Mechanism of Specificity in the Fos-Jun Oncoprotein Heterodimer

Erin K. O'Shea, *† Rheba Rutkowski, † and Peter S. Kim †*†

*Howard Hughes Medical Institute
Whitehead Institute for Biomedical Research
Cambridge, Massachusetts 02142
†Department of Chemistry
†Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

Fos and Jun, the protein products of the nuclear proto-oncogenes c-fos and c-jun, associate preferentially to form a heterodimer that binds to DNA and modulates transcription of a wide variety of genes in response to mitogenic stimuli. Both Fos and Jun contain a single leucine zipper region. Previous studies have shown that the leucine zippers of Fos and Jun are necessary and sufficient to mediate preferential heterodimer formation. The leucine zipper regions from Fos and Jun are also known to fold autonomously, most likely as two-stranded, parallel coiled coils. We show here that 8 amino acids from Fos and from Jun are sufficient to mediate preferential heterodimer formation. The leucine zipper regions from Fos and Jun are described here differ slightly from those used previously (O'Shea et al., 1989b). The Fos and Jun leucine zipper peptides preferentially form a heterodimer, demonstrating that these sequences are sufficient to mediate preferential heterodimer formation. The Fos-Jun leucine zipper peptides provide a simple system for studying the specificity underlying a protein–protein interaction: two helices that prefer to interact with each other rather than with themselves. The structure of both components of this system is simple, and a relatively small region of primary sequence contains the determinants for specific heterodimer formation (O'Shea et al., 1989b). Furthermore, the preferential interaction between the Fos and Jun leucine zipper peptides mimics the specificity seen with the intact proteins (Smeal et al., 1989).

The goal of this study is to understand the mechanism of specificity in the Fos–Jun peptide system and to outline the basis for preferential heterodimer formation in both structural and energetic terms.

We demonstrate that 8 amino acids from Fos and from Jun are sufficient to mediate preferential heterodimer formation. Furthermore, the thermodynamic driving force for preferential heterodimer formation appears to be destabilization of the Fos homodimer by acidic residues. The charged residues in the Fos leucine zipper that are important for specificity are those known to be adjacent to the hydrophobic interface of coiled-coil proteins.

Results

pH Dependence of Stability in the Fos and Jun Leucine Zipper Peptides

The relative stabilities of the Fos and Jun peptide heterodimer and homodimers (Table 1) indicate that the Fos peptide homodimer is substantially less stable than the Fos–Jun heterodimer and the Jun homodimer (O'Shea et al., 1989b). The Fos and Jun peptides used in the studies described here differ slightly from those used previously (O'Shea et al., 1989b); the new peptides are shorter, lacking both an N-terminal residue and 7 amino acids following...
the last leucine of the leucine repeat (see Experimental Procedures). As a first step toward identifying sources of stabilization and destabilization in the new Fos and Jun leucine zipper dimers, the pH dependence of the disulfide-bonded peptide pairs was studied (Figure 1A).

The thermal stability of the Fos and Jun homodimers is pH dependent in a dramatic way; the \( T_m \) (melting temperature determined from the midpoint of the thermal unfolding transition, see Experimental Procedures) of the Fos homodimer increases \( \sim 40^\circ C \) from neutral to acidic pH, and the stability of the Jun homodimer increases \( \sim 20^\circ C \) from neutral to basic pH (Figure 1A). The stability of the heterodimer changes approximately as expected from an average of the pH dependences for the homodimers. This result suggests that the Fos–Jun leucine zipper lacks dominant stabilizing electrostatic interactions that are unique to the heterodimer; in such a case, a bell-shaped pH dependence curve would be expected. In contrast, the stability of the heterodimer increases at acidic pH values, suggesting that intrahelical repulsion (expected from the Fos sequence) is strong.

Electrostatic effects provide a possible explanation for preferential heterodimer formation in the Fos–Jun system. The data suggest that the peptide homodimers are destabilized at neutral pH by residues of like charge—the Fos homodimer by acidic residues, and the Jun homodimer, to a lesser extent, by basic residues. The interhelical component of this electrostatic destabilization is relieved in the heterodimer because the Fos and Jun monomers are of opposite charge.

### The Inside Residues Are Responsible for Specificity and pH-Dependent Stability

To probe the specificity of the Fos–Jun leucine zipper interaction further, structurally based hybrid peptides were made by replacing portions of the Fos and Jun sequences with sequence from GCN4. A peptide corresponding to the leucine zipper region from GCN4 forms very stable homodimers (O’Shea et al., 1989a). As Fos and Jun are likely to fold as coiled coils, the boundary between the Fos or Jun sequence and the GCN4 sequence was set by dividing the helical wheel diagram into two groups of residues: the “inside” group, consisting of the predominantly hydrophobic residues (positions a and d) and the predominantly charged residues at positions e and g, and the “outside” group, consisting of residues from positions b, c, and f (Figure 2A).

Two sets of hybrid leucine zipper peptides were constructed. One set of peptides has native sequence (N) from Fos or Jun at the inside positions and outside sequence from GCN4; these peptides are referred to as \( N_d \) (Figure 2A). The other set of peptides contains GCN4 sequence inside and Fos or Jun sequence outside; these peptides are referred to as \( N_o \) (Figure 2A). The preference for heterodimer formation was quantitated from a redox experiment in which an equimolar mixture of the cysteine-containing Fos and Jun peptides is equilibrated in a redox buffer that facilitates disulfide bond formation. \( K_{red} \) is determined from the ratio of disulfide-bonded heterodimer to homodimers (Figure 3). The free

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<th>Peptide Pair</th>
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<td>N(_o)</td>
<td>61</td>
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<tr>
<td>N(_o)</td>
<td>72</td>
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Conditions: 50 mM NaCl, 10 mM sodium phosphate (pH 7.0).
Mechanism of Specificity in the Fos-Jun Heterodimer

Figure 1. Native Fos and Jun Disulfide-Bonded Leucine Zipper Peptide Dimers

(A) pH dependence of stability for the native Fos peptide dimer (NFF), Jun dimer (NJJ), and heterodimer (NFJ).

(B) Coiled-coil helical wheel representation of the sequences of the Fos N peptide homodimer, Jun N homodimer, and Fos N-Jun N heterodimer. The Fos N peptide homodimer has a net charge of -10 at pH 7, and the Jun homodimer has a net charge of +2.

The energy of specificity for heterodimer formation ($\Delta G_{\text{spec}}$) is equal to $-RT\ln K_D + RT\ln 2$ (Figure 3). These experiments indicate that there is $-2.3 \text{kcal/mol}$ (~100:1) preference for heterodimer in the native peptides (Figure 4).

The Nₙ peptides also form heterodimers preferentially, but with reduced specificity ($\Delta G_{\text{spec}}$ is $-1.2 \text{kcal/mol}$). The decrease in specificity of the Nₙ peptides appears to arise from a decrease in stability of the Nₙ heterodimer; the Nₙ heterodimer is less stable than the native heterodimer but the stabilities of the Nₙ homodimers are the same as the
Figure 2. Structurally Based Inside-Outside Hybrid Dimers

(A) Helical wheel representation of the sequence of the N_in and N_out Fos-Jun heterodimers. Residues within the shaded box are from the GCN4 leucine zipper sequence, and unshaded residues are from the native Fos and Jun leucine zippers.

(B) pH dependence of stability for the N_in hybrid hetero- and homodimers. The pH dependence of stability for these hybrid peptides is similar to the pH dependence of stability for the native Fos and Jun peptides, especially for the N_in Fos homodimer.

(C) Comparison of the pH dependence of stability for the N_out hybrid dimers and the disulfide-bonded GCN4 leucine zipper peptide. The pH dependence of stability for the N_out dimers resembles that of the GCN4 leucine zipper dimer.
Mechanism of Specificity in the Fos-Jun Heterodimer

A thermodynamic cycle describing equilibria between the disulfide-bonded Fos and Jun peptide dimers is shown. U represents the unfolded state; N is the folded state; FF, JJ, and FJ are the disulfide-bonded Fos-Fos, Jun-Jun, and Fos-Jun peptide dimers, respectively. K_{redox} is the equilibrium constant measured by redox experiments, as described in the Experimental Procedures. K_{fold} represents the equilibrium constant for random pairing of Fos and Jun peptides, the value of K_{fold} is 2 because there are two ways to form the heterodimer and only one way to form each homodimer. K_{fold} is the folding equilibrium constant (for the disulfide-bonded peptide pairs).

\[
\Delta G_{\text{spec}} = \Delta G_{\text{redox}} + R T \ln 2
\]

\[
= \Delta G_{\text{fold}} (N^{FJ}) - 1/2 [\Delta G_{\text{fold}} (N^{FF}) + \Delta G_{\text{fold}} (N^{JJ})]
\]

Figure 3. Quantitative Representation of the Preference for Heterodimers

A quantitative representation of the preference for heterodimers.

Figure 4. Values for \(\Delta G_{\text{spec}}\) and \(\Delta T_m\) for Various Peptide Combinations

\(\Delta G_{\text{spec}}\) and \(\Delta T_m\) were determined as described in the text. The reported value of \(\Delta G_{\text{spec}}\) is the mean of at least six redox experiments, and the error reported is \pm one standard deviation.

\[
\Delta G_{\text{spec}} = -R T \ln K_{\text{redox}} + R T \ln 2
\]

\[
\Delta T_m = T_m (\text{heterodimer AB}) - 1/2 [T_m (\text{homodimer AA}) + T_m (\text{homodimer BB})]
\]
Figure 6. Sequence Determinants of Specificity

(A) Matrix of $T_m$s for 28 disulfide-bonded dimers. Melting temperatures reported are those determined by curve fitting (see Experimental Procedures).

Greater than 75% of the folded CD signal is recovered in all melting curves upon cooling, with the exception of those dimers marked with an asterisk. The peptides marked with an asterisk have undergone some degradation and/or modification during the melting curve determination, as judged by reverse-phase HPLC. Those dimers marked with a number sign have a $T_m$ that is slightly dependent upon peptide concentration, suggesting...
ences in Tm (∆Tm ≤ -8°C), and the additive class consists of peptide pairs in which the stability of the heterodimer is intermediate between that of the homodimers (+8°C > ∆Tm > -8°C).

All peptide dimers combining Jun sequence inside with Fos sequence inside fall into the specificity class (Figure 5C). Although the mechanism of antispecificity is not apparent at this time, all members of the antispecificity class have Fos sequence inside combined with GCN4 sequence inside. The other peptide combinations fall into the additive class, with one exception (the Jun Nα-Jun Nβ heterodimer falls into the specificity class, for reasons that are not readily apparent). The most striking result is that specific heterodimer formation is observed with all peptide pairs containing Fos sequence inside combined with Jun sequence inside (Figure 5), regardless of the sequences at the outside positions, reinforcing the previous conclusion that the inside residues (positions a, d, e, and g) are the major determinant of peptide pairing.

The inside residues consist of the predominantly hydrophobic positions (a and d) and predominantly charged positions (e and g). GCN4-based hybrid peptides containing native Fos or Jun sequence at the hydrophobic positions (Nα) or the predominantly charged positions (Nβ) were made to evaluate the contribution of these groups of residues to specificity (Figure 6A). The Nα peptides form heterodimers with specificity (∆Tm and ∆Gspec) at least as great as that of the native sequences (Table 1; Figure 4). In contrast, the Nβ peptides are slightly antispecific.

Thus, 8 residues at positions e and g of Fos and Jun are sufficient to mediate preferential heterodimer formation. Although the residues from Fos and Jun that comprise the hydrophobic interface between the helices (positions a and d) are undoubtedly important for stability (Smeal et al., 1989; Ransone and Verma, 1990), these residues do not appear to be important for specificity. We conclude that van der Waals packing differences do not have a dominant role in the discrimination between the Fos–Jun heterodimer and the corresponding homodimers. Rather, the mechanism of specific heterodimer formation appears to be predominantly electrostatic in nature.

This conclusion is supported further by the finding that the Nα hybrids show pH-dependent stability similar to that observed with the native peptides (Figure 6B). In particular, the Fos Nα homodimer exhibits very strong pH-dependent stability (Tm = 41°C at pH 7 and >90°C at pH 4). In contrast, the pH dependence of the Nα hybrid dimers does not resemble that of the native peptides (Figure 6C).

Thus, residues at positions e and g in the Fos and Jun sequences also account in large part for the dramatic pH-dependent stabilities observed with the native peptides.

Discussion

The requirements for specificity in the Fos–Jun system appear to be simple: 8 residues from Fos and from Jun, in a background of the GCN4 leucine zipper, are sufficient to mediate preferential heterodimer formation. pH dependence studies suggest a mechanism for specificity in which destabilization of the Fos homodimer by acidic residues (at positions e and g) shifts the dimerization equilibrium toward the Fos–Jun heterodimer. Therefore, preferential heterodimer formation by the Fos and Jun leucine zipper peptides is largely a thermodynamic consequence of Fos homodimer instability (O’Shea et al., 1989b). Destabilization of a homodimer is also used to provide specificity in the case of the tropomyosin αβ heterodimer (Lehrer et al., 1989; Lehrer and Stafford, 1991).

The coupling of the ionization state of residues at positions e and g to the stability of the Fos and Jun leucine zippers can be rationalized by using the crystal structure of a peptide corresponding to the GCN4 leucine zipper (O’Shea et al., 1991). In this crystal structure, the methylene groups of the predominantly charged residues at positions e and g pack against the predominantly hydrophobic residues at positions a and d (Figure 7). Thus, the hydrophobic interface is actually formed by side chains from 4 residues of the heptad repeat. Additionally, terminal charged groups of residues at positions e and g of the preceding heptad are close to each other. It is likely that the close proximity of negatively charged residues at positions e and g of opposing Fos monomers would disrupt the complementary packing seen at the dimer interface of the coiled coil, accounting for the instability of the Fos homodimer at neutral pH.

Studies of the requirements for specificity in the Fos and Jun proteins reinforce the conclusions drawn from the peptide studies described here. Schueremann et al. (1991) have systematically substituted residues from each position of the heptad repeat of Jun (a, b, c, e, f, and g) into the Fos leucine zipper and assayed for preferential heterodimer formation by coimmunoprecipitation. Although these authors reach a slightly different conclusion, their results demonstrate that only substitutions at positions e and g affect substantially the amount of heterodimer obtained.

The agreement between the studies of leucine zipper
Figure 6. N_e,g and N_a,d Hybrid Dimers
(A) Helical wheel representation of the sequence of the N_e,g and N_a,d Fos-Jun heterodimers. Residues within the shaded box are from the GCN4 leucine zipper sequence, and unshaded residues are from the native Fos and Jun leucine zippers.
(B) The pH dependence of stability for the hybrid N_e,g dimers resembles the pH dependence of stability for the native Fos and Jun leucine zipper peptides (Figure 1A).
(C) The pH dependence of stability for the hybrid N_a,d dimers does not resemble that of the native Fos and Jun peptides (Figure 1A).

Figure 7. Side and End Views of a Coiled Coil Illustrating Interactions Seen in the Structure of the GCN4 Leucine Zipper
For simplicity, the supercoiling of the helices is not depicted. Prime refers to the opposing helix. The dimer interface is composed of residues from the 4-3 hydrophobic repeat (positions a and d), as well as the predominantly charged residues (positions e and g). Methylene groups in the side chains of residues at positions e and g pack against residues at positions a and d. Additionally, the terminal charged groups of residues at position e' and position g of the preceding heptad repeat are seen to be close to one another in the crystal structure of a peptide corresponding to the GCN4 leucine zipper (O'Shea et al., 1991).
peptides and the Fos and Jun proteins suggests that the conclusions derived from studies of isolated leucine zipper peptides are applicable to the intact proteins. The ability to study small pieces of proteins simplifies greatly many problems of protein structure and stability. It is likely that this peptide approach will be useful in studying homo- and heterotypic interactions between other transcription factor domains. Principles of specificity of the type learned from the Fos-Jun peptide system should assist in the design and prediction of coiled-coil sequences that preferentially form homo- or heterodimers.

Experimental Procedures

Peptide Synthesis and Purification

Peptides were synthesized using t-BOC chemistry on an Applied Biosystems model 431A peptide synthesizer with standard reaction cycles modified to include acetic anhydride capping (for a review, see Kent, 1988). Peptide Jun N corresponds to residues 286-317 of the c-Jun protein (Bohmann et al., 1987; Maxi et al., 1987), and peptide Fos N corresponds to residues 162-193 of the c-Fos protein (Van Beveren et al. 1983; van Straaten et al., 1983). Ser-295 of c-Jun and Ser-177 of c-Fos have been replaced with tyrosine to facilitate concentration determination by UV absorbance measurements. Peptide GCN4 N consists of residues 269-298 of the GCN4 protein (Hinnenbush, 1984). All peptides have undergone chemical modification and/or deglycosylation, and the Fos and Jun proteins suggest that these peptides are applicable to the intact proteins. The ability to study small pieces of proteins simplifies greatly many problems of protein structure and stability. It is likely that this peptide approach will be useful in studying homo- and heterotypic interactions between other transcription factor domains. Principles of specificity of the type learned from the Fos-Jun peptide system should assist in the design and prediction of coiled-coil sequences that preferentially form homo- or heterodimers.

Circular Dichroism Studies

Circular dichroism (CD) studies were performed using a 1 cm or 1 mm cuvette (Helma or Uvonic) on an Aviv CD spectrophotometer (model 60DS or model 62DS) equipped with a thermoelectric controller. The CD signal was determined by tyrosine absorbance (Edelhoch, 1967) at 275.5 nm and by curve fitting the thermal denaturation curve to a two-state, described by an equilibrium between unfolded and folded peptide, and the enthalpy and entropy of unfolding are independent of temperature. Because some of the Fos peptide homodimers and Fos N-GCN4 N peptide heterodimers are not completely folded at 0°C, the slope of the folded baseline and the value for the CD signal of the unfolded peptide extrapolated linearly to 0 K is the slope of the temperature dependence of the CD signal for the folded peptide, and the temperature dependence of the CD signal for the unfolded peptide, and the temperature dependence of the CD signal for the unfolded peptide extrapolated linearly to 0 K is the value for the CD signal of the unfolded peptide extrapolated linearly to 0 K. DH is the enthalpy of unfolding at the midpoint of the thermal denaturation curve, and DS is the entropy of unfolding at the midpoint of the thermal denaturation curve. The Tm is the temperature at which the fraction unfolded is equal to the fraction folded (ΔS = 0), and ΔH/ΔS is independent of temperature.

For each peptide the Tm was also determined by taking the first derivative of the CD signal (θ) with respect to temperature (temperatures in K) and finding the minimum of this function (Cantor and Schimmel, 1980). All reported values of Tm are those determined from curve fitting. The error in the measurement of Tm is ±0.5°C except in cases in which 20°C > Tm > 80°C, where the error is ±5°C. The determinations of Tm were accompanied by curve fitting to within the estimated errors. Additionally, the Tm for each disulfide-bonded dimer was measured as a function of peptide concentration (over at least a 2.5-fold range of peptide concentration in the low micromolar range), as estimated by the ratio of the CD signal at low and high concentrations to determine if the dimers were associating to higher order oligomers. Redox Experiments

The disulfide-bonded heterodimer was incubated in redox buffer consisting of 100-500 μM reduced glutathione (GSH), 100-500 μM oxidized glutathione (GSSG), 50 mM NaCl, 10 mM sodium phosphate (pH 7.4) at ~23°C in an anaerobic chamber (Coy Laboratory Products, Inc.). Reactions were equilibrated at a total peptide concentration of ~40-50% and then reduced as estimated by the ratio of the CD signal at low and high concentrations to determine if the dimers were associating to higher order oligomers. Reactions were equilibrated at a total peptide concentration of ~40-50% and then reduced as estimated by the ratio of the CD signal at low and high concentrations to determine if the dimers were associating to higher order oligomers. Each redox reaction was determined to be at equilibrium by repeating the reaction using an equimolar mixture of reduced peptides as the starting material. The values for AGdimer obtained from these two different starting points agreed to within 0.1 kcal/mole.

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References


