Different requirements for formation of Jun : Jun and Jun : Fos complexes

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The cFos proto-oncoprotein associates with cJun to form a heterodimer with increased DNA binding and transcriptional activities. It has been suggested that dimerization of these proteins is mediated by the interdigitation of an orderly repeat of leucine residues forming a leucine zipper. In agreement with this model, we find that binding to the AP-1 site requires dimerization of these proteins. Although cFos, itself, does not seem to dimerize and bind to the AP-1 site, Jun : Fos heterodimers have higher stability than Jun homodimers, which accounts for their increased DNA binding activity. Mutational analysis indicates that at least three of the repeated leucines of cJun are important for homodimer formation. However, these residues can be mutated without affecting formation of Jun : Fos heterodimers. In addition, several other residues present between the leucines are also important for both homo- and heterodimerization. These findings provide support for the recent proposal that these proteins dimerize via formation of a coiled coil and suggest that residues other than leucines provide specificity for this interaction. Assuming that dimerization is required for proper alignment of the DNA recognition sites, we generated a cJun mutant containing a small insertion between the dimerization and the DNA recognition domains. This mutant fails to bind DNA, but it acts as a *trans*-dominant inhibitor of cJun and cFos because it still dimerizes with the wild-type proteins.

[Key Words: Transcription factor; Jun; Fos; AP-1; protein–DNA interactions]

Received April 28, 1989; revised version accepted October 11, 1989.

Transcription factor AP-1 mediates gene induction by phorbol ester tumor promoters (Angel et al. 1987; Chiu et al. 1987; Lee et al. 1987), transforming oncogenes (Schönthal et al. 1988; Wasylyk et al. 1988), and polypeptide hormones (Brenner et al. 1989). AP-1 is a complex whose major components are the products of the *c-jun* and *c-fos* proto-oncogenes, the cJun and cFos proteins (Bohmann et al. 1987; Angel et al. 1988a; Chiu et al. 1988; Rauscher et al. 1988a; Allegretto et al. 1989). cJun and the related vJun protein, the product of the retroviral oncogene *v-jun* (Maki et al. 1987), are sequence-specific DNA-binding proteins that recognize the same sequence motif, termed the TPA-responsive element (TRE), as AP-1 (Bohmann et al. 1987; Angel et al. 1988a; Bos et al. 1988). On the other hand, the cFos protein does not bind to the TRE in the absence of Jun. When added to either of the Jun proteins, Fos associates with them to form a heteromeric complex, exhibiting higher DNA binding activity than Jun alone (Halazonetis et al. 1988; Kouzarides and Ziff 1988; Nakabeppu et al. 1988; Sassone-Corsi et al. 1988; Rauscher et al. 1988b; Allegretto et al. 1989). These studies also suggested that Jun binds the TRE as a homodimer, with low affinity, whereas together, Jun and Fos form a heterodimer that binds to the TRE with higher affinity. However, the molecular basis for the increased affinity of the Jun : Fos heterodimer was unexplainable.

Dimerization of Jun and Fos was suggested to occur by the leucine zipper motif (Kouzarides and Ziff 1988; Landschulz et al. 1988; Schuermann et al. 1989). The 'leucine zipper' model was originally proposed by Landschulz et al. (1988), who detected a heptad repeat of leucine residues in several proteins involved in transcriptional regulation, including C/EBP, Jun, Fos, and GCN4. The key feature of the leucine zipper model is the interdigitation of leucine residues extending from one α -helix with those displayed by a similar α -helix on a second protein molecule. This type of hydrophobic interaction is supposed to be unique to leucine residues and to be the major contributor for dimerization of proteins containing this motif. Evidence supporting the role of the leucine residues in Jun : Fos interaction was recently provided (Kouzarides and Ziff 1988; Gentz et al. 1989; Schuermann et al. 1989; Turner and Tjian 1989).

The leucine zipper model, however, fails to explain how the specificity in Jun : Fos interaction is derived. For example, Myc, which also has a leucine zipper, fails to interact with either cJun or cFos (Halazonetis et al. 1988). To shed more light on these interactions and to understand how cFos increases the activity of the complex, we undertook further biochemical and mutational analyses of these proteins. We found that mutations in the heptad repeat of leucine residues that interfere with dimerization and DNA binding of cJun do not affect its

interaction with cFos. Furthermore, these mutant Jun : Fos heterodimers exhibit wild-type DNA binding and transcriptional activities. Mutations of several residues between the leucines were found to have detrimental effects on both homo- and heterodimerization. These results indicate that amino acid residues between the leucines also play a very important role and are likely to provide the specificity for Jun : Fos interaction. These and recent findings regarding the activation functions of cJun and cFos (Angel et al. 1989) indicate that the increased DNA binding and transcriptional activity of the Jun : Fos heterodimer is attributable to formation of a more stable complex than the cJun homodimer.

Results

Mutants in the leucine heptad repeat are defective in DNA binding

The presence of a leucine repeat within the DNA-binding domain of the Jun proteins predicts that they bind DNA as dimers (Landschulz et al. 1988). Indeed, this notion is supported by experiments using in vitro-translated Jun proteins (Halazonetis et al. 1988; Nakabeppu et al. 1988). We used a different approach for testing this possibility. Both the full-length vJun protein and the carboxy-terminal 138 amino acids of cJun were expressed as trpE fusion proteins in *Escherichia coli* (Fig. 1A,B). In mobility-shift assays, these proteins, trpE-vJun and trpE-cJDBD, form specific complexes with a TRE probe whose mobilities reflect the difference in their sizes (Fig. 1C). We have shown elsewhere that these complexes are specific because they are competed by the wild-type TRE sequence but not by a nonfunctional TRE point mutant (Allegretto et al. 1989). Mixing and preincubation of the two proteins, prior to addition of the TRE probe at either 4°C or 25°C, did not generate complexes of intermediate mobility, indicative of a multimer that is most likely a dimer composed of both the full-length and the truncated protein (Hope and Struhl 1987). However, if the two proteins were mixed and preincubated at 37°C prior to incubation with the DNA probe at 4°C, a complex with intermediate mobility appeared (denoted as vJ/cJ in Fig. 1C). The simplest interpretation of these results is that the Jun proteins exist as stable dimers prior to binding DNA. These dimers appear to melt between 25°C and 37°C, allowing the exchange of different size monomers which, upon cooling, will reassociate.

To test whether dimerization is mediated by interactions between the repeated leucine residues, we replaced the second and third leucines of cJun by phenylalanine and histidine residues, respectively. Phenylalanine is hydrophobic in nature but is considerably more bulky than leucine, whereas histidine is a polar residue having the same mean volume as leucine (Chothia 1984). In addition, a double-point mutant that affects both of these leucines was generated, as well as a control mutant in which threonine 286 was converted to a serine (Fig. 2A). Mutations of the second and third leucines, which lie in the middle of the leucine repeat, were expected to be

most disruptive to the leucine zipper. The expression and transcriptional activities of these mutants were assayed by their ability to *trans*-activate a reporter gene -73Col·CAT, constructed by fusion of the minimal promoter of the human collagenase gene to chloramphenicol acetyltransferase (CAT) structural sequences, after transfection into F9 cells. Expression of this reporter is AP-1 dependent (Angel et al. 1988a; Chiu et al. 1988). Although all of the mutants were expressed at levels similar to the wild-type protein (Figs. 2B and 5B) and localized to the nucleus (not shown), some of them exhibited greatly reduced transcriptional activity. At low input levels, the control serine 286 mutant was al-

Figure 1. Expression of trpE-Jun fusion proteins in *E. coli* and formation of wild-type homodimers. (A) Structure of trpE-Jun fusion proteins. [Hatched boxes] DNA-binding domains of vJun and cJun; [dotted boxes] trpE-coding region; [solid boxes] vJun *trans*-activation regions. The molecular masses of these proteins are trpE, 37 kD; trpE-vJun, 75 kD; trpE-cJDBD, 55 kD; trpE-cJAV4, 50 kD. (B) Partially purified and renatured fusion proteins were analyzed by SDS-PAGE and immunoblotted with anti-Jun antiserum (Chiu et al. 1988). Approximately 10 ng of cJDBD, M14, M9, and M8, 20 ng of cJAV4, and 5 ng of vJun were analyzed; however, because these are renatured proteins, only a fraction of this amount (20–30%) is active. The faster migrating bands correspond to degradation products of these proteins formed in *E. coli*. Because the antibodies are directed against the DNA-binding domain of cJun, these degradation products are likely to result from proteolysis within the trpE sequence and retain a functional DNA-binding domain. (C) Partially purified trpE-vJun (30 nM) and trpE-cJDBD (30 nM) fusion proteins were mixed and incubated at the indicated temperatures for 30 min and then chilled on ice prior to the addition of the TRE probe (0.1 nM; ~10,000 cpm). Protein-DNA complexes were resolved by nondenaturing polyacrylamide gel electrophoresis. The complexes formed by the homodimer and heterodimer are indicated. The unbound probe was run off the gel to increase resolution.

Figure 2. Analysis of mutants in the leucine repeat. (A) Structure of point mutants. The structure of cJun and the sequences of the various point mutants at the region containing the second and third leucines of the heptad repeats are shown. (Solid boxes) *trans*-activation domains; (dotted box) a proline- and glutamine-rich region; (hatched box) DNA recognition site; (L) leucine repeat. Activities of the various constructs were determined by transient transfection into F9 cells, using 2 μ g of the -73-Col-CAT reporter and the indicated amounts of expression vector. Fold *trans*-activation is the ratio of CAT activity after transfection with the indicated expression vectors to CAT activity in cells transfected with RSV-cJun, which lacks a functional DNA-binding domain (Angel et al. 1988b). The mutants were expressed by the same RSV expression vector used for expression of wild-type cJun (Angel et al. 1988b). Numbers shown are the averages of three independent experiments. (ND) Not determined. (B) Expression of wild-type and mutant cJun proteins in transiently transfected F9 cells. Jun proteins were labeled and immunoprecipitated with anti-Jun antibodies as described in the Materials and methods. (cJ) Wild-type cJun; (NT) not transfected. (For M14 expression, see Fig. 5.)

most as active as wild type, the phenylalanine 287 mutant (M8) had 40% of wild-type activity, the histidine 294 mutant (M9) exhibited an eightfold drop in activity, and the double-point mutant (M14) was almost completely inactive (Fig. 2A). At higher input levels, the activities of M8 and M9 approached that of the wild-type cJun, whereas M14 was still ineffective. Increasing the amount of the wild-type expression vector beyond 1.0 μ g per plate did not lead to further increase in *trans*-activation (not shown).

To determine the basis for their reduced *in vivo* activity, the carboxy-terminal halves containing the

DNA-binding and dimerization domains of these mutants were expressed as trpE fusion proteins in *E. coli* (see Fig. 1A and Materials and methods), and their DNA binding activities were assayed *in vitro* (Fig. 3A). The point mutants displayed reduced DNA binding activity which paralleled the loss of transcriptional activity. M8 displayed a 2.5-fold reduction in DNA binding activity, and M9 displayed a 7.6-fold reduction in DNA binding (determined by quantitating the fraction of the probe bound at various protein concentrations). As expected by

Figure 3. *In vitro* activities of leucine mutants. (A) A fixed amount of the TRE probe (10,000 cpm; 0.1 nM) was incubated with increasing concentrations (10 nM to 80 nM) of cJDBD (wild-type), M8, and M9. Binding was analyzed by the mobility shift assay. (F) Free probe, (B) protein-DNA complex. (B) cJDBD, M8, M9, and M14 binding activities were assayed using 1 nM probe and 100 nM protein. (C) The truncated wild-type and mutant cJun proteins (30 nM) were incubated with full-length trpE-vJun (30 nM) at 37°C and analyzed by a mobility-shift assay for formation of heterodimers. Note that the lanes on which the mutants and vJun were analyzed represent a threefold longer exposure than the wild-type lanes to demonstrate the formation of a cJ/vJ heterodimer. (+ and -) the presence or absence of trpE-vJun during the preincubation. C and Fig. 1C were part of the same experiment and are shown separately for convenience.

its nearly complete loss of *in vivo* activity, the double-point mutant (M14) failed to bind DNA, even at higher probe and protein concentrations (Fig. 3B).

When mixed with full-length trpE-vJun and preincubated at 37°C, the single-point mutants formed greatly reduced amounts of heterodimers with v-jun, in comparison to the truncated wild-type protein, trpE-cJDBD. No heterodimers could be detected upon mixing of trpE-vJun with M14 (Fig. 3C)

Increased stability of Jun : Fos heterodimers

The leucine repeat of the Jun proteins was also proposed to mediate formation of Jun : Fos heterodimers via an interaction with a similar motif in cFos (Landschulz et al. 1988). Experimental evidence demonstrating the formation of Jun : Fos heterodimers was recently provided (Halazonetis et al. 1988; Kouzarides and Ziff 1988; Nakabeppu et al. 1988; Rauscher et al. 1988b; Sassone-Corsi et al. 1988; Allegretto et al. 1989). For example, mixing of a truncated fusion protein containing the 99 carboxy-terminal amino acids of cJun (trpE-cJAV) with extracts of insect cells expressing cFos protein after infection with a baculovirus-fos recombinant (S. Agarwahl, T. Curran, and T.M. Roberts, *in press*), leads to formation of a heteromeric complex, involving cFos and cJun (Allegretto et al. 1989). In contrast to the vJun : cJun heterodimer (see Fig. 1), formation of the putative cFos : cJun heterodimer occurred on mixing of the two protein extracts at 4°C (Fig. 4A). cFos, itself, does not bind DNA. One possible explanation for these re-

sults is that, in contrast to the Jun proteins, cFos does not exist in solution as a stable dimer. Additionally, we propose that the cFos monomer has higher affinity to the Jun monomer than two Jun monomers have to each other.

These postulates predict that the heterodimer should have higher stability than the homodimer. To test this prediction, a small amount of the full-length trpE-vJun protein was preincubated with a subsaturating amount of cFos. To the resulting heterodimer, we added trpE-cJAV. After preincubation at various temperatures, the mixtures were cooled to 4°C, incubated with a TRE probe, and analyzed by mobility shift. As shown previously, upon direct incubation with cFos, the cJAV : cFos heterodimer formed at 4°C (Fig. 4B). When incubated with the vJun : cFos heterodimer, however, formation of the cJAV : cFos heterodimer required preincubation at 42°C. These results confirm that the Jun : Fos heterodimer has higher thermal stability, melting between 37°C and 42°C, than the Jun homodimers, which melt between 25°C and 37°C (see Fig. 5). Further evidence for the increased stability of the heterodimer is provided by the experiment showing that the Jun : Fos heterodimer is still active at 2.4 M urea, whereas the Jun homodimer loses activity at 1.5 M urea (Fig. 4C).

Leucine mutations do not affect interaction with Fos

We examined the leucine residues of cJun to determine whether they are involved in heterodimerization. To our

Figure 4. Interactions between Jun and Fos. (A) The effect of temperature on heterodimer formation between Jun and Fos. Partially purified trpE-cJAV (20 nM) was mixed with extracts of cFos-expressing insect cells (~4 nM Fos protein) and preincubated at the indicated temperatures for 30 min prior to addition of the TRE probe and incubation at 4°C. In this experiment and the following, a subsaturating amount of cFos was used (at saturating levels of cFos, essentially all of the cJun fusion protein is bound to cFos to form the heteromeric complex; not shown). The migration position of the protein-DNA complexes corresponding to the Jun homodimer and the Jun : Fos heterodimer are indicated. (B) trpE-vJun (5 nM) and cFos (4 nM) were mixed at 4°C and the resulting heteromeric complex was incubated with trpE-cJAV (20 nM) at the indicated temperature prior to chilling on ice and addition of the TRE probe. The same conditions were used in lanes 1–4 except that the indicated proteins were preincubated at 37°C. (C) The effect of urea on Jun and Jun : Fos complexes. trpE-cJAV (20 nM) and cFos (4 nM) were mixed and incubated at the indicated concentrations of urea (0–3.0 M) at 4°C for 30 min prior to addition of the TRE probe. (NS) Nonspecific complex formed by a contaminating bacterial protein.

surprise, all of the leucine mutants interacted efficiently with cFos, forming heterodimers that have the same DNA binding activity and mobility as the wild-type heterodimer (Fig. 5A). Most striking were the results obtained with the double-point mutant. Although neither this mutant nor cFos were capable of binding the TRE, when mixed together, a DNA-binding activity was generated that did not differ from that of the wild-type heterodimer.

Similar results were obtained by examining the ability of these mutants to bind cFos *in vivo*. A cFos expression vector (pSV-Fos; Schönthal et al. 1988) was cotransfected with expression vectors specifying production of wild-

type cJun and each of the leucine point mutants. Formation of stable complexes between these proteins and cFos was determined by their ability to coimmunoprecipitate with cFos after incubation of transfected cell extracts with anti-Fos antibodies (Chiu et al. 1988). All of the leucine mutants interacted with cFos as efficiently as the wild-type cJun protein (Fig. 5B).

These results indicate that the leucine residues altered by these mutations are not as important for formation of the Jun : Fos heterodimer as they are for the homodimer.

The importance of residues between the leucines.

As shall be discussed below, the behavior of the leucine mutants was inconsistent with the original version of the leucine zipper model, according to which all of the interaction and specificity is provided by the leucine residues. Therefore, we examined the contribution of other amino acid residues found within the leucine zipper region to Jun : Jun and Jun : Fos interaction. We were also concerned about the effects of the mutations on the most important function of cJun and cFos, namely transcriptional activation. For this purpose, we tested the ability of the various cJun mutants to activate transcription of the -73 Col·CAT reporter in the absence and presence of cotransfected cFos expression vector. As described previously (Chiu et al. 1988), wild-type cJun showed a considerable level of transcriptional activity in the absence of cFos, which was further increased by cotransfection of a cFos expression vector (Fig. 6). The M14 double-point mutant had very little activity by itself, but its activity was strongly stimulated by cFos, reaching half the level observed with wild-type cJun in the presence of cFos (see Fig. 8A, below). Another cJun mutant, I10 (see below), which is defective in DNA binding, did not activate, and together with cFos, the observed activity was not higher than the basal activity obtained with cFos alone. This basal activity probably results from the effect of cFos on endogenous Jun expression in F9 cells (Chiu et al. 1988). Thus, the results obtained in this transfection assay were in excellent agreement with the results of the *in vitro* DNA binding assays described above and clearly demonstrate that the M14 double-point mutant regains almost full biological activity in the presence of cFos.

We generated another series of mutants affecting amino acids lying between the leucines and tested their effect on the transcriptional activity of cJun in the absence and presence of cFos. In addition, we generated additional mutants affecting the last leucine in the zipper region. In M3, the lysine at position 288 was changed to a glutamate (see Fig. 8A). This mutant had higher transcriptional activity than wild-type cJun and was no longer stimulated by cFos. Conversion of valine 284 to phenylalanine (M4) also increased cJun's activity, but this mutant, in contrast to M3, was still responsive to cFos. Conversion of the fifth leucine (308) in the zipper region to phenylalanine (M5) led to a small decrease in cJun activity but did not affect the interaction with cFos. However, a double-point mutant (M15), in which both

Figure 5. Interaction between the leucine point mutants and Fos. (A) *In vitro* analysis of heterodimer formation. Wild-type and mutant trpE-cJDBD proteins (20 nM) were incubated in the absence or presence of cFos (4 nM) for 30 min prior to addition of the TRE probe (0.1 nM). Subsaturating amounts of cFos were used to allow detection of both homodimers and heterodimers. The migration positions of the various complexes are indicated. (F) cFos. (B) *In vivo* analysis of heterodimer formation. F9 cells were cotransfected with the various expression vectors specifying wild-type and mutant cJun proteins (8 μ g each) and a cFos expression vector (14 μ g), where indicated. Formation of Jun : Fos heterodimers was analyzed by immunoprecipitation of labeled cell extracts with anti-Fos antibodies. In the absence of cFos expression, these antibodies do not precipitate the cJun proteins (see cJ lane). The migration positions of the cJun and Fos proteins are indicated.

Figure 6. *Trans*-activation by cJun mutants in the absence and presence of cFos. F9 cells were transfected with 0.5 μ g of WT and various mutant RSV-cJun expression vectors in the presence and absence of 1.0 μ g of pSV-Fos, as indicated. Each precipitate also contained 2.0 μ g of the reporter plasmid $-73\text{Col} \cdot \text{CAT}$ and was brought to a total of 12.0 μ g of DNA with pUC18. The precipitate was removed after 6 hr and the cells were harvested 8 hr later and CAT activity determined. Shown are results of two separate CAT assays. For quantitation of the results and structure of mutants see Fig. 8A.

the second and fifth leucines were replaced by phenylalanines, exhibited very similar activity to M14: very low in the absence of cFos and similar to wild-type in the presence of cFos. Replacements of asparagine 291 and alanine 298 by phenylalanines (M16) also had a detrimental effect on the activity of cJun in the absence of cFos, but this mutant was also severely defective in *trans*-activation in the presence of cFos. Replacement of alanine 298 and valine 305 by phenylalanines (M17) led to a small decrease in activity in the absence of cFos but was identical to wild-type in the presence of cFos. All of the mutants were found to give rise to stable proteins in levels similar to that of the wild-type construct (data not shown).

A dominant negative Jun allele

The results described above suggest that binding of cJun and cFos to DNA requires their dimerization. On dimerization, the DNA recognition sites of the two monomers are likely to be oriented in a way favorable for binding of DNA. Therefore, a mutant, in which the space between the dimerization site and the DNA-binding site is disturbed by more or less than an integral number of helical repeats, should form stable dimers with the wild-type monomers that are incapable of binding DNA.

To test this prediction, we examined the properties of a cJun mutant with a duplication of five amino acids in the amino-terminal part of the leucine repeat region. This insertion creates part of an additional heptad repeat, shifting the first two heptads and the putative DNA-binding site out of alignment with the four carboxy-terminal heptad repeats (Fig. 7A). When tested *in vivo*, this mutant (I10) not only failed to activate transcription but actually inhibited transcriptional activation by the wild-type cJun protein (Fig. 7B). In contrast, a deletion mutant, which lacks the *trans*-activation domain but contains a wild-type DNA-binding domain (cJDBD) was an ineffective inhibitor. Although it exhibited no DNA-binding activity of its own, the insertion

mutant interacted with cJun and cFos *in vitro* and dramatically inhibited their binding to DNA (Fig. 7C). As expected from the higher affinity of cFos to cJun, the Jun : Fos heterodimer is more sensitive to inhibition by the insertion mutant than the cJun homodimer, despite its higher stability. These results further support the contention that the region containing the leucine repeats is involved in both homo- and heterodimerization.

Discussion

Mechanism and role of dimerization

As expected from the palindromic nature of its recognition site and similarity to GCN4, cJun was demonstrated to interact with the DNA as a dimer (Halazonetis et al. 1988; Nakabeppu et al. 1988). The experiments described here show that the Jun proteins exist as stable dimers prior to binding of DNA. They also provide evidence suggesting that cFos exists as either a monomer or a very unstable dimer. On the other hand, the Jun : Fos heterodimers are more stable than the Jun : Jun homodimers, as indicated by their respective melting temperatures: 37–42°C and 25–37°C. The lower melting temperature of Jun homodimers may explain why some investigators failed to detect the binding of the Jun homodimer to DNA in mobility-shift experiments performed at room temperature (Sassone-Corsi et al. 1988; Turner and Tjian 1989). In addition, the Jun : Fos heterodimer is significantly more resistant to urea than the Jun homodimer. Extrapolation of these *in vitro* observations suggests that within the cell, the Jun : Fos heterodimer is exceedingly more stable than the Jun : Jun homodimer and may form at lower protein concentrations. Because mutations that reduce dimerization interfere with DNA binding, it appears that the active cJun DNA-binding species is a dimer. Thus, the differential stability of the Jun and Fos complexes explains the earlier observations indicating that the Jun : Fos heterodimer exhibits higher affinity to the TRE than the cJun homodimer, whereas

Figure 7. A dominant negative Jun mutant. (A) The internal sequence duplication that generated the I10 mutant. (B) I10 inhibits *trans*-activation by wild-type cJun. F9 cells were cotransfected with 2 μ g of -73Col · CAT or -63Col · CAT (lacking an AP-1 site) and the indicated amounts of the wild-type cJun or the I10 and cJDBD mutant expression vectors. Transfected cell extracts were analyzed for CAT activity. An autoradiogram of a typical TLC plate is shown. (C) I10 inhibits DNA binding by Jun homodimers and Jun : Fos heterodimers. trpE-cJAV (10 nM) was mixed with subsaturating amount of cFos (3 nM) in the presence of increasing amounts (20–200 nM) of trpE-I10 at 37°C and DNA binding activity was determined after incubation with the TRE probe at 4°C and resolution of the complexes by electrophoresis. In addition, I10 alone (200 nM) was also incubated with the TRE probe (I10 lane).

cFos, by itself, does not bind to the TRE. This differential stability resulting in differences in the DNA binding activities of the two complexes may play an important role in cellular regulation, as the composition of the AP-1 complex may change from one moment to another. Such changes are attributable to the differential kinetics of *c-jun* and *c-fos* induction and mRNA accumulation (Brenner et al. 1989).

Leucine zipper versus a coiled coil

Landschulz et al. (1988) proposed that the conserved leucine zipper motif, which is present within the DNA-binding domains of C/EBP, GCN4, Jun, and Fos, could be responsible for their dimerization and, in the case of

Jun and Fos, may also mediate heterodimer formation. Mutational analysis of the leucine repeat in cJun indicates that although several of the leucines of the heptad repeat are important for formation of Jun homodimers, they can be mutated without significant effect on the formation and function of Jun : Fos heterodimers. The effect of the leucine mutations on dimerization of cJun is inferred from their effect on formation of protein–DNA complexes with intermediate mobility upon mixing of full-length wild-type trpE–vJun protein with truncated fusion proteins that contain these mutations. Although these results, based on DNA binding assays, can also be interpreted to suggest that the leucine residues affected by these mutations are directly interacting with the DNA, we consider this interpretation unlikely because the Jun : Fos heterodimers formed by these mutants exhibited identical DNA binding activity to the wild-type heterodimer.

A single point mutation in cJun is, in fact, equivalent to two point mutations when the homodimer is concerned. It is noteworthy, however, that, although single point mutations have significant effects on homodimerization and *trans*-activation by cJun, double point mutations affecting two leucines have little or no effect on heterodimerization and *trans*-activation in the presence of cFos. The lack of effect of the leucine mutations on binding of cJun to cFos cannot be explained easily by the original leucine zipper model, according to which the predominant force stabilizing the dimers is provided by hydrophobic interactions between interdigitated leucine residues. Furthermore, we find that residues between the leucines play a role that is as important in homodimerization as that of the leucines themselves. Our results are best explained by the model proposed recently by O'Shea et al. (1989a). By studying the interaction between synthetic peptides corresponding to the leucine repeat of GCN4, these workers concluded that these peptides dimerize as a parallel coiled coil. The leucine repeat region of the Jun proteins, like that of GCN4, contains an additional repeat of hydrophobic residues at the 'a' position of an idealized α helix interspersed within the leucine heptad repeat (Fig. 8B). This 4-3 repeat is the hallmark of coiled coils (Cohen and Parry 1986). Indeed, we find that a double point mutation affecting the third and fourth residues in the a position has a detrimental effect on the activity of both Jun : Jun homodimers and Jun : Fos heterodimers. However, a double point mutation affecting the fourth and fifth residues at the a position do not have a major effect on activity. Therefore, asparagine 291 present at the third position appears to play an essential role in both homo- and heterodimerization. Interestingly, this asparagine residue is conserved among all members of the Jun family, including GCN4 and CREB, that are capable of homodimerization (Vogt and Bos 1989). Although this residue is the least hydrophobic of all the residues at the a position, it may play a very important role in conferring specificity to the hydrophobic interactions holding these complexes together.

In contrast to cJun, cFos does not contain a hydro-

Figure 8. (A) The sequence of the leucine zipper region of wild-type cJun and the various point mutants. Relative transcriptional activity of the various cJun proteins in the presence and absence of cFos is shown and represents the average of three different determinations. (ND) Not determined. (B) The leucine zipper regions of cJun (Angel et al. 1988a) and cFos (Van Beveren et al. 1983) are presented as parallel coiled coils. The two regions are viewed down the helix axis from the amino-terminal end of the first heptad repeat. All of the amino acids occupying the a, d, e, and g positions are shown, whereas only the amino acids occupying the first heptad are shown at the other positions.

phobic repeat at the a position and instead, contains two positively charged and two polar residues (Fig. 8B). The decreased number of hydrophobic interactions and the likely electrostatic repulsions between the glutamates at the internal 'e' and 'g' positions may explain why cFos does not form stable homodimers. On the other hand, the increased stability of the Jun : Fos heterodimers may be attributable to ionic interactions between positively charged residues at the e and g positions of cJun with negatively charged residues at the equivalent positions of cFos. The number of such interactions is lower in Jun : Jun homodimers. In support of such interactions, we find that replacement of lysine 288 of cJun with glutamate increases the activity of the Jun : Jun homodimer while decreasing the interaction between cJun and cFos both in vivo (Fig. 6) and in vitro, using bacterially expressed Jun protein (data not shown). Thus, the coiled-coil model also offers an explanation for the different stabilities of the various cJun and cFos complexes. Because of the decreased number of potential ionic interactions, the Jun : Jun dimer is more dependent on hydrophobic interactions and therefore is less tolerant than the Jun : Fos heterodimer of the substitution of leucines with the bulkier phenylalanine or the polar histidine residues.

Substitutions of some of the repeated leucines in cFos by arginine, isoleucine, valine, or glycine residues were shown to interfere with its binding to cJun. Although

these findings were interpreted in favor of the original leucine zipper hypothesis (Kouzarides and Ziff 1988; Gentz et al. 1989; Schuermann et al. 1989; Turner and Tjian 1989), it is noteworthy that the majority of single changes did not have very significant effects on the interaction between Fos and Jun; therefore, these results are more consistent with the coiled-coil model. According to this model, replacement of leucines in cFos by either the smaller valine or positively charged arginine residues can have detrimental effects on hydrophobic interactions with cJun, leading to destabilization of the Jun : Fos heterodimer. Additional evidence in favor of a coiled-coil interaction was recently obtained by O'Shea et al. (1989b), analyzing the dimerization of synthetic peptides containing the leucine heptad repeats of Jun and Fos.

Generation of trans-dominant inhibitor

Interestingly, immediately upstream of their leucine repeat, GCN4, cJun, and cFos contain a highly conserved basic region (Vogt et al. 1987; Kouzarides and Ziff 1988; Vogt and Bos 1989). The presence of this motif in three proteins that interact with the same target sequence suggests that it may be the region involved in direct interaction with the DNA. This role is supported by mutations in this region of cFos, which do not interfere with its binding to cJun but which impair DNA binding

(Kouzarides and Ziff 1988; Turner and Tjian 1989). The results discussed above regarding the importance of dimerization mediated by the hydrophobic repeats suggest that dimerization orients the basic regions of cJun and cFos in a way favorable for DNA binding. Support for this model was provided by the analysis of the I10 insertion mutant, in which the spacing between the leucine zipper and the basic motif was altered by more than an integral number of α -helical repeats. As expected, this mutant did not bind DNA and activate transcription. However, it was found to act as a dominant negative (Herskowitz 1987), probably because of its ability to interact with wild-type monomers to form heterodimers in which the spatial orientation of the two recognition sites is unfavorable to DNA binding. This insertion mutant provides further and significant support to the idea that the region containing the leucine zipper cJun is the site used for dimerization. In contrast to the leucine mutants in cJun and cFos that either have no effect on or lead to loss of the dimerization function, this mutant retains the ability to dimerize with the wild-type proteins and exhibits a distinct phenotype.

This and similar dominant negative Jun alleles will be useful for investigating the role of the AP-1 complex in various processes such as cellular proliferation, differentiation, and malignant transformation. In addition, we believe that the approach used to construct this mutant could be applicable to construction of dominant-acting negative mutants of many other DNA-binding proteins, which require dimerization for their activity, by simply changing the spatial arrangement of the dimerization and DNA recognition regions.

Materials and methods

Cells and transfections

F9 thymidine kinase negative embryonal carcinoma stem cells were grown in F12-Dulbecco's modified Eagle medium (DMEM) (1:1) supplemented with 10% FCS and 10^{-4} M β -mercaptoethanol. As described in Angel et al. (1988b) and Chiu et al. (1988), each 100-mm culture dish was incubated with a calcium phosphate/DNA coprecipitate containing the various expression and reporter plasmids at varying amounts, as indicated in the figure legends. Coprecipitates were removed after 6 hr and cells were harvested either 6–8 hr after removal of precipitate in the cJun:cFos cotransfection experiments (see Angel et al. 1989) or 16–18 hr after removal of precipitate in other experiments.

Immunoprecipitation

For immunoprecipitations, cell proteins were labeled for 60–90 min using L-[35 S]methionine (800 μ Ci/ml, Amersham) in methionine-free DMEM. Cells were swelled and homogenized, and crude nuclei was isolated in 20 mM HEPES–NaOH (pH 7.9), 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM DTT, 0.1% NP-40, and 1 mM PMSF. Crude nuclei were washed and then lysed in RIPA buffer (see Chiu et al. 1988). Fos protein complexes were immunopurified using rabbit antiserum directed against trpE–Fos, kindly provided by J. Feramisco (University of California at San Diego). Jun proteins were immunoprecipitated using rabbit antiserum raised against the DNA-binding domain of cJun, as described in Angel et al. (1988b).

Mobility-shifts assay

In general, the modifications of Welch et al. (1989) and Carthew et al. (1985) of the original protocol of Fried and Crothers (1981) were followed. Binding reactions were carried out at 4°C for 30 min in 20 μ l of 12% glycerol, 12 mM HEPES–NaOH (pH 7.9), 5 mM MgCl₂, 4 mM Tris–Cl (pH 7.9), 60 mM KCl, 0.6 mM EDTA, and 0.6 mM DTT. For mixing experiments, samples were preincubated in the absence of DNA at 37°C for 30 min or at other temperatures as specified, before cooling and incubation with DNA at 4°C. Samples were loaded onto 5% polyacrylamide gels that had been prerun for 3–4 hr at 4°C in 0.25 $\times$ TBE buffer [22.5 mM Tris–borate and 0.5 mM EDTA (pH 8.3)]. Protein–DNA complexes were resolved by electrophoresis at 4°C. The probe used in these experiments was a 173-bp EcoRI–PvuI fragment from TRE-TKCAT (Angel et al. 1987) containing the TRE, labeled by filling in with avian myeloblastosis virus reverse transcriptase.

TrpE fusion proteins used in these experiments were partially purified, renatured, and quantitated by Western analysis and silver staining (see Angel et al. 1988a). Lysates of SF9 cells infected with the Fos-expressing baculovirus recombinant were kindly provided by Sadhana Agarwahl and Tom Roberts. These extracts contained ~20 ng Fos protein per microliter, as determined by Western blotting.

Plasmid constructions

Mutants in the leucine zipper of cJun were generated using the indicated oligodeoxynucleotides.

M8, M9, M14, Ser, and M15: 5'-GAAAAGCTT^CAAAGCGCAGAAATTCGGAGC^ACGGCTC-3'
 M3: 5'-GCCCGGCTC^CAGGAGAAGGTGAAGACCTTG^AAGCTCAGAAC-3'
 M5, M15: 5'-GTGGCACAGTTTAAGCAGAAAGTC-3'
 M4: 5'-CTGGAGGAAAAATTTAAAGCTTGAAGCTCAGTTCTCGGAGCTGGCG-3'
 M16: 5'-TTGAAAGCTCAGTTTTCGGAGCTGGCGTCCAGCTTCAACATGCTCA-3'
 M17: 5'-CGGTCCACGTTTAACTGCTCAGGGAACAGTTCCGACAGCTTAAAC-3'

The insertion mutant I10 was generated during the course of mutagenesis by 'slipping' of the mutagenic oligodeoxynucleotide. Site-directed mutagenesis was done according to Angel et al. (1988b), except that pBluescript (Stratagene) was used instead of M13mp18. The expression and construction of the trpE fusion proteins trpE–vJun, trpE–cJDBD, and trpE–cJAVa are described in Angel et al. (1988a). The point mutants of cJun were cloned into pATH2 by inserting the *NaeI*/*Bam*HI fragment of the mutants into the *SmaI*/*Bam*HI site of pATH2 and are equivalent to trpE–cJDBD. –73Col·CAT –63Col·CAT, and pSV-Fos are described in Angel et al. (1988a) and Schöththal et al. (1988).

Note added in proof

Scatchard plot analysis indicates that both the Jun:Jun and Jun:Fos dimers have the same affinity ($\sim 2 \times 10^{-10}$ M) for the AP1 consensus sequence (TGAGTCAG) as quantitated by mobility shift experiments performed at 4°C. Hence, the increased binding activity of the heterodimer is likely to be the result of its increased thermostability.

Acknowledgments

We thank J. Feramisco, J. Lipsick, C.I. Buchman, and B. Boyle for providing antiFos antibodies, trpE and T7 expression vectors, help with band-shift assays, and advice on immunoprecipitations, respectively. We are also indebted to S. Argarwahl, B. Spiegelman, and T.M. Roberts for the generous supply of baculovirus-fos extracts, and P. Kim for sharing his unpublished

results and helpful discussions. Finally, we thank K. Hattori and F. Mercurio for oligodeoxynucleotide synthesis and D. Caruso and P. Bucher for typing and editing this manuscript. This work was supported by grants from the National Institutes of Health, Department of Energy, California Task Force on AIDS, and Council for Tobacco Research. P.A. was supported by a postdoctoral fellowship from the Deutscher Akademischer Austauschdienst.

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Different requirements for formation of Jun: Jun and Jun: Fos complexes.

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Genes Dev. 1989, **3**:

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