The mechanism of phosphorylation-inducible activation of the ETS-domain transcription factor Elk-1

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Protein phosphorylation represents one of the major mechanisms for transcription factor activation. Here we demonstrate a molecular mechanism by which phosphorylation by mitogen-activated protein (MAP) kinases leads to changes in transcription factor activity. MAP kinases stimulate DNA binding and transcriptional activation mediated by the mammalian ETS-domain transcription factor Elk-1. Phosphorylation of the C-terminal transcriptional activation domain induces a conformational change in Elk-1, which accompanies the stimulation of DNA binding. C-terminal phosphorylation is coupled to activation of DNA binding by the N-terminal DNA-binding domain via an additional intermediary domain. Activation of DNA binding is mediated by an allosteric mechanism involving the key phosphoacceptor residues. Together, these results provide a molecular model for how phosphorylation induces changes in Elk-1 activity.

Keywords: conformational change/Elk-1/ETS-domain/
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Introduction

Eukaryotic transcription factors typically consist of functionally separable domains for directing DNA binding and transcriptional activation or repression. All these activities are often tightly regulated by post-translational modification and/or ligand binding (Hunter and Karin, 1992; Hill and Treisman, 1995; Mangelsdorf and Evans, 1995; Treisman, 1996). Recently, the concept of transcription factor modularity has been challenged by the observation that DNA can act as an allosteric activator (Lefstin and Yamamoto, 1998), thereby implying structural coupling between the DNA-binding and transcriptional activation domains. Transcription factor activation is also often accompanied by conformational changes. For example, interaction with the VP16 transcriptional activation domain (TAD) triggers a structural change in TFIIB (Roberts and Green, 1994). Alternatively, allosteric effectors such as DNA and hormones can trigger conformational changes, as observed with nuclear hormone receptors (Lefstin and

Yamamoto, 1998). Similarly, DNA binding can allosterically induce conformational changes in the ETS-domain family member Ets-1 (Petersen *et al.*, 1995; Jonsen *et al.*, 1996). Hence, an understanding of how transcription factors are activated within their natural context, rather than as isolated domains, is of key importance.

DNA binding and transcriptional activation by several members of the ETS-domain transcription factor family are subject to autoinhibitory regulation (Sharrocks *et al.*, 1997; Graves and Petersen, 1998). In the best studied example, Ets-1, DNA-binding inhibition is mediated by cooperatively acting N- and C-terminal inhibitory motifs that act to suppress DNA binding by the ETS-domain (Jonsen *et al.*, 1996). Activation of DNA binding is accompanied by a structural change in these motifs, but to date the activating trigger is unknown (Petersen *et al.*, 1995). A member of the ternary complex factor (TCF) subfamily of ETS-domain proteins, Elk-1, is activated following phosphorylation by mitogen-activated protein kinases (MAPKs) (Cavigelli *et al.*, 1995; Gille *et al.*, 1995; Price *et al.*, 1996; Whitmarsh *et al.*, 1997; reviewed in Treisman, 1996; Yang *et al.*, 1998c). Similarly, the other TCFs, SAP-1 and SAP-2, are activated by MAPKs (reviewed in Yang *et al.*, 1998c). Phosphorylation takes place at multiple sites in the C-terminal TAD and results in enhancement of both DNA binding and transcriptional activation mediated by Elk-1 (Cavigelli *et al.*, 1995; Gille *et al.*, 1995; Sharrocks, 1995; Whitmarsh *et al.*, 1995; Shore *et al.*, 1996; reviewed in Treisman, 1994; Yang *et al.*, 1998c). Furthermore, upon phosphorylation of Elk-1, DNA-bound complexes exhibit a change in mobility that is suggestive of a conformational change in the complex (Marais *et al.*, 1993; Shore *et al.*, 1996). Part of this mobility change may be due to a change in the structure of the DNA in the ternary complex (Sharrocks and Shore, 1995). Two additional Elk-1 domains, the B-box and D-domain, are located between the ETS DNA-binding domain and TAD, and act as protein–protein interaction motifs for serum response factor (SRF) and MAPKs, respectively (Shore and Sharrocks, 1994; Yang *et al.*, 1998a,b). In the context of C-terminally truncated proteins, the B-box also appears to act as a DNA-binding inhibitory motif (Dalton and Treisman, 1992; Janknecht *et al.*, 1994). A model has been proposed in which Elk-1 exists in two conformations (Treisman *et al.*, 1992; Sharrocks, 1995). In the ‘closed’ conformation, Elk-1 is unable to bind DNA efficiently. Either phosphorylation of the Elk-1 TAD or SRF binding to the B-box is then thought to convert Elk-1 to an open conformation that can efficiently bind DNA in both binary and ternary complexes.

In this study, we provide evidence to support several key features of this model and new insights into the mechanism of phosphorylation-inducible activation of

Fig. 1. The role of the Elk-1 B-box in inhibiting DNA binding and mediating intramolecular transduction of the phosphorylation signal. (A) Diagrammatic representation of the wild-type (WT) and mutant Elk-1 proteins. (B) Gel-retardation analysis of Elk1-168 in the presence of increasing amounts (0, lanes 1 and 6; 20 μ M, lanes 2 and 7; 80 μ M, lanes 3 and 8; 100 μ M, lanes 4 and 9; 350 μ M, lanes 5 and 10) of peptides corresponding to the Elk-1 B-box (Ling *et al.*, 1997) or D-domain (Yang *et al.*, 1998b). The asterisk represents complexes derived from C-terminal degradation products. (C) and (D) Gel-retardation analysis of wild-type and mutant Elk-1 derivatives with deletions of the B-box (C) or point mutations in the B-box (D) binding to the E74 site. Prior phosphorylation of Elk-1 by ERK2 is indicated (P_i).

Elk-1. We have investigated how C-terminal phosphorylation of Elk-1 leads to activation of DNA binding by the N-terminal ETS-domain and demonstrate that allosteric activation with concomitant conformational changes takes place. Thus, although the DNA-binding domain and TAD act as discrete modules, structural coupling is required for phosphorylation-inducible activation of Elk-1.

Results

The Elk-1 B-box plays a key role in phosphorylation-inducible relief of DNA binding inhibition

DNA binding by Elk-1 is inhibited by an intramolecular mechanism involving residues located C-terminally to the ETS-domain (Janknecht *et al.*, 1994). C-terminal deletion analysis suggests that the Elk-1 B-box represents a DNA-binding inhibitory region (Janknecht *et al.*, 1994). In order to investigate this phenomenon further, the DNA-binding activity of the C-terminally truncated Elk-1 protein Elk1-168 (comprising the ETS-domain and the B-box region; Figure 1A) was determined in the presence of increasing amounts of peptides corresponding to the Elk-1 B-box region or the Elk-1 D-domain. The presence of the B-box peptide causes a stimulation of DNA binding by Elk1-168 (Figure 1B, lanes 1–5), whereas no such stimulation is elicited by the D-domain peptide (Figure 1B, lanes 6–10). In contrast, the B-box peptide has no effect on DNA binding by Elk1-93 (containing the ETS-domain alone; data not shown). Thus, when supplied *in trans*, the B-box motif stimulates DNA binding, presumably by competing with the *cis*-linked B-box and hence relieving the intramolecular inhibition.

To assess the role of the B-box in phosphorylation-inducible DNA binding in the presence of the C-terminal

regulatory domain, internal deletions and point mutations were introduced into Elk-1 (Figure 1A). Proteins were phosphorylated to equivalent levels (monitored by 32 P incorporation and detection of phosphorylated Ser383 residues by Western blotting; S.-H. Yang and A.D. Sharrocks, data not shown) and tested for binding to the E74 site. Phosphorylation of Elk-1 by MAPKs results in an increase in its DNA binding activity (Figure 1C, lanes 1 and 2). However, deletion of the B-box blocks the activation of DNA binding by Elk-1 following phosphorylation (Figure 1C, lanes 3 and 4). Similarly, the introduction of a proline residue into the B-box blocks the activation of DNA binding by Elk-1 following phosphorylation (Figure 1D, lanes 5 and 6). Other mutations in the B-box have little effect on this activation process (Figure 1D, lanes 3 and 4; data not shown).

Collectively, these results demonstrate that the B-box plays a role in inhibiting DNA binding in the context of truncated Elk-1 proteins and is required for the induction of DNA binding following Elk-1 phosphorylation.

Elk-1 undergoes a phosphorylation-inducible conformational change

The mechanism by which phosphorylation induces DNA binding by Elk-1 is unknown, although one attractive mechanism would be to induce a conformational change that unmasks or alters the DNA binding surface of Elk-1. Indeed, upon phosphorylation of Elk-1, DNA-bound complexes exhibit a change in mobility that is suggestive of a conformational change in the complex (Marais *et al.*, 1993; Shore *et al.*, 1996). To probe for conformational changes within the protein following phosphorylation, C-terminally Flag-epitope-tagged Elk-1 was phosphorylated *in vitro* by ERK2 and subjected to limited proteolytic digestion (Figure 2A, B and C). Of the

Fig. 2. Phosphorylation of Elk-1 induces a change in the tryptic cleavage pattern of Elk-1. (A and C) Western blot analysis of phosphorylated and non-phosphorylated Elk-1 following partial proteolytic digestion with trypsin, in which times of either digestion (A) or phosphorylation of Elk-1 (C) were varied. C-terminally Flag-tagged fragments were detected with an anti-Flag antibody. The locations of the major proteolytic cleavage sites in Elk-1 are indicated. # represents a band resulting from the shift in mobility of band 5 which is induced in all C-terminal fragments following phosphorylation of Elk-1. (B) Graph of the relative mobilities of the five major proteolytic fragments derived from phosphorylated Elk-1, plotted against the mobilities of the five major proteolytic fragments derived from non-phosphorylated Elk-1. The identities of the bands contributing to the points on the graph are indicated. (D) Western blot analysis, using anti-Flag antibodies, of phosphorylated (lanes 9–16) and non-phosphorylated (lanes 1–8) wild-type and mutant Elk-1 (L158P) following partial proteolytic digestion with trypsin for the indicated times. Bands resulting from enhanced cleavage of phosphorylated Elk-1 in the L158P mutant are indicated by an arrow. The band labelled with an asterisk was not resolved from band 2 in (A) and (C). Band 6 is not present on these gels. Proteolytic fragments were separated on SDS-PAGE using either standard 12% acrylamide (A and C) or 10% ProSieve™ acrylamide (D). (E) Locations of constitutive (below) and variable (above) proteolytic cleavage sites in wild-type Elk-1. Sites present in the non-phosphorylated protein (N), or induced by phosphorylation (P), are indicated.

proteases tested, only trypsin produced a good ladder of fragments (S.-H. Yang and P. Shore, data not shown). Preliminary experiments demonstrated that generation of the tryptic ladder was dependent on the addition of trypsin

rather than a contaminating protease (S.-H. Yang and P. Shore, data not shown). Five major bands are detected in the tryptic digests of both phosphorylated and non-phosphorylated Elk-1 by Western blotting with an anti-Flag antibody (Figure 2A). A summary is shown in Figure 2E where sites are assigned based on the relative mobilities of the C-terminally Flag-tagged fragments and the presence of a tryptic cleavage site in the sequence. Following phosphorylation, a general reduction in the mobility of all the C-terminal fragments is observed. However, the relative mobilities of four of these bands remain unchanged, but significantly, one major band is lost (band 5) and a new one is gained (band 4) (Figure 2A and B). The phosphorylation-inducible shift in the mobilities of the bands is better visualized over a time course (Figure 2C). Initially, the mobility of band 5 is reduced in an analogous manner to the other bands following phosphorylation (Figure 2C; compare lanes 2–4). A further shift is then observed in this band upon increased phosphorylation, whereas no further changes are observed in the other bands (Figure 2C, lanes 4–6). An additional minor cleavage product is also induced upon phosphorylation (band 7) (Figure 2A). Although it is possible that a band might be lost due to a phosphate group interfering with trypsin cleavage, the appearance of an additional band cannot be attributed to this effect. The changes we observe are, therefore, most likely interpreted as demonstrating a conformational change in the protein upon phosphorylation.

The proteolytic digestion products generated by trypsin cleavage of Elk-1 were also analysed by gel-retardation analysis using the E74 site. In this case, all fragments contain the N-terminal ETS DNA-binding domain and additional C-terminal sequences. Elk-1 is partially phosphorylated after 5 min, but fully phosphorylated after 60 min (Yang *et al.*, 1998a; S.-H. Yang and A.D. Sharrocks, data not shown). Several cleavage products are observed in both the phosphorylated and non-phosphorylated protein. However, a new cleavage product (band A) is produced following phosphorylation of Elk-1 (Figure 3A, lane 6). This novel band is only detected at high levels of phosphorylation required for maximal stimulation of DNA binding (see Figure 3A, lanes 1–3), thereby providing a clear link between the stimulation of DNA binding and a conformational change in Elk-1. The location of this inducible site towards the N-terminus of the protein (Figure 3B), far away from the phosphoacceptor motifs, is indicative of a phosphorylation-inducible conformational change being transduced from the C- to the N-terminal end of Elk-1.

As the mutant protein Elk-1(L158P) is defective in the phosphorylation-inducible activation process (Figure 1D), it is possible that the conformational change induced following phosphorylation is also disrupted. Partial proteolysis experiments demonstrate that although the unphosphorylated Elk-1(WT) and Elk-1(L158P) proteins exhibit similar cleavage patterns (Figure 2D, lanes 1–8), a series of hypersensitive cleavage sites occur in the phosphorylated Elk-1(L158P) protein (Figure 2D, lanes 13–16).

Collectively, these results demonstrate that a change in the proteolytic cleavage pattern of Elk-1 occurs following phosphorylation, which most likely reflects a conformational change in the protein. This conformational change

Fig. 3. Analysis of phosphorylation-inducible tryptic digestion products of Elk-1 by gel-retardation analysis. (A) Gel-retardation analysis of phosphorylated and non-phosphorylated Elk-1 following partial proteolytic digestion with trypsin. Elk-1 was phosphorylated with ERK2 for the indicated times (0–60 min). Bands represent C-terminally truncated fragments that retain the intact N-terminal ETS DNA-binding domain. Constitutive cleavage products are asterisked. (B) Location of the phosphorylation-inducible proteolytic cleavage site in wild-type Elk-1. A novel cleavage site induced in the phosphorylated protein (P) is indicated. The location of this site was estimated based on the fact that the complex mobility of this Elk-1 fragment is less than Elk1–168 (which contains the B-box), but much greater than the ETS-domain alone (S.-H. Yang, data not shown).

appears to be partially defective in the mutant protein Elk-1(L158P).

To further establish that these proteolytic changes reflect a conformational change in Elk-1, circular dichroism (CD) and fluorescence emission spectroscopy were employed (Figure 4). In the wild-type protein, phosphorylation causes a shift in both its CD spectrum, indicating an increase in α -helicity, and in its fluorescence emission spectrum, indicating a change in the environment of tryptophan residues. In contrast, little change is observed in the fluorescence emission spectrum of the isolated C-terminal TAD contained in glutathione *S*-transferase (GST)–Elk-310 upon phosphorylation (S.-H. Yang and A.D. Sharrocks, data not shown). Elk-1 contains four Trp residues; three in the ETS-domain and one close to the key phosphoacceptor motifs (Ser383 and Ser389) in the TAD (Figure 6A); therefore, C-terminal phosphorylation of Elk-1 causes a change in intramolecular interactions with either the ETS-domain or the C-terminal activation domain.

The mutant protein Elk-1(L158P) was also analysed by CD and fluorescence emission spectroscopy. Although an increase in α -helicity can still be observed using CD (compare Figure 4A and B), little change in the fluores-

Fig. 4. Phosphorylation induces changes in the secondary and tertiary structure of Elk-1. (A and B) CD spectra of phosphorylated and non-phosphorylated wild-type Elk-1 and the mutant Elk-1(L158P). (C and D) Fluorescence emission spectra of phosphorylated and non-phosphorylated wild-type Elk-1 and the mutant Elk-1(L158P). Spectra representing the phosphorylated (+P) and non-phosphorylated forms (–P) of each Elk-1 derivative are indicated.

cence emission spectrum is detected following phosphorylation (Figure 4D). Thus, whilst secondary structure changes can still be observed, the tertiary structural changes induced by phosphorylation of Elk-1 are lost in the Elk-1(L158P) mutant protein.

Together, these results therefore demonstrate that a significant conformational change is induced in both the secondary and tertiary structure of Elk-1 following phosphorylation. However, the phosphorylation-induced structural change in the mutant protein Elk-1(L158P) is partially defective.

Regulation of Elk-1 DNA binding by phosphorylation-regulatable effector peptides

Phosphorylation of the Elk-1 TAD plays a pivotal role in the activation of its DNA binding activity. Furthermore, the C-terminal TAD appears sufficient to inhibit DNA binding in the absence of the B-box (Figure 1). To investigate how the inhibition and activation of DNA binding by the C-domain occur in response to phosphorylation, DNA binding experiments were carried out in the presence of phosphorylated (+P) and non-phosphorylated (–P) peptides from the C-domain that encompass the key phosphoacceptor motifs Ser383/Ser389 (Figure 5A). Significantly, the non-phosphorylated peptide inhibits, whereas the phosphorylated peptide slightly enhances, DNA binding by phosphorylated Elk-1 (Figure 5B). In contrast, neither peptide affects DNA binding by non-phosphorylated Elk-1 (Figure 5C). Moreover, a different peptide corresponding to the Elk-1 D-domain does not alter the efficiency of DNA binding by phosphorylated Elk-1 (Figure 5D). The inhibitory activity of the C-domain peptides is therefore specific, regulatable by phosphorylation and directed towards the phosphorylated form of Elk-1.

In order to analyse whether the peptides act as allosteric

Fig. 5. Inhibition of DNA binding occurs by an allosteric mechanism. (A) Diagrammatic illustration of domains contained in full-length and truncated Elk-1 proteins. The sequence of phosphorylated (+P) and non-phosphorylated (-P) C-domain peptide is indicated. (B) Gel-retardation analysis of phosphorylated full-length Elk-1, non-phosphorylated Elk-1 (C) or Elk1-93 (E) binding to the E74 site in the absence (lane 1) or presence of increasing amounts of phosphorylated (+P) and non-phosphorylated (-P) C-domain peptides (lanes 2 and 5, 7 μM; lanes 3 and 6, 70 μM; lanes 4 and 7, 350 μM). (D) Gel-retardation analysis of phosphorylated full-length Elk-1 in the presence of increasing amounts (lane 1, 0 μM; lane 2, 50 μM; lane 3, 150 μM) of D-domain peptide.

or steric regulators of DNA binding, competition experiments were carried out with the isolated ETS DNA-binding domain (Elk1-93). Neither peptide affects DNA binding by Elk1-93 (Figure 5E). Similarly, neither peptide affects the efficiency of DNA binding by a longer Elk-1 derivative, Elk1-168, that encompasses the B-box (data not shown). These results indicate that these peptides do not regulate DNA binding by sterically blocking protein-DNA interactions by the ETS-domain, but rather act via an allosteric mechanism mediated by the C-terminal region of Elk-1.

Intramolecular interactions in Elk-1

A molecular model emerges for how DNA binding by Elk-1 is regulated by phosphorylation (Figure 7; see Discussion). Phosphorylation at the C terminus of the protein triggers a structural change in the protein that results in the activation of DNA binding by the N-terminal ETS-domain. An additional domain (the B-box) appears to be involved in both the inhibition process and coupling of the C-terminal phosphorylation event with the activation

Fig. 6. Intramolecular interactions in Elk-1. (A) Diagrammatic illustration of the Elk-1 domains contained in the GST fusion proteins. (B) GST pull-down assay of binding of Elk1-93 and Elk1-168 to GST fusion proteins containing the non-phosphorylated and phosphorylated C-terminal domain of Elk-1. Prior phosphorylation of Elk-1 by ERK2 is indicated (P_i). (C) GST pull-down assay of binding of Elk1-93 to GST fusion proteins containing the Elk-1 B-box or D-domain in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of B-box competitor peptide (7 μM). (D) GST pull-down assay of binding of full-length Elk-1 (lanes 1 and 2) and Elk1-168 (lanes 3 and 4) to GST-core^{SRF} fusion proteins in the presence and absence of prior phosphorylation by ERK2. (E) Diagrammatic illustration of the Elk-1 domains contained in the GST fusion proteins. Numbers above each construct represent the N- and C-terminal amino acids in the Elk-1 moiety. (F) GST pull-down assays of the binding of full-length phosphorylated (³²P-labelled) Elk-1 (Elk1-428) to GST fusion proteins containing either non-phosphorylated or phosphorylated parts of the C-terminal domain of Elk-1. Prior phosphorylation of Elk-1 by ERK2 is indicated (P_i). Bound proteins were visualized by autoradiography.

process. In order to demonstrate direct structural coupling between the C-terminal end and the N-terminal ETS DNA-binding domain of Elk-1, GST pull-down experiments were carried out. Both Elk1-93 and Elk1-168 (containing the ETS DNA-binding domain) bind to GST-Elk-310 (containing the Elk-1 TAD). Phosphorylation of GST-Elk-310 reduces binding to Elk1-93, but enhances binding to Elk1-168 (Figure 6B), indicating a shift in the interactions of the C- and N-terminal regions. As the B-box

Fig. 7. Model depicting how phosphorylation relieves the inhibition of DNA binding by Elk-1. (A) The C-terminal transcription activation domain (TAD) and B-box interact with the ETS-domain and inhibit DNA binding. Phosphorylation causes a local change in the TAD that is transmitted into a conformational change in the rest of the protein and leads to loss of inhibition and stimulation of DNA binding. Arrows denote the positions of the changes in protease sensitivity that occur following phosphorylation. (B) Peptides encompassing the key phosphoacceptor motifs act as phosphorylation-regulatable allosteric switches which bind to phosphorylated Elk-1 and inhibit its DNA binding. The binding site of the peptide is unknown, although it requires the presence of the C-terminal TAD and it may act indirectly by binding to this domain, thereby altering its interactions with the N-terminal end of the protein. The black box represents the phosphorylation-regulatable effector motif in the TAD. Phosphoacceptor motifs (serines 383 and 389) are indicated by S (unphosphorylated) or P (phosphorylated).

plays a role in inhibiting DNA binding (Janknecht *et al.*, 1994; Figure 1), this suggests that interactions occur between the ETS-domain and the B-box. Indeed, Elk1-93 binds to GST-ElkB (containing the B-box), but not to a different GST fusion protein (GST-ElkD; Figure 6C).

As phosphorylation also stimulates the formation of ternary complexes between Elk-1 and DNA-bound SRF, it is also possible that in addition to stimulating DNA binding, phosphorylation might also stimulate protein-protein interactions with SRF. However, GST pull-down assays demonstrate that phosphorylated and non-phosphorylated Elk-1 bind to SRF with similar efficiencies (Figure 6D).

An additional prediction from our model (Figure 7) and the peptide competition experiments (Figure 5) is that the part of the Elk-1 TAD which encompasses the regulatory peptide will interact *in trans* with Elk-1 in a phosphorylation-dependent manner. This interaction would presumably mimic the *cis* interactions normally observed in Elk-1. In order to address this question, GST fusion proteins were constructed that either contain the intact TAD [Elk(310–399)] or the N- [Elk(310–375)] or C-terminal [Elk(375–399)] parts of this region (Figure 6E). GST pull-down assays were carried out with phosphorylated full-length Elk-1 and each of the GST fusion proteins, which were either unphosphorylated or had been previously phosphorylated by ERK2 (Figure 6F). Elk-1 bound to GST-Elk(310–399) and the N-terminally deleted protein GST-Elk(375–399) (Figure 6F, lanes 1 and 5). The latter

construct contains only the sequence included in the effector peptides used previously (see Figure 5A). In contrast, the C-terminally truncated derivative GST-Elk(310–375) which lacks this sequence shows much reduced binding (Figure 6F, lane 3), indicating that this sequence is necessary and sufficient for interaction with full-length Elk-1. Upon phosphorylation of the GST fusion proteins, much reduced binding of Elk-1 to GST-Elk(310–399) and GST-Elk(375–399) was observed (Figure 6F, lanes 2 and 6). Thus the inability of the phosphorylated C terminus to interact with Elk-1 is consistent with the observation that the phosphorylated effector peptide is unable to inhibit DNA binding (Figure 5B). These results are consistent with an independent study in which an internal deletion within the TAD (amino acids 375–394) blocked homodimerization of Elk-1, although the deleted region was not shown to be sufficient for the interaction, and the role of phosphorylation in the process was not addressed (Gille *et al.*, 1996).

The intact C-terminal TAD can bind to the N-terminal part of the protein (Figure 6B). In order to identify the part of Elk-1 to which the effector peptides bind, the interaction of C- and N-terminally truncated Elk-1 proteins with GST-Elk(375–399) was investigated. However, no reproducible binding could be observed to these truncated Elk-1 proteins (data not shown), indicating that the peptides bind to an epitope that is only present in full-length Elk-1.

Together, these results demonstrate that, consistent with our model, both the B-box and Elk-1 TAD can interact directly with the ETS DNA-binding domain. These interactions appear to be modified by phosphorylation, which correlates with the activation of DNA binding and conformational changes induced in the protein. Similarly, a short peptide surrounding the phosphoacceptor motifs is sufficient for binding to Elk-1 in a phosphorylation-dependent manner, although binding is also dependent upon the integrity of the protein.

Discussion

Eukaryotic transcription factors are usually modular in design and consist of functionally separable domains for directing DNA binding, and transcriptional activation or repression. The latter two types of activity are often associated with protein-protein interactions leading to the recruitment of co-activator or co-repressor proteins. It is becoming apparent that these 'independent' domains are actually intimately interlinked rather than functioning in isolation (reviewed in Lefstin and Yamamoto, 1998). In this study, we demonstrate that phosphorylation signals received at the C-terminal end of Elk-1 are transmitted via an intermediary domain to permit activation of DNA binding by the N-terminal ETS DNA-binding domain. This phosphorylation-dependent switch is accompanied by conformational changes in the protein and results in a change in the intramolecular interactions between the different domains in Elk-1.

Mechanism of phosphorylation-inducible activation of DNA binding by Elk-1

Phosphorylation of Elk-1 regulates both its DNA-binding activity and transcriptional activation properties (reviewed

in Treisman, 1996; Yang *et al.*, 1998c). Based on the results of this study, we propose a model for the mechanism of phosphorylation-inducible Elk-1 activation (Figure 7). In the unphosphorylated state, DNA binding by Elk-1 is inhibited by a combination of the B-box and C-terminal TAD (Figure 7A). Evidence to implicate these two regions is provided in this (Figure 1) and a previous study (Janknecht *et al.*, 1994). A regulatory region is located in the TAD, which encompasses the key phosphoacceptor motifs (Ser383/Ser389) and acts as a phosphorylation-inducible switch which controls DNA binding. Phosphorylation initiates both a local change in the intramolecular interactions (see Figure 6) and a complex conformational change in the whole protein that relieves the inhibitory interactions (Figure 6B; see Figures 2, 3 and 4). The addition of inhibitory peptides can then reverse this activation process by binding to phosphorylated Elk-1 and acting as a phosphorylation-regulatable allosteric switch (Figure 6B; see Figure 5). These peptides are, however, unable to gain access to the unphosphorylated protein due to its 'closed' conformation.

Although we and others (Gille *et al.*, 1995) have demonstrated that phosphorylation of Ser383/Ser389 is a key regulatory event in DNA binding, it is clear that the overall stoichiometry of phosphorylation is important in inducing both DNA binding and conformational changes in Elk-1. Other phosphoacceptor motifs are, therefore, likely to play a role in this process. First, high levels of phosphorylation are required to gain maximal DNA binding (Yang *et al.*, 1998a; Figure 3A) and induce structural changes in Elk-1 (Figure 2C). Under the conditions we used, Elk-1 is maximally phosphorylated after 60 min, at which point maximal stimulation of DNA binding and conformational changes are observed. Secondly, Elk-1(S383A/S389A) can still undergo a phosphorylation-inducible conformational change, as detectable by partial proteolysis (S.-H. Yang and A.D. Sharrocks, data not shown), although further differences in the digestion patterns indicate that the conformational change is defective. This scenario is very similar to that detected for the B-box mutant L158P (Figure 1E), indicating that multiple determinants act to determine the precise conformational change that is induced by phosphorylation.

Multiple roles of the Elk-1 B-box

Deletion analysis (Janknecht *et al.*, 1994) and peptide competition experiments (Figure 1B) indicate that the B-box acts as an inhibitory motif in the context of a truncated Elk-1 protein. However, a comprehensive mutagenic study of the B-box did not identify any amino acids whose mutation caused a stimulation of DNA binding (Ling *et al.*, 1997). Furthermore, although the insertion of a proline residue into the B-box was shown to disrupt its propensity for α -helix formation and abolish interactions with SRF, no stimulation of DNA binding was observed (Ling *et al.*, 1997; S.-H. Yang and A.D. Sharrocks, data not shown). In the context of the full-length protein, an internal deletion of the B-box does not cause a complete loss of DNA binding inhibition, as might be predicted from previous studies (Figure 1). This indicates that the C-terminal TAD is sufficient to inhibit DNA binding. Together, these results therefore suggest that in the context of truncated proteins, the inhibitory effect of the B-box

might be non-specific and be driven by the requirement to exclude the hydrophobic residues in the B-box from the solvent. However, in the context of the full-length protein, the B-box clearly plays a role in the inhibitory process. First, the B-box binds to the ETS-domain (Figure 6C) and is also involved in interactions with the phosphorylated TAD (Figure 6B). Secondly, deletion or mutation of the B-box disrupts the ability of C-terminal phosphorylation to stimulate DNA binding (Figure 1C and D). Thirdly, the phosphorylation-inducible conformational change is partially disrupted when the B-box is mutated in the Elk-1(L158P) protein (Figures 2D and 4).

Hence, the B-box plays a key role in coupling C-terminal phosphorylation signals with the enhancement of DNA binding via the N-terminal ETS-domain. In addition, the B-box has also been shown to bind to SRF (Shore and Sharrocks, 1994). Therefore, the Elk-1 B-box plays a pivotal role in the formation of the ternary Elk-1-SRF-SRE complex. As phosphorylation stimulates ternary complex formation as well as autonomous DNA binding (Gille *et al.*, 1995; Sharrocks, 1995), the conformational change might be expected to unmask the B-box for interaction with SRF. However, this does not seem to be the case as protein-protein interactions with SRF are not stimulated by phosphorylation (Figure 6D). Thus, the major effect of phosphorylation on ternary complex formation is in stimulating protein-DNA interactions.

Comparison with other DNA binding inhibitory mechanisms

As observed with Elk-1, DNA binding by SAP-1 is stimulated by phosphorylation of the C-terminal TAD (Shore *et al.*, 1996; Strahl *et al.*, 1996). Furthermore, deletion analysis indicates that DNA binding by SAP-2 is also regulated by an intramolecular inhibitory process (Maira *et al.*, 1996; Price *et al.*, 1996), although in this case phosphorylation does not appear to be sufficient to stimulate high-affinity DNA binding. In SAP-1, deletion analysis has implicated sequences including the B-box as important inhibitory determinants (Dalton and Treisman, 1992), and in SAP-2 further sequences located C-terminal to the B-box have been implicated in the inhibitory process (Maira *et al.*, 1996; Price *et al.*, 1996). A short motif known as the NID (net inhibitory domain) represents a *cis*-acting inhibitory motif in SAP-2 (Maira *et al.*, 1996). Thus, although several similarities exist in the autoinhibitory regions and regulatory mechanisms used to control DNA binding by the different TCFs, there are also key differences.

DNA binding by other more diverse members of the ETS-domain transcription factor family is also regulated by autoinhibitory mechanisms (Sharrocks *et al.*, 1997; Graves and Petersen, 1998). In Ets-1, DNA-binding inhibition is mediated by cooperatively acting N- and C-terminal inhibitory motifs that act to suppress DNA binding by the ETS-domain (Jonsen *et al.*, 1996). DNA binding is accompanied by the unfolding of an α -helix in the N-terminal inhibitory motif (Petersen *et al.*, 1995). In contrast, in Elk-1, phosphorylation induces α -helix formation and subsequent activation of DNA binding. Further changes in the tertiary structure of Elk-1 are observed upon phosphorylation. Similarly, further changes in Ets-1

might also be detected once the activation trigger is identified for this transcription factor.

Finally, it is becoming clear that many members of diverse families of transcription factors are subject to intramolecular inhibition, which leads to reductions in their DNA binding capacity. In many cases, the regulatory trigger that relieves this inhibition is unknown, although DNA has been shown to be able to act in the reciprocal manner as an allosteric activating trigger (reviewed in Lefstin and Yamamoto, 1998). The tumour suppressor p53 exists in an inhibited state that, like Elk-1, can be stimulated to bind DNA by phosphorylation. However, in p53, peptides were identified that act cooperatively with casein kinase II to stimulate DNA binding (Hupp *et al.*, 1995). In this study, we have used peptides that cause the opposite phenomenon, and act to antagonize the effect of phosphorylation on DNA binding by Elk-1. Similar approaches can be applied to other transcription factors to help understand the mechanisms of autoinhibition in the context of full-length proteins.

Conclusions

In addition to activating DNA binding, the conformational changes we detect are also likely to reflect the enhanced ability of phosphorylated Elk-1 to activate transcription by enhancing/modifying interactions with co-regulatory proteins. Our results significantly enhance the current understanding of how Elk-1 functions at the molecular level. Direct evidence is provided for an allosteric switch that transduces the phosphorylation signal through intimately interacting modules in Elk-1 to cause conformational changes and the concomitant activation of DNA binding. More generally, this study provides significant insights into how transcription factor activity might be regulated by phosphorylation.

Materials and methods

Plasmid constructs

The following plasmids were used for expressing GST fusion proteins in *Escherichia coli*. pAS545 (encoding GST–Elk310; Elk-1 amino acids 310–428), pAS767 (encoding GST–ElkD; Elk-1 amino acids 307–329) (Yang *et al.*, 1998a), pAS77 (encoding GST–ElkB; Elk-1 amino acids 139–168) and pAS58 (encoding GST–core^{SRF}; SRF amino acids 132–222) have been described previously (Shore and Sharrocks, 1994). pAS1352 [encoding GST–Elk(310–399); Elk-1 amino acids 310–399], pAS1353 [encoding GST–Elk(310–375); Elk-1 amino acids 310–375] and pAS1354 [encoding GST–Elk(375–399); Elk-1 amino acids 375–399] were constructed by inserting *Bam*HI–*Xho*I, *Bam*HI–*Xho*I and *Eco*RI–*Xho*I cleaved PCR products, respectively, into the same sites of pGEX-KG (Guan and Dixon, 1991). The following plasmids were constructed for expressing Elk-1 derivatives as C-terminal His₆/Flag-tagged proteins in *E. coli*. pAS278 (encoding full-length Elk-1) was described previously (Yang *et al.*, 1998a). pAS323, pAS326, pAS382 [encoding the His/Flag-tagged Elk-1 derivatives; Elk1–93 (amino acids 1–93), Elk-1ΔB (internal deletion of amino acids 148–218) and Elk1–168 (amino acids 1–168)] were constructed by inserting the *Nco*I–*Xho*I fragments derived by either PCR, by combining a *Nco*I–*Bam*HI fragment from pAS186 and a *Bam*HI–*Xho*I fragment from pAS327 or from pAS381, respectively, into the same sites in pET-Hnef-PFH (Zhao and Narayan, 1993). pAS853 and pAS854 (encoding His/Flag-tagged full-length Elk-1 L158P and Y159E, respectively) were constructed by ligating *Nco*I–*Stu*I fragments from pAS851 and pAS852 into the same sites in pAS382. The following intermediate subclones were constructed. pAS381 is pBS-SK⁺ with a *Hind*III–*Xho*I-cleaved PCR fragment of Elk-1 (encoding amino acids 1–168). pAS851 and pAS852 (encoding Elk-1 amino acids 1–205) are derivatives of pAS197 (Ling *et al.*, 1997)

with the site-directed mutations L158P and Y159E, respectively. All plasmid constructs encoding Elk-1-derived proteins made by PCR were verified by automated dideoxy sequencing. Details of PCR primers can be supplied on request.

Protein production and kinase reactions

GST fusion and His₆-tagged proteins were expressed in the *E. coli* strains JM101 and BL21(DE3), respectively, and purified as described previously (Shore *et al.*, 1995; Yang *et al.*, 1998a). Two bands (occasionally three) were typically detected by Western blotting with an anti-Flag antibody following purification of Elk-1, the smaller of which represent N-terminally deleted products (see zero time points in Figure 2A, C and D). Kinase reactions were carried out as described previously using recombinant, active ERK2 (NEB) (Yang *et al.*, 1998a). The relative stoichiometry of kinase to Elk-1 substrate used was typically <1:50.

Peptides

All peptides were synthesized in the molecular biology facility at Sheffield University. The peptides were purified by HPLC, and their purity and molecular weights were verified by mass spectroscopy. The phosphorylated (+P) and non-phosphorylated (–P) C-domain peptides were synthesized in parallel, but the +P peptide was further modified chemically prior to purification. Each peptide was synthesized by standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry, and in the case of the +P peptide, triphenylmethyl serine was used at positions 383 and 389. The peptide was acetylated on the resin and treated with 1% trifluoroacetic acid (TFA)/5% triisopropylsilane in dichloromethane to remove the triphenylmethyl groups. The resin was dried and treated with a dimethylformamide (DMF)–tetrazole–dibenzyl-*N,N*-diisopropylphosphoramidite, followed by washing with DMF and cleavage of the peptide from the resin.

B-box peptide (N-GLARSSRNEYMRSLYSTFTIQLQ-C), D-domain peptide (N-KGRKPRDLEPLSPSLGGPGPE-C) and C-domain peptide (–P) (N-SIHFWSTLSPAPRSPAKLSFQFPS-C). The sites modified with phosphate groups in the C-domain peptide (+P) are underlined.

Proteolytic digests

Partial proteolytic digests were carried out by adding trypsin (10–40 ng) to purified Elk-1 derivatives (1–5 pmol) in 20 µl reaction volumes. Digestions were carried out at 30°C for either 10 min or over a 60 min time course, and were terminated by the addition of SDS loading buffer and immediate boiling at 100°C for 10 min. Proteolytic fragments were fractionated on SDS–PAGE containing 12% standard acrylamide or 10% ProSieve® acrylamide (FMC). C-terminally tagged fragments were visualized by Western blot analysis using anti-M2 Flag-tag antibodies (Yang *et al.*, 1998a) and molecular weights estimated by comparison to known standards. Samples were also analysed by gel-retardation analysis in which the ETS-domain acts as an N-terminal tag. For digestion in the presence of the E74 binding site, protein samples were incubated with the DNA for 10 min at room temperature prior to protease addition.

Circular dichroism and fluorescence spectra

CD spectra were obtained from 100–250 µg/ml samples in 0.2 mm quartz cuvettes on a Jobin–Yvon CD6 spectrophotometer. Curve fitting and α -helical content were calculated according to the method in Manavalan and Johnson (1987). The CD spectra of Elk-1(L158P) showed a similar phosphorylation-inducible change to the WT protein (Figure 1E) [26–31% (WT), 26–34% (L158P)]. Fluorescence emission spectra were obtained in ratio mode on an SLM 8100 spectrophotometer from 500 µl samples (10–25 µg/ml) in a 5-mm-pathlength cuvette using an excitation wavelength of 280 nm and cross-polarizing prisms to remove scattering light. Samples for CD and fluorescence emission spectroscopy were measured in 1× phosphate-buffered saline (100 mM NaCl, 1 mM KCl, 5 mM Na₂HPO₄, 1 mM KH₂PO₄, pH 7.4).

GST pull-down assays

Pull-down assays for protein–protein interactions with GST fusion proteins were carried out essentially as described previously (Yang *et al.*, 1998a). Samples were subjected to SDS–PAGE and detected by Western blot analysis using anti-M2 Flag epitope antibodies.

Gel-retardation assays

Gel-retardation assays were performed in 10 µl reaction volumes with a ³²P-labelled E74 site and analysed as described previously (Yang *et al.*, 1998a). In peptide competition experiments, different concentrations (final concentrations ~7–350 µM) of peptides were pre-incubated with the proteins for 30 min at room temperature prior to the addition of DNA.

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