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Preferential Heterodimer Formation by Isolated Leucine Zippers from Fos and Jun

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The products of the nuclear oncogenes *fos* and *jun* are known to form heterodimers that bind to DNA and modulate transcription. Both proteins contain a leucine zipper that is important for heterodimer formation. Peptides corresponding to these leucine zippers were synthesized. When mixed, these peptides preferentially form heterodimers over homodimers by at least 1000-fold. Both homodimers and the heterodimer are parallel α helices. The leucine zipper regions from Fos and Jun therefore correspond to autonomous helical dimerization sites that are likely to be short coiled coils, and these regions are sufficient to determine the specificity of interaction between Fos and Jun. The Fos leucine zipper forms a relatively unstable homodimer. Instability of homodimers provides a thermodynamic driving force for preferential heterodimer formation.

FOS AND JUN EACH CONTAIN A LEUcine zipper region in which five leucine residues are spaced periodically at every seventh residue (*1, 2*). The leucine zipper regions of these proteins are known to be important for heterodimer formation (*3–7*), as originally proposed in the leucine zipper model (*2*). Jun can also form a homodimer (*3, 4, 8*), whereas there has been no evidence for dimerization of Fos (*3, 4, 7, 8*).

A synthetic peptide, corresponding to the isolated leucine zipper of the yeast transcriptional activator GCN4, self-associates to form a very stable dimer of parallel α helices, probably as a short, parallel coiled coil (*9*). Both Fos and Jun show sequence homology to GCN4 in regions within and adjacent to the leucine zipper (*10*). We investigated whether the leucine zipper regions of Fos and Jun are sufficient by themselves to mediate specific heterodimer formation.

Peptides corresponding to the leucine zippers (Fos-p1N and Jun-p1N) were synthesized (*11*). In each case, a Cys residue (to permit S–S bond formation) and two Gly residues (for flexibility) were added at the NH_2-terminus (Fig. 1A). Equimolar amounts of the peptides were mixed in a redox buffer (which favors S–S bond formation), and the amounts of S–S homodimers and heterodimer were measured by high-performance liquid chromatography (HPLC).

The results (Fig. 1B) show that only heterodimers are detected. This reaction is at equilibrium since an equimolar mixture of S–S Fos-p1N and Jun-p1N homodimers also rearranges to give only heterodimer (Fig. 1C). In both cases, a homodimer would have been detected if it were present at 1/1000 the concentration of the heterodimer (*12*). Only when oxidation of the peptides is performed in conditions where the peptides are unfolded [6.8*M* guanidine hydrochloride (GuHCl)] are homodimers observed.

Circular dichroism (CD) spectra of all three S–S dimers display minima at 208 and 222 nm (Fig. 2A), indicating that they are predominantly α helical (*13*). All of the S–S dimers are also resistant to thermal denaturation (*14*). Thus the leucine zippers of Fos and Jun, like that of GCN4 (*9*), appear to be helical dimerization sites (*15*).

The orientation of the helices in the peptide dimers was determined by monitoring the stability of the S–S species as a function of peptide concentration. The S–S bond joins the peptides in a parallel manner. If the helix orientation is parallel, then the stability of the S–S dimers should be independent of peptide concentration. If the orientation is antiparallel, S–S dimers should associate and show concentration-dependent stability. [Such higher order association is observed when leucine zippers of GCN4 are forced to be antiparallel (*16*).] The stabilities of the S–S Fos-p1N homodimer, Jun-p1N homodimer, and Fos-p1N–Jun-p1N heterodimer are independent of peptide concentration [(*14*) Fig. 2B], indicating that the helix

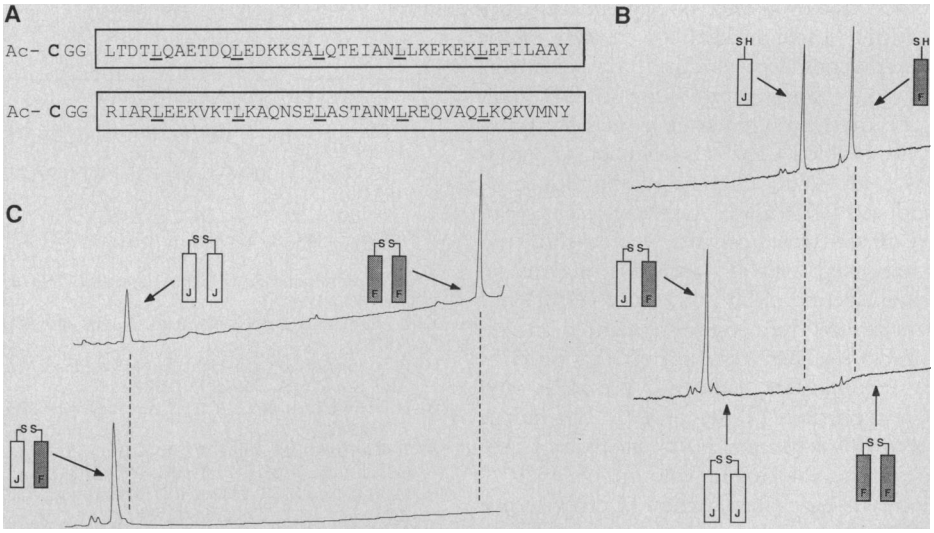

Fig. 1. Preferential heterodimer formation by isolated leucine zipper peptides from Fos and Jun. (**A**) Sequences of the peptides (Fos-p1N, top, and Jun-p1N, bottom) used in the S–S assay (*11*). Residues defining the leucine repeat are underlined. (**B**) HPLC analyses of reduced Fos-p1N + reduced Jun-p1N in 2 m*M* HCl (top) and redox buffer (bottom) (*29*). In redox buffer (where reshuffling of S–S bonds is permitted) only the heterodimer is detected (*12*). The arrows at the bottom indicate the elution positions for the two homodimers. (**C**) HPLC analyses of the S–S Fos-p1N homodimer + Jun-p1N homodimer in 2 m*M* HCl (top) and in redox buffer (bottom) (*29*). Only heterodimer is observed in redox buffer, confirming that the reaction mixtures are at equilibrium. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.

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orientation is parallel in all cases (*17*).

The stability of the Jun-p1N homodimer is comparable to that of the heterodimer (Fig. 2B). The absence of Jun homodimer in the previously described redox experiments (Fig. 1) can be understood in terms of the relative instability (Fig. 2B) of the Fos homodimer (*18*). A thermodynamic consequence of homodimer instability is to shift the equilibrium toward heterodimer formation (Fig. 2C).

Because there has not been evidence for dimerization of the Fos protein (*3, 4, 7, 8*), we also studied the Fos leucine zipper sequence without appended Cys-Gly-Gly residues (Fos-p1). Substantial α-helical content (Fig. 3A) and concentration-dependent stability in the micromolar concentration range (Fig. 3B) were observed with Fos-p1, indicating that it self-associates in the absence of S–S bonding. Moreover, sedimentation equilibrium studies indicate that Fos-p1 is a dimer at concentrations greater than 40 μ*M* (*19*).

Previous studies of Fos dimerization (*3, 4, 7, 8*) used in vitro transcription-translation reactions that typically produce nanomolar concentrations of protein or less (*20*). We estimate that the dissociation constant for dimerization of Fos-p1 is 6 μ*M* at *p*H 7 (*21*), a concentration much higher than those normally obtained with in vitro translation reactions. Failures to detect dimerization of Fos may therefore be the result of low protein concentrations. Alternatively, there may be an active mechanism to prevent dimerization of the Fos protein.

Our working model (*9*) for the structure of the Fos-Jun leucine zipper complex is that of a parallel, heterodimeric, coiled coil (Fig. 4). Coiled coils result from two right-handed α helices wrapping around one another with a slight left-handed superhelical twist (*22*), and represent the exception to antiparallel packing of α helices in proteins. In an ideal α helix, there are 3.6 residues per turn, whereas there are only 3.5 residues per turn in a coiled coil. Residues in a heptad repeat therefore align integrally every two turns in a coiled coil. The interaction surface between helices in a coiled coil is formed from two interspersed heptad repeats of hydrophobic residues, the 4-3 repeat (*23*).

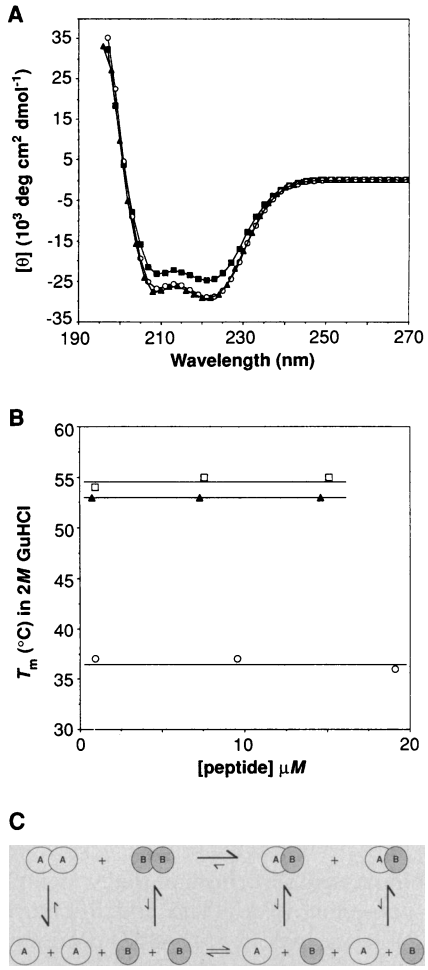

Fig. 2. (**A**) Circular dichroism (CD) spectra of S–S Fos-p1N homodimer (○), Jun-p1N homodimer (▲), and Fos-p1N–Jun-p1N heterodimer (■) at 0°C (*30*). The values for $[\theta]_{222}$ indicate that the peptides are >75% helical at 0°C (*13*). (**B**) Midpoint of the thermal unfolding transition (T_m) versus peptide concentration for the homodimers (○, Fos, and ▲, Jun) and the heterodimer (□) (*30*). Stability is independent of concentration, indicating that the helix orientation within each dimer is parallel. (**C**) Thermodynamic cycle illustrating how instability of a homodimer contributes to the specificity of heterodimer formation. The stability of one homodimer is shown to be equal to that of the heterodimer, whereas the second homodimer is relatively unstable.

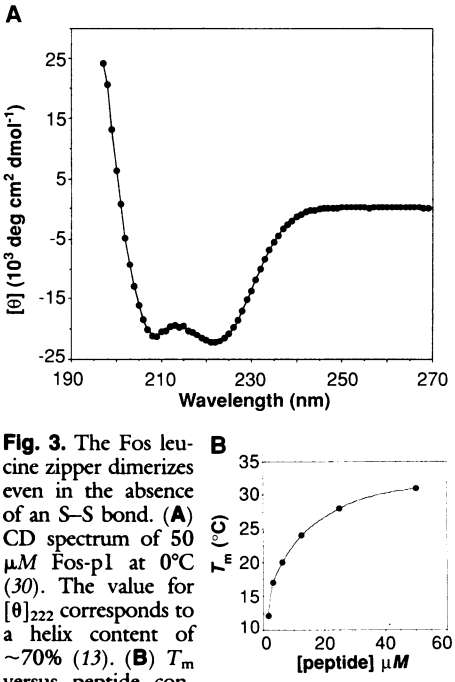

Fig. 3. The Fos leucine zipper dimerizes even in the absence of an S–S bond. (**A**) CD spectrum of 50 μ*M* Fos-p1 at 0°C (*30*). The value for $[\theta]_{222}$ corresponds to a helix content of ~70% (*13*). (**B**) T_m versus peptide concentration for Fos-p1 (*30*). Concentration-dependent stability is observed, indicating that the peptide self-associates [see also (*19*)].

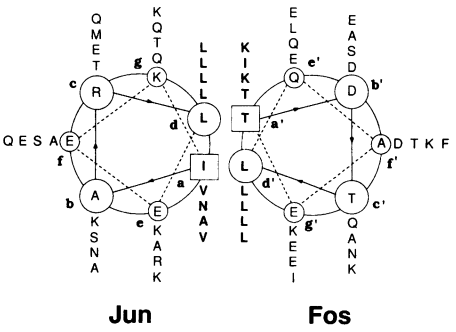

Fig. 4. Fos-Jun leucine zipper heterodimer modeled as a parallel coiled coil. A view down the helix axis from the NH_2-terminus is shown (*23*). Hydrophobic interactions (the 4-3 repeat) that contribute to stability are shown as **a** and **d**. Electrostatic interactions that may contribute to the specificity of interaction (*25*) are shown as **e** and **g**′ (also **e**′ and **g**). Residues in the first turn of each helix are enclosed and residues in subsequent turns of the helix are shown in order.

The 4-3 repeat is made up by residues at positions **a** and **d**, in the nomenclature for coiled coils [Fig. 4; see also figure 5A of (*9*)]. Fos appears to violate the hydrophobic nature of the 4-3 repeat, since it contains two Lys residues at the **a** position (Fig. 4). However, Lys residues are often found at the **a** position of myosin coiled coils, presumably because the methylene groups of the Lys side chain can make hydrophobic contacts while the charge is exposed to solvent (*24*).

Interactions within the 4-3 repeat (positions **a** and **d**) and interhelical charge interactions (between positions **e** and **g**′, or **e**′ and **g** in Fig. 4) are likely to be important for determining the specificity of interaction between Fos and Jun. In addition, the Fos leucine zipper has a net charge of negative four, whereas the Jun leucine zipper has a net charge of positive four. Electrostatic effects are likely both to stabilize the heterodimer and destabilize the homodimers (*25*).

The Fos-Jun leucine zipper peptides probably represent the simplest model system known for specific protein-protein interactions. Both Fos and Jun are members of larger families of related proteins, containing leucine zippers, that are known to form DNA-binding heterodimers (*4, 8, 26, 27*). In addition, the expression of Fos and Jun is temporally regulated (*26, 28*). Our results suggest that additional dimensions of regulation and diversity of transcription control are provided by specific interactions between leucine zippers.

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11. Reviewed by S. B. H. Kent, *Annu. Rev. Biochem.* **57**, 957 (1988). The peptide synthesis and purification procedures used here have been described (*9*). Jun-p1N contains residues 285 to 324 from the c-Jun protein (*1*); Fos-p1N and Fos-p1 contain residues 162 to 201 from the v-Fos protein (*1*). The COOH-terminal His in each peptide was replaced with a Tyr to permit peptide concentration measurements by absorbance at 275.5 nm in 6*M* GuHCl [H. Edelhoch, *Biochemistry* **6**, 1948 (1967)]. All peptides were acetylated at the NH_2-terminus and the COOH-terminus was left free. Fast-atom-bombardment mass spectrometry (FAB-MS) yielded molecular weights M_r of 4892.8 for Jun-p1N (calculated, 4892.9), 4826.3 for Fos-p1N (calculated, 4826.6), and 4609.5 for Fos-p1 (calculated, 4609.4).
12. Although there might be slight differences in the propensity to form an S–S bond in the heterodimer and homodimers, the Gly-Gly linker should provide enough flexibility to minimize these differences [see (*9*)].
13. The inference of α helices can be made from CD spectra obtained in aqueous solution with much higher confidence than for other secondary structures; see R. W. Woody, in *The Peptides*, S. Udenfriend, J. Meienhofer, J. R. Hruby, Eds. (Academic Press, New York, 1985), vol. 7, pp. 15–114; Y.-H. Chen, J. T. Yang, K. H. Chau, *Biochemistry* **13**, 3350 (1974).
14. In the absence of GuHCl, S–S Fos-p1N homodimer, Jun-p1N homodimer, and Fos-p1N–Jun-p1N heterodimer have T_m values of 59°, 69°, and 71°C, respectively, that are independent of peptide concentration from ~0.7 to ~15 μ*M*. The melting transition is reversible for the Fos-p1N homodimer, but some hysteresis is observed with the Jun-p1N homodimer and the heterodimer. In the presence of 2*M* GuHCl (Fig. 2B), reversible thermal unfolding transitions are observed for all three species.
15. Preliminary results suggest that the isolated Jun leucine zipper peptide, without appended Cys-Gly-Gly residues, may not form a stable unique dimer. Thus, regions outside the leucine zipper may be important for homodimer formation by the intact Jun protein. Nevertheless, isolated Fos and Jun leucine zippers form a specific heterodimer even in the absence of an S–S bond (unpublished results): (i) An equimolar mixture of the Fos and Jun peptides has a CD spectrum that is not equivalent to the simple sum of the individual peptide spectra. Such a mixture exhibits concentration-dependent stability and is more stable than either peptide alone; and (ii) Sedimentation equilibrium experiments (4°C) indicate that an equimolar mixture of the Fos and Jun peptides exists as a single species with M_r = 9400 in the concentration range from 20 μ*M* to 0.25 m*M* (expected M_r for heterodimer = 9285). Note that any instability of the Jun homodimer not detected here would enhance heterodimer formation even further (see Fig. 2C).
16. It is likely that in the antiparallel orientation, S–S dimers associate to form higher order species in which individual helices can pair in a parallel manner (*9*).
17. Others have concluded that the leucine repeat regions in the Fos-Jun heterodimer are parallel, based on mutagenesis studies [(*3*, *5*); see also W. H. Landschulz, P. F. Johnson, S. L. McKnight, *Science* **243**, 1681 (1989)].
18. In experiments with intact proteins, the Fos-Jun heterodimer is more stable than the Jun homodimer under conditions where the Fos homodimer is not detected (T. Smeal and M. Karin, personal communication), suggesting that the instability of the Fos homodimer also contributes to the specificity of heterodimer formation with the intact proteins. The tropomyosin heterodimer from *R. esculenta* appears to be favored thermodynamically in an analogous manner (S. S. Lehter, Y. Qian, S. Hvidt, personal communication).
19. The M_r for Fos-p1 is 9300 (constant over the concentration range from 40 μ*M* to 0.5 m*M*) as determined by sedimentation equilibrium studies at 4°C, demonstrating that it is dimeric. A Model E centrifuge was used at 52,000 rpm to make measurements in phosphate buffered saline (PBS: 0.15*M* NaCl, 10 m*M* phosphate, *p*H 7.0) as described in P. Gaceffa, C.-L. A. Wang, W. F. Stafford [*J. Biol. Chem.* **263**, 14196 (1988)].
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21. If we assume that the Fos-p1 dimer is the only species that contributes to the helical signal at 222 nm, the dissociation constant can be estimated from the concentration dependence of $[\theta]_{222}$. The dissociation constant in PBS estimated in this manner is 3.2 μ*M* at 0°C and 5.6 μ*M* at 25°C. These values represent upper limits since at low concentrations the peptide adheres to glass.
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25. The stability of the Fos-p1 dimer is *p*H dependent, with maximum stability at low *p*H, suggesting that ionized acidic residues destabilize the Fos-p1 homodimer (E. K. O'Shea and P. S. Kim, unpublished results). Residues at the **e** and **g** positions in Fos are likely to contribute to this destabilization.
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29. The peptides were mixed and incubated in either 2 m*M* HCl (control) or in redox buffer (250 μ*M* oxidized glutathione, 250 μ*M* reduced glutathione, 0.2*M* KCl, 0.1*M* tris, 1 m*M* EDTA, *p*H 8.7) for 1 hour at 23°C at a total reduced peptide concentration of 30 μ*M* and then analyzed by HPLC (an acetonitrile-H_2O gradient with segments of 0.1% and 0.2% acetonitrile increase per minute was used in the presence of 0.1% trifluoroacetic acid with an analytical Bio-Rad or Vydac C18 column). The redox conditions were chosen to minimize the amount of mixed disulfide (that is, between peptide and glutathione) and reduced peptide, while maximizing the amount of peptide S–S species. The redox experiments were repeated at 4° and 37°C with identical results.
30. CD spectra were recorded in a 1-mm pathlength cell at a total peptide concentration of 50 μ*M* in PBS. All peptide concentrations were determined by tyrosine absorbance (*11*). Thermal unfolding studies were performed in PBS with a 1-cm path length cell. An Aviv Model 60DS CD spectrophotometer with an HP Model 89100A Peltier temperature control unit was used.
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NMDA Antagonists Differentiate Epileptogenesis from Seizure Expression in an in Vitro Model

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In an electrographic model of seizures in the hippocampal slice, both of the *N*-methyl-D-aspartate (NMDA) antagonists 2-amino-5-phosphonovaleric acid and 5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine maleate (MK-801) prevented the progressive development of seizures but did not block previously induced seizures. Thus, a process dependent on the NMDA receptor–ionophore complex establishes a long-lasting, seizure-prone state; thereafter the seizures depend on non-NMDA receptor–ionophore mechanisms. This suggests that there is an important distinction between epileptogenesis and seizure expression and between antiepileptogenic and anticonvulsant pharmacological agents.

TO BETTER UNDERSTAND AND TREAT human epilepsy, it is important to understand not only the expression of individual seizures but the development of a lasting seizure-prone state, that is, epileptogenesis. In epilepsy, excessive electrical activity in one region of the brain can induce

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