Differential roles for Fos and Jun in DNA-binding: redox-dependent and independent functions

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ABSTRACT
The Fos and Jun family of transcription factors contain an invariant sequence motif lysine-cysteine-arginine (KCR) in the highly conserved DNA-binding region. Reduction of the cysteine residue is necessary to facilitate DNA-binding. Here, we examined the potential dual roles of the flanking lysine and arginine residues in influencing the redox reactivity of the cysteine and the DNA-binding activity of Fos and Jun. Two sets of Fos and Jun mutants were generated: the KCR and KSR series representing proteins capable of redox-dependent and redox-independent DNA-binding activity, respectively. Mutation of the lysine in Fos-Jun heterodimers had no obvious effect on the redox reactivity of the cysteine, suggesting that lysine is not essential in this respect. However, mutation of the arginine but not lysine, in both the KCR and the KSR series abolished DNA-binding activity. Thus, the arginine but not the lysine residue in the KCR motif is critical for both redox-dependent and redox-independent functions in DNA-binding. Surprisingly, the triple substitution, ISI, exhibited high levels of DNA-binding activity. This demonstrates that the effects of amino acid substitutions can be highly dependent on context and that non-basic amino acids can function efficiently in DNA-binding. Analysis of combinations of wild-type and mutant Fos and Jun proteins indicated that Fos was dominant in dictating the DNA-binding ability of Fos-Jun heterodimers. This suggests that the lysine and arginine residues in the KCR region of Fos are not equivalent to those in Jun and that they interact with DNA differently.

INTRODUCTION
The proto-oncogenes c-fos and c-jun encode proteins that function as transcription factors in nuclear signal transduction processes. Their expression can be induced by a variety of agents associated with proliferation, differentiation, neuronal excitation and even cell death [1—3]. Fos and Jun form dimeric complexes that bind to DNA sequences related to the AP-1 (Activator protein-1) and CRE (cyclic AMP responsive element) motifs [4]. Dimerization occurs through a leucine zipper structure that serves to juxtapose basic amino acid regions in each protein that form a bipartite DNA binding surface [5—8]. Both c-fos and c-jun are members of gene families that share several of these properties. Fos and Fos-related proteins can form heterodimers with Jun- and selected ATF/CREB proteins but they do not form stable homodimers, whereas Jun-related proteins can also form homodimers [9—12]. Thus, a relatively large number of protein dimers can be generated from a small number of subunits. Each of these complexes may have distinct properties in terms of their affinity and specificity for DNA, their transcriptional activity and their effects on DNA topology [9, 11, 13—19].

Given the ubiquitous nature of the cellular-immediate early response, it is likely that Fos- and Jun-related proteins regulate different target genes in distinct cell types. Therefore, mechanisms must exist that regulate the target gene specificity of Fos- and Jun-related proteins. Several potential regulatory processes have been suggested. For example, phosphorylation has been proposed to affect both the DNA-binding and transcriptional activities of Fos and Jun [20—24]. However, the situation is complex, since multiple kinases are involved and the state of proteins i.e. whether they are monomers, dimers or bound to DNA, can determine kinase specificity and efficiency [23, 25]. A further level of regulation may involve DNA topology. Fos and Jun have been shown to cause DNA-bending in opposite directions [15, 26]. It is possible that each leucine-zipper dimer may recognize a unique conformation of the AP-1 target sequence that contributes to specifying the protein-DNA interaction [3]. In addition, an unusual reduction/oxidation (redox) mechanism has been uncovered that can regulate Fos-Jun DNA-binding activity [27].

Redox regulation of Fos and Jun is mediated by a conserved cysteine residue located in the DNA-binding domain [27]. This cysteine is converted spontaneously in vitro to an inactive state that does not involve disulphide bond formation. Conversion to the active state can be achieved by incubation with high levels of reducing agents or with a cellular protein, named Ref-1 (Redox Factor-1) [28—31]. Substitution of the cysteine residue with a serine results in a gain-of-function phenotype, in that mutant proteins can bind to DNA efficiently even under oxidizing conditions [27]. Interestingly, this substitution has occurred spontaneously during the generation of the viral jun oncogene and may contribute to its oncogenic properties [32, 33]. Analysis

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of mutated fos genes in cell transformation assays suggests that redox regulation also affects Fos activity in vivo [34].

The critical cysteine residue (Cys154 in Fos and Cys272 in Jun) is situated in an invariant lysine-cysteine-arginine sequence motif within the basic DNA-binding domains of Fos and Jun [35]. Biochemical studies on synthetic polypeptides have shown that the exchange rate of a sulphydryl group on a cysteine with a disulphide bond is influenced by the net charge of the neighbouring amino acids [36]. Therefore, it is possible that the flanking lysine and arginine residues influence redox control of the cysteine. It has been suggested that these positively charged flanking amino acids elevate the reactivity of the cysteine, making it susceptible to oxidation and therefore redox regulation [27].

The basic region is a common DNA-binding motif in leucine-zipper proteins and in other transcription factors [35, 37-39]. Initial models suggested that the basic region and the leucine zippers were α-helical, with the conserved amino acid residues that interact with DNA being located on one helical surface [35, 37]. The helical conformation of the basic region and the zippers was confirmed by circular dichroism analysis and more recently, in the case of GCN4, by X-ray crystallography [40-42]. GCN4 is a b-ZIP protein that contains the sequence arginine-serine-arginine (Arg241-Ser242-Arg243) at the equivalent position of lysine-cysteine-arginine in Fos and Jun. Arg241 forms hydrogen bonds with the phosphates of the DNA backbone, Ser242 interacts through van der Waal’s forces and Arg243 can form hydrogen bonds with specific bases in the AP-1 recognition site. This suggests that these residues are important in stabilizing the protein-DNA complex and in specifying the interaction with the nucleotide sequence of the AP-1 site. Here we have conducted a mutagenesis analysis of Fos and Jun to examine the potential roles of the lysine-cysteine-arginine sequence motif in DNA-binding and redox control.

MATERIALS AND METHODS

Proteins
Regions of the rat c-fos gene (amino acids 118–211) and of the rat c-jun gene (amino acids 225–334) were expressed in E.coli. and purified by nickel-chelate chromatography [28]. Mutations were engineered into c-fos and c-jun by the polymerase chain reaction using synthetic oligonucleotide primers containing specific mutations as reported before [27]. The oligonucleotide sequence of the mutants was confirmed by dideoxynucleotide sequencing. The apparent molecular weights of the recombinant truncated Fos and Jun proteins were 15 kD and 19 kD, respectively, as determined by SDS-PAGE.

Recombinant Ref-1 protein was kindly provided by S. Xanthoudakis. The human ref-1 cDNA was previously cloned and expressed in E.coli., and the protein was purified by nickel-chelate chromatography and characterized as described [30]. Extracts of 208F rat fibroblasts were prepared as described previously [43, 44]. Briefly, three volumes of cold hypotonic buffer containing 10 mM HEPES, pH 7.5, 1 mM EDTA and 10 mM b-mercaptoethanol were added and the cells were allowed to swell for fifteen minutes before Dounce homogenization. NaCl and Nonidet P-40 were added to the cell lysate to a final concentration of 0.5 M and 0.5 %, respectively. The lysate was agitated gently for 30 minutes at 4°C. Then following centrifugation in an eppendorf microfuge, the supernatant was taken and glycerol was added to a final concentration of 5%. The supernatant was stored in −70°C in aliquots.

AP-1 probe
The AP-1 probe consisted of synthetic oligonucleotides representing the human metallothionein II A AP-1 site [45]. The probe was labeled with [γ-32P]ATP using polynucleotide kinase to a specific activity of approximately 1×10^6 cpm/pmol, following the instructions from the supplier (Promega).

DNA-binding and gel-shift assays
The DNA-binding reaction and gel-retardation assays were performed essentially as described previously [28, 30]. Proteins were incubated for 15 minutes at 37°C in 10 μl of reaction buffer, containing 50 mM Tris—Cl, pH 8, 5 mM MgCl2, 1 mM EDTA, 5 % glycerol, 5 % sucrose, 0.05–0.1 % Nonidet P-40 and 0.2 mg/ml bovine serum albumin. Other reagents, such as dithiothreitol (DTT), β-mercaptoethanol (β-Me), Ref-1 or cell extract, were sometimes included in the reaction, as indicated in the figure legends. A typical incubation contained 10 ng of Fos and Jun which is equivalent to final concentrations of 0.07 μM and 0.05 μM, respectively. 1 μg of poly (dl-dC)-(dl-dC) was added and the incubation was continued for 5 minutes at 25°C. 0.2 ng of labeled AP-1 probe was added and the incubation was continued for 20 minutes. The protein—DNA complex was then subjected to electrophoresis on a native 4.5 % polyacrylamide gel in a buffer of 0.5×Tris-borate-EDTA. Gels were dried and visualized by autoradiography.

RESULTS

Mutagenesis of the lysine-cysteine-arginine motif
The amino acid sequence lysine-cysteine-arginine (KCR) motif is identical in all known members of the fos and jun gene families. It is also present in other b-ZIP proteins where the arginine is the most conserved amino acid, the cysteine is frequently replaced by serine and the lysine is often substituted by arginine [35]. Previous analysis of unrelated synthetic peptides had revealed that the reactivity of a cysteine can be increased significantly by one flanking basic amino acid [36]. Therefore, we hypothesized that the flanking positively-charged lysine and arginine may influence the reactivity of the cysteine of Fos and Jun by contributing to the local electrostatic environment. To address this question, we generated different combinations of mutations in one or both of the flanking lysine and arginine residues in Fos and Jun, as shown in Fig. 1. We chose to replace both lysine and arginine with isoleucine to preserve a four-carbon hydrophobic side chain in these positions. Computer analysis predicted that all mutants can adopt an α-helical structure similar to that of the proteins containing the wild-type lysine-cysteine-arginine motif (data not shown). For simplicity, and for purification purposes, the mutations were all made in the context of poly (dl-dC)-(dl-dC) was added and the incubation was continued for 5 minutes at 25°C. 0.2 ng of labeled AP-1 probe was added and the incubation was continued for 20 minutes. The protein—DNA complex was then subjected to electrophoresis on a native 4.5 % polyacrylamide gel in a buffer of 0.5×Tris-borate-EDTA. Gels were dried and visualized by autoradiography.

An alternative hypothesis that does not exclude the above proposal, is that the lysine and arginine residues may perform a direct function in DNA binding. Therefore, since replacement of the critical cysteine by serine renders DNA-binding by Fos and Jun independent of redox control, we compared the consequences of mutations in the flanking residues both in the
context of proteins retaining the critical cysteine (the KCR series) or with a serine substitution in this position (the KSR series; Fig. 1). In this way, we aimed to distinguish between the direct effect of the flanking residues on the DNA-binding activity of Fos and Jun from their influence on the redox control of DNA-binding.

**Requirement for the arginine but not lysine for redox-responsive DNA-binding**

We first examined the DNA-binding ability of the various KCR mutants. In the presence of DTT, homodimers of different Jun mutants displayed varying behaviour (Fig. 2A). Jun(ICR) could be stimulated to bind DNA with a similar affinity to wild-type Jun. In contrast, neither Jun(KCI) nor Jun(ICI) homodimers showed any specific DNA interaction, even when a great excess of protein was used in the binding assay. We also examined the DNA-binding activity of the heterodimers formed between mutated Fos and Jun proteins in which each subunit contained the same substitution (Fig. 2B). Considerably less protein was necessary when analyzing Fos and Jun proteins in which each subunit contained the same substitution (Fig. 2B). In the presence of DTT, as little as 5 ng of the Fos-Jun heterodimer could bind to DNA as previously reported [28]. Fos(ICR)-Jun(ICR) responded to stimulation by DTT and bound to DNA efficiently, although 25 ng of the protein dimer was required for efficient DNA-binding. This indicated that Fos(ICR)-Jun(ICR) had a lower affinity for DNA than the wild-type proteins. As observed with the Jun(KCI) and Jun(ICI) homodimers, Fos(KCI)-Jun(KCI) and Fos(ICI)-Jun(ICI) heterodimers lost the ability to bind DNA. These experiments showed that mutation of arginine but not the lysine in the lysine-cysteine-arginine motif (the KCI and ICI mutants) severely impaired the redox-responsive DNA-binding activity of Jun homodimers and Fos-Jun heterodimers. This suggests that the arginine residue is critically required for DNA-binding activity, whereas the lysine is less important.

**Redox-responsiveness of lysine mutants**

Although the lysine in the KCR motif was apparently not essential for DNA-binding in the presence of 10 mM DTT (Fig. 2), it
arginine in DNA binding could reflect either an indirect role of DNA-binding activity by DTT (Fig. 2). The requirement for the lysine residue in the KCR motif is critical for stimulation of the wild-type proteins (data not shown) [27]. Thus, there was no detectable change in the redox reactivity of the cysteine caused by mutation of the conserved lysine as measured by gel-shift analysis. Nevertheless, the reduced efficiency of Ref-1 in stimulating DNA-binding by Fos(ICR)-Jun(ICR) suggests that there may be some requirement for lysine in mediating optimal stimulation by Ref-1.

Participation of the arginine residue in the protein–DNA interaction

The preceding experiments indicated that the arginine but not the lysine residue in the KCR motif is critical for stimulation of DNA-binding activity by DTT (Fig. 2). The requirement for arginine in DNA binding could reflect either an indirect role of this residue on the redox reactivity of the cysteine or a direct participation in DNA-binding. We sought to distinguish between these possibilities by utilising the KSR series of mutants that are capable of binding to DNA in the absence of reducing agents (Fig. 1C) [27]. The DNA-binding activity of heterodimers formed between various Fos mutants and their Jun counterparts measured in the absence of DTT is shown in Fig. 4. As was observed with the KCR series of mutants (Fig. 2), mutation of lysine to isoleucine was permissive for DNA binding since Fos(ISR)-Jun(KSI) heterodimers were capable of binding to DNA, although with a slightly lower efficiency than Fos(KSR)-Jun(KSR) heterodimers. However, Fos(KSI)-Jun(KSI) exhibited no DNA-binding activity even when using high levels of protein, suggesting that mutation of arginine to isoleucine was incompatible with DNA binding. A similar trend was also observed when the same mutations were analyzed in the context of Jun homodimers. Jun(ISR) exhibited comparable DNA-binding activity to Jun(KSR) whereas Jun(KSI) did not bind to DNA. Thus, substitution of arginine but not lysine with isoleucine in this region abolished DNA binding activity.

Since the KSR series of proteins did not rely on reduction by DTT for protein-DNA interaction, the loss of DNA-binding activity of the KSI mutants suggested that the arginine itself plays an essential role in the protein-DNA interaction, irrespective of its potential involvement in redox regulation. In addition, the ability of the ISR mutants to bind to DNA indicated that the lysine residue is not important for the protein-DNA interaction of Fos and Jun. Thus, the lysine is apparently not essential for either redox stimulation of DNA binding or for redox-independent functions in the protein–DNA interaction.

Unusually, in contrast to all other proteins where mutation of arginine to isoleucine (KI, ICI and KSI) abolished DNA binding, the ISI mutants bound to DNA with a comparable efficiency to the wild-type proteins (Fig. 4). Thus, mutation of arginine in the context of the triple mutant ISI, restored significant DNA binding activity. At present, it is difficult to interpret this

![Figure 3. Redox-responsiveness of the Fos(ICR)-Jun (ICR) heterodimer. 25 ng Fos and 25 ng Jun were incubated in the presence of one of the following: no addition (NA), 10 mM β-mercaptoethanol (β-Me), 10 mM DTT, 2 μg Ref-1 or 2 μg cell extracts of 208F rat fibroblasts (CE), as indicated. Parallel incubations were also performed containing 25 ng Fos(ICR) and 25 ng Jun(ICR). The DNA-binding activity was then measured by gel-shift assays. In the lanes containing the cell lysate, the slower-migrating minor bands represent the protein-DNA complexes formed by endogenous Fos and Jun proteins.](image)

![Figure 4. Requirement for the arginine but not lysine in redox-independent DNA-binding. Increasing amounts of Fos(KSR) and Jun(KSR) as indicated were incubated in the absence of DTT and subjected to subsequent gel-shift analysis. Parallel incubations included one of the followings: Fos(KSI) and Jun(KSI), Fos(ISR) and Jun(ISR), or Fos(ISI) and Jun(ISI).](image)
amounts of protein and Fos(ICI)-Jun had no DNA-binding activity of Fos(KCI)-Jun was detected even when using excessive Fos-Jun heterodimer. However, only minimal DNA-binding could bind to DNA with a slightly lower affinity than wild-type heterodimers formed between various Fos mutants with wild-type Jun, the DNA-binding activity of these mutant heterodimers also showed the same dose-response to stimulation by various concentrations of DTT as wild-type heterodimers (data not shown). Thus, in clear contrast to the Fos mutants, Jun mutants exerted little or no influence on the DNA-binding activity of the heterodimers.

To assess the dominance of Fos in DNA-binding independently of its effect on redox control, the experiments described above were repeated with the KSR series of mutants. In general, a similar trend was observed in the context of the serine substitution. Taken together, these results suggest that Fos is dominant in determining DNA-binding by Fos-Jun heterodimers and that the arginine and lysine residues dictate this dominance regardless of any role they may have in contributing to the redox reactivity of the cysteine.

**DISCUSSION**

The *in vitro* interaction of Fos and Jun with the AP-1 recognition site has been well characterized but the underlying mechanisms that govern the recognition of and interaction with DNA are largely unknown. The DNA-binding domains of Fos and Jun, like other members of the basic-zipper family of transcription factors contain highly conserved amino acid residues that appear to have specific roles in the protein–DNA interaction. In addition, DNA binding by Fos and Jun is regulated by oxidation/reduction at a cysteine flanked by basic lysine and arginine residues (the KCR motif) near the center of their DNA-binding domains [27]. Previous studies have indicated that the presence of a neighbouring, positively-charged amino acid can increase the reactivity of a cysteine by a factor of 10⁶ [36]. This is a consequence of the elevation of the activation energy contributed by the increase in local electrostatic charges. Here, we have investigated the potential dual roles of the basic residues in the KCR motif of Fos and Jun in DNA-binding and influencing the redox reactivity of the cysteine.

**Redox-responsive DNA-binding**

Analysis of the KSR series of proteins which exhibit redox-dependent DNA-binding, revealed that the lysine but not the arginine was dispensable for DNA binding. Thus, the ICR mutants of Fos and Jun could be stimulated by DTT to bind DNA efficiently, as Jun homodimers or as Fos-Jun heterodimers, albeit with a lower apparent affinity than the wild-type proteins. In contrast, the KCI proteins did not bind to DNA. Therefore, the lysine does not make an obvious contribution to redox-responsive DNA-binding. However, it is interesting to note that while the ICR proteins could be stimulated to bind DNA efficiently by various general reducing agents or whole cell extracts, they did display somewhat reduced binding when incubated with recombinant Ref-1, the cellular factor involved in redox control of DNA-binding activity [30]. Therefore, it remains possible that the lysine residue influences the recognition of Fos and Jun by Ref-1.

The requirement for the arginine observed with the KCR series of proteins did not distinguish between a possible indirect role...
through influencing the redox reactivity of the cysteine or a more direct role in the interaction with DNA. Further analyses with mutants containing a serine in place of the cysteine (the KSR series) that bind DNA constitutively in the absence of reducing agents, demonstrated a similar trend to that seen with the KCR proteins, namely that the ISR but not the KSI proteins, were capable of binding to DNA. Therefore, we conclude that the arginine possesses some redox-independent function in DNA binding. However, since the requirement for the arginine is absolute as measured on both the KCR and KSR background, our results do not rule out the possibility that it may also influence the redox reactivity of the cysteine.

It is intriguing that the ICR proteins had only minimal effect on the redox reactivity of the cysteine. This suggests that the redox reactivity of the cysteine is not maintained by a single residue, but it may involve a number of factors. The electron density on the sulfur atom is affected by proximal and distal charged residues through inductive effects and Coulombic forces, respectively [36]. Computer analysis of the b-ZIP proteins predicts that the conserved cysteine is surrounded by positively-charged amino acids in the DNA-binding a-helix [35, 37]. Such a highly charged environment may make it extremely difficult to reduce the cysteine. Therefore, the structure of Fos and Jun may tend to keep them inactive, that is maintaining the cysteine in its reversible, presumed oxidized state [27], unless it is specifically activated. This may prevent activation by marginal changes in the reducing environment and it could reflect a mechanism that suppresses the transcriptional function of Fos and Jun in the absence of specific stimulation by extra-cellular signals.

Role of the lysine and arginine residues in DNA binding

It has been predicted that the basic regions of the b-ZIP proteins form a-helices that track along the major groove of DNA, with the highly conserved residues, including the cysteine and arginine lying on the same helical surface that faces DNA [37, 47]. For Fos and Jun, we observed that mutation of the arginine abolished DNA-binding, whereas, mutation of the lysine had only a modest effect on DNA-binding. When the arginine of a transforming viral Fos was replaced by lysine which carries a similar positive charge, the heterodimer formed with wild-type Jun had significantly reduced DNA-binding activity [48], suggesting that the interaction of the arginine residue with DNA is specific. Considering that the arginine is absolutely conserved among the Fos and Jun families and other b-ZIP proteins, we speculate that it be used to make base-specific contacts with DNA in most b-ZIP transcription factors. This is in agreement with crystallographic studies of the GCN4 protein in which Arg243 of the two subunits interact with DNA differentially [42]. By extrapolation, it may be predicted that in Fos-Jun heterodimers, Fos but not Jun assumes a conformation that allows its lysine and arginine residues to establish important contacts with the phosphodiester backbone and guanine base, respectively. In the case of the Jun homodimer, it is reasonable to propose that one monomer of Jun assumes a similar, ‘dominant’ conformation as does Fos in the heterodimer and therefore, the lysine and arginine residues of this Jun subunit would constitute an important determinant in DNA-binding.

Recently, it was suggested that substitution of the amino acids in GCN4 that contact DNA (Asp235, Ala238, Ala239, Ser242 and Lys246) causes an alteration in DNA-binding specificity [49]. Since the DNA-binding region of GCN4 is an a-helical chain which is not constrained by other domains, it can probably adopt different conformations to accomodate amino acid changes on binding to DNA. This flexibility of the DNA-binding domain may be an important determinant of the specificity of the protein-DNA interaction. In this regard, it is interesting that the ISI mutants can bind to DNA efficiently. Although the mutations were predicted not to alter radically the a-helical conformation of the DNA-binding region, this region of the ISI mutants may be shifted so that other amino acid residues can interact with DNA. Recently, Fos and Jun have been shown to bind to an unrelated DNA sequence in association with NFAT transcription factor [50, 51]. It is conceivable that different amino acid residues would be involved in direct protein-DNA contact and even redox regulation, depending on the actual DNA sequence of the response element of the particular target gene.

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REFERENCES