

Differential targeting of MAP kinases to the ETS-domain transcription factor Elk-1

Shen-Hsi Yang, Alan J. Whitmarsh¹,
Roger J. Davis¹ and Andrew D. Sharrocks²

Department of Biochemistry and Genetics, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, UK and ¹Program in Molecular Medicine, Department of Biochemistry and Molecular Biology, Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA 01605, USA

²Corresponding author
e-mail: a.d.sharrocks@ncl.ac.uk

The activation of MAP kinase (MAPK) signal transduction pathways results in the phosphorylation of transcription factors by the terminal kinases in these cascades. Different pathways are activated by mitogenic and stress stimuli, which lead to the activation of distinct groups of target proteins. The ETS-domain transcription factor Elk-1 is a substrate for three distinct classes of MAPKs. Elk-1 contains a targeting domain, the D-domain, which is distinct from the phosphoacceptor motifs and is required for efficient phosphorylation and activation by the ERK MAPKs. In this study, we demonstrate that members of the JNK subfamily of MAPKs are also targeted to Elk-1 by this domain. Targeting via this domain is essential for the efficient and rapid phosphorylation and activation of Elk-1 both *in vitro* and *in vivo*. The ERK and JNK MAPKs use overlapping yet distinct determinants in the D-domain for targeting to Elk-1. In contrast, members of the p38 subfamily of MAPKs are not targeted to Elk-1 via this domain. Our data therefore demonstrate that different classes of MAPKs exhibit differential requirements for targeting to Elk-1.

Keywords: Elk-1/ETS-domain proteins/MAP kinase/TCFs/transcription factor

Introduction

The MAP kinase (MAPK) pathways play major roles in converting mitogenic and stress stimuli into nuclear responses (reviewed in Treisman, 1996; Whitmarsh and Davis, 1996). In humans, at least three parallel pathways exist which can be classified according to the sequence conservation in the terminal MAPKs. The ERK pathway primarily transmits mitogenic and differentiation stimuli, whereas the JNK and p38 pathways primarily transduce stress and cytokine stimuli to the nucleus. These pathways are conserved in a diverse range of organisms including *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Caenorhabditis elegans* (reviewed in Treisman, 1996). Several distinct MAPKs have been identified in each class of pathway. For example, the ERK subclass contains ERK1 and ERK2, the JNK subclass contains JNK1, JNK2 and JNK3, and the p38 subclass contains p38 α , p38 β ,

p38 γ and p38 δ (Gupta *et al.*, 1996; Jiang *et al.*, 1996; Lechner *et al.*, 1996; Stein *et al.*, 1997; Cuenda *et al.*, 1997; Goedert *et al.*, 1997; Wang *et al.*, 1997; Enslin *et al.*, 1998; reviewed in Whitmarsh and Davis, 1996; Robinson and Cobb, 1997). In addition, multiple MAPK isoforms are generated by alternative splicing which is best illustrated by the JNK MAPKs (Gupta *et al.*, 1996). The large number of MAPKs potentially allows differential phosphorylation of nuclear substrates and thereby specific responses to upstream signals. However, the substrate specificity determinants for these related kinases are just beginning to be understood.

A combination of *in vitro* and *in vivo* approaches has led to the identification of several nuclear targets for MAPK pathways. For example, c-Myc (Gupta and Davis, 1994) and Spi-B (Mao *et al.*, 1996) are ERK substrates, c-Jun (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994) and NFAT4 (Chow *et al.*, 1997) represent JNK targets, whereas CHOP (Wang and Ron, 1996) and MEF2C (Han *et al.*, 1997) are targets for the p38 pathways. ATF-2 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; Van Dam *et al.*, 1995) and ATF α (Gupta *et al.*, 1995; Bocco *et al.*, 1996) are targets for both the JNK and p38 stress-activated pathways. Members of the ternary complex factor (TCF) sub-family of ETS-domain transcription factors are also targets of MAPK pathways (reviewed in Treisman, 1996; Whitmarsh and Davis, 1996). The TCF Elk-1 is a target for all three pathways (Price *et al.*, 1996; Janknecht and Hunter, 1997a; Whitmarsh *et al.*, 1997; reviewed in Treisman, 1996). However, another family member, SAP-1, appears to only be phosphorylated and activated efficiently by the ERK and p38 pathways (Price *et al.*, 1996; Whitmarsh *et al.*, 1995, 1997; Strahl *et al.*, 1996), although it can also act as a JNK substrate (Janknecht and Hunter, 1997b). Phosphorylation of the TCFs takes place at multiple residues in the conserved C-terminal transcriptional activation domain (C-domain) which leads to enhanced DNA binding and TCF-mediated transcriptional activation (reviewed in Treisman, 1994, 1996; Price *et al.*, 1996).

It is becoming apparent that the substrate specificity of MAPKs is determined by two components: the sequence and local context of the phosphoacceptor motifs, and binding of the kinase to docking sites on the substrate. For example, in the case of phosphorylation of c-Jun by the JNK MAPKs, the local context of the phosphoacceptor motifs plays a major role in substrate specificity determination in combination with targeting via a kinase docking domain on the transcription factor (Derijard *et al.*, 1994; Kallunki *et al.*, 1994, 1996; Sluss *et al.*, 1994; Dai *et al.*, 1995; Gupta *et al.*, 1996). A short region of c-Jun, the δ -domain, appears to be sufficient for this interaction (Derijard *et al.*, 1994; Kallunki *et al.*, 1994, 1996; Sluss *et al.*, 1994; Dai *et al.*, 1995; Gupta *et al.*, 1996). Similar

interactions occur between JNK MAPKs and ATF-2 via a short motif which is distinct from the phosphoacceptor sites (Gupta *et al.*, 1995, 1996; Livingstone *et al.*, 1995). It has recently been demonstrated that the ERK MAPKs are also targeted to a nuclear substrate, Elk-1, by a docking motif known as the D-domain which is conserved amongst the TCFs (Yang *et al.*, 1998). The D-domain is located N-terminally from the transcriptional activation domain and is required for efficient phosphorylation of Elk-1 within this adjacent domain and hence enhancement of its transcriptional activation potential (Yang *et al.*, 1998).

In this study, we have investigated the targeting of a panel of mitogen- and stress-activated MAPKs to Elk-1. Elk-1 has been implicated as a substrate for members of all three classes of MAPKs and hence represents an excellent candidate to investigate targeting of MAPKs. The ERK and JNK MAPKs are both targeted to Elk-1 via the D-domain. Targeting is essential for efficient phosphorylation of Elk-1 *in vitro* and *in vivo*. However, different residues in the D-domain are important for ERK and JNK binding. In contrast, the p38 MAPKs are not targeted to Elk-1 via the D-domain. Our results therefore demonstrate that Elk-1 contains a docking site that contains specificity determinants which allow recognition by two different classes of MAPKs but exclude binding of a third class. Such specificity determinants within transcription factor substrates are likely to play a pivotal role in producing unique nuclear responses to the activation of diverse MAPK signal transduction pathways.

Results

Differential requirement for the Elk-1 D-domain for phosphorylation by MAPKs *in vitro*

Elk-1 has been shown to be an *in vitro* substrate for all three classes of MAPKs and to be regulated by these kinase cascades *in vivo* (Price *et al.*, 1996; Janknecht and Hunter, 1997a; Whitmarsh *et al.*, 1997; reviewed in Treisman, 1996; Whitmarsh and Davis, 1996). It has recently been demonstrated that Elk-1 contains an ERK targeting motif that maps to the D-domain which is located N-terminally from the transcriptional activation domain (Figure 1A). The integrity of this domain is required to allow efficient Elk-1 phosphorylation *in vitro* (Yang *et al.*, 1998). In order to investigate whether the efficiency of Elk-1 phosphorylation by members of the stress-activated MAPKs is enhanced by the presence of the D-domain, we investigated the ability of a representative member of each subclass, JNK-1 and p38 γ , to phosphorylate a series of truncated Elk-1 derivatives (Figure 1).

N-terminal truncations of Elk-1 up to amino acid 310 (Elk-310) did not alter the efficiency of Elk-1 phosphorylation by ERK2. However, truncations up to and beyond amino acid 330 (Elk-330 and Elk-349), which delete the D-domain, resulted in a significant reduction in Elk-1 phosphorylation (Figure 1A; Figure 1B, lanes 1–4; Yang *et al.*, 1998). Similarly, the efficiency of phosphorylation of the proteins Elk-330 and Elk-349 by JNK-1 was dramatically reduced (Figure 1A; Figure 1B, lanes 5–8). In contrast, the efficiency of phosphorylation of all the Elk-1 deletion proteins by p38 γ was virtually identical (Figure 1A; Figure 1B, lanes 9–12). Taken together, these results indicate that the integrity of the D-domain is

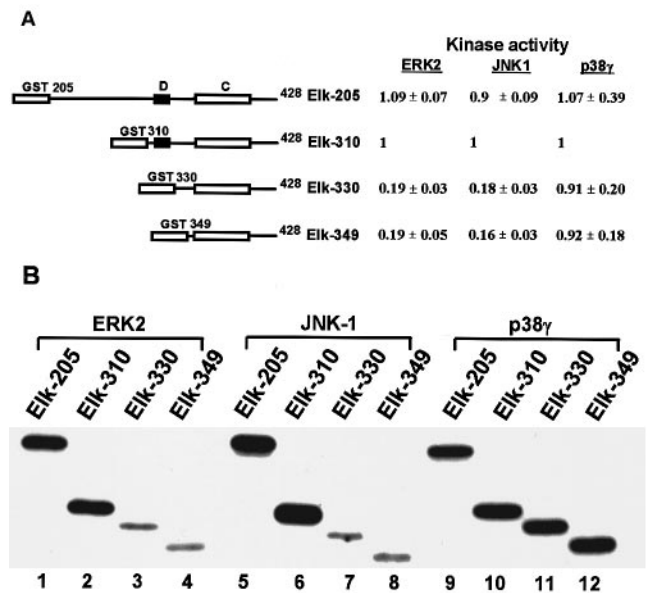


Fig. 1. Differential requirement for the D-domain for efficient Elk-1 phosphorylation by MAPKs. (A) A diagram illustrating a series of truncated Elk-1 proteins fused to GST. The black box represents the D-domain of Elk-1 (amino acids 312–334), and the numbers of the N- and C-terminal amino acids in the Elk-1 moiety are indicated. The degree of phosphorylation of each protein in the immune complex protein kinase assay (relative to Elk-310) is indicated. (B) The phosphorylation of GST–Elk fusion proteins by MAPKs was examined using either activated ERK2 or epitope-tagged JNK-1 and p38 γ which had been immunopurified from UV-activated COS-1 cells. The activity of each protein kinase towards the substrate GST–Elk205 was standardized with respect to activated ERK2 (1U, NEB). Kinase assays were performed for 15 min at 30°C with equal molar quantities (5 pmol) of GST–Elk-1 fusion proteins as substrates.

required for efficient phosphorylation of Elk-1 by the ERK and JNK MAPKs but not by p38 γ .

In order to determine whether the differential requirement for the D-domain is conserved within each class of MAPK, we tested the ability of several members of each subfamily to phosphorylate full-length Elk-1 in the presence or absence of the D-domain. The activity of each MAPK towards Elk-1 was initially standardized (Figure 2B, lanes 1–4; Figure 2C). The kinetics of phosphorylation of wild-type Elk-1 were virtually indistinguishable (Figure 2B, lanes 1–4), although graphical analysis indicated that phosphorylation by ERK-2, JNK-1 and, to a lesser extent, JNK-2 was non-linear and rapidly reached maximal levels (Figure 2C). In contrast, differential phosphorylation of Elk-1 Δ D (which lacks the D-domain) by individual MAPKs was observed (Figure 2B, lanes 5–8; Figure 2C). The kinetics and overall efficiency of phosphorylation of Elk-1 Δ D by p38 α , p38 β and p38 γ were virtually indistinguishable from wild-type Elk-1 (compare Figure 2B, lanes 1–4 and 5–8; Figure 2C). In contrast, the kinetics of Elk-1 Δ D phosphorylation by ERK2, JNK-1 and JNK-2 were delayed and the overall efficiency greatly reduced. It appears, therefore, that the differential requirement for the D-domain for efficient phosphorylation of Elk-1 is a common property of all members of a particular subclass of MAPK.

Identification of important residues in the D-domain

The D-domain plays an important role in enhancing Elk-1 phosphorylation by the ERK and JNK MAPKs. In order

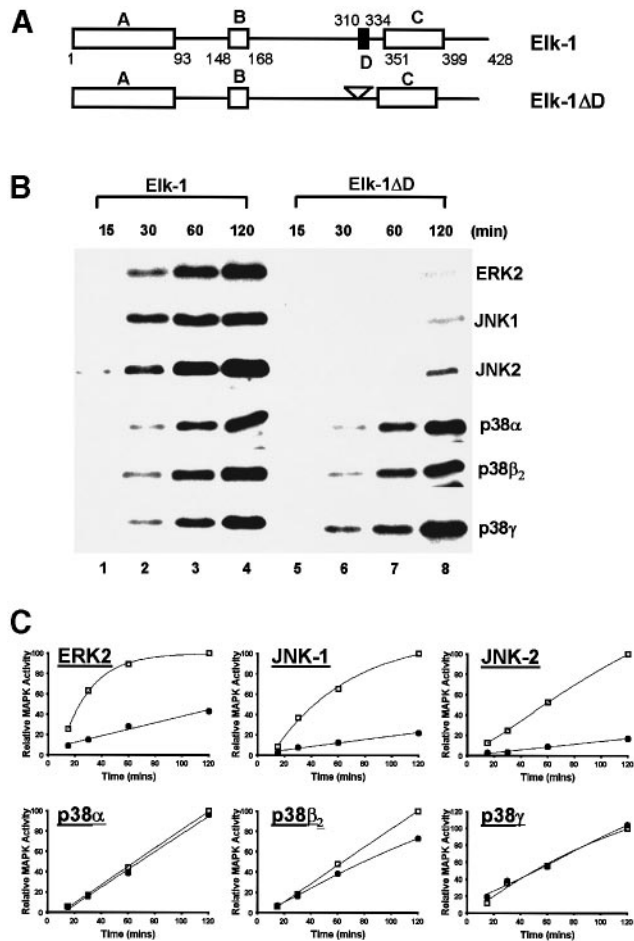


Fig. 2. Kinetic analysis of Elk-1 phosphorylation by MAPKs in the presence and absence of the D-domain. (A) Diagrammatic illustration of full-length Elk-1 and Elk-1 with a nine amino acid deletion in the D-domain (Elk-1ΔD). Bacterially expressed FLAG epitope-tagged full-length Elk-1 and Elk-1ΔD proteins were purified and equimolar concentrations were used in all assays. (B) Full-length Elk-1 (lanes 1–4) and Elk-1ΔD (lanes 5–8) (3.75 pmol of each) were phosphorylated by MAPKs (as indicated on the right panel of the figure) for the times indicated above each lane. The activity of each kinase was standardized to ERK-2 as described in Figure 1. (C) Graphical representation of the data from (B). Open and closed squares denote wild-type Elk-1 and Elk-1ΔD respectively used as MAPK substrates. Data are presented relative to phosphorylation of wild-type Elk-1 after 120 min (taken as 100).

to investigate whether identical residues within this domain are involved in this function, pairs of amino acids were mutated to alanine residues (Figure 3A). Such mutations should preserve any structural motifs which are present but remove side chains which are available for intermolecular interactions. The mutant proteins were tested as substrates for a panel of MAPKs (Figure 3B). Mutations in the N-terminal half of the D-domain resulted in significant reductions in the efficiency of phosphorylation by ERK2 (Figure 3B, mutants M1 and M2; Yang *et al.*, 1998). Similarly, reductions in the efficiency of phosphorylation of the M1 and M2 mutants by JNK-1 and JNK-2 were observed (Figure 3B, lanes 2 and 3). However, in the case of JNK-1 and JNK-2, additional important residues were identified by the M3 mutant which was also phosphorylated to a reduced level (Figure 3B, lane 4). In contrast, none of the mutations resulted in large decreases in the efficiency

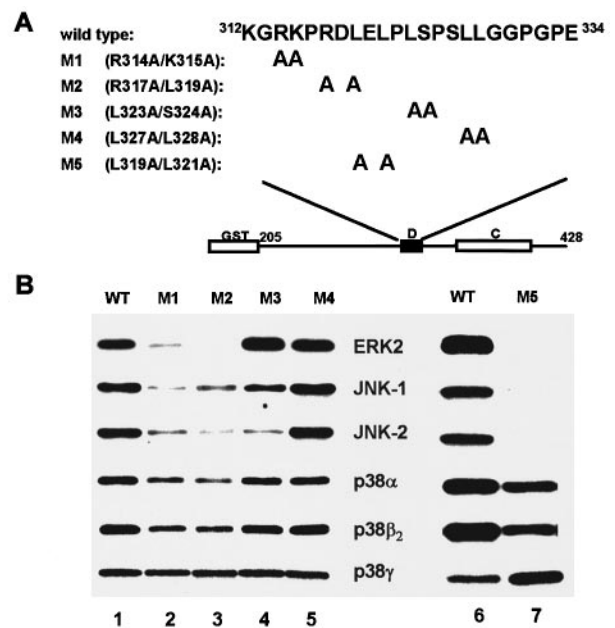


Fig. 3. Identification of residues within the D-domain required for efficient phosphorylation by MAPKs. (A) Amino acid sequence of the wild-type (WT) and D-domain mutants R314A/K315A (M1), R317A/L319A (M2), L323A/S324A (M3), L327A/L328A (M4) and L319A/L321A (M5) are indicated. Numbers above the sequence represent the N- and C-terminal residues in the D-domain. (B) Kinase assays of wild-type and mutant GST-Elk205 fusion proteins by MAPKs were carried out as described in Figure 1. Amounts of phosphorylation of the mutant M1, M2, M3, M4 and M5 Elk-1 proteins relative to WT Elk-1 (taken as 1) are 0.47 ± 0.06 , 0.40 ± 0.04 , 1.10 ± 0.22 , 1.12 ± 0.28 and 0.23 ± 0.11 (ERK2; Yang *et al.*, 1998); 0.45 ± 0.06 , 0.39 ± 0.11 , 0.63 ± 0.10 , 1.19 ± 0.20 and 0.21 ± 0.06 (JNK-1); 0.39 ± 0.04 , 0.28 ± 0.06 , 0.46 ± 0.09 , 1.01 ± 0.01 and 0.14 ± 0.05 (JNK-2); 0.79 ± 0.07 , 0.72 ± 0.04 , 1.0 ± 0.09 , 1.17 ± 0.24 and 0.75 ± 0.35 (p38α); 0.71 ± 0.15 , 0.66 ± 0.04 , 0.97 ± 0.01 , 1.05 ± 0.07 and 0.77 ± 0.09 (p38β₂); and 0.97 ± 0.08 , 0.9 ± 0.18 , 0.98 ± 0.17 , 1.08 ± 0.18 and 1.22 ± 0.31 (p38γ).

of Elk-1 phosphorylation by p38γ and, in comparison with the effect on ERK and JNK MAPKs, only small differences were seen for phosphorylation of the M1 and M2 mutants by p38α and p38β₂ (Figure 3B). Finally, an additional mutant was created (M5) which has mutations in both of the leucine residues which make up the conserved 'LXL' central core of several MAPK targeting motifs (Yang *et al.*, 1998). The efficiency of phosphorylation of the M5 mutant by the ERK and JNK MAPKs is severely reduced whereas little effect is seen on phosphorylation by the p38 MAPKs (Figure 3B, lane 7).

These results demonstrate that residues in the D-domain play different roles in directing phosphorylation of Elk-1 by MAPKs. Residues in the N-terminal end of this domain are important for both the ERK and JNK MAPKs, with further C-terminal residues also being important for the JNKs. In contrast, mutations within the D-domain have minimal effects on phosphorylation by the p38 MAPKs, consistent with the lack of effect of deleting this domain.

Differential binding of MAPKs to the Elk-1 D-domain

It previously has been demonstrated that the ability of ERK2 to phosphorylate Elk-1 correlates with its ability to bind to its substrate via the D-domain (Yang *et al.*, 1998). Moreover, JNKs have also been demonstrated to

interact physically with their substrate c-Jun via the δ -domain (Derijard *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994; Dai *et al.*, 1995) and with Elk-1 (Gille *et al.*, 1996), although in the latter case the binding site was not determined. In comparison with ERK2, binding of JNKs to Elk-1 was barely detectable using the GST pull-down assay (Gupta *et al.*, 1996; data not shown), suggesting that the kinetics of interaction differ between these two different classes of kinases. In order to investigate binding of the kinases to Elk-1 under the same conditions, we therefore adopted a different approach using a peptide competition assay. Peptides were synthesized which correspond to Elk-1 amino acids 311–328 and encompass the D-domain (Figure 4A). Increasing amounts of these peptides were included in kinase assays to compete for binding of the MAPKs to Elk-1 via the D-domain. Mutant D-domain peptides and a peptide from JIP-1, a specific JNK inhibitor (Dickens *et al.*, 1997), were used as controls (Figure 4A).

The wild-type and M3 mutant D-domain peptides both acted as inhibitors of ERK2 in a concentration-dependent manner (Figure 4B, lanes 2–4 and 8–10). In contrast, the M5 and JIP peptides did not act as competitors (Figure 4B, lanes 5–7 and 12–13). These results are consistent with the observation that the M3 mutation affects neither the efficiency of phosphorylation nor binding by ERK2, whereas the M5 mutation inhibits both these functions (Figure 3; Yang *et al.*, 1998; data not shown). Similarly, the wild-type peptide inhibits phosphorylation by the JNK kinases whereas the M5 peptide is an ineffective competitor. In comparison with ERK2, the wild-type peptide inhibits phosphorylation by the JNK kinases whereas the M5 peptide is an ineffective competitor. In comparison with ERK2, the wild-type peptide inhibits phosphorylation by JNK-1 and JNK-2 at a 10-fold lower concentration (Figure 4B, compare lanes 3 and 4). Significantly, inhibition of JNK-1 and JNK-2 by the M3 peptide is reduced in comparison with the wild-type peptide (Figure 4B, compare lanes 3 and 9), which correlates with the reduction in phosphorylation observed in the M3 mutant protein (Figure 3). The control JIP peptide acts as an efficient JNK inhibitor in this assay (Figure 4B, lanes 12 and 13). In contrast, none of the peptides used act as efficient inhibitors of the p38 MAPKs (Figure 4B).

In order to confirm that the peptides are blocking substrate binding rather than impairing the catalytic activity of the MAPKs, they were used as competitors in kinase reactions containing ERK2 and JNK-2 and the substrates myelin basic protein (MBP) and c-Jun respectively. The binding of MAPKs to a docking domain on MBP is not thought to occur and, consistent with this concept, phosphorylation of MBP by ERK2 was not inhibited by the presence of either the wild-type, M3 or M5 peptides (Figure 4C). The JIP peptide inhibited phosphorylation of c-Jun by JNK-2 (Figure 4D, lanes 8 and 9). This inhibition previously has been attributed to competition for binding of the kinase to the c-Jun δ -domain (Dickens *et al.*, 1997). Similarly, the wild-type D-domain peptide inhibited phosphorylation of c-Jun by these kinases, albeit to a lesser extent (Figure 4D, lanes 2 and 3). Mutant D-domain peptides which are defective in JNK binding are also defective in inhibiting phosphorylation of c-Jun by JNKs (Figure 4D, lanes 4–7). Thus, the D-domain peptide is acting as expected to disrupt inter-

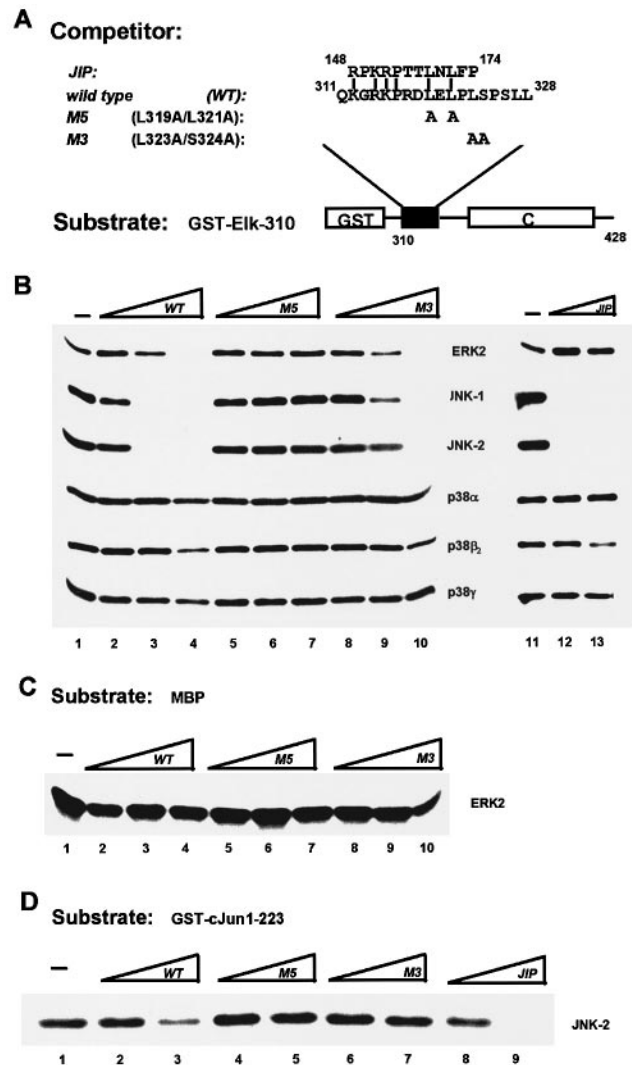


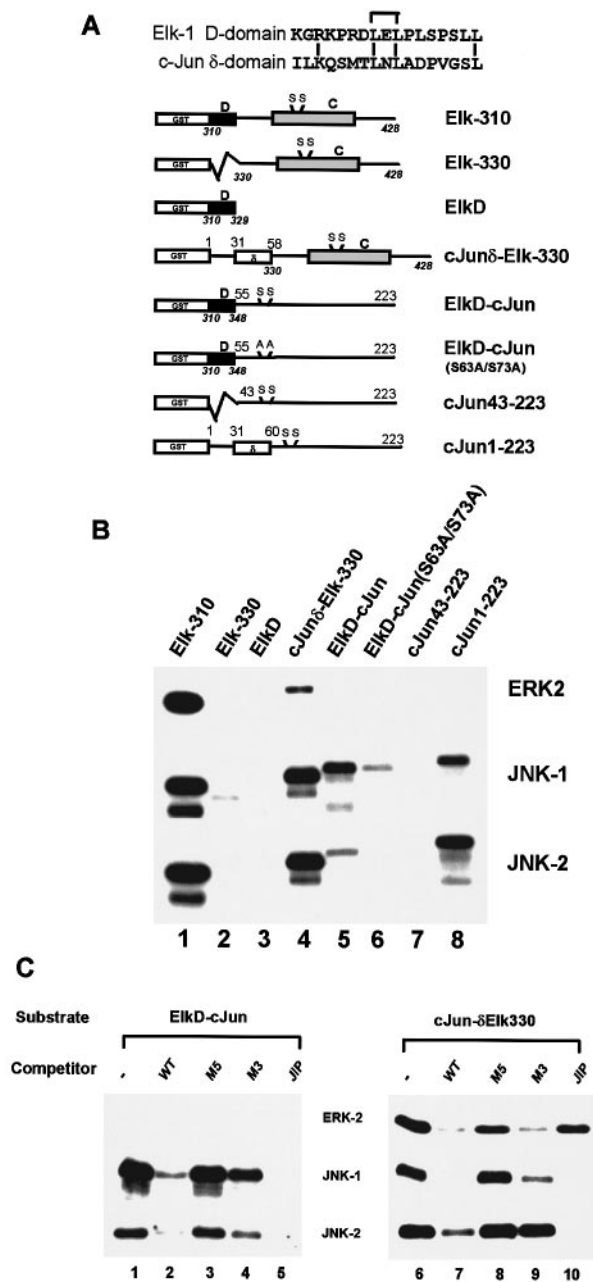
Fig. 4. The Elk-1 D-domain acts as a binding site for the ERK and JNK MAPKs. (A) The sequences of peptide competitors and a diagrammatic illustration of the substrate (GST-Elk310, 5 pmol) used in the kinase reactions. Identical or highly conserved (Arg/Lys) residues conserved in the Elk-1- and JIP-derived peptides are indicated by dashes. (B) Phosphorylation of Elk-1 by MAPKs in the presence of competitor peptides. The peptide competition assay was based on the kinase assays described in Figure 1 except that the MAPKs were pre-incubated in the absence (lanes 1 and 11) or presence of competitor peptides (10- to 1000-fold excess over Elk-1 substrate), 50 pmol (lanes 2, 5, 8 and 12), 500 pmol (lanes 3, 6, 9 and 13) and 5 nmol (lanes 4, 7 and 10), respectively. (C) and (D) Competition assays were carried out as in (B) by using 5 pmol of MBP and GST-c-Jun as substrates, respectively. The assays were performed in the absence (C and D, lane 1) or presence of peptide competitors, 50 pmol (C, lanes 2, 5 and 8; D, lanes 2, 4, 6 and 8), 500 pmol (C, lanes 3, 6 and 9; D, lanes 3, 6, 9 and 12) and 5 nmol (C, lanes 4, 7 and 10), respectively. Increases in the concentration of added competitor peptides are indicated schematically above each set of lanes.

actions between the JNKs and c-Jun but is an ineffective competitor of phosphorylation of MBP by ERK2.

Collectively, these data demonstrate that the D-domain acts as a binding site for both the ERK and JNK MAPKs, but phosphorylation of Elk-1 by the p38 MAPKs does not take place via this mechanism.

The JNK targeting domains of Elk-1 and c-Jun act as discrete interchangeable modules

The Elk-1 D-domain is sufficient to bind to ERK2 in a heterologous context when fused to another protein (Yang *et al.*, 1998). In order to investigate whether the D-domain can act in a similar manner to allow JNK targeting, reciprocal chimeric proteins were created in which the MAPK targeting domains of c-Jun and Elk-1 were exchanged (Figure 5A). Deletion of the Elk-1 D-domain (Elk-330; Figure 1; Figure 5B, lanes 1 and 2) or the δ -domain from c-Jun (cJun43–223; Figure 5B, lanes 7 and 8) resulted in a loss in the efficiency of phosphorylation by both JNK-1 and JNK-2. However, addition of the reciprocal targeting domains to these truncated proteins converted them back to efficient JNK substrates (cJun δ -Elk-330 and ElkD-cJun; Figure 5B, lanes 4 and 5). Interestingly, of the two chimeric proteins, only cJun δ -Elk-330 exhibited enhanced phosphorylation by ERK2



despite the D-domain in the ElkD-cJun chimera representing an ERK-binding motif (Figure 5B, top panel, lanes 3 and 4). This is in agreement with the observation that the local context of the phosphoacceptor motifs also plays a major role in determining the specificity of c-Jun phosphorylation (Gupta *et al.*, 1996; Kallunki *et al.*, 1996). Finally, in order to rule out that the D-domain itself was phosphorylated by the MAPKs, two control constructs were made and tested. Neither ElkD (which contains the D-domain alone) nor ElkD-cJun(S63A/S73A) (which lacks the primary JNK phosphoacceptor motifs) represent good ERK or JNK substrates (Figure 5B, lanes 3 and 6).

In order to investigate binding of the kinases and the relative strengths of the different binding motifs, peptide competition assays were carried out with the chimeric Elk-1-c-Jun proteins (Figure 5C). The wild-type D-domain and JIP peptides acted as efficient competitors of ElkD-cJun phosphorylation by JNKs (Figure 5C, lanes 2 and 5). Competition was also exhibited by the M3 peptide, albeit to a lesser extent with JNK-2, whereas the M5 peptide was an ineffectual competitor (Figure 5C, lanes 3 and 4). These results are essentially the same as observed with wild-type Elk-1 (Figure 4), thereby demonstrating that the D-domain acts in a similar manner in a heterologous context. Competition assays were also carried out with cJun δ -Elk-330, and again the JIP peptide acted as an efficient competitor of phosphorylation by JNKs (Figure 5C, lane 10). The wild-type and M3 D-domain peptides also acted as competitors, but to a lesser extent than observed with ElkD-cJun (Figure 5C, compare lanes 7 and 9 with lanes 2 and 4), indicating that the δ -domain acts as a stronger JNK-binding motif. Furthermore, competition for JNK-1 was more pronounced than for JNK-2 (Figure 5C, lanes 7 and 9; compare JNK-1 and JNK-2 panels). This is consistent with the observation that the c-Jun δ -domain preferentially binds JNK-2 (Kallunki *et al.*, 1994; Sluss *et al.*, 1994; Gupta *et al.*, 1996) whereas the Elk-1 D-domain preferentially binds JNK-1 (Figure 4; data not shown). Residual ERK phosphorylation of cJun δ -Elk-330 was inhibited by the wild-type and M3 peptides

Fig. 5. The JNK-binding domain of Elk-1 (D-domain) and c-Jun (δ -domain) can be functionally interchanged. (A) A diagram illustrating fusions of GST to Elk-310 (amino acids 310–428), Elk-330 (amino acids 330–428), ElkD (amino acids 310–329), cJun δ -Elk-330 chimera (amino acids 1–58 of c-Jun, Elk-1 amino acids 330–428), ElkD-cJun chimera (amino acids 321–348 of Elk-1, c-Jun amino acids 55–223), ElkD-cJun (S63A/S73A), cJun43–223 (c-Jun amino acids 43–223) and c-Jun (amino acids 1–223). The numbers of the N- and C-terminal c-Jun (normal type) and Elk-1 (italics) amino acids are indicated above and below each construct respectively. The locations of the major JNK phosphorylation sites in c-Jun (Ser63 and Ser73) are indicated as the most functionally important phosphoacceptor sites in Elk-1 (Ser383 and Ser389). The sequences of the Elk-1 D-domain and c-Jun δ -domain are shown and are indicated by solid and open boxes, respectively. The Elk-1 transcriptional activation domain (C-) is shown by a grey box. Identical or highly conserved (Arg/Lys) residues conserved between the two domains are indicated by dashes. The central 'LXL' motif is bracketed. (B) Kinase assays of GST fusion proteins as substrates for the indicated MAPKs were carried out using 5 pmol of each protein for 30 min reactions as described in Figure 1. (C) Phosphorylation of chimeric Elk-1-c-Jun chimeras by MAPKs in the presence of competitor peptides. Peptide competition assays were carried out as described in Figure 4. Five pmol of each chimeric protein were used in the reactions with 500 pmol of the indicated peptides. The exposure of the ERK2 panel was selected in order to show equivalent ERK and JNK phosphorylation of cJun δ -Elk-330.

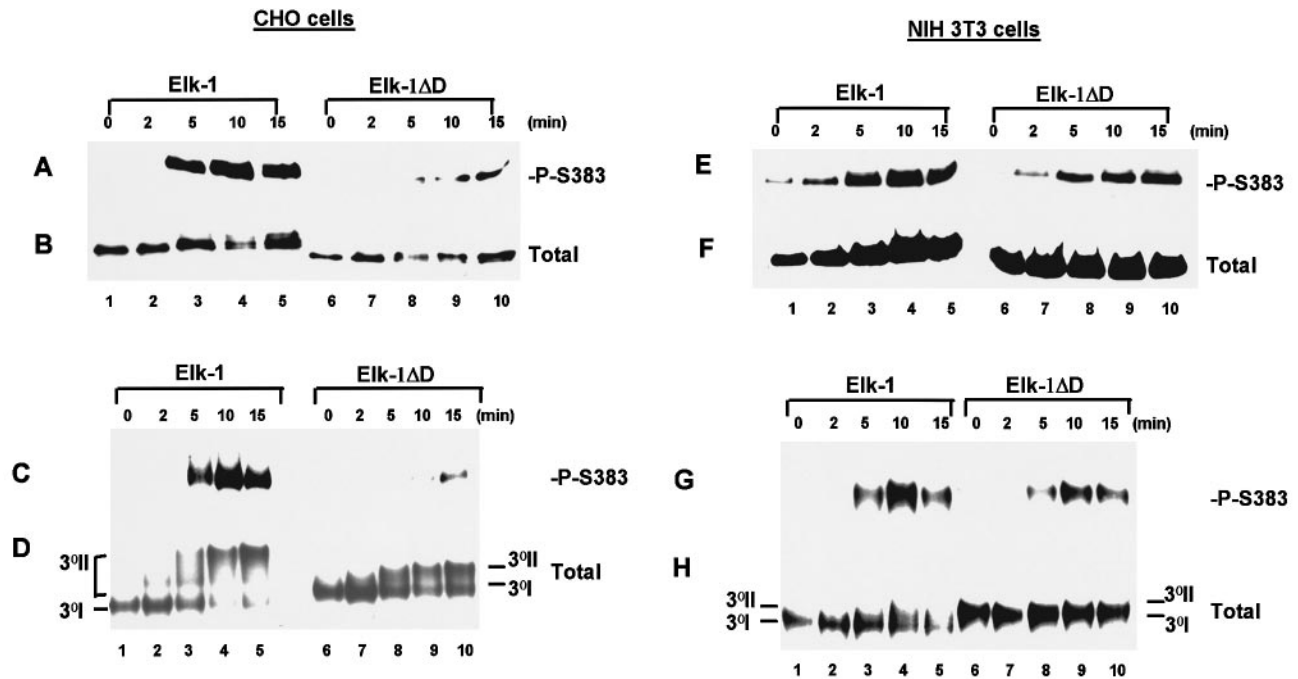


Fig. 6. Role of the D-domain in Elk-1 phosphorylation *in vivo* in IL-1-stimulated CHO and NIH-3T3 cells. CHO (A–D) or NIH-3T3 (E–G) cells were transfected with 2 μ g of CMV-driven expression vectors encoding either wild-type Elk-1 or Elk-1 Δ D. Total cell extracts were taken at the indicated times after IL-1 stimulation and analysed by Western blot using the anti-Phospho Elk-1(383) antibody to detect phosphorylation of Ser383 (A and E) or anti-FLAG antibody to examine the total levels of Elk-1 and Elk-1 Δ D in each sample (B and F). Samples were also analysed by gel retardation analysis in the presence of core^{SRE} and the *c-fos* SRE (SRE*) (C, D, G and H). Anti-phospho Elk-1(383) antibody was also included. Supershifted bands representing complexes containing phosphorylated Elk-1 derivatives are shown in (C) and (G). Ternary complexes with core^{SRE} and a 134 bp fragment of *c-fos* promoter containing the SRE (SRE*) are shown in (D) and (H). The locations of unphosphorylated ternary complex (3^I) and multiple phosphorylated forms of ternary complex (3^{II}) are indicated. (C and D) and (G and H) are from separate experiments using the same extracts.

but not by the M5 and JIP peptides (Figure 5C, top panel, lanes 6–10) as predicted from previous experiments (Figure 4).

Collectively, these data demonstrate that the Elk-1 D-domain can act as a JNK-binding motif in a heterologous context. However, differences in the relative strength of kinase binding are apparent, with the *c-Jun* δ -domain acting as a stronger JNK-2-binding site than the Elk-1 D-domain and the Elk-1 D-domain preferably binding JNK-1 rather than JNK-2.

Differential targeting of the JNK and p38 MAPKs to Elk-1 *in vivo*

Evidence has been gathered to suggest that Elk-1 is a substrate for both the JNK (Cavigelli *et al.*, 1995; Gille *et al.*, 1995; Whitmarsh *et al.*, 1995, 1997) and p38 (Price *et al.*, 1996; Whitmarsh *et al.*, 1997) MAPK subclasses *in vivo*. However, the response of Elk-1 to these different stress-activated MAPK pathways differs depending on the cell type and stimulus. For example, UV activates Elk-1 via the p38 pathway in HeLa and NIH-3T3 cells (Price *et al.*, 1996). In contrast, interleukin-1 (IL-1) activates Elk-1 via the JNK pathway in CHO cells, but by a combination of the JNK and p38 pathways in NIH-3T3 cells (Whitmarsh *et al.*, 1997). We took advantage of this cell type-specific response to IL-1 to investigate the role of the D-domain in targeting stress-activated MAPKs to Elk-1 *in vivo*.

CHO and NIH-3T3 cells were transfected with either Elk-1 or Elk-1 Δ D, stimulated with IL-1, and cell extracts subsequently were prepared over a 15 min time period.

The phosphorylation status of the Elk-1 derivatives was monitored indirectly by gel retardation analysis (Figure 6D and H) and directly by supershift analysis of the Elk-1-containing ternary complexes using an anti-phospho Elk-1(Ser383) antibody (Figure 6C and G) which was also used in Western blotting experiments (Figure 6A and E). In CHO cells, the phosphorylation of Ser383 in Elk-1 occurred rapidly within 5 min of stimulation (Figure 6A and C, lane 3), and was accompanied by a shift in the mobility of the ternary DNA-bound complex (Figure 6D, lane 3). In comparison, the speed and degree of phosphorylation of Ser383 and induction of a lower mobility ternary complex containing Elk-1 Δ D was reduced. Maximal phosphorylation of Ser383 was not reached for 15 min (Figure 6A and C), and the stimulation of a lower mobility DNA-bound complex was reduced in comparison with wild-type Elk-1 (Figure 6D, compare lanes 1–5 and 6–10). Taken together with the observation that JNKs represent the major IL-1-activated protein kinases in CHO cells that activate Elk-1 (Whitmarsh *et al.*, 1997), these data provide strong evidence for a role of the D-domain in targeting these cascades to Elk-1 *in vivo*.

In contrast, in NIH-3T3 cells, phosphorylation of Ser383 in response to IL-1 stimulation took place in Elk-1 and Elk-1 Δ D with virtually identical kinetics (Figure 6E and G). However, in comparison with CHO cells, the magnitude of Ser383 phosphorylation was reduced, which is consistent with the observation that minimal effects on the mobility of ternary complexes containing either Elk-1 or Elk-1 Δ D were observed (Figure 6H). Together with the observation that p38 is a major effector of IL-1 signalling

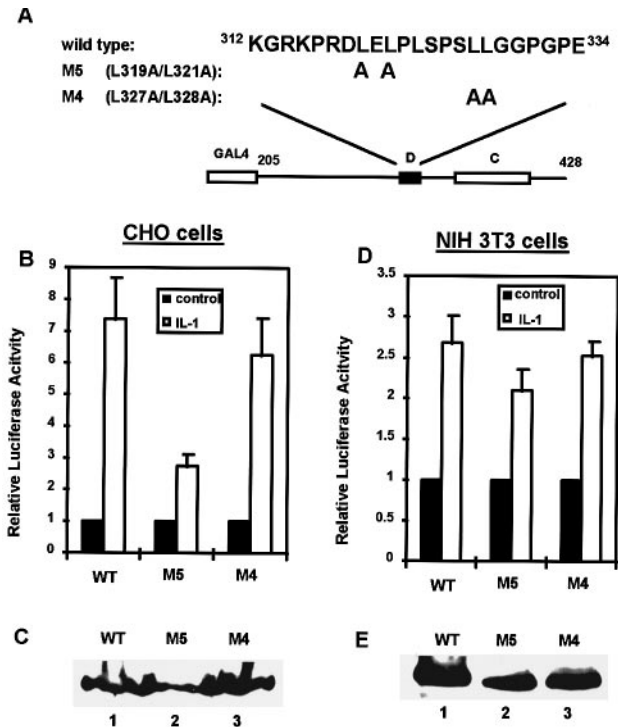


Fig. 7. The role of the Elk-1 D-domain in IL-1-inducible transcriptional activation *in vivo*. (A) A diagrammatic representation of wild-type and mutant Elk-1 proteins fused to the DNA-binding domain of GAL4. CHO (B and C) or NIH-3T3 cells (D and E) were transfected with CMV promoter-driven constructs encoding GAL4 fusions to either wild-type or mutant Elk-1 derivatives and a GAL4-driven luciferase reporter plasmid. Cells were either unstimulated or stimulated with IL-1. Transfection efficiency was monitored by using the β -galactosidase expression vector pCH110. The luciferase activities relative to the unstimulated cells of each wild-type or mutant (means \pm standard deviations; $n = 3$) are presented. Expression levels of the GAL4 fusion proteins in CHO (C) and NIH-3T3 (E) cell lines were examined by Western blotting using total cell extracts with an anti-GAL4 antibody.

to Elk-1 in NIH-3T3 cells (Whitmarsh *et al.*, 1997), these results are consistent with the notion that the Elk-1 D-domain is not required for targeting by p38 *in vivo*.

The response of GAL4 fusion proteins containing the Elk-1 transcriptional activation domain to IL-1 stimulation in these cell types was also investigated. Wild-type Elk-1 and proteins containing mutations in the D-domain were constructed which either did not affect (M4) or reduced (M5) the efficiency of phosphorylation by the JNKs *in vitro* (Figure 7A). These proteins were all expressed at equivalent levels in CHO and NIH-3T3 cells (Figure 7C and E). In CHO cells, the response of the M5 mutant to IL-1 stimulation was severely reduced (65% reduction) whereas, in comparison, the M4 mutant was virtually unaffected (15% reduction; Figure 7B). However, in NIH-3T3 cells, the response of the M5 mutant was only slightly reduced (20% reduction) and again the M4 mutant was virtually unaffected (Figure 7D). These results therefore add further weight to the notion that the D-domain is required for targeting of JNK MAPKs but not the p38 MAPKs to Elk-1 *in vivo*.

Discussion

MAPK signalling cascades play a pivotal role in converting extracellular signals into specific nuclear responses

(reviewed in Treisman, 1996; Whitmarsh and Davis, 1996). In order to achieve such specific responses, MAPKs must recognize their substrates with high specificity. A general mechanism is emerging to generate such specificity in which initial binding of the kinases to a docking domain is followed by recognition of the local context of the phosphorylation motifs (Gupta *et al.*, 1996; Kallunki *et al.*, 1996; Yang *et al.*, 1998). In the case of c-Jun, the δ -domain directs binding by the JNK subclass of MAPKs (Derijard *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994; Dai *et al.*, 1995). Similarly, the Elk-1 D-domain directs binding of the ERK subclass of MAPKs (Yang *et al.*, 1998). However, in this study, we demonstrate that the Elk-1 D-domain possesses dual specificity and is also a target for the JNK MAPKs. This domain does not, however, allow promiscuous MAPK binding but discriminates against binding by the p38 MAPK subclass. This is the first reported example of such a motif which allows diverse MAPK pathways to be integrated via a single transcription factor.

MAP kinase targeting domains

Short motifs which bind to the JNK MAPKs have been identified in the JNK inhibitor protein JIP-1 (Dickens *et al.*, 1997) and the transcription factor c-Jun (Derijard *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994; Dai *et al.*, 1995; Gupta *et al.*, 1996). In this study, we have identified a further motif in Elk-1 which targets JNKs to transcription factors. Similarities between the binding motifs from c-Jun and Elk-1 (Figure 5A) and JIP-1 and Elk-1 (Figure 4A) are apparent, with the consensus motif $^R_KXXXL^N/E$ representing a core binding site. The JNK-binding site on ATF-2 also contains a motif which loosely conforms to this consensus (KHEMTLKF) (Gupta *et al.*, 1995; Livingstone *et al.*, 1995). However, examination of the binding motifs of several JNK substrates reveals little similarity outside this central core motif although more extensive similarities between pairs of proteins can be observed (e.g. c-Jun/JIP-1 and Elk-1/NFAT4; Figure 8). Moreover, c-Jun preferentially binds to JNK-2 (Kallunki *et al.*, 1994; Sluss *et al.*, 1994; Gupta *et al.*, 1996) whereas Elk-1 preferentially binds to JNK-1 (Figures 4 and 5). Similarly, ATF-2 exhibits preferential binding of certain JNK isoforms (Gupta *et al.*, 1996). The non-conserved residues surrounding this central core motif must dictate these subtly different binding preferences. Further specificity determinants must be built into the Elk-1 D-domain as this is also bound efficiently by the ERK MAPKs (Yang *et al.*, 1998). Similarly, further specificity determinants must be built into the ATF-2 docking site as this serves as both a JNK- and p38-binding motif (N.Jones, personal communication). Binding of the ERK and JNK MAPKs to Elk-1 probably occurs with different kinetics as, although binding can be detected by a competition assay (Figure 4), only ERK binding can be detected by a GST pull-down assay (Yang *et al.*, 1998; data not shown). Moreover, as wild-type D-domain peptides displace JNKs more readily than ERK-2 from Elk-1, this suggests that the JNKs may bind with high affinity but rapidly associate and dissociate from the substrate in the presence of ATP as observed with p38 binding to MEF2C (Han *et al.*, 1997). Stable complexes between Elk-1 and JNKs have been demonstrated previously (Gille *et al.*, 1996), but we

33 **ILKQSMT-LNLADPVGSLKPHLR**AKN 57 c-Jun
 34 **LLKPSLA-VNLADPYRSLKAPGARG**P 58 JunB
 153 **RPKRPTT-LNLFQVPRSQDTLNNNS** 187 JIP-1
 312 **KGRKPRD-LELPL-SPSLLG-GPGPE** 334 Elk-1
 LERPSRDHLYLPL-EPYRESSLSPS 165 NFAT4
 28 **KHKHEMT-LKFGPARNDSVIVADQTP** 52 ATF-2
 28 **KHKHEMT-LKFGPARTDSVIIADQTP** 52 ATFa
 318 **RSKKPKG-LGL---APTLVITSSDPS** 339 SAP-1*

Fig. 8. Similarity amongst JNK-binding motifs. The sequences of the JNK-binding domains found in Elk-1, c-Jun, JunB, JIP-1, NFAT4, ATF-2 and ATFa are shown. The sequence of the D-domain of SAP-1 is also shown. The asterisk denotes that SAP-1 is predominantly an ERK substrate and is poorly phosphorylated by JNKs. The MAPK-binding motifs in Elk-1 and ATF-2 exhibit dual specificity with both JNK and ERK MAPKs targeted to Elk-1 (this study) and both JNK and p38 MAPKs targeted to ATF-2 via a single motif (N.Jones, personal communication). Amino acid numbers of the N- and C-terminal residues are given based on their location in either human c-Jun, JunB, Elk-1, NFAT4, ATF-2, ATFa, SAP-1 or mouse JIP-1 proteins. Identical or highly conserved (Arg/Lys) amino acids comprising the R/KXXXXLXL motif are highlighted. Brackets indicate the conserved central MAPK-binding motif.

are unable to detect such complexes under conditions in which we detect ERK2 binding. However, although we can detect stable complexes of ERK2 and Elk-1 both *in vitro* (Yang *et al.*, 1998) and *in vivo* when both partners are overexpressed (data not shown), the physiologically relevant binding event is likely to involve a transient interaction of the kinase and substrate after cellular stimulation. An emerging theme amongst MAPK targeting motifs is that variations on a simple core consensus can dictate binding of specific subsets of transcription factors. Specific rules are difficult to discern from the currently characterized motifs, although all are short sequences (18 amino acids in the case of the D-domain) and the central 'LXL' motif clearly plays a pivotal role in kinase targeting to transcription factors (e.g. M5 mutant, Figures 3, 4 and 7). However, MAPKs may also be targeted to substrates by different motifs, as binding of the ERK and p38 MAPKs to Mnk1 and Mnk2 kinases does not appear to be mediated by sequences related to the Elk-1 D-domain (Waskiewicz *et al.*, 1997).

In contrast to the targeting of ERK and JNK MAPKs to Elk-1 via the D-domain, this domain does not play a role in the binding of p38 MAPKs (Figure 4). The D-domain, therefore, does not represent a promiscuous MAPK targeting motif but allows discrimination between different classes of MAPKs.

Stress-activated MAPKs and Elk-1 activation

Elk-1 can be phosphorylated and activated by both the JNK (Cavigelli *et al.*, 1995; Gille *et al.*, 1995; Whitmarsh *et al.*, 1995, 1997) and p38 (Price *et al.*, 1996; Whitmarsh *et al.*, 1997) MAPK subclasses *in vitro* and *in vivo*. However, the response of Elk-1 to these different stress-activated MAPK pathways differs depending on the cell type and stimulus. IL-1 activates Elk-1 via the JNK pathway in CHO cells but by a combination of the JNK and p38 pathways in NIH-3T3 cells (Whitmarsh *et al.*,

1997), whereas UV activates Elk-1 via a combination of the p38 and ERK pathways in HeLa and NIH-3T3 cells (Price *et al.*, 1996). In the present study, several lines of evidence indicate that phosphorylation of Elk-1 requires targeting of the kinase by binding to the D-domain for the JNK but not the p38 MAPKs. First, deletions of the D-domain reduce the efficiency of Elk-1 phosphorylation by members of the JNK but not the p38 MAPK subclasses (Figures 1 and 2). Secondly, point mutations in the D-domain cause minimal reductions in the efficiency of Elk-1 phosphorylation by the p38 subclass in comparison with the JNK MAPKs (Figure 3). Thirdly, peptide competition assays demonstrate a role for the D-domain in binding the JNK but not the p38 MAPKs (Figure 4). Interestingly, although not as marked as the effect on the ERK and JNK MAPKs, subtle changes in the proficiency of Elk-1 as a substrate for p38 α and, to a lesser extent, for p38 β 2 are uncovered in the M1 and M2 mutants (Figure 3). As deletion of the D-domain does not affect phosphorylation of Elk-1 by the p38 MAPKs (Figures 1 and 2), this result may reflect that these mutations cause subtle conformational changes in Elk-1 which may restrict kinase access to the phosphoacceptor motifs. However, as the D-domain peptide acts as a weak competitor of Elk-1 phosphorylation by p38 β 2 (Figure 4), this might reflect that the Elk-1-binding site on p38 MAPKs retains some similarity to the other MAPKs and hence binds weakly to Elk-1 via the D-domain. Finally, *in vivo*, the efficiency of Elk-1 phosphorylation and activation by p38 MAPK is not dependent upon the integrity of the D-domain (Figures 6 and 7). The latter result is also consistent with the observation that stimulation of Elk-1 phosphorylation and activity *in vivo* by the p38 MAPK pathways is not as pronounced as activation by either the JNK (compare Figure 6D and H and Figure 7B and D; Whitmarsh *et al.*, 1997) or ERK pathways (Yang *et al.*, 1998). Furthermore, in response to UV stimulation in HeLa and NIH-3T3 cells, the p38 pathway appears insufficient for full Elk-1 activation and requires cooperation with the ERK pathway (Price *et al.*, 1996). Finally, in 293 cells, Elk-1 does not appear to be activated by p38 MAPKs (Janknecht and Hunter, 1997a). The reduced activation by and lack of targeting of p38 MAPKs to Elk-1 *in vitro* and *in vivo* in comparison with the strong activation elicited by the ERK and JNK MAPKs suggests that the residual activation by p38 may be a consequence of overexpressing pathway components and overriding normal specificity determinants. The p38 MAPKs may, however, phosphorylate Elk-1 in a more constitutive manner which does not require rapid and efficient kinase targeting to the substrate. Alternatively, sustained p38 activation may be required to activate Elk-1. Further studies are required to differentiate between these possibilities. Finally, p38 may bind to a docking site located elsewhere on Elk-1. However, none of the deleted proteins we have tested exhibit markedly reduced phosphorylation by p38 isoforms (Figure 1; data not shown). Moreover, no binding of p38 α , β 2 and γ to Elk-1 could be detected in GST pull-down assays carried out in the absence of ATP (data not shown). Together these results strongly suggest that p38 does not require or bind to a docking motif on Elk-1.

In conclusion, our data contribute to the understanding of how MAPKs recognize and phosphorylate their nuclear

targets. Elk-1 contains a MAPK-binding motif which allows efficient targeting and subsequent transcription factor activation by both the ERK and JNK subclasses of MAPKs. Similarly, both JNK and p38 subclasses of MAPKs are targeted via a single binding motif to ATF-2 (N. Jones, personal communication). Future studies are likely to uncover further motifs which direct binding of the p38 subclass of MAPKs to transcription factors (e.g. MEF2C; Han *et al.*, 1997). Investigation of these targeting domains will provide significant insights into how the specificity of signalling via MAPK cascades is achieved.

Materials and methods

Plasmid constructs

The following plasmids were used for expressing GST fusion proteins in *Escherichia coli*. pAS407 (encoding GST–Elk205; Elk-1 amino acids 205–428), pAS545 (encoding GST–Elk310; Elk-1 amino acids 310–428), pAS406 (encoding GST–Elk330; Elk-1 amino acids 330–428), pAS405 (encoding GST–Elk349; Elk-1 amino acids 349–428) and pAS547 (encoding GST–ElkD–cJun; Elk-1 amino acids 310–348 fused to c-Jun amino acids 55–223) have been described previously (Yang *et al.*, 1998). Plasmids encoding GST–cJun1–223 and GST–cJun43–223 have been described previously (Hibi *et al.*, 1993). pAS767, encoding GST fused to Elk-1 amino acids 307–329 (GST–ElkD), was constructed by inserting a *Bam*HI–*Eco*RI-cleaved PCR-derived fragment into the same sites of pGEX-3X. pAS768, encoding c-Jun amino acids 1–58 fused to Elk-1 amino acids 330–428 (cJun δ –Elk-330), was constructed by ligating a *Bam*HI–*Bgl*II-cleaved PCR fragment (encoding c-Jun amino acids 1–58) into the *Bgl*II site of pAS406. pAS548, pAS549, pAS550, pAS564 and pAS769 (encoding GST–Elk205 mutants) are derivatives of pAS407 with the site-directed mutations R314A/K315A (GST–Elk205M1), R317A/L319A (GST–Elk205M2), L323A/S324A (GST–Elk205M3), L327A/L328A (GST–Elk205M4) and L319A/L321A (GST–Elk205M5), respectively. pAS565 (GST–Elk307M2), pAS566 (GST–Elk307M3), pAS567 (GST–Elk310^{S383A/S389A}) and pAS568 (GST–Elk310^{M2/S383A/S389A}) were described previously (Yang *et al.*, 1998). Mutations were introduced by a two-step PCR protocol using a mutagenic primer and two flanking primers as described previously (Shore *et al.*, 1996).

pAS278 and pAS380 [encoding full-length His/FLAG-tagged Elk-1 and the same protein with an internal deletion of amino acids 312–321 (Elk-1 Δ D)] were used for expression of Elk-1 derivatives in *E. coli* (Yang *et al.*, 1998).

The following plasmids were constructed for use in mammalian cell transfections. pG5E1b contains five GAL4 DNA-binding sites cloned upstream of a minimal promoter element and the firefly luciferase gene (Seth *et al.*, 1992). pSG424 encodes the GAL4 DNA-binding domain (Sadowski and Ptashne, 1989). The vectors pCDNA3-F-JNK1 (Derijard *et al.*, 1994), pCDNA3-F-JNK2 (Sluss *et al.*, 1994), pCMV5-F-p38 α (Raingeaud *et al.*, 1995), pCDNA3-F-p38 β_2 (Enslin *et al.*, 1998) and pCDNA3-F-p38 γ (J. Raingeaud and R.J. Davis, unpublished data) encoding flag-tagged MAPKs have been described previously. pAS572 (pCMV-GAL4-Elk205) and pAS577 (pCMV-GAL4-Elk205M4) were described previously (Yang *et al.*, 1998). pAS770 (GAL4-Elk205M5) was constructed by ligating *Bam*HI–*Xba*I fragments from pAS769 into the same sites of pSG424. pAS771 (pCMV-GAL4-Elk205M5) was constructed by ligating the *Hind*III–*Xba*I fragments from pAS770 into the same sites of pCMV5. The cytomegalovirus (CMV) promoter-driven expression vectors, pAS383 and pAS387, encoding full-length Elk-1 and Elk-1 Δ D, respectively, with C-terminal FLAG tags were described previously (Yang *et al.*, 1998). All plasmid constructs encoding Elk-1-derived proteins made by PCR were verified by automated dideoxy sequencing.

Protein expression and purification

GST fusion proteins were expressed in the *E. coli* JM101 strain and purified as described previously (Shore *et al.*, 1995). Full-length hexahistidine-tagged polypeptides were expressed in *E. coli* BL21(DE3)pLysS with the pET vector system and quantified as described previously (Yang *et al.*, 1998).

Tissue culture, cell transfection and reporter gene assays

COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco-

BRL). CHO cells were maintained in F12 medium supplemented with 5% FBS. NIH-3T3 cells were maintained in DMEM supplemented with 10% FBS. Transfection experiments were carried out using Superfect transfection reagent (Qiagen) as described previously (Yang *et al.*, 1998).

For reporter gene assays, GAL4-driven promoters were co-transfected with various vectors encoding GAL4–Elk-1 fusion proteins. The activities of the GAL4 DNA-binding domain (amino acids 1–147) and GAL4–Elk-1 deletion fusion proteins (50 ng of plasmid DNA) were measured in co-transfection assays in all cell lines using 1 μ g of reporter plasmid pG5E1bLuc. Transfection efficiencies were normalized by measuring the activity from a co-transfected plasmid (1 μ g) which expresses β -galactosidase (pCH110, Pharmacia KB Biotechnology Inc.). Cell extracts were prepared, and luciferase and β -galactosidase assays were carried out as described previously (Yang *et al.*, 1998).

Protein kinase assays

In order to prepare recombinant JNK and p38 MAPKs, COS-1 cells were transfected with constructs encoding FLAG epitope-tagged MAPKs. Kinases were activated by stimulation with UV light for 30 min. Purified kinases were then eluted from beads by competing with 0.1 mg/ml of FLAG peptide. Recombinant active ERK2 was obtained from New England Biolabs (NEB) and MBP was obtained from Sigma. The kinase assays were carried out in 20 μ l reaction volumes as described previously (Yang *et al.*, 1998). The phosphorylation of substrate proteins was examined following SDS–PAGE by autoradiography, and quantified by phosphorimaging (Fuji BAS1500; TINA 2.08e software). Data were quantified by phosphorimaging and the data presented graphically after curve fitting with the appropriate equation using BIOSOFT Fig.P or Microsoft Excel software. Peptide competition experiments were carried out essentially as described above, except that pre-incubation with 50–5000 pmol of the peptide competitors with MAPKs was carried out before the kinase reactions. Final peptide concentrations were 2.5–25 μ M (10- to 100-fold excess over Elk-1 substrate).

Western blot analysis

FLAG-tagged Elk-1 and Elk-1 Δ D in extracts from CHO or NIH-3T3 cells were detected by immunoblot analysis using a mouse monoclonal anti-M2 FLAG antibody (Kodak) or anti-phosphopluTM Elk-1 (S383) antibody (NEB). GAL4 fusion proteins were detected using the anti-GAL4 antibody directed against the amino-terminal DNA-binding domain (Santa Cruz). Immune complexes were detected by using horseradish peroxidase-conjugated secondary antibody followed by ECL (Amersham).

Gel retardation assays

Gel retardation assays were performed with a ³²P-labelled 134 bp *c-fos* promoter fragment containing the SRE (SRE*) as described previously (Whitmarsh *et al.*, 1995). Total cell extracts from transfected cells containing ~0.02 pmol of Elk-1 or Elk-1 Δ D were used in DNA-binding reactions. Binding reactions on SRE-containing sites also contained purified bacterially expressed core^{SRE} (Shore and Sharrocks, 1994). Antibody supershift experiments were described previously (Yang *et al.*, 1998). Protein–DNA complexes were analysed on non-denaturing 5% polyacrylamide gels cast in 0.5 \times Tris–borate–EDTA and visualized by autoradiography and phosphorimaging.

Figure generation and data quantification

All figures were generated electronically from scanned images of autoradiographic images by using Picture Publisher (Micrografix) and Powerpoint version 7.0 (Microsoft) software. Final images are representative of the original autoradiographic images. Phosphorimager data were quantified using Tina software (version 2.08e).

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