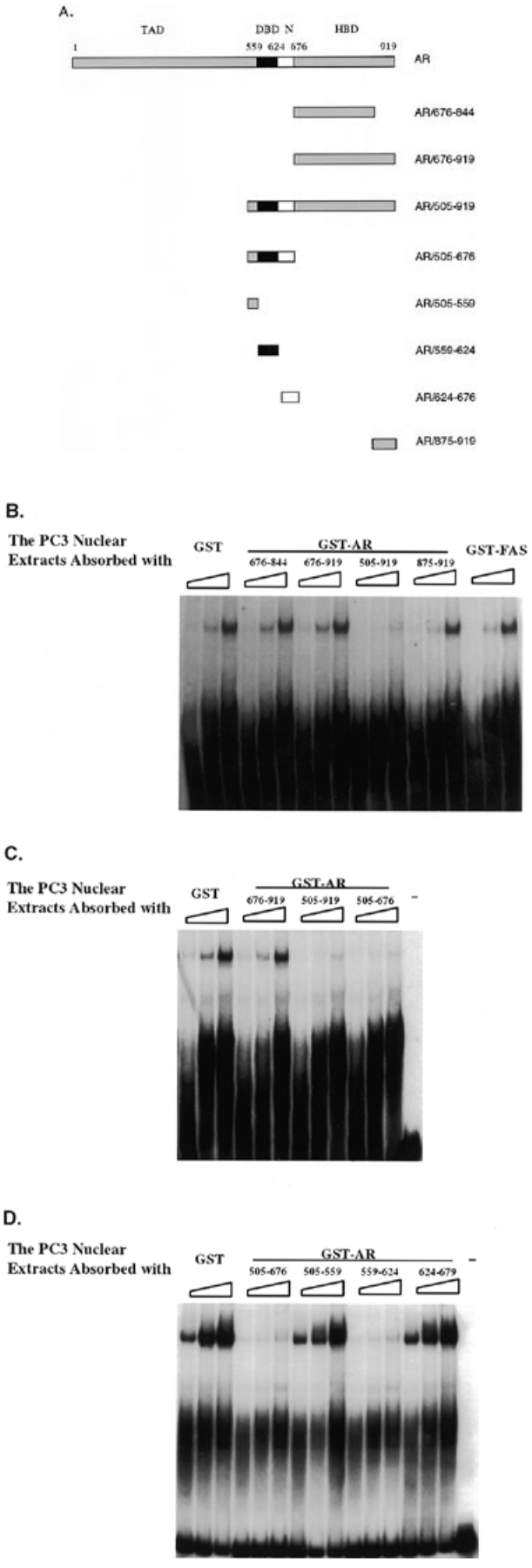

Figure 4. Absorption of PC3 nuclear proteins by GST–AR fusion proteins. (**A**) Different portions of the human AR are shown. They are the transactivation domain (TAD), DNA binding domain (DBD), nuclear localization signal region (N) and hormone binding domain (HBD) respectively. The corresponding amino acids are labeled above. The AR fragments were cloned downstream of the GST gene in the pGEX-2TK vector (Pharmacia). (**B**)–(**D**) Fifty microliters of PC3 crude nuclear extract (1 µg/µl) were incubated with equal amounts of GST–AR fusion proteins or GST on agarose beads at 4°C for 12–14 h. Then 1, 2 and 4 µl depleted PC3 nuclear extract were used in EMSA experiments to detect the remaining binding activity with the labeled KC fragment. The constructs which contained the DBD were able to specifically deplete the DNA binding activities in PC3 nuclear extracts. These were AR/505–919 (B), AR/505–919 and AR/505–676 (C) and AR/505–676 and AR/559–624 (D).

c-Fos family proteins are components of the AR-associated DNA binding protein complex

Regulatory interactions between Fos/Jun (AP-1) and nuclear receptors have been described previously (25–27) and these interactions may involve c-Fos protein and the DBD of the nuclear receptor (28). To determine whether a c-Fos protein is a component of this AR-associated complex, a polyclonal antibody recognizing c-Fos family proteins, including c-Fos, FosB, Fra1 and Fra2, was used (29–32). In initial control experiments an AP-1 protein complex was detected in PC3 nuclear extracts with a ^{32}P-labeled double-stranded AP-1 site oligonucleotide (upper band labeled C in gel in Fig. 6A). This DNA–protein complex was specifically blocked by excess cold AP-1 oligonucleotide, but not by an ARE oligonucleotide (Fig. 6A). The specificity of the c-Fos polyclonal antibody was confirmed by supershifting this AP-1 complex. A second lower band was also observed which was competed by both AP-1 and ARE oligonucleotides and was not supershifted by the c-Fos antibody, indicating that it was non-specific.

The possible involvement of c-Fos protein in the AR-associated protein complex was then assessed with this antibody. The c-Fos family antibody generated a supershifted band in EMSA with PC3 nuclear extracts and a labeled KC probe (Fig. 6B, lanes 4 and 5). Preincubation of the PC3 nuclear extracts with the c-Fos antiserum inhibited formation of the DNA–protein complex (Fig. 6B, lanes 2 and 3). These results indicate that a c-Fos protein was in the complex and was also required for complex formation. Control rabbit anti-CBP antibody (lanes 6 and 7) and non-immune rabbit antiserum (data not shown) had no effect.

GST–AR absorption experiments and immunobloting were used to further assess whether a Fos protein(s) was part of the protein complex which associated with the AR DBD. PC3 nuclear extracts were absorbed with a series of GST–AR fusion proteins and the absorbed proteins were analyzed by immunoblotting with the c-Fos antiserum. Figure 6C shows that the AR DBD fusion protein (GST–AR/559–624) specifically precipitated a series of immunoreactive proteins with molecular weights of ~40 kDa, consistent with FosB (29), Fra1 (33) and/or Fra2 (31). c-Fos (62 kDa) was detected by the antibody in PC3 whole nuclear extracts, but not in the AR-precipitated material (Fig. 6C).

Although sequence analysis of the PL and KC fragments from the PSA and KLK2 promoters did not reveal any definite AP-1 binding sites, detection of Fos in the protein–DNA complex still suggested that DNA binding could be AP-1 mediated. To address this, the AP-1 binding site oligonucleotide was used in EMSA competition experiments. Figure 6D shows that this oligonucleotide

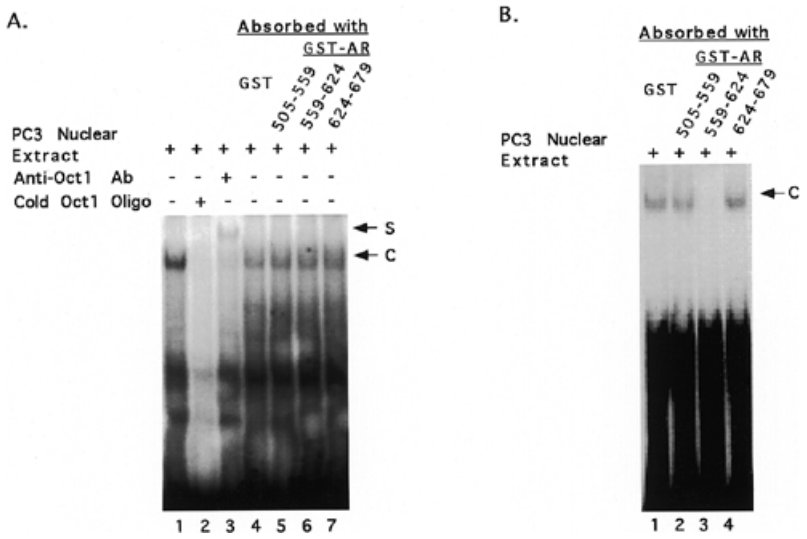

Figure 5. Specificity of depletion by the DNA binding domain of the AR. Fifty microliters of PC3 nuclear extracts (1 μg/μl) were absorbed with equal amounts of GST and GST–AR fusion proteins on agarose beads exactly as described in Figure 4. (**A**) One microliter of undepleted PC3 nuclear extract (lanes 1–3) and depleted extract as labeled above (lanes 4–7) were incubated with 1 μg poly(dI·dC) and a labeled Oct double-stranded oligonucleotide (tgtcgaATGCAAATcactagaa). A specific Oct-1 DNA–protein complex was detected, labeled C. It was competed by excess cold Oct oligonucleotide (lane 2) and supershifted with 0.5 μl anti-Oct-1 antibody (catalog no. sc-232X; Santa Cruz) (lane 3). There was no decrease in complex formation with the nuclear extracts depleted by GST or by three GST–AR fusion proteins. (**B**) Equal amounts of the depleted PC3 nuclear extracts used in (A) were analyzed in EMSA with a labeled KC fragment from the human KLK2 promoter. The specific DNA binding activity to KC fragments was depleted by incubation with GST–AR DBD fusion protein (559–624) in lane 3.

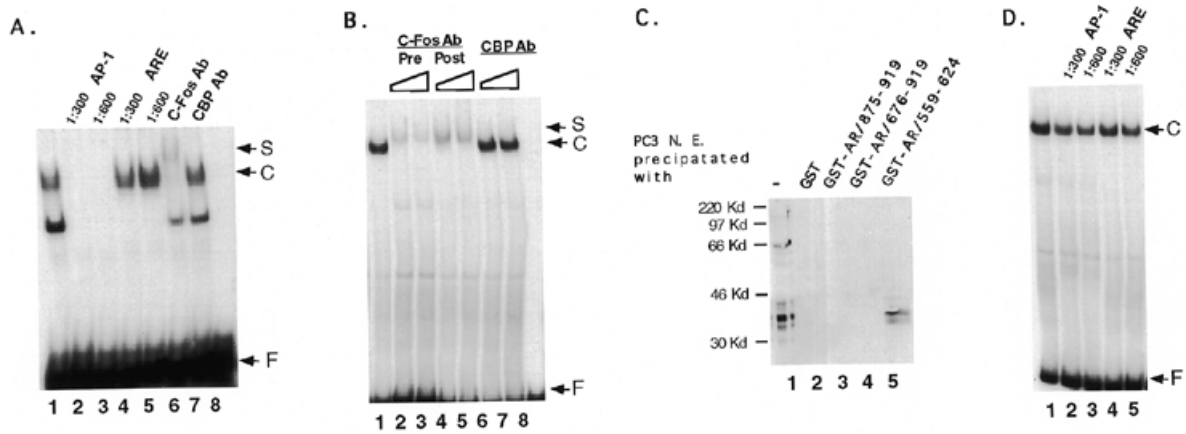

Figure 6. Involvement of Fos in the protein complex which interacts with the DBD of the AR and binds upstream of the ARE in the KLK2 promoter. (**A**) The crude PC3 nuclear extracts were incubated with ^{32}P-labeled AP-1 oligonucleotide (GGCTTGATGACTCAGCCGGA) and 1 μg poly(dI·dC) (lane 1). The specific DNA–protein interaction was competed by adding excess cold AP-1 (lanes 2 and 3) but not ARE (lanes 4 and 5) oligonucleotide. A polyclonal antibody for c-Fos family members (lane 6) supershifted the DNA–protein complex, while a negative control CBP antibody had no effect (lane 7). (**B**) The EMSA was performed with PC3 nuclear extracts which were incubated with 0.5 or 1 μl c-Fos family antibody before adding the ^{32}P-labeled KC fragment probe (lanes 2 and 3) or after adding the KC probe (lanes 4 and 5). CBP antiserum was used as a control (lanes 5 and 6). (**C**) The nuclear extracts of PC3 were absorbed with several distinct GST–AR fusion protein beads as described previously. After washing, 2× SDS loading buffer was added and 10 μl each sample were analyzed by 10% SDS–PAGE. The same c-Fos family antibody described above was then used for Western blotting. A series of ~40 kDa protein bands were detected in lane 5, which was incubated with the AR–DBD (559–624) construct (see Materials and Methods). In lane 1, 10 μg unabsorbed PC3 nuclear extract were used as a control. (**D**) The ^{32}P-labeled KC fragment was used in EMSA with PC3 crude nuclear extracts. Excess cold AP-1 (lanes 2 and 3) and ARE (lanes 4 and 5) oligonucleotides were used as competitors. Neither oligonucleotide competed with the KC fragment for DNA–protein complex formation.

does not compete with the KC probe for binding, even at a 600-fold excess (lanes 2 and 3), demonstrating that AP-1 does not mediate DNA binding by this complex. An ARE oligonucleotide similarly had no effect (lanes 4 and 5), indicating that complex binding to the KC fragment is not mediated by the AR or the glucocorticoid receptor (GR).

DISCUSSION

Understanding in detail how the PSA gene is regulated by androgen is of particular interest as activity of the PSA promoter parallels activity of prostate cancer cells during the androgen-dependent and androgen-independent phases of prostate cancer in

the vast majority of patients. Previous functional studies showed that sequences located between –539 and –320 in the PSA promoter were required for full induction by the AR in COS cells (15). Similar studies of the KLK2 promoter identified the region between –411 and –171 as necessary for induction by the AR in PC3 cells (14). The objective of the current study was to identify and characterize factors which bind to these functionally identified regions of the PSA and KLK2 genes. Using EMSA, a single protein or protein complex binding specifically to the PF fragment (–539 to –317) from the PSA promoter was identified. Under the same conditions a protein or protein complex of the same apparent size was also identified binding to the KF fragment (–411 to –173) from the KLK2 promoter. Importantly, cross-competition experiments demonstrated that the same factor(s) was binding to both the PF and KF fragments. This suggested that a common regulatory protein(s) binding upstream of the AREs in the PSA and KLK2 promoters may interact directly or indirectly with the AR to regulate expression of these genes.

The sequences for the common *trans*-acting factor were further mapped by EMSA to a 140 bp PSA fragment and a 126 bp KLK2 fragment. However, discrete binding sites could not be further mapped using several different strategies, including EMSA, with smaller PCR fragments and double-stranded oligonucleotides, methylation interference and DNase I footprinting. This suggests that the specific DNA–protein interactions which were observed in the PSA and KLK2 promoters may be mediated by multiple weak binding sites. Similar observations have been made with the SWI/SNF complex, which is required for enhancement of transcription by many transcriptional activators in yeast, including the GR (34–36). The SWI/SNF binds DNA in a sequence-specific manner, but requires a relatively large DNA fragment and no consensus sequences have yet been identified (37). Using an antibody against BRG-1 protein, the human homolog of SWI2 (38), we have not been able to show that BRG-1 is a component of the common *trans*-factor complex which binds to the PSA and KLK2 promoters.

The detection of one specific protein or protein complex binding to the PF and KF fragments suggested that this complex may interact with the AR. A series of GST–AR fusion proteins in conjunction with a sensitive absorption assay indicated that there was a specific binding interaction between this protein complex and the DBD of the AR. It should be emphasized that the absorption assay used a large excess of GST–AR fusion protein to facilitate the detection of what one expects to be a weak binding interaction. Consistent with this expectation, we have not been able to detect binding of GST–AR fusion proteins to this complex directly in EMSA supershift experiments (data not shown). Nonetheless, it is reasonable to suggest that such a weak interaction may be significant *in vivo*, in which case DNA binding would tether both of these factors in close proximity. Finally, it should be noted that an intermediary protein mediating the AR association with this factor has not been ruled out.

Using an antibody against c-Fos family members, it was demonstrated that a Fos protein is one component of the AR-associated DNA binding complex by both EMSA and immunoblotting experiments. The Fos protein(s) that was identified in this study does not appear to be c-Fos (62 kDa) (18,29), but is consistent with one or more of the other known Fos proteins based upon their molecular weights (29–32). Importantly, there were no consensus AP-1 sites present in the PL or KC fragments of the PSA or KLK2 promoters. Moreover, competition experiments with an AP-1 binding site oligonucleotide demonstrated that DNA binding by the protein complex was not mediated by AP-1. These data indicate that a Fos-containing protein complex distinct from, or possibly in addition to, AP-1 may modulate AR function on the PSA and KLK2 genes.

Previous studies have shown mutual inhibitory interactions between AP-1 and nuclear hormone receptors. In the case of the glucocorticoid receptor, this interaction has been mapped to the DBD (28). It is not yet clear whether this interaction is indirect, possibly through CBP, or reflects a direct association with Fos or Jun (26,39,40). The protein complex described here was detected in nuclear extracts from AR^+ and AR^- prostate epithelial cell lines (LNCaP and PC3 respectively) and U937 and HeLa cells, indicating that it is not prostate specific and may be ubiquitous. Therefore, similarly to AP-1, it is likely that this complex interacts with additional factors, perhaps other nuclear hormone receptors, and possible that Fos mediates these interactions. Studies are underway to determine whether the DBD of other nuclear hormone receptors also interact with this complex.

A recent report indicates that the PSA promoter contains a second weak ARE located at –393 to –378, just 3′ of the PL fragment (41). A reporter gene regulated by this ARE in conjunction with the initially described downstream ARE in the PSA promoter was strongly androgen inducible in LNCaP cells, but only weakly induced in a series of other AR-transfected cells (41). In preliminary functional studies in PC3 cells we have not been able to reproduce the activity of this putative second ARE, but have observed a 3-fold enhancement of androgen inducibility by the PL fragment (data not shown). Further functional studies in a variety of cell types and under a variety of conditions will be necessary to determine the functional properties of these *cis*-acting elements and the corresponding *trans*-acting factors *in vivo*. However, the data presented here provides a potential link between the AR and other transcription factors in the regulation of androgen-responsive genes.

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