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c-Fos Dimerization with c-Jun Represses c-Jun Enhancement of Androgen Receptor Transactivation

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The transcriptional activity of the human androgen receptor (hAR), like other nuclear receptors, is dependent on accessory factors. One such factor is c-Jun, which has been shown to have a selective function of mediating androgen receptor-dependent transactivation. This c-Jun activity is inhibited by c-Fos, another protooncoprotein that can dimerize with c-Jun to form the transcription factor AP-1. Here we show that c-Jun mediates hAR-induced transactivation from the promoter of the androgen-regulated gene, *human kallikrein-2* (*hKLK2*), and c-Fos blocks this activity. Using c-Fos truncation mutants and measuring *hKLK2*-dependent transcription, we have determined that the bZIP region of c-Fos is required and sufficient for inhibiting c-Jun enhancement of hAR transactivation. Further truncation analysis of the bZIP shows that the c-Fos dimerization function, mediated through the leucine zipper, is essential for the negative activity, whereas DNA binding, mediated through the basic region, is dispensable. These results suggest that heterodimerization by c-Fos with c-Jun blocks c-Jun's ability to enhance hAR-induced transactivation.

Key Words: c-Fos; c-Jun; androgen receptor; transactivation.

Introduction

Nuclear receptors comprise a large family of transcription factors that modulate transcription in a ligand-dependent manner (reviewed in refs. *1–5*). These receptors mediate the activities of steroid and thyroid hormones, retinoids, vitamin D_3, and many other unidentified ligands by binding these ligands and stimulating (or sometimes repressing) the rate of transcription of their target genes (reviewed in ref. *1*). This alteration in gene expression leads to modulation of cellular differentiation (reviewed in refs. *6* and *7*).

The androgen receptor (AR) *(8)*, like other nuclear receptors, is believed to regulate transcription via interactions with accessory factors collectively called coactivators (reviewed in ref. *9*). Coactivators have been proposed to sit on a ligand-responsive promoters by virtue of their associations with both the nuclear receptor and the RNA polymerase II transcription machinery (reviewed in ref. *9*). Some of these coactivators, including TIF-2 *(10)*, SRC-1 *(11)*, and CBP/P300 *(12,13)*, act on several different nuclear receptors, including the receptors for progesterone (PR), glucocorticoids (GR), estrogens (ER), retinoids (RARs and RXRs), and thyroid hormones (TR), whereas others, such as ARA-70 *(14)*, appear to be AR-specific.

The AR is unique in the kind of interaction it has not only with ARA-70, but also c-Jun, a bZIP-containing protooncoprotein that can heterodimerize with another bZIP protein, c-Fos, to form AP-1 (reviewed in ref. *15*). Although c-Jun almost exclusively represses the transcriptional activity of the AR-related GR *(16–19)*, it strongly potentiates AR-mediated transcription *(20,21)*. Our results indicate that the c-Jun positive effect on AR is general, occurring without any apparent dependency on cell-specific or promoter-specific factors (*20*; Oberfield and Shemshedini, unpublished results). This is in contrast to the positive interaction between c-Jun and GR, which has been observed only in T-cell lines *(22)*. Furthermore, we *(21)* have recently demonstrated that AR is stimulated by both exogenous and endogenous c-Jun, that c-Jun can relieve AR self-squelching, and that the c-Jun effect is primary, all of which are consistent with c-Jun serving a coactivator role in AR-mediated transactivation.

The transcriptional activity of AP-1 on AP-1-responsive promoters is highly regulated. Numerous external stimuli, such as cytokines and growth factors, can activate many MAP kinase cascades and thus ultimately lead to the upregulation of transcriptional activities on the c-Fos promoter, increasing c-Fos expression and AP-1 activity in the cell (reviewed in ref. *23*). Additionally, these growth factors and cytokines can activate the MAP kinase Jun N-terminal

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kinase (JNK), resulting in the phosphorylation of the N-terminal activation domains of c-Jun and, thereby, activation of c-Jun as a sequence-specific DNA binding-dependent transcription factor *(24,25)*. Furthermore, c-Fos is phosphorylated on its C-terminal activation domain by another MAP kinase known as Fos regulatory kinase (FRK), resulting in the activation of c-Fos as a transcriptional activator when dimerized with activated c-Jun *(26)*. Interestingly, both c-Fos and c-Jun must be phosphorylated in order to activate transcription from a TPA-responsive element (TRE), but phosphorylation of c-Jun is not required for its modulation of AR activity *(27)*. In addition, a functional analysis of c-Jun has revealed that multiple regions of this protooncoprotein, including the bZIP region and an amino-terminal domain not required on AP-1-responsive promoters, are involved in mediating AR activity *(27)*.

In contrast to its cooperative role on c-Jun's activity on AP-1-responsive promoters (reviewed in ref. *15*), c-Fos negatively regulates AR-mediated transcription in both the presence and absence of transfected c-Jun *(21)*. Like the c-Jun positive effect, the c-Fos negative effect occurs independent of cell type (Tillman and Shemshedini, unpublished results). We have recently begun to analyze this negative effect of c-Fos. Here it is demonstrated that c-Jun can stimulate AR transactivation of the promoter of *human kallikrein-2* (*hKLK2*), a gene expressing a serine protease that is androgen-inducible *(28)*, and that c-Fos can inhibit this stimulatory effect. Furthermore, we show that c-Fos dimerization with c-Jun, mediated by an intact c-Fos bZIP, is necessary for the inhibitory effect, and that the basic region of c-Fos may be playing a critical role.

Results

Experimental Design

To study the role of c-Fos in c-Jun-activated hAR, we tested a series of c-Fos truncation and point mutants (Fig. 1A). These proteins were expressed in Cos cells from the expression plasmid pSG5 *(29)*. Note that all c-Fos mutants, except for Δ1-159, that could be detected by Western blot analysis are expressed to a significantly higher level than wild-type c-Fos (Fig. 1B, compare lanes 2, 3, 6, and 7 with lane 1). Both wild-type c-Fos (lane 1) and Δ1-159 (lane 8) are very weakly expressed based on this Western analysis. These Fos proteins were analyzed for effects on hAR-induced transactivation of a reporter construct containing the CAT gene under the control of the *hKLK2* promoter *(28)* in the presence of transfected c-Jun. They were also tested for the ability to dimerize with c-Jun, using a mammalian two-hybrid assay *(30,31)*. In this assay, two fusion proteins are expressed, one with the GAL4 DBD fused to the bZIP region of c-Fos (GAL-c-Fos[bz]) and the other with the VP16 activation domain fused to the bZIP of c-Jun (VP16-c-Jun[bz]). Neither fusion protein alone had significant activity, but the two proteins together greatly stimulated the expression of the GAL4-inducible reporter plasmid 17M-tk-CAT (Fig. 1C, compare lane 4 with 2 and 3), indicative of strong in vivo dimerization between the bZIP regions of c-Fos and c-Jun. When the GAL4(DBD) and VP16, without the bZIP regions of c-Jun and c-Fos, were coexpressed, no significant CAT activity was detected (Tillman and Shemshedini, unpublished results), excluding a possible interaction between the GAL4(DBD) and VP16. As expected, transfected c-Fos inhibited the activity of the two fusion proteins (Fig. 1C, compare lane 4 with 5–7), strongly suggesting that c-Fos is competing with GAL-c-Fos(bz) for interacting with VP16-c-Jun(bz). Thus, any c-Fos mutant able to dimerize with c-Jun should be able to disrupt the interaction between GAL-c-Fos(bz) and VP16-c-Jun(bz) measured using this mammalian two-hybrid assay. Note that all c-Fos mutants, both those that were active in inhibiting hAR-mediated transcription and those that were not, were significantly more highly expressed than wild-type c-Fos in transient transfections of Cos cells (*see* Fig. 1B).

hAR-Induced Transactivation of hKLK2 Is Modulated by c-Jun and c-Fos

We have previously demonstrated that c-Jun can modulate hAR transactivation of the MMTV promoter irrespective of cell type *(20)* and that c-Fos could inhibit this stimulatory effect *(21)*. Additionally, our data have indicated that coexpression of c-Jun does not affect expression of hAR *(21)*. To look for a possible promoter-specific nature of these effects, we tested the promoter for the naturally occurring androgen-responsive gene, *hKLK2 (28)*. As shown before on MMTV-CAT *(21)*, transfected hAR has a weak dihydroxytestosterone- (DHT) dependent activity on hKLK2-CAT (Fig. 2A, compare lanes 1 and 2), and this is greatly stimulated by coexpression of c-Jun (compare lanes 2 and 4). Also, as with MMTV, coexpression of increasing amounts of c-Fos inhibits c-Jun-stimulated hAR activity on hKLK2-CAT (Fig. 2B, compare lane 3 with 4–6). Similar results have been obtained with another androgen-regulated promoter, that of prostate-specific antigen (PSA) (Oberfield and Shemshedini, unpublished results). The positive effect of c-Jun and the negative effect of c-Fos have been observed on these androgen-responsive promoters in various cell lines, including Cos, HeLa, CV-1, and the prostate cell line PC-3 (Tillman et al., unpublished results). Therefore, the modulatory effects of c-Jun and c-Fos on hAR-induced transactivation appear to be general, without any apparent cell or promoter specificity.

The c-Fos bZIP Region Is Required for Inhibition of c-Jun-Enhanced hAR Transactivation of hKLK2

To determine which region of c-Fos is required for inhibition of c-Jun-enhanced hAR transactivation, a series of c-Fos truncation mutants (described in Fig. 1A) were tested in transient transfection. As shown previously (*see*

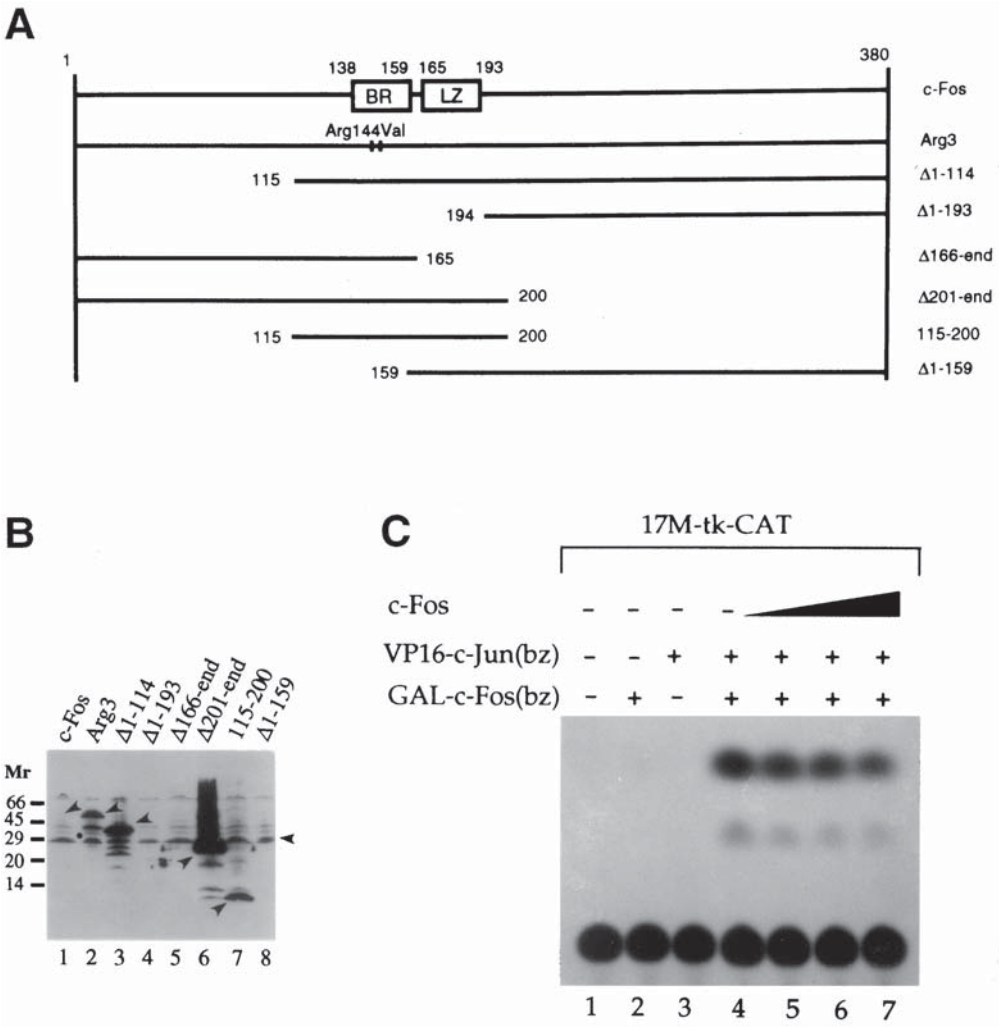

Fig. 1. Experimental design. **(A)** Schematic structure of c-Fos mutants. The 380 amino acids of c-Fos are represented by a black bar. The basic region (BR, amino acids 138–159) and the leucine zipper (LZ, amino acids 165–193) are shown in open boxes. The point mutant, c-Fos(Arg3), has a one amino acid substitution of valine for arginine at residue 144. The deletion mutants are shown with their N- and/or C-terminal amino acids numbered (when different than 1 or 380). **(B)** Expression of c-Fos mutants in transfected cells. Cos cells were transfected with 5 μg each of c-Fos plasmids and then subjected to Western blot analysis. Note that Fos mutants Δ1-193 and Δ166-end are not recognized by the antibody used and Δ1–159 comigrates with the nonspecific bands (indicated by an asterisk) detected by the antibody. The positions of the various Fos proteins are indicated by arrowheads. Molecular-weight markers are given in kilodaltons. **(C)** In vivo dimerization between c-Fos and c-Jun can be measured using a mammalian two-hybrid assay. Cos cells were transfected with 2 μg of the 17M-tk-CAT reporter plasmid, 1 μg of GAL-c-Fos(bz), 1 μg of VP16-c-Jun(bz), and increasing amounts (0.5, 1, or 3 μg) of c-Fos (lanes 5–7). The following relative activities were measured: lane 1, 1.0; lane 2, 1.7; lane 3, 0.7; lane 4, 128.3; lane 5, 92.6; lane 6, 81.5; lane 7, 61.4.

Fig. 2B), full-length c-Fos is capable of repressing activation of hKLK2-CAT induced by hAR and c-Jun (Fig. 3A, compare lane 3 with 4–6). Deletion of either the N-terminus (Δ1-114) or C-terminus (Δ201-end) from c-Fos resulted in mutants that were actually stronger than full-length c-Fos in their negative activities (compare lanes 4–6 with 7–9 or 16–18). The higher activity of the mutants is likely owing to their higher expression levels when compared to full-length c-Fos (*see* Fig. 1B). Larger truncations of c-Fos, which removed either the entire bZIP region (Δ1-193) or only the leucine zipper (Δ166-end), almost completely abolished the negative activity (compare lanes 4–6 with 10–12 or 13–15). These results strongly suggest an essential role of the bZIP region of c-Fos for inhibiting c-Jun-stimulated hAR.

The involvement of the c-Fos bZIP region suggests that dimerization between c-Fos and c-Jun is required to block hAR activity. To evaluate this, the mammalian two-hybrid system was used to measure the abilities of c-Fos mutants to dimerize with c-Jun (*see* Fig. 1C). The c-Fos truncation mutants with intact bZIP regions (Δ1-114 and Δ201-end) were able to disrupt the dimerization between GAL-c-Fos(bz) and VP16-c-Jun(bz) at least as well as can full-length c-Fos (Fig. 3B, compare lanes 2–4 with 5–7 and

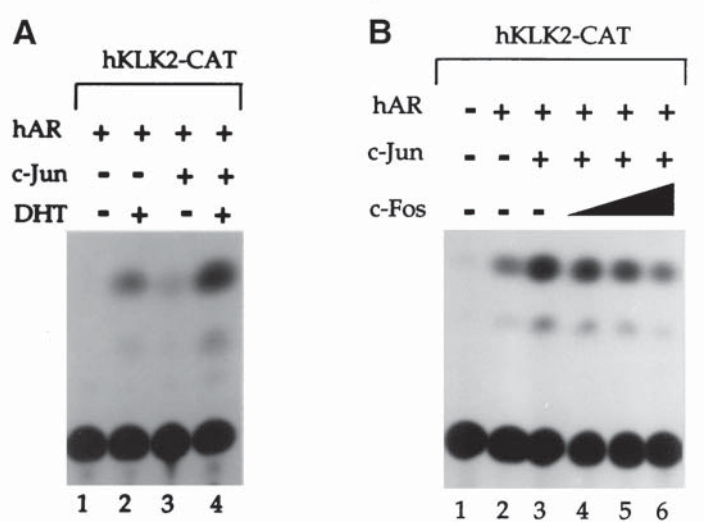

Fig. 2. c-Jun and c-Fos modulate hAR-induced transactivation of hKLK2-CAT. Cos cells were transfected with 2 μg of the hKLK2-CAT reporter plasmid, 1 μg of hAR, and 1 μg of c-Jun; 100 n*M* DHT were used in all lanes. **(A)** Cells were transfected with or without hAR in the presence or absence of c-Jun. **(B)** Cells were transfected with 1 μg of c-Jun (lanes 3–6) and increasing amounts (0.5, 1, or 3 μg) of c-Fos (lanes 4–6). The following relative activities were measured: A lane 1, 1.0; lane 2, 10.0; lane 3, 36.2; B lane 1, 1.0; lane 2, 13.1; lane 3, 37.3; lane 4, 25.4; lane 5, 19.3; lane 6, 11.3.

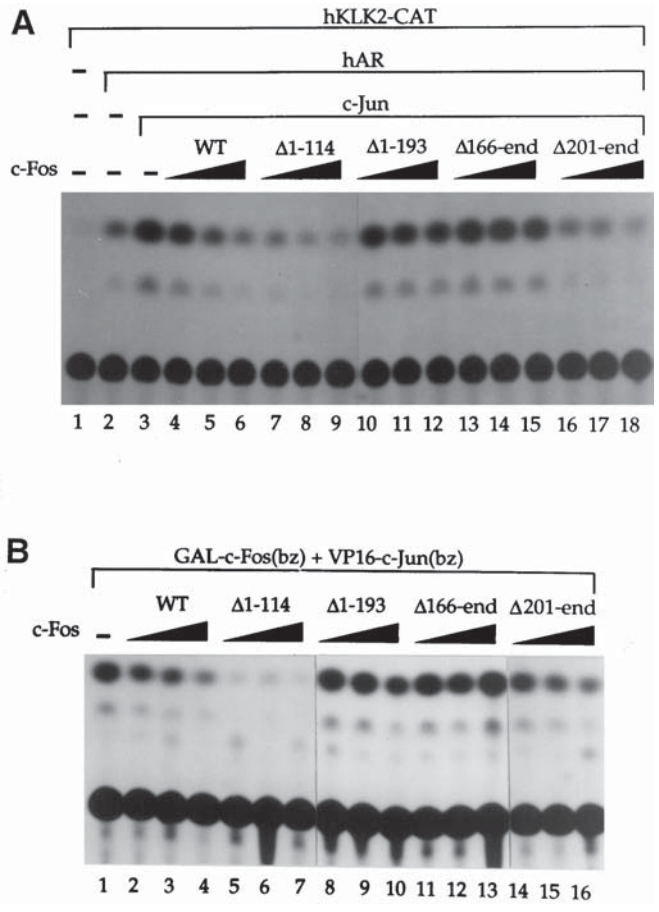

Fig. 3. The bZIP region of c-Fos is necessary for inhibition of c-Jun- enhanced hAR transactivation. **(A)** Cos cells were transfected with 2 μg of the hKLK2-CAT reporter plasmid, 1 μg of hAR in lanes 2–18, 1 μg of c-Jun in lanes 3–18, and increasing amounts (0.5, 1, or 3 μg) of wild-type (WT) c-Fos (lanes 4–6), c-Fos(Δ1–114) (lanes 7–9), c-Fos(Δ1–193) (lanes 10–12), c-Fos(Δ166-end) (lanes 13–15), or c-Fos(Δ201-end) (lanes 16–18); 100 n*M* DHT were used in all lanes. **(B)** Cos cells were transfected with 2 μg of the 17M-tk-CAT reporter plasmid, 1 μg of GAL-c-Fos(bz), 1 μg of VP16-c-Jun(bz), and increasing amounts (0.5, 1, or 3 μg) of WT c-Fos (lanes 2–4), c-Fos(Δ1-114) (lanes 5–7), c-Fos(Δ1–193) (lanes 8–10), c-Fos(Δ166-end) (lanes 11–13), or c-Fos(Δ201-end) (lanes 14–16). The following relative activities were measured: (A) lane 1, 1.0; lane 2, 8.8; lane 3, 24.5; lane 4, 17.7; ; lane 5, 11.5; lane 6, 8.6; lane 7, 7.8; lane 8, 5.9; lane 9, 4.2; lane 10, 22.5; lane 11, 21.5; lane 12, 21.9; lane 13, 23.6; lane 14, 21.1; lane 15, 21.1; lane 16, 11.7; lane 17, 10.0; lane 18, 4.9; (B) lane 1, 1.0; lane 2, 0.6; lane 3, 0.5; lane 4, 0.3; lane 5, 0.04; lane 6, 0.06; lane 7, 0.03; lane 8, 0.9; lane 9, 0.8; lane 10, 0.6; lane 11, 0.9; lane 12, 0.9; lane 13, 1.0; lane 14, 0.7; lane 15, 0.4; lane 16, 0.3.

14–16), but those mutants lacking an intact bZIP region (Δ1-193 and Δ166-end) failed to have significant effects (compare lanes 2–4 with 8–10 and 11–13). Taken together, these results demonstrate a direct correlation between the ability of c-Fos mutants to inhibit c-Jun-activated hAR and their ability to dimerize with c-Jun.

The c-Fos bZIP Region Is Sufficient for Repression of c-Jun-Enhanced hAR Transactivation of hKLK2

The truncation analysis above (s*ee* Fig. 3) shows that the c-Fos bZIP region is necessary for c-Fos-induced repression of hAR/c-Jun transactivation of hKLK2-CAT. To examine if the bZIP region is sufficient for the activity, a c-Fos mutant expressing the bZIP region (115–200; *see* Fig. 1A) was made. c-Fos(115–200) was able to repress hKLK2-CAT activity as well as full-length c-Fos can, but less than c-Fos(Δ1–114) (Fig. 4A, compare lanes 1–3 with 4–6 and 7–9). As expected, c-Fos(115–200) is able to dimerize with c-Jun as well as full-length c-Fos can (Fig. 4B, compare lanes 2 and 3 with 4 and 5). Thus, the c-Fos bZIP region is not only necessary, but sufficient for inhibiting c-Jun-enhanced hAR transactivation from the hKLK2 promoter.

The c-Fos Basic Region Has a Role in Addition to Dimerization That Is Critical for c-Fos Repression of c-Jun-Enhanced hAR Transactivation of hKLK2

The c-Fos bZIP region can be divided into two functionally interdependent domains, the basic region and leucine zipper (reviewed in Angel and Karin, 1991). Our data show that the leucine zipper portion of this region is necessary for hAR repression, since removal of this part along with the C-terminus abolishes the c-Fos activity (*see* c-Fos mutant Δ166-end of Fig. 3). To determine if the basic region of c-Fos is essential for inhibition of c-Jun-enhanced hAR activity, a basic region point mutant (c-Fos[Arg3]) was tested for its activity. Surprisingly, c-Fos(Arg3), a mutant deficient in binding to an AP-1 DNA element (Neuberg et al.,

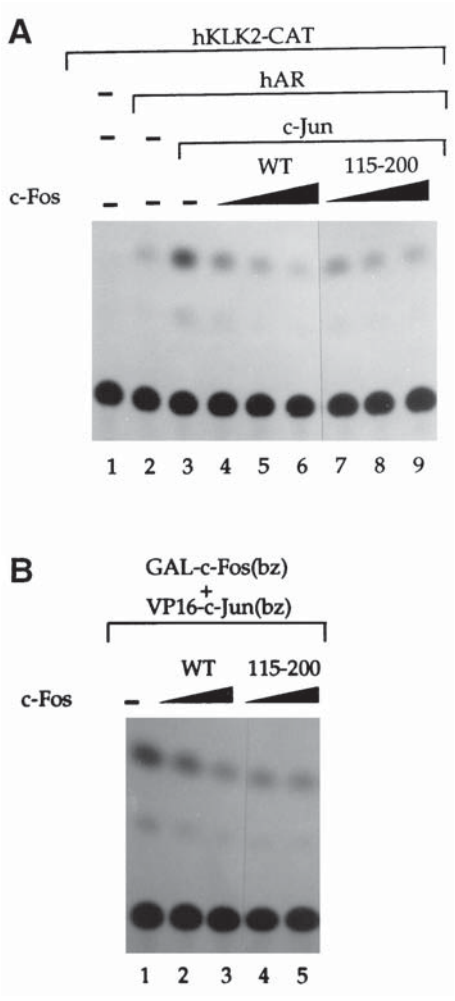

Fig. 4. The bZIP region of c-Fos is sufficient to repress c-Jun-enhanced hAR transactivation. **(A)** Cos cells were transfected with 2 μg of the hKLK2-CAT reporter plasmid, 1 μg of hAR expression plasmid in lanes 2–15, 1 μg of c-Jun, and increasing amounts (0.5, 1, or 3 μg) WT c-Fos (lanes 4–6) or c-Fos (115–200) (lanes 13–15); 100 n*M* DHT were used in all lanes. **(B)** Cos cells were transfected with 2 μg of the 17M-tk-CAT reporter plasmid, 1 μg of GAL-c-Fos(bz), 1 μg of VP16-c-Jun(bz), and increasing amounts (0.5 or 1 μg) of WT c-Fos (lanes 2, 3) or c-Fos(115–200) (lanes 4 and 5). The following relative activities were measured: (A) lane 1, 1.0; lane 2, 13.6; lane 3, 82.3; lane 4, 35.9; lane 5, 14.6; lane 6, 5.2; lane 7, 31.0; lane 8, 16.2; lane 9, 11.2; (B) lane 1, 1.0; lane 2, 0.6; lane 3, 0.4; lane 4, 0.4; lane 5, 0.4.

1991), weakly, if at all, inhibits the c-Jun activity on hAR transactivation of hKLK2-CAT (Fig. 5A, compare lane 3 with 7-9), even though it can very efficiently dimerize with c-Jun (Fig. 5B, compare lane 1 with 5–7). However, removal of the N-terminus and basic region of c-Fos (*see* c-Fos[Δ1–159] in Fig. 1A) does not affect the ability of c-Fos either to inhibit transcriptional activity of hAR (Fig. 5C, compare lane 3 with 7–9) or to dimerize with c-Jun (Fig. 5D, compare lane 1 with 5–7). Taken together, these data suggest that dimerization of c-Fos with c-Jun may be required, but is not sufficient for inhibition of c-Jun-enhanced hAR transactivation and, interestingly, that the basic region of c-Fos may have another role, in addition to dimerization, important for c-Fos repression of c-Jun/hAR transactivation.

hAR Targets c-Jun When Repressing AP-1 Transactivation

Like most nuclear receptors, hAR can inhibit AP-1 transactivation in a ligand-dependent fashion (*20*; reviewed in ref. *33*). Although the mechanism of this action is not understood, it is conceivable that hAR is targeting c-Jun and/or c-Fos. To address this, we examined the effect of exogenous c-Jun or c-Fos on hAR-induced inhibition of AP-1 activity. Endogenous AP-1 activity was repressed by hAR in a ligand- and dose-dependent manner (Fig. 6, compare lane 1 with 2–4). Interestingly, this repression was completely relieved by exogenously expressed c-Jun, but only weakly by c-Fos (Fig. 6, compare lanes 5–7 with 8–10), suggesting that hAR is sequestering c-Jun away from c-Fos and AP-1-responsive promoters.

Discussion

The bZIP region of c-Fos appears to have multiple functions in this protooncoprotein's role as a transcriptional regulator. The best studied is this region's direct involvement in the in vivo dimerization that occurs between c-Fos and c-Jun and in DNA binding that follows formation of this heterodimer (reviewed in Angel and Karin, 1991). Recently, it has been shown that the bZIP regions of both c-Fos and c-Jun mediate a physical association between these proteins and the general transcription factors TBP *(34)* or TFIIE and TFIIF *(35)*. In this study, we demonstrate that the bZIP region of c-Fos is essential for inhibition of c-Jun-supported hAR transactivation of the *hKLK2* promoter.

Based on our analysis of truncation mutants of c-Fos, the regions flanking the bZIP region, which have previously been shown to have domains required for transcriptional activation and cellular transformation (reviewed in ref. *15*), are not required for c-Fos-induced repression of hAR. Moreover, these results suggest that phosphorylation of c-Fos by FRK, which acts on the carboxy-terminal activation domains *(26)*, is not required for the c-Fos effect on hAR. In fact, a mutant that expresses only the bZIP region (with a few flanking amino acids) (c-Fos[115–200]) is capable of the same repression as full-length c-Fos, clearly showing that the bZIP region is not only required, but is sufficient for the negative activity.

The bZIP region of c-Fos consists of two domains, the leucine zipper and basic region. Though these domains do not have completely distinct functions, the basic region is responsible for DNA–protein interactions during site-specific DNA binding of AP-1 *(36–38)* and the leucine zipper is involved in protein-protein interactions during dimerization of c-Fos with c-Jun *(38)*. This study strongly suggests that the dimerization function of c-Fos is required for inhibition of c-Jun-enhanced hAR transactivation, since any

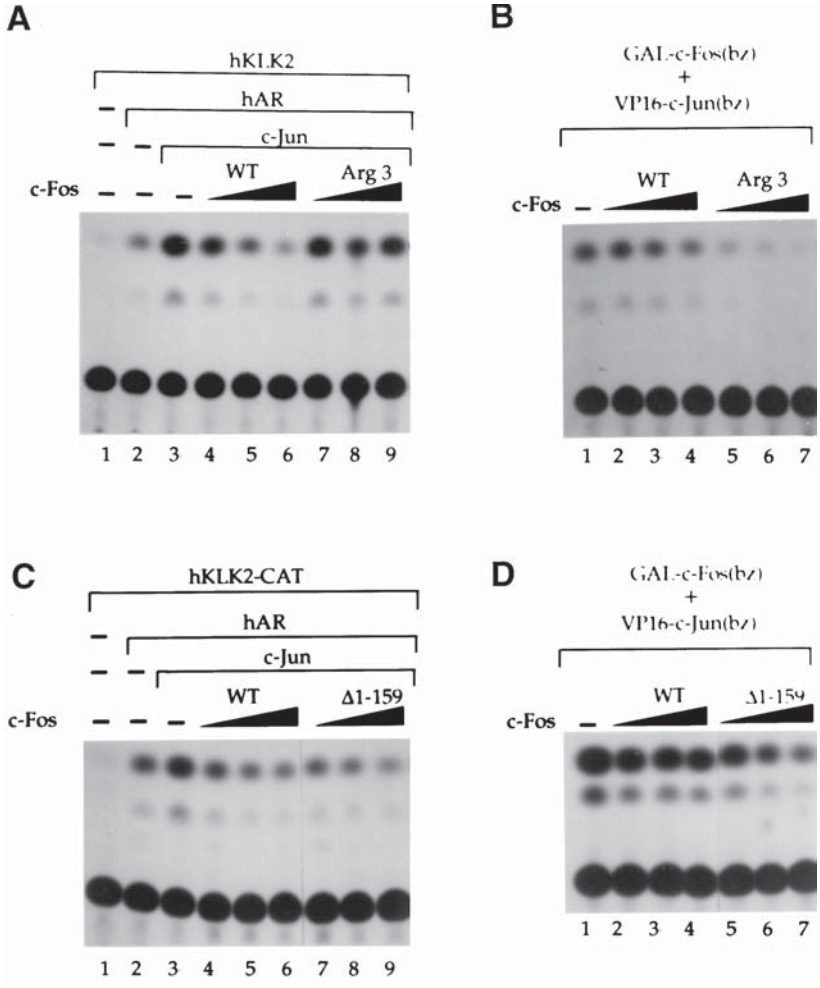

Fig. 5. The c-Fos basic region has a role in addition to dimerization that is critical for c-Fos repression of c-Jun-enhanced hAR transactivation. **(A,C)** Cos cells were transfected with 2 μg of the hKLK2-CAT reporter plasmid, 1 μg of hAR, 1 μg of c-Jun, and increasing amounts (0.5, 1.0, or 3.0) μg of WT c-Fos (lanes 4–6) and c-Fos(Arg3) (lanes 7–9) (A) or c-Fos(Δ1–159) (C); 100 n*M* DHT were used in all lanes. **(B,D)** Cos cells were transfected with 2 μg of the 17M-tk-CAT reporter plasmid, 1 μg of GAL-c-Fos(bz), 1 μg of VP16-c-Jun(bz), and increasing amounts (0.5, 1, or 3 μg) of WT c-Fos (lanes 2–4) and c-Fos(Arg3) (lanes 5–7) (B) or c-Fos(Δ1–159) (D). The following relative activities were measured: (A) lane 1, 1.0; lane 2, 45.8; lane 3, 120.4; lane 4,93.4; lane 5, 59.1; lane 6, 27.1; lane 7, 119.6; lane 8, 95.5; lane 9, 113.5; (B) lane 1, 1.0; lane 2, 1.0; lane 3, 0.9; lane 4, 0.5; lane 5, 0.2; lane 6, 0.2; lane 7, 0.1; (C) lane 1, 1.0; lane 2, 74.9; lane 3, 136.6; lane 4, 94.3; ; lane 5, 64.3; lane 6, 47.0; lane 7, 66.2; lane 8, 45.5; lane 9, 36.2; (D) lane 1, 1.0; lane 2, 76.0; lane 3, 70.8; lane 4, 62.0; lane 5, 60.7; lane 6, 41.9; lane 7, 25.0.

mutation within the leucine zipper of c-Fos that disrupts its ability to dimerize with c-Jun also abolishes its ability to inhibit hAR activity. However, deletion of the basic region, in mutant c-Fos(Δ1–159), does not affect the negative activity of c-Fos on hAR, strongly arguing that c-Fos DNA binding is not required and, thus, in agreement with earlier work *(20)*.

The requirement for an intact leucine zipper in c-Fos for inhibition of c-Jun-enhanced hAR activity is not surprising. PR-induced transcription can also be blocked by c-Fos, and this activity also requires an intact c-Fos leucine zipper *(20)*. Interestingly, however, the c-Fos negative effect on GR transcriptional activity is dependent on a region amino-terminal of the leucine zipper (and basic region) *(17)*. From these results, it is clear that different regions of c-Fos are involved in inhibiting these three closely related steroid receptors, making it possible that c-Fos may regulate nuclear receptors via multiple mechanisms. In this regard, it is interesting to note that the dimerization partner of c-Fos, c-Jun, has different effects on these three receptors: almost exclusively positive effects on AR *(20,21)*, either positive or having no effect on PR depending on the promoter and cell type *(20)*, and almost entirely negative on GR *(16–19)*. These results suggest that the region of c-Fos required for repressing nuclear receptor activity depends on the nature of the c-Jun effect on that receptor. Thus, it is possible that the leucine zipper of c-Fos is required for negating a positive effect of c-Jun, perhaps by dimerizing with the bZIP region of c-Jun as suggested by the results of this study, and this region of c-Fos becomes dispensable

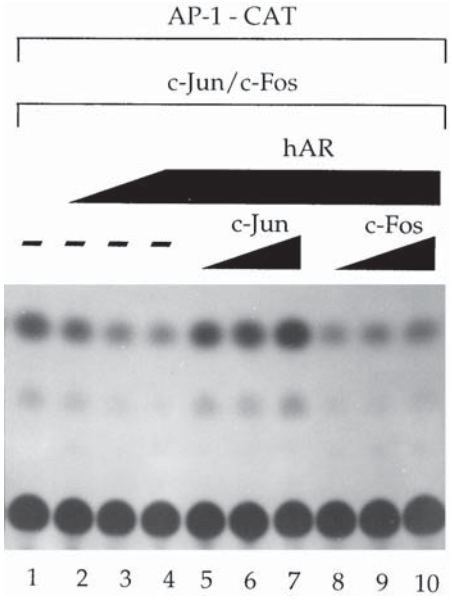

Fig. 6. hAR targets c-Jun when repressing AP-1 transactivation. Cos cells were transfected with 2 μg of the TRE-tk-CAT reporter plasmid, 1 μg of c-Jun, 1 μg of c-Fos, and increasing amounts (0.1, 1, or 3 μg) of hAR. In lanes 4–10, hAR amount was kept constant at 3 μg, and additional amounts (0.5, 1, or 3 μg) of c-Jun (lanes 5–7) or c-Fos (lanes 8–10) were transfected; 100 n*M* DHT were used in all lanes. The following relative activities were measured: lane 1, 1.0; lane 2, 0.7; lane 3, 0.3; lane 4, 0.2; lane 5, 0.9; lane 6, 1.0; lane 7, 1.6; lane 8, 0.4; lane 9, 0.5; lane 10, 0.6.

when c-Jun has a negative effect. Support for this comes from our earlier finding that the c-Jun bZIP region is necessary for this protein's enhancing activity on hAR *(27)*.

Although c-Fos dimerization with c-Jun is required for this protein's negative effect on c-Jun-activated hAR, it may not be sufficient. A point mutation in the c-Fos basic region has produced a mutant, c-Fos(Arg3), which has previously been shown to be deficient in DNA binding, but efficient in dimerization with c-Jun *(32)*. As expected, this mutant is capable of dimerizing with c-Jun in our mammalian two-hybrid assay, but it is incapable of inhibiting c-Jun-enhanced hAR transactivation. This finding suggests that the Arg to Val mutation of c-Fos in the Arg3 mutant generates a particular conformation in the protein that is not suitable for c-Fos inhibition of c-Jun/hAR, but does not disrupt the c-Jun/c-Fos dimerization. This conformational change may in turn influence the manner in which the c-Fos/c-Jun heterodimer interacts with either hAR or coactivators acting on hAR.

Recent results from our lab suggest that the c-Jun action on hAR depends on additional factors that can be squelched by high amounts of c-Jun *(27)*. Although the identity of such factors is unknown, recent results have reported that CREB binding protein (CBP) can enhance AR-dependent transcription through a physical association between the two proteins *(39)*. Since CBP has previously been shown to mediate AP-1-induced transactivation presumably by interacting with both c-Jun *(12,40)* and c-Fos *(41)*, it is conceivable that CBP may play an important role in the regulatory effects of these protooncoproteins on hAR activity. In view of the nearly universal modulatory effects of c-Jun and c-Fos on AR activity, a complex consisting c-Jun, c-Fos, and CBP may have important regulatory functions on AR control of gene expression. Future work will test the validity of this hypothesis.

Materials and Methods

Cell Culture, Transfections, and CAT Assays

Cos cells were maintained in DMEM (Sigma, St. Louis, MO) supplemented with 7.5% FBS (HyClone, Logan, UT). Cells were plated at approx 60% confluency in 60-mm dishes and transiently transfected using the calcium phosphate precipitation method *(42)*. Cells were washed with PBS 24 h after the introduction of DNA and subjected to fresh DMEM with 7.5% FBS containing 100 nM DHT or ethanol carrier. CAT assays were standardized by β-galactosidase activity originating from the transfected pCH110 plasmid and performed as previously described *(42)*. For all transfections, we used different amounts of expression plasmids, 2 μg of reporter plasmid (hKLK2-CAT), 2 μg of pCH110, and enough Bluescript to bring the final plasmid amount to 10 μg/dish. Note that all cells received the same amount parental expression vector (pTL1-NLS) in order to exclude the possible effects of expression vector. All transfections were conducted at lease three times, and the results shown are representative. CAT assay results were quantified by densitometric scanning (420 oe scanner, PDI, Inc., Huntington Station, NY).

Plasmids

Expression plasmids for hAR, c-Jun, and c-Fos are derived from the pSG5 parental plasmid as previously described *(29)*. c-Fos truncation mutants Δ1-114, Δ1-193, Δ166-end, and Δ201-end along with c-Fos(Arg3) were previously described *(20)*. c-Fos(115–200) was generated by polymerase chain reaction (PCR) amplification using the upstream oligo 5'-ATAGAATTCCACCATGAC AGGAGG-CCGAGACGCAG-3' and downstream oligo 5-TATGGATCCTCAGTGAGCTGCCAG-GATGAACTC-3'. The PCR fragment was digested with *Eco*RI and *Bam*HI and ligated in-frame into the *Eco*R1/*Bg*lII sites of pTL1-NLS. This plasmid was made by inserting a PCR fragment encoding the T-antigen nuclear localization signal (NLS) with an AUG into *Pst*I/*Kpn*I sites of pTL1 *(21)*. c-Fos(Δ1–159) was generated by PCR amplification using the upstream oligo 5'-GATCGATATCGAGCTGACAGATACA-3' and the downstream oligo 5'-GATCGGATCCTCACAGGG CCAGCAGCGT-3'. The PCR fragment was digested with *Eco*RV and *Bam*HI and ligated in-frame into the *Eco*RV/*Bg*lII sites of pTLI-NLS. VP16-c-Jun(bz), which has the VP16 activation domain fused to the bZIP (amino acids 237–331) of c-Jun, was generated by PCR amplification

using upstream oligo 5'-GATCGAATTCCTCGAGAT GGGCGAGACACCGCCC-3' and downstream oligo 5'-GATCGGATCCTCAAAATGTTTGCAA-3'. The PCR fragment was digested with *Eco*RI and *Bam*HI, and ligated into the *Eco*RI/*Bgl*II site of pTLI. pGAL0 and GAL4-c-Fos(bz) were previously described *(43,44)*. All c-Fos mutants were much better expressed in Cos cells than wild-type c-Fos (Tillman and Shemshedini, unpublished results).

The CAT reporter plasmids hKLK2-323-CAT *(28)*, TRE-*tk*-CAT, and 17M-*tk*-CAT *(20)* have been previously described. Cotransfected pCH110 provided the expression of β-galactosidase for standardization of transfection efficiency for CAT assays *(41)*.

SDS-PAGE and Western Blot

Cos cells transfected with various c-Fos plasmids were lysed by boiling cells directly in sodium dodecyl sulfate (SDS) sample buffer. Equal β-galactosidase units of cell extract were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were electrotransferred onto nitrocellulose (Micron Separations Inc., Westbrough, MA). After blocking with nonfat dry milk, the nitrocellulose blots were probed with an anti-c-Fos polyclonal antibody (K-25, Santa Cruz Biotechnology, Santa Cruz, CA). The blots were developed using the chemiluminescence detection kit from Amersham.

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