Molecular characterization of the B-box protein–protein interaction motif of the ETS-domain transcription factor Elk-1

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The ternary complex factor (TCF) subfamily of ETS-domain transcription factors form ternary complexes with the serum response factor (SRF) and the *c-fos* SRE. Extracellular signals are relayed via MAP kinase signal transduction pathways through the TCF component of the ternary complex. Protein–protein interactions between TCFs and SRF play an essential role in formation of this ternary complex. A 30 amino acid sequence encompassing the TCF B-box is sufficient to mediate interactions with SRF. In this study we have identified amino acids which are critical for this interaction and derived a molecular model of the SRF binding interface. Alanine scanning of the Elk-1 B-box reveals five predominantly hydrophobic residues which are essential for binding to SRF and for ternary complex formation *in vitro* and *in vivo*. These amino acids are predicted to lie on one face of an α -helix. Peptides encompassing the B-box retain biological activity and have helix-forming propensity. α -Helix and ternary complex formation is disrupted by the introduction of helix-breaking proline residues. Our results are consistent with a model in which the Elk-1 B-box forms an inducible α -helix which presents a hydrophobic face for interaction with SRF. We discuss the wider applicability of our results to similar short protein–protein interaction motifs found in other transcription factors.

Keywords: Elk-1/ETS-domain/protein–protein interactions/transcription factors

Introduction

Multi-component transcription factor complexes play important roles in integrating the transduction of extracellular signals into promoter-specific transcriptional responses. One such complex is composed of the serum response factor (SRF) and the ternary complex factors (TCFs). This ternary complex plays a pivotal role in activation of the *c-fos* serum response element (SRE) by a series of mitogenic and stress stimuli (reviewed in Cahill *et al.*, 1996). Indeed, ternary complex formation is stimulated by MAP kinase phosphorylation of the TCFs (Gille *et al.*, 1992, 1995a,b; Whitmarsh *et al.*, 1995).

The TCFs comprise a subfamily of ETS-domain transcription factors (reviewed in Treisman, 1994). Three

human TCFs (Elk-1, SAP-1 and SAP-2) have been identified (Rao *et al.*, 1989; Dalton and Treisman, 1992; Price *et al.*, 1995). Murine Elk-1 and SAP-2 (ERP/Net) homologues have also been identified (Giovane *et al.*, 1994; Lopez *et al.*, 1994). Each protein shares three major regions of primary amino acid sequence similarity. The minimal ETS DNA binding domain is located at the N-terminus of the protein (A box), whereas the C-terminal domain (C box) is a target for phosphorylation by MAP kinases and acts as an autonomous transcriptional activation domain in a heterologous context (reviewed in Treisman, 1994). The third conserved region, the B-box, is required for ternary complex formation with SRF and the *c-fos* SRE (Dalton and Treisman, 1992; Janknecht and Nordheim, 1992; Marais *et al.*, 1993; Janknecht *et al.*, 1994; Giovane *et al.*, 1994; Price *et al.*, 1995). Furthermore, in combination with a heterologous DNA binding domain, the B-box is also required for ternary complex formation (Hill *et al.*, 1993) as predicted from the ‘grappling hook’ model, which proposed a role for the Elk-1 B-box in SRF binding (Treisman *et al.*, 1992). Indeed, the B-box makes direct protein–protein interactions with SRF and a 30 amino acid segment containing the 21 amino acid B-box is necessary and sufficient for interaction with SRF in the absence of DNA-bound SRF (Shore and Sharrocks, 1994). The minimal DNA binding domain of SRF is sufficient to mediate these protein–protein interactions with the TCF B-box (Mueller and Nordheim, 1991; Shaw, 1992). Residues on an extended surface in the C-terminal half of the minimal DNA binding domain of SRF play important roles in this interaction (Shaw, 1992).

Dimerization domains represent some of the best studied protein–protein interaction motifs in transcription factors. For example, the function of the leucine zipper motif has been elucidated at the molecular level (see for example Pu and Struhl, 1993; Vinson *et al.*, 1993). In this case, each dimerization partner contributes a related structural motif to the protein–protein interaction interface. However, to date, protein–protein interaction motifs between different classes of transcription factors have been poorly characterized. Members of the ETS-domain transcription factor family are often involved in interactions with members of other transcription factor families (reviewed in Crepieux *et al.*, 1994). In particular, interactions between the TCFs and SRF serve as a paradigm for such combinatorial binding between heterologous classes of transcription factors.

In this study we have investigated the B-box motif of the TCF ETS-domain proteins and its interaction with SRF. Alanine scanning mutagenesis revealed that four hydrophobic residues play critical roles in this interaction *in vitro* and are necessary for transcriptional activation *in vivo*. Moreover, the Elk-1 B-box adopts an α -helical

Fig. 1. Schematic structure of human TCFs. The locations of the three regions conserved between Elk-1, SAP-1 and SAP-2 are indicated as A (ETS DNA binding domain), B (SRF interaction domain) and C (MAP kinase phosphorylation domain) respectively. The sequence of the 21 amino acid conserved region in the B-box is shown below. Identical residues are shaded.

conformation in a hydrophobic environment. This transition is inhibited by the introduction of a proline residue, which also abolishes interactions with SRF. Taken together, these data strongly suggest a model in which the Elk-1 B-box forms an inducible α -helix which presents a hydrophobic face for interaction with SRF. Our data provide a framework which can be used to help understand how similar short peptide motifs mediate specific protein–protein interactions between different transcription factors.

Results

Alanine scanning of the Elk-1 B-box

Interactions between Elk-1 and SRF are mediated by a 30 amino acid peptide which encompasses the B-box of Elk-1 (Shore and Sharrocks, 1994). The 21 amino acid B-box is highly conserved between the three human TCFs (Figure 1). In order to identify residues that are important for interaction with SRF, we employed an alanine scanning approach (Cunningham and Wells, 1989). The substitution of target residues with alanine removes side chains that are available for mediating intermolecular interactions whilst minimizing structural perturbations (Bordo and Argos, 1991). Mutations were firstly introduced into a truncated Elk-1 derivative (Elk1–168) (Figure 2A) that constitutively binds to DNA-bound SRF in the absence of prior MAP kinase phosphorylation.

Mutant Elk-1 proteins containing single alanine substitutions were initially tested for their ability to form complexes with SRF and the *c-fos* SRE. Most mutant proteins formed ternary complexes with SRF and the SRE with similar efficiency to the wild-type protein (Figure 2B and C). However, five mutations severely decreased the interactions with SRF. The mutation S156A and mutations in four hydrophobic residues (Y153A, Y159A, F162A and I164A) reduced binding to <50% (Figure 2B, lanes 7, 10, 13, 16 and 18, and C). Of these mutations, substitution of the three aromatic residues had the most severe effect on ternary complex formation (<10% wild-type binding). Gel retardation assays were carried out under conditions in which maximum wild-type Elk-1 binding was observed (Figure 2B, lane 23) in order to detect residual binding by deleterious Elk-1 mutants. However, with lower Elk-1 concentrations, within the linear binding range, the pattern of binding was virtually identical and no further residues were identified which decreased binding to <50% in comparison with the wild-type protein (data not shown).

Both protein–DNA and protein–protein interactions are involved in ternary complex formation with SRF at the *c-fos* SRE (Janknecht and Nordheim, 1993; Shore and Sharrocks, 1994). Elk-1 can also efficiently bind to sites related to the *Drosophila* E74 binding site in an SRF-independent manner (Janknecht and Nordheim, 1992; Shore and Sharrocks 1995b). In order to assess the effect of each mutation on DNA binding by Elk-1, the mutant proteins were tested for binding to the E74 site. All mutant proteins efficiently bound to this site with the exception of R150A and R155A, which showed a large reduction in binding efficiency (Figure 2D, lanes 3 and 8, and E; see Discussion). These data suggest that the mutations that affect ternary complex formation (Y153A, S156A, Y159A, F162A and I164A) reduce protein–protein interactions with SRF rather than DNA–protein contacts in the context of the ternary complex.

In order to directly assess the role of each amino acid in mediating protein–protein interactions with SRF, the mutant proteins were tested using the GST pull-down assay. *In vitro* translated wild-type and mutant Elk1–168 proteins were tested for interaction with GSTcore^{SRF}. The results of this assay show an excellent correlation with the ternary complex formation assay (compare Figures 2C and 3B). Most mutant proteins bound SRF with similar efficiency to the wild-type protein (Figure 3A and B). However, the five mutations identified as defective in ternary complex formation (Y153A, S156A, Y159A, F162A and I164A) also showed severely decreased interaction with SRF (Figure 3A, lanes 6, 9, 12, 15 and 17, and B). Again, mutations of the three aromatic amino acids showed the greatest reduction in binding. Taken together, these results demonstrate that five predominantly hydrophobic residues in Elk-1 play a key role in mediating protein–protein interactions with SRF and ternary complex formation at the *c-fos* SRE.

Role of critical amino acids in phosphorylation-inducible ternary complex formation

Phosphorylation of Elk-1 by MAP kinases enhances its ability to bind DNA (Sharrocks, 1995; Whitmarsh *et al.*, 1995) and also to be recruited into ternary complexes with SRF and the *c-fos* SRE (Gille *et al.*, 1995a,b; Whitmarsh *et al.*, 1995). The requirement for prior phosphorylation for efficient DNA binding and ternary complex formation can be circumvented by using truncated Elk-1 proteins which lack regulatory C-terminal residues (see for example Figure 2). However, in order to assess the roles of individual B-box residues in mediating phosphorylation-inducible ternary complex formation, mutations were introduced into the B-box of full-length Elk-1. Binding of each mutant protein to SRF–SRE binary complexes was investigated in the presence and absence of prior phosphorylation by the ERK2 MAP kinase. Phosphorylation-inducible ternary complex formation was observed with both the wild-type and mutant proteins R150A, R155A and I164A (Figure 4A, lanes 2, 6, 12 and 14). However, ternary complex formation was severely disrupted in the proteins containing mutations in the aromatic residues (Y153A, Y159A and F162A; Figure 4A, lanes 4, 8 and 10). In order to exclude potential effects on DNA binding rather than interaction with SRF, each mutant protein was tested for phosphorylation-inducible binding to the E74

Fig. 2. Identification of Elk-1 amino acids that are critical for ternary complex formation. **(A)** Diagrammatic representation of Elk-1 and the truncated derivative Elk1-168. The N-terminal and C-terminal residues of each domain are indicated by numbers at the top and the sequence of the 21 amino acids within the B-box is shown at the bottom. **(B)** Gel retardation assay of the effect of alanine mutations in Elk1-168 (indicated above the lanes) on ternary complex formation with core^{SRF} at the SRE. The locations of binary (2°) (SRF-SRE) and ternary (3°) (Elk1-168-SRF-SRE) complexes are indicated. Equal molar amounts of ³⁵S-labelled Elk1-168 mutants were used in each reaction. **(C)** Graphical representation of the effect of Elk1-168 mutations (indicated below the columns) on ternary complex formation. Data are expressed relative to wild-type Elk1-168 (100%). The data given represent the average of three experiments with different sample preparations. **(D)** Gel retardation assay of the effect of alanine mutations in Elk1-168 (indicated above the lanes) on autonomous binding to the E74 site. The location of binary (2°) complexes (Elk1-168-E74) are indicated. Equal molar amounts of ³⁵S-labelled Elk1-168 derivatives were used in each reaction. **(E)** Graphical representation of the effect of Elk1-168 mutations (indicated below the columns) on autonomous DNA binding to the E74 site. Data are expressed relative to wild-type Elk1-168 (100%). The data and standard errors given represent the average of three experiments with different sample preparations.

site. All mutant Elk-1 proteins bound the E74 site in a phosphorylation-inducible manner (Figure 4B). The aromatic residues Y153, Y159 and F162 therefore play key roles in interactions with SRF in the context of phosphorylated full-length Elk-1.

Role of critical amino acid residues in ternary complex formation *in vivo*

We have demonstrated that several amino acid residues play important roles in interactions between Elk-1 and SRF *in vitro*. We used an *in vivo* transient transfection assay to assess the roles of these amino acids in the recruitment of Elk-1 to SRF-SRE complexes *in vivo*. Two reporter constructs were used in which transcription of

the chloramphenicol acetyltransferase gene (CAT) was regulated by either the *c-fos* promoter (SRE-CAT) or a series of reiterated E74-like sites (E74-CAT) (Figure 5A). Elk-1 would be predicted to require interaction with SRF to bind the SRE-CAT reporter but should bind in an SRF-independent manner to the E74-CAT reporter. In order to circumvent problems associated with competition with endogenous TCFs and the requirement for MAP kinase pathway activation, constitutively active fusion proteins containing the VP16 activation domain fused to Elk-1 were utilized (Elk-VP16) (Figure 5A; Price *et al.*, 1995).

The mutant Elk-VP16 proteins R150A and R155A activated transcription of the SRE-CAT reporter to a similar extent as wild-type Elk-1. However, transcriptional

activation of SRE-CAT by the mutant Elk-1 proteins Y153A, Y159A, F162A and I164A was severely compromised (Figure 5B). In contrast, the wild-type and all

the mutant Elk-1 proteins tested activated the E74-CAT constructs to similar extents (Figure 5C).

Taken together, these data demonstrate that, as observed *in vitro*, the hydrophobic residues Y153, Y159, F162 and I164 all play major roles in ternary complex formation with SRF and the SRE *in vivo* but are unimportant for DNA binding.

Fig. 3. The effect of alanine mutations in the Elk-1 B-box on protein-protein interactions with SRF. (A) Pull-down assay of ^{35}S -labelled Elk1-168 derivatives (indicated above the lanes) by immobilized GSTcore^{SRF}. Equal molar amounts of ^{35}S -labelled protein were used in each reaction. (B) Graphical representation of the effect of mutations of the Elk-1 B-box on protein-protein interactions with SRF. Data are presented relative to the binding of wild-type Elk1-168 (100%) and represent the average of three experiments with different sample preparations.

The Elk-1 B-box forms an inducible α -helix

Five residues (Y153, S156, Y159, F162 and I164) play critical roles in the interaction of Elk-1 and SRF *in vitro*. The spacing of these residues is consistent with a model in which they all fall on one face of an α -helix (Figure 6A). In this model, a predominantly hydrophobic interface would be presented for interaction with SRF. In order to test these predictions, a further series of mutations were introduced into the B-box (Figure 6B) in Elk1-168. First, the amino acids Y153 and Y159, which are critical for interaction with SRF, were substituted with phenylalanine residues. Protein-protein interactions with SRF (Figure 6C, lanes 1 and 2) and ternary complex formation (Figure 6D, lanes 1 and 2) by the mutant proteins Y153F and Y159F were equivalent to that observed with the wild-type protein (Figure 6C and D, lane 5). This further underlines the importance of an aromatic amino acid at these positions.

Secondly, proline residues were introduced into the Elk-1 B-box in order to disrupt the formation of an α -helical structure (Williams and Deber, 1991). Proline residues were introduced in place of leucine residues at

Fig. 4. The effect of B-box mutations on phosphorylation-inducible ternary complex formation. (A) Gel retardation analysis of ternary complex formation by full-length Elk-1 mutant proteins. Proteins were incubated with activated ERK2 in the presence (+) or absence (-) of ATP (indicated above lanes). The locations of binary (2°) (SRF-SRE) and ternary (3°) (Elk1-168-SRF-SRE) complexes are indicated. Equal molar amounts of Elk-1 derivative proteins were used in each reaction. (B) Gel retardation analysis of Elk-1 mutants (indicated above the lanes) on autonomous DNA binding to the E74 site. Proteins were incubated with ERK2 in the presence (+) and absence (-) of ATP (indicated above lanes). The locations of binary (2°) (Elk-1-E74) complexes are indicated. The asterisk represents the position of a complex derived from a degraded, C-terminally truncated Elk-1 protein. Equal molar amounts of Elk-1 derivative proteins were used in each reaction.

Fig. 5. Critical B-box mutations influence ternary complex formation *in vivo*. (A) Structure of reporter gene systems. The *ets* binding motif and SRE in SRE-CAT (left) and E74 *ets* binding sites in E74-CAT (right) are located upstream of the CAT gene (shaded box). Constitutively active Elk-1 derivatives are shown diagrammatically as clear ovals, joined to the transcriptional activation domain of (HSV) VP16 (hatched oval). Elk-1 binds to either the *ets* motif located upstream from the SRE or in the E74 site. SRF dimers are indicated by two shaded circles. The CAT reporter gene constructs were transiently transfected into NIH 3T3 cells and CAT activities were determined after 24 h. (B) Quantification of CAT activity induced by Elk-8 mutants (indicated below the columns) with SRE-CAT reporter constructs. The CAT activities were calculated relative to wild-type Elk-1-VP16. The data and standard errors given represent the average from four independent transfection experiments. (C) Quantification of the CAT activity induced by Elk-8 mutants (indicated below the columns) with E74-CAT reporter constructs. The CAT activities were calculated relative to wild-type Elk-1-VP16. The data and standard errors given represent the average from four independent transfection experiments.

positions 158 and 167, which are located in the centre and towards the C-terminal end of the B-box respectively (Figure 6B). The introduction of alanine residues at these positions has minimal effects on interactions between Elk-1 and SRF (Figures 2 and 3). The interaction of the Elk1-168 derivative L167P with SRF (Figure 6C, lane 4) was similar to wild-type Elk1-168. In contrast, SRF binding (Figure 6C, lane 3) and ternary complex formation (Figure 6D, lane 3) by Elk1-168 (L158P) were severely decreased. Autonomous DNA binding by Elk1-168 (L158P) was not affected (Figure 6E, lane 3). The introduction of a proline residue into the centre of an α -helix would be expected to disrupt its secondary structure and hence abolish interactions with SRF, whereas the introduction of a proline residue at the end would be expected to result in minimal structural and functional perturbation. These data are therefore consistent with the view that an α -helix structure is formed in the B-box which is essential for interaction with SRF.

To probe the structure of the B-box further, three 25 amino acid peptides were synthesized corresponding to the wild-type and two B-box mutants (Figure 7A). The ability of these peptides to bind to SRF was tested in a competition assay. Ternary complexes were formed between SRF, Elk1-168 and the *c-fos* SRE in the presence of increasing amounts of B-box competitor peptide. A

gradual decrease in the amount of ternary complex gained was observed with increasing concentrations of the wild-type peptide (Figure 7B, lanes 11–15). Fifty per cent inhibition of binding was observed upon addition of 300 pmol wild-type peptide. Little effect is observed on the efficiency of binary SRF-SRE complex formation (Figure 7B, lanes 11–15) or DNA binding by other transcription factors (data not shown). In contrast, the two mutant peptides (L158P and Y159A) caused minimal disruption of ternary complex formation over an identical concentration range (Figure 7B, lanes 1–5 and 6–10). These data therefore demonstrate that a 25 amino acid peptide encompassing the Elk-1 B-box retains biological activity and is sufficient for interaction with SRF in the absence of additional protein sequences.

Circular dichroism (CD) spectroscopy was used to probe the structure of the B-box peptides. This method allows sensitive detection of secondary structure elements in native proteins and peptides (Lehrman *et al.*, 1990; Van Hoy *et al.*, 1993). The CD spectra of the wild-type and mutant peptides (L158P, Y159A) in aqueous solution (Figure 7C; data not shown) were identical to the wild-type peptide, with a recognizable random coil signal characterized by a clear minimum at 197 nm. However, upon addition of trifluoroethanol (TFE) to create a hydrophobic environment, both the wild-type and Y159A mutant

Fig. 6. Proline residue insertion abolishes B-box interactions with SRF. (A) Helical wheel representation of B-box residues. Residues that play critical roles in interaction with SRF are indicated in large, bold type. The putative SRF interaction interface of the α -helix is shaded. (B) The amino acid sequence of the 21 amino acid region encompassing the Elk1 B-box is given at the top and point mutant derivatives are indicated below the wild-type sequence. SRF binding, ternary complex formation and E74 DNA binding are summarized on the right. (C) GST pull-down assay of Elk1-168 mutants (indicated above the lanes) binding to GSTcore^{SRF}. Equal amounts of ³⁵S-labelled proteins were used in each reaction. (D) Gel retardation analysis of ternary complex formation between Elk1-168 mutants (indicated above the lanes), core^{SRF} and the *c-fos* SRE. The locations of binary (2°) (SRF-SRE) and ternary (3°) (Elk1-168-SRF-SRE) complexes are indicated. Equal molar quantities of each mutant protein were used in each binding reaction. (E) Gel retardation analysis of autonomous DNA binding to the E74 site by wild-type and mutant Elk1-168 proteins (indicated above the lanes). The location of binary complexes (2°) are indicated. Equal quantities of each mutant protein were used in each binding reaction.

peptides underwent a structural transition in which a high degree of α -helical structure was induced. The helix content of the wild-type peptide increased as a linear function of TFE concentration, with no apparent cooperativity, and no significant changes were induced by lowering either the temperature or the pH (data not shown). In 50% TFE, the wild-type and Y159A peptides were characterized by apparently pure α -helical spectra with features at 193, 208 and 222 nm (Figure 7C). The intensities reveal that the absolute amount of α -helix was 16 and 18% for the wild-type and Y159A respectively. In contrast, the mutant peptide L158P shows severely reduced induction of α -helical content upon TFE addition (Figure 7C). Reductions in α -helical content are observable by changes at 222 nm (11% α -helix), but are most noticeable at 193 nm, where the intensity of signal from the L157P peptide is one fifth of the wild-type peptide. This large difference may be due to a residual random coil signal which is fully suppressed in the wild-type sequence at this concentration of TFE. These data are consistent with the notion that the substitution of a proline residue for L158 disrupts the α -helical structure of the B-box.

The data therefore indicate that a significant induction of α -helix in the wild-type and Y159A peptides occurs in a

hydrophobic environment in comparison with the aqueous state. However, the low overall intensity indicates that the B-box peptide is still flexible. The α -helical content of the L157P peptide is significantly lower than the wild-type peptide, but not entirely suppressed, which would be expected from inserting a single proline residue. Taken together, our data are entirely consistent with a model in which protein-protein recognition relies on α -helix-forming propensity which, in conjunction with the inhibitory properties of the wild-type and mutants peptides (Figure 7B), suggests an induced fit of the Elk-1 B-box to the correct site on SRF.

Discussion

Formation of the ternary complex of SRF, TCFs and the *c-fos* SRE requires both protein-DNA and protein-protein interactions from both protein partners. The DNA binding domain of SRF (Mueller and Nordheim, 1991; Shaw, 1992) and the 30 amino acid B-box from the TCFs (Shore and Sharrocks, 1994) are sufficient for these protein-protein interactions. Five predominantly hydrophobic residues in the B-box play major roles in mediating this interaction. Several lines of evidence indicate that the

Fig. 7. Elk-1 B-box inducibly forms an α -helix. (A) Sequence of wild-type and mutant B-box peptides are given. The conserved region of the B-box is bracketed. (B) Disruption of ternary complex formation by wild-type and mutant B-box peptides. Increasing amounts of competitor wild type and mutant peptides (shown schematically above lanes) were incubated with core^{SRF} and the *c-fos* SRE. Reactions contained 0 (lanes 1, 6 and 11), 102 (lanes 2, 7 and 12), 204 (lanes 3, 8 and 13), 408 (lanes 4, 9 and 14) or 816 (lanes 5, 10 and 15) pmol peptide. The locations of binary (2°) (SRF-SRE) and ternary (3°) (Elk1-168-SRF-SRE) complexes are indicated. (C) CD spectra of wild-type and mutant B-box peptides in aqueous and hydrophobic environments. Each curve represents the average of 10 accumulated spectra from each peptide measured at a concentration of 0.2 mg/ml (~75 μ M) (in a 0.2 mm path length cell) in either 50 mM Tris-HCl, pH 7.4, alone or diluted with 50% (v/v) TFE. Wild-type peptide in aqueous solution (---), wild-type peptide in 50% TFE (—), Y158P peptide in 50% TFE (—), Y159A peptide in 50% TFE (.....). The identity of the spectra of the wild-type and mutant peptides in the presence of TFE are indicated on the figure. Each spectrum was corrected by subtraction of a comparable blank. The abscissa is represented in units of $\Delta\epsilon$ (per M/cm, where M is the molar concentration of amino acid residues).

Elk-1 B-box adopts an α -helical structure for interaction with SRF. First, the spacing of the amino acids which are critical for interaction with SRF suggests that they may form one face of an α -helix. Furthermore, the introduction of a 'helix-breaking' proline residue into the middle of this putative α -helix abolishes interactions with SRF. A 25 amino acid peptide encompassing the B-box is biologically active and inducibly forms an α -helix upon exposure to a hydrophobic environment. This transition is also abolished by the incorporation of a proline residue into the peptide. Taken together, these data strongly suggest a model whereby the B-box in the TCFs inducibly forms an α -helix upon interaction with SRF. In this model, the SRF binding face of this α -helix is composed of several hydrophobic residues which play a major role in driving this interaction.

Four hydrophobic residues in Elk-1 (Y153, Y159, F162 and I164) have been identified as major contributors to the interaction interface with SRF by alanine scanning mutagenesis. This approach relies on the removal of critical side chains that are involved in intermolecular interactions whilst maintaining the structural integrity of the protein. The hydrophobic side chains of these residues are therefore likely to be involved in interaction with SRF, rather than forming a structural scaffold. This conclusion is supported by the observation that a mutant peptide containing the Y159A mutation retains an identical structure whilst losing its biological activity. This is in stark contrast to the L158P mutant peptide, which loses both its secondary structure and ability to interact with SRF. S156 is also implicated in contributing to the SRF binding

interface as, in this case, removal of a hydroxyl group causes a significant reduction in binding to SRF. The mutant proteins Y153F and Y159F also differ from the wild-type protein by the loss of a hydroxyl group but, in this case, little reduction in SRF binding is observed. This further underlines the importance of the hydrophobic aromatic side chains in interaction with SRF. Possible effects of point mutations in disrupting the overall structure of Elk-1 can be ruled out, firstly by the observation that SRF-independent DNA binding is unaffected and secondly that the mutations have similar effects in isolated peptides (Shore and Sharrocks, 1994; this study). However, although our results identify five residues as critical mediators of protein-protein interactions with SRF, mutations in other residues have lesser effects on SRF binding. Moreover, the strict conservation of most of the residues in the B-box (Figure 1A) suggests that these conserved residues also have an important role. Further mutagenesis is required to address whether these residues play a role in determining the specificity and affinity of the interactions of Elk-1 with SRF.

The B-box also appears to play a role in inhibiting autonomous binding of SAP-1a to certain ets motifs, such as the *c-fos* SRE (Dalton and Treisman, 1992), although similar inhibitory effects are not observed on higher affinity sites (Shore and Sharrocks, 1995b). A role for the B-box in inhibiting autonomous DNA binding by Elk-1 has also been proposed (Janknecht *et al.*, 1994). However, none of the mutations introduced into the Elk-1 B-box increased its DNA binding affinity in the context of the truncated Elk1-168 protein. Such increases would be

expected upon disruption of inhibitory contacts between the B-box and the ETS-domain. In particular, disruption of the overall B-box structure by the introduction of a proline residue would be expected to alleviate the putative inhibitory effects of this motif. Indeed, mutation of two residues (R150 and R155) results in a decrease in DNA binding by truncated Elk-1 derivatives (Figure 2C), although protein–protein interactions are unaffected. It is not clear why these mutations decrease DNA binding, but it may reflect the fact that the B-box contacts DNA via these residues when not bound to SRF or that these mutant B-boxes adopt conformations that inhibit DNA binding. In the context of full-length proteins, mutations R150A and R155A disrupt the induction of ternary complex formation by phosphorylation (Figure 4). One explanation for this phenomenon might be that these two residues are involved in coupling inhibitory and stimulatory signals from the C-terminal domain to effects on DNA binding by the N-terminal ETS-domain.

In yeast, the SRF-related MADS box transcription factor MCM1 interacts with several proteins, including $\alpha 2$ (reviewed in Shore and Sharrocks, 1995a). As MCM1 and SRF show significant primary amino acid sequence similarity within their DNA binding domains, it is likely that they will present similar interaction faces to other proteins. A region of limited homology between the Elk-1 B-box and the N-terminal tail of the $\alpha 2$ homeodomain has been noted previously (Shore and Sharrocks, 1994), suggesting that the conserved residues may form an SRF interaction surface. Recently, alanine scanning mutagenesis has been used to identify a short contiguous stretch of eight predominantly hydrophobic amino acids which are critical for the $\alpha 2$ –MCM1 interaction (Mead *et al.*, 1996). Three of these hydrophobic amino acids in $\alpha 2$ are spaced equivalently to the critical Elk-1 amino acids Y153, Y159 and I164, although none of these amino acids are identical. Furthermore, in contrast to the Elk-1–SRF interaction, all the intervening amino acids play key roles in the interaction between $\alpha 2$ and MCM1. Taken together, these data suggest that although TCFs and $\alpha 2$ both use hydrophobic residues as the major binding components with their MADS box protein partners, the identity and presentation of these residues differs significantly. It is also likely that differences also exist in the interaction surfaces recognized on MCM1 and SRF.

Short hydrophobic amino acid motifs appear to be an increasingly common mode used for interactions between eukaryotic transcription factors. The yeast protein $\alpha 2$ uses the 21 amino acid C-terminal tail of its homeodomain to interact with $\alpha 1$ (Stark and Johnson, 1994). Hydrophobic amino acids play important roles in this interaction and are presented on the face of an α -helix which forms upon interaction with $\alpha 1$ (Li *et al.*, 1995). This mode of interaction is comparable with the model we propose to describe Elk-1 B-box binding to SRF. In humans, the Hox homeodomain proteins utilize hexapeptide motifs to interact with pbx proteins. These peptides are also rich in hydrophobic amino acids (Chang *et al.*, 1995; Knoepfler and Kamps, 1995). Moreover, a cluster of hydrophobic amino acids is important for the interaction of p53 with mdm-2 and adenovirus 5 E1B (Lin *et al.*, 1994). Hydrophobic amino acids are also important for synergistic transcriptional activation by Pit-1 (Holloway *et al.*, 1995)

and TCR α enhancer activation by LEF-1 (Carlsson *et al.*, 1993). In both cases three critical tyrosine residues are spaced identically to Y153, Y159 and F162 in the TCF B-box, suggesting that they may form a similar protein–protein interaction motif which is important in mediating transcriptional activation. Our detailed studies on the B-box provide the basis to test this hypothesis.

In conclusion, our detailed molecular analysis of the protein–protein interaction motif in Elk-1 has led to a model in which an inducible α -helix forms an interface with SRF. This interaction is dependent upon several critical, predominantly hydrophobic amino acids. This data further refines the molecular basis to the ‘grappling hook’ model which initially suggested a critical role for the Elk-1 B-box in mediating interactions with SRF (Treisman *et al.*, 1992). Moreover, this study provides a framework to aid our understanding of how related short peptide motifs dictate specific protein–protein interactions between transcription factors.

Materials and methods

Plasmid constructs

The following plasmids were constructed for making proteins by *in vitro* transcription/translation. pAS197 was made by replacement of the *Bam*HI–*Sac*I fragment of pAS186 (Shore and Sharrocks, 1994) with the *Bam*HI–*Sac*I fragment from pAS185 (Shore and Sharrocks, 1994) to introduce an additional *Bam*HI cleavage site in the linker region between the ETS DNA binding domain and the B-box. The resulting plasmid encodes the ETS DNA binding domain and B-box of Elk-1 (Elk1–168, amino acids 1–168).

pAS198, pAS199, pAS200, pAS201, pAS201, pAS203, pAS204, pAS205, pAS206, pAS207, pAS208, pAS209, pAS210, pAS211, pAS212, pAS213, pAS99, pAS214, pAS215, pAS216 and pAS217 are point mutant derivatives from pAS197 with site-directed mutations S148A, S149A, R150A, N151A, E152A, Y153A, M154A, R155A, S156A, G157A, L158A, Y159A, S160A, T161A, F162A, T163A, I164A, Q165A, S166A, L167A and Q168A respectively. Mutations were introduced by a two-step PCR protocol using a mutagenic primer and two flanking primers as described previously (Shore *et al.*, 1996). Details of mutagenic primers can be supplied upon request.

The following plasmids were used for protein expression in *Escherichia coli*.

pAS58 encodes GSTcore^{SRF} (SRF amino acids 132–222) (Shore and Sharrocks, 1994). pAS276 was constructed in pBS-SK⁺ by inserting a PCR fragment to introduce *Sac*I and *Xho*I sites flanking the sequences encoding Elk-1 amino acids 305–428. pAS413 was constructed by ligating the *Nco*I–*Bgl*II fragment from pQE6/16Elk (Gille *et al.*, 1995a) and the *Bgl*II–*Xho*I fragment from pAS276 into the *Nco*I and *Xho*I sites of pET21d. The resulting vector encodes full-length Elk-1 (amino acids 1–428) fused to a C-terminal hexahistidine tag.

pAS414, pAS415, pAS416, pAS418, pAS419 and pAS420 are derivatives of pAS413 with the site-directed mutations R150A, Y153A, R155A, Y159A, F162A and I164A respectively. pAS413b is identical to pAS413 apart from the insertion of a *Bam*HI site into the DNA encoding the linker between the ETS-domain and B-box. The subclones pAS478, pAS479, pAS480, pAS481, pAS483, pAS484 and pAS485 were constructed by ligating an *Eco*RI/*Xba*I-cleaved PCR fragment (encoding Elk-1 amino acids 34–207) into the same sites in pUC19. pAS413 was used as a PCR template with *Pst*I fragments from pAS197, pAS200, pAS203, pAS205, pAS209, pAS212 and pAS214 respectively acting as 3′ primers. pAS413b–pAS420 were constructed by replacing the *Eco*RI–*Sst*I fragment in pAS413 with the *Eco*RI–*Sst*I fragments from pAS478–pAS485 respectively.

The following plasmids were constructed for use in mammalian cells. pF711CAT (SRE–CAT) contains human *c-fos* promoter –711 to +42 in front of the CAT gene (Treisman, 1985). pAS346 (E74–CAT) was constructed by ligating a double-stranded oligonucleotide containing four ets DNA binding sites into the *Hind*III and *Bam*HI sites of the polylinker of pBLCAT5 (Boshart *et al.*, 1992). This site was constructed by annealing the two complementary oligonucleotides ADS305 and

ADS307 (top strand 5'-AGCTTAAACCGGAAGTACAAACCGGAA-GTACAAACCGGAAGTACAAACCGGAAGTACG-3'). The *ets* sites (underlined) are similar to the *Drosophila* E74 site and were based on the high affinity S27 Elk-1 binding site selected from a random DNA pool (Shore and Sharrocks, 1995b). The vector comprises the minimal tk promoter linked to a *CAT* reporter gene (see Figure 5).

pElk-1.VP16 (Price *et al.*, 1995; kindly provided by R. Treisman) encodes Elk-1 (amino acids 1–405) fused to the VP16 activation domain (amino acids 410–490) at its C-terminus. pAS421, pAS422, pAS423, pAS424b, pAS425, pAS426 and pAS427 are derivatives of pElk-1.VP16 with the site-directed mutations and R150A, Y153A, R155A, Y159A, F162A and I164A respectively. pAS421–pAS427 were constructed by replacing the *NcoI*–*StuI* fragment of pElk-1.VP16 with *NcoI*–*StuI* fragments from pAS414, pAS415, pAS416, pAS418, pAS419 and pAS420 respectively.

Protein production

In vitro transcription and translation of Elk-1 derivatives were carried out with the TNTTM-coupled reticulocyte lysate system (Promega) according to the manufacturer's recommendations. ³⁵S-Labelled proteins were analysed by electrophoresis through 0.1% SDS–12% PAGE before visualization and quantification of bands representing intact proteins by phosphorimaging (Fuji BAS-1500 phosphorimager and TINA 2.08e software).

The GSTcore^{SRF} fusion protein and core^{SRF} were purified from *E. coli* containing pAS58 as described previously (Shore and Sharrocks, 1994). Wild-type and mutant full-length Elk-1 proteins were purified as hexahistidine tagged proteins from *E. coli* BL21 cells that had been transformed with pAS413 or its mutant derivatives using the Ni-NTA spin kit (Qiagen). The purity of protein samples was estimated by SDS–PAGE and their concentrations were routinely estimated using the BioRad protein assay reagent.

Expression and purification of MAP kinase (p42/ERK2) as a fusion with GST and the preparation of cell extracts containing activated MEK have been described previously (Sharrocks, 1995).

Phosphorylation of wild-type and mutant Elk-1 derivatives by GST–ERK2 was carried out in 30 µl reaction mixtures containing ~5 ng GST–ERK2, 1.5 µl MEK extract, 0.1 mM ATP, 0.1 mM sodium vanadate, 40 mM Tris–HCl, pH 7.4, 1 mM dithiothreitol, 10 mM MgCl₂ and 15 ng bacterially expressed Elk-1 derivatives. The presence of ATP, MEK and MAP kinase was required for maximal stimulation of Elk-1 phosphorylation.

Gel retardation and GST pull-down assays

Pull-down assays for protein–protein interactions with GSTcore^{SRF} were carried out essentially as described previously (Shore and Sharrocks, 1994).

Gel retardation assays were performed in 12 µl reaction volumes as described previously (Sharrocks *et al.*, 1993). In competition assays with B-box peptides, bacterial core^{SRF} and the *c-fos* SRE (Shore and Sharrocks, 1994) were allowed to bind for 10 min on ice. Different concentrations of B-box peptides (1.06–8.16 × 10^{–10} M) were then added in combination with *in vitro* translated Elk-1–168 and the reactions allowed to proceed for a further 20 min at room temperature. Peptide concentrations were determined at 280 nm according to their molar extinction coefficients (Gill and von Hippel, 1989).

Cell culture, transfection and extract preparation

NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Gibco). Transfection experiments were carried out with LipofectamineTM reagent according to the manufacturer's recommendations. Twenty four hours after transfection, the cells were washed three times with phosphate-buffered saline (Gibco), scraped into 1.6 ml TEN (40 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl), pelleted by centrifugation and resuspended in 150 µl 0.25 M Tris–HCl, pH 7.8. Following three freeze–thaw cycles, the cell lysate was centrifuged for 10 min and the supernatant was assayed for CAT activity using a TLC assay (Promega *Protocols and Application Guide*, 1996). Transfection efficiency was monitored by measuring the β-galactosidase activity from co-transfected pCH110 plasmids (Pharmacia). β-Galactosidase activities varied <1.5-fold between different transfection samples.

Circular dichroism

CD spectra were recorded at 20°C using a Jobin-Yvon CD6 spectrometer. Mean residue Δε values were calculated using concentrations derived from absorption measurements at 280 nm on a Cary 4E absorption spectrometer. Theoretical molar extinction coefficients were calculated

from the amino acid sequence using ε₂₈₀Tyr = 1280/M/cm. Spectra were measured in aqueous solution (50 mM Tris–HCl, pH 7.4) or TFE (Aldrich). Helical content of the peptides was calculated from the Δε₂₂₂ by assuming that a 100% α-helical peptide has Δε₂₂₂ = –9.545/M/cm (= a molecular ellipticity of –31 500° cm²/dmol; Chen *et al.*, 1972).

Acknowledgements

We thank Margaret Bell and Catherine Pyle for excellent technical and secretarial assistance, Bob Liddell for DNA sequencing and oligonucleotide synthesis and Arthur Moir for advice and peptide synthesis. We are grateful to members of our laboratory and Paul Shore for stimulating discussions and comments on the manuscript. We are grateful to Richard Treisman for reagents. This work was supported by the North of England Cancer Research Campaign, the Wellcome Trust and a Medical Research Council Studentship to C.E.R.

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Received on December 10, 1996; revised on January 17, 1997