Activation of ternary complex factor Elk-1 by stress-activated protein kinases

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Background: The mammalian response to stress results in the activation of stress-activated protein kinases (also known as cJun N-terminal kinases; SAPKs or JNKs), which are a sub-group of the mitogen-activated protein (MAP) kinase family. The SAPKs are involved in the upregulation of activity of the transcription factor AP-1 by post-translational modification of two of its components, cJun and ATF2. AP-1 activity can also be elevated by increased expression of the Fos protein, a further AP-1 component. Elk-1 (also called $p62^{TCF}$), a transcription factor involved in the induction of the expression from the c-*fos* promoter through the promoter's serum response element, is known to be activated as a result of phosphorylation by the MAP kinases ERK1 and ERK2. However, induction of c-*fos* expression in response to noxious agents takes place in the absence of ERK activation. We therefore investigated whether SAPKs similarly upregulate c-*fos* expression by phosphorylating Elk-1.

Results: Elk-1 is activated in response to stimuli other than mitogenic signals. Both $p46^{SAPK}$ and $p54^{SAPK}$ interact physically with, and phosphorylate, Elk-1. The capacity of Elk-1 to form a ternary complex with serum response factor *in vitro* is thereby elevated. *In vivo*, selective activation of SAPKs stimulates formation of the ternary complex containing Elk-1, serum response factor and the serum response element, and enhances Elk-1-dependent transcription. Expression of the SAPK upstream-activator kinase, MEKK1, induces SAPK activation and c-*fos* transcription in the absence of ERK activity. Phosphopeptide mapping of Elk-1 phosphorylated with $p46^{SAPK}$ or $p54^{SAPK}$ reveals Ser383, a residue critical for ternary complex formation and transcriptional activation, to be the major phosphorylation site.

Conclusion: Elk-1 responds to stress-induced, as well as mitogenic, signals by stimulating c-*fos* transcription through the serum response element. Phosphorylation of Elk-1 by SAPKs and the ensuing expression of Fos protein thus constitutes an additional mechanism by which cells can upregulate AP-1 activity in response to stress.

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Background

Upon exposure to noxious agents, cells initiate a stress-response programme that helps them to overcome any damage incurred to their proteins or DNA. Several of the cellular genes that are induced in response to unfavourable biological conditions contain AP-1 sequence elements, and indeed, under such conditions, AP-1 transcription factor activity is upregulated [1]. Activation of AP-1 is known to occur by at least two distinct mechanisms. Firstly, the amino-terminal domains of the AP-1 components cJun and ATF2 become phosphorylated in response to extracellular stimuli, increasing their transactivation potential [2,3]. A second mechanism known to increase AP-1 activity is transcriptional, for example by increased expression of the gene encoding the AP-1 component Fos. Growth factors cause a rapid and transient induction of expression of the c-*fos* gene. This response is mediated primarily by the ternary complex factor known as $p62^{TCF}$ or Elk-1, which is phosphorylated and activated by the ERK1 and ERK2 mitogen-activated protein (MAP) kinases, themselves part of a conserved signalling cascade [4–8]. The response can be blocked by expression of dominant-interfering mutant forms of ERK1 and ERK2, further implicating these enzymes in the transmission of mitogenic signals leading to c-*fos* induction [9].

Induced expression of c-*jun* and c-*fos* is also a response of mammalian cells exposed to alkylating agents, the protein-synthesis inhibitor anisomycin, or ultraviolet light [10–13]. The stress-activated protein kinases (also known as cJun N-terminal kinases; SAPKs or JNKs) are members of a MAP kinase sub-group capable of phosphorylating Ser63 and Ser73 of the cJun amino-terminal domain, as well as sites in ATF2 that are necessary for AP-1 activity [2,14–16]. SAPKs are also implicated in the transcriptional response which leads to an increase in AP-1 activity by making Fos protein available [12]. These kinases are strongly activated by ultraviolet light, pro-inflammatory cytokines or anisomycin. Among these agents, anisomycin is reported to activate SAPKs highly selectively, notably in the absence of ERK1 and ERK2 activation [12]. We therefore investigated whether these enzymes are involved in the activation of c-*fos* transcription.

Treatment of NIH3T3 cells with anisomycin leads to c-*fos* transcriptional induction and activates endogenous SAPKs but not ERKs. The capacity of Elk-1 to form a ternary complex with the serum response factor (SRF) and the serum response element (SRE) is also stimulated by treatment of cells with anisomycin. *In vitro*, both $p46^{SAPK}$ and $p54^{SAPK}$ stably interact with, and phosphorylate, Elk-1, leading to an increase in ternary

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complex formation. Selective stimulation of SAPKs correlates with an increase in Elk-1-mediated transcription: Elk-1-dependent reporter gene transcription is activated in anisomycin-treated cells or in cells expressing the SAPK upstream activator MEKK1, which, like anisomycin, fails to activate ERKs. Thus, phosphorylation of Elk-1 by SAPKs leads to the induction of the c-*fos* promoter in response to stress signals.

Results

The c-*fos* stress response correlates with SAPK activation

In resting fibroblasts, c-*fos* expression is undetectable, but it is induced to maximal levels within 30 minutes of stimulation by growth factors or phorbol esters. The kinetics of c-*fos* induction in NIH3T3 cells in response to noxious agents differ from those observed upon mitogenic stimulation (Fig. 1a) [10]. The onset of transcription after treatment of cells with 1.0 or 0.1 $\mu g\ ml^{-1}$ anisomycin is delayed, as c-*fos* mRNA levels peak only after 60 minutes. This observation suggests that a distinct signalling pathway may be involved.

Superinduction of 'immediate-early' gene transcription in response to inhibition of protein synthesis is a well-documented phenomenon [17]. However, the induction of immediate-early gene expression by anisomycin has been documented to occur independently of protein synthesis inhibition [11,18]. Several observations confirm that the induction of c-*fos* expression by anisomycin under our experimental conditions occurs in the absence of translational inhibition. If c-*fos* induction resulted from inhibition of protein synthesis, one would expect the induction to be enhanced with increasing concentrations of anisomycin. However, the same delayed kinetics are

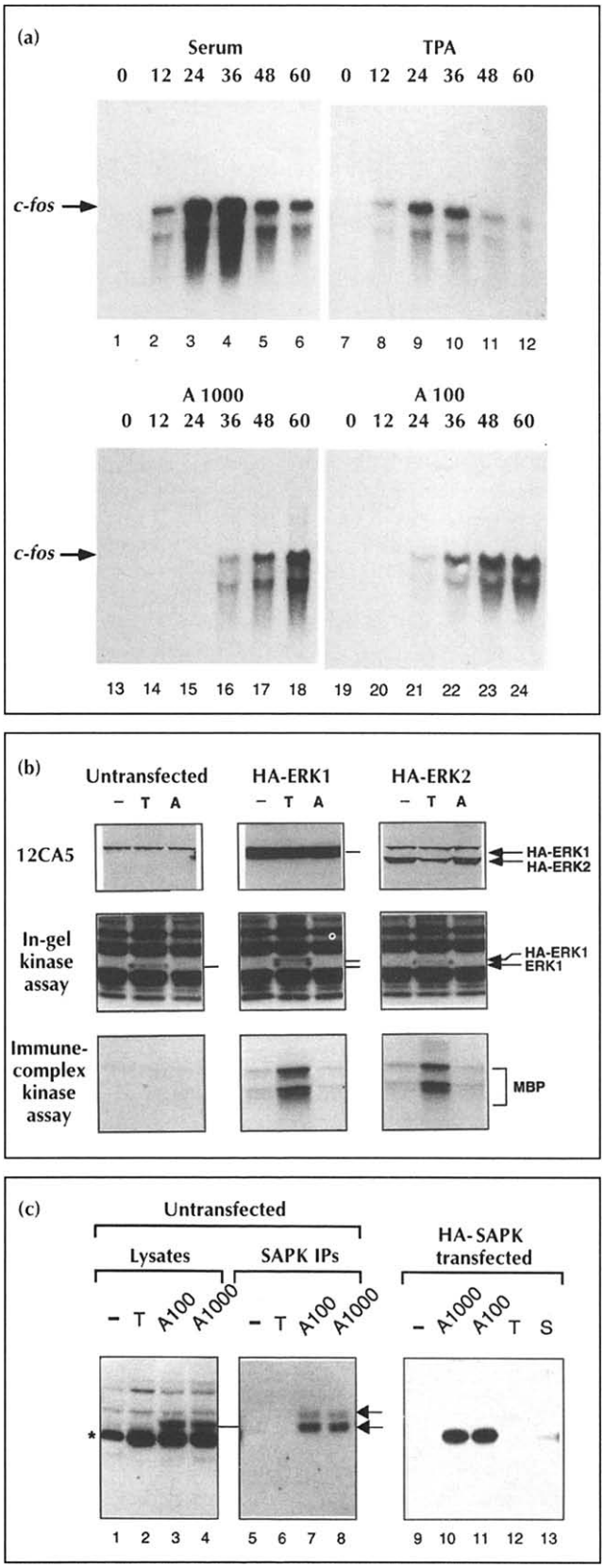

Fig. 1. (a) Kinetics of c-*fos* induction. Northern hybridization analysis of c-*fos* expression in response to treatment of NIH3T3 cells with 15 % serum, 100 $\mu g\ ml^{-1}$ TPA (top), or 1 $\mu g\ ml^{-1}$ or 100 $ng\ ml^{-1}$ anisomycin (A 1 000 and A 100, lower panels). The times after treatment at which RNA was isolated are given in minutes. Cells were serum-starved for 24 h prior to treatment. RNA was isolated and analyzed as described [5]. **(b)** Activation of ERKs by phorbol ester (TPA) but not anisomycin. Untransfected NIH3T3 cells (left panels) or cells over-expressing haemagglutinin-tagged (HA-) ERK1 (middle) or ERK2 (right) were serum-starved (–) or starved and treated with 100 $ng\ ml^{-1}$ TPA (T) or 100 $ng\ ml^{-1}$ anisomycin (A). Cell lysates were tested for myelin basic protein (MBP) kinase activity, either directly, by in-gel kinase assays (middle panels), or by preparing immune complexes, which were assayed in solution (lower panels). The haemagglutinin-tagged ERKs migrate more slowly than endogenous ERKs, as indicated. The top panels are immunoblots using a mouse monoclonal anti-haemagglutinin antibody 12CA5. **(c)** Activation of SAPKs by anisomycin but not TPA. NIH3T3 cells were starved and treated with nothing (–) or 100 $ng\ ml^{-1}$ anisomycin (A 100), 1 $\mu g\ ml^{-1}$ anisomycin (A 1 000) or 100 $ng\ ml^{-1}$ TPA (T), as indicated, and lysates were either loaded directly onto a polyacrylamide gel co-polymerized with GST–ElkC fusion protein (left), or immunoprecipitated with an antiserum against SAPKs prior to SDS–PAGE (middle panel). The position of a constitutive kinase activity that is independent of co-polymerized substrate is denoted by an asterisk. The positions of the endogenous $p46^{SAPK}$ and $p54^{SAPK}$ are indicated by arrows in the middle panel. NIH3T3 cells over-expressing haemagglutinin-tagged $p46^{SAPK}$ (HA-SAPK; right panel) were treated as above or with 10 % FCS (S). Cell lysates were prepared and $p46^{SAPK}$ activity was tested in immune-complex kinase assays with GST–cJun fusion protein.

observed with anisomycin concentrations ranging from 1 $\mu g\,ml^{-1}$ to 50 $ng\,ml^{-1}$. Furthermore, 1 $\mu g\,ml^{-1}$ anisomycin induces the *de novo* production of Fos protein to levels comparable to those induced by 12-O-tetradecanoylphorbol-13-acetate (TPA; data not shown). It also stimulates the expression of chloramphenicol acetyltransferase (CAT) from reporter constructs (see below) and fails to reduce β-galactosidase expression from a co-transfected gene. Therefore, in agreement with published data [11,12,18], we conclude that the observed induction of c-*fos* expression by anisomycin occurs independently of protein synthesis inhibition. The transcriptional response observed one hour after anisomycin treatment is comparable to the TPA response that is already apparent after 24 minutes (Fig. 1a). However, neither TPA nor anisomycin is as potent as serum, which exerts its effects through multiple pathways.

In order to determine which MAP kinases are activated in response to stress signals, we examined their activity in serum-starved NIH3T3 cells treated with either TPA or anisomycin. As expected, the activation of endogenous ERKs in response to TPA could be detected in cell lysates by an in-gel kinase assay (Fig. 1b, middle panels, lanes marked T). In contrast, endogenous ERKs were not activated by anisomycin (Fig. 1b, middle panels, lanes marked A). The activation of endogenous SAPKs was analyzed in a similar manner. NIH3T3 cells were treated with TPA or anisomycin and cell lysates were analyzed using as a substrate a fusion protein made up of glutathione-S-transferase and an amino-terminally truncated Elk-1 protein (GST–ElkC; Fig. 1c). The two SAPK isoforms are 46 and 54 kDa. A 46 kDa kinase activity was induced in response to anisomycin but not in response to TPA. The presence of a constitutive activity, which was independent of substrate (data not shown), precluded analysis in the 52–56kD range (Fig. 1c, left panel). Therefore, in order to confirm the identity of the 46 kDa activity and to determine whether $p54^{SAPK}$ is also activated, endogenous SAPKs were immunoprecipitated from the lysates and analyzed in parallel (Fig. 1c, middle panel). The 46 kDa kinase could thus be identified as $p46^{SAPK}$, and in the absence of background phosphorylation, the activation of $p54^{SAPK}$ by anisomycin treatment was also apparent. Thus, although we cannot exclude the existence of additional, anisomycin-induced Elk-1 kinases that do not renature under these experimental conditions or are obscured by the presence of constitutive activities, $p46^{SAPK}$ and $p54^{SAPK}$ are clearly detected as endogenous Elk-1 kinases that are activated upon treatment of NIH3T3 cells with anisomycin.

Regulation of epitope-tagged kinases was also investigated under these conditions. NIH3T3 cells were transfected with vectors for haemagglutinin-tagged ERKs and SAPKs and their expression was confirmed by western immunoblot analyses (Fig. 1b and data not shown). Cells were serum-starved and treated with either TPA or anisomycin. The in-gel kinase assays with whole cell lysates (Fig. 1b, middle panels) show that the activity of epitope-tagged ERK1 is stimulated by TPA but not by anisomycin, as is its endogenous counterpart. In addition, immune-complex kinase assays (Fig. 1b, lower panels) revealed the stimulation of both epitope-tagged ERK1 and epitope-tagged ERK2 by TPA but not by anisomycin.

The enzyme activity in lysates of cells transfected with haemagglutinin-tagged $p46^{SAPK}$ was also assayed in an immune-complex kinase assay. Anisomycin at 1.0 and 0.1 $\mu g\,ml^{-1}$ strongly activated $p46^{SAPK}$, whereas TPA and serum were ineffective (Fig. 1c, right panel). Thus, the activation pattern of the transfected SAPKs and ERKs faithfully reflects that of the endogenous MAP kinases. The observation that TPA and anisomycin activate distinct MAP kinase sub-groups without detectable overlap further implies that the anisomycin-induced c-*fos* expression in NIH3T3 cells is mediated by a signalling pathway distinct from the one used by TPA.

Induction of c-*fos* by stress signals is mediated by Elk-1 acting through the SRE

The induction of c-*fos* expression in response to TPA is mediated by the SRE [19,20]. Two factors, SRF and Elk-1, bind to the SRE as a ternary complex. Phosphorylation of Elk-1 by ERKs is known to stimulate ternary complex formation [5,8]. Transfection of NIH3T3 cells with an SRE–CAT reporter gene construct and subsequent treatment with TPA or anisomycin revealed that the SRE responds similarly to both reagents (data not shown).

In order to test whether anisomycin leads to stimulation of ternary complex formation by Elk-1, lysates were prepared from either NIH3T3 cells over-expressing Elk-1 that had been serum-starved, or such cells subsequently treated with either serum, anisomycin or TPA for 30 minutes. Analysis of ternary complex activity in the lysates by mobility-shift assays with the SRE and a truncated variant of SRF ($core^{SRF}$) confirmed that all three agents stimulate the formation of a ternary complex by Elk-1 (Fig. 2, lanes 5, 7 and 9). Addition of a polyclonal antiserum raised against Elk-1 disrupted the complex formed (Fig. 2, lanes 6, 8 and 10), thus confirming its identity. These results show that ternary complex formation by Elk-1 is stimulated in cells under conditions that activate SAPKs but not ERKs, implying that SAPKs phosphorylate and activate Elk-1 in intact cells in response to stress signals. Comparable amounts of ternary complex activity are induced by TPA and anisomycin, although neither of these is as potent as serum. Indeed, the levels of ternary complex formation induced by these agonists correlate with the induced strength of c-*fos* mRNA expression observed in Figure 1a.

SAPKs directly phosphorylate and activate Elk-1

We wanted to assess whether the activation of Elk-1 observed *in vivo* under conditions that activate SAPKs is the result of direct phosphorylation by SAPKs. To this end, epitope-tagged $p46^{SAPK}$ and $p54^{SAPK}$ were over-expressed in COS cells and their ability to phosphorylate

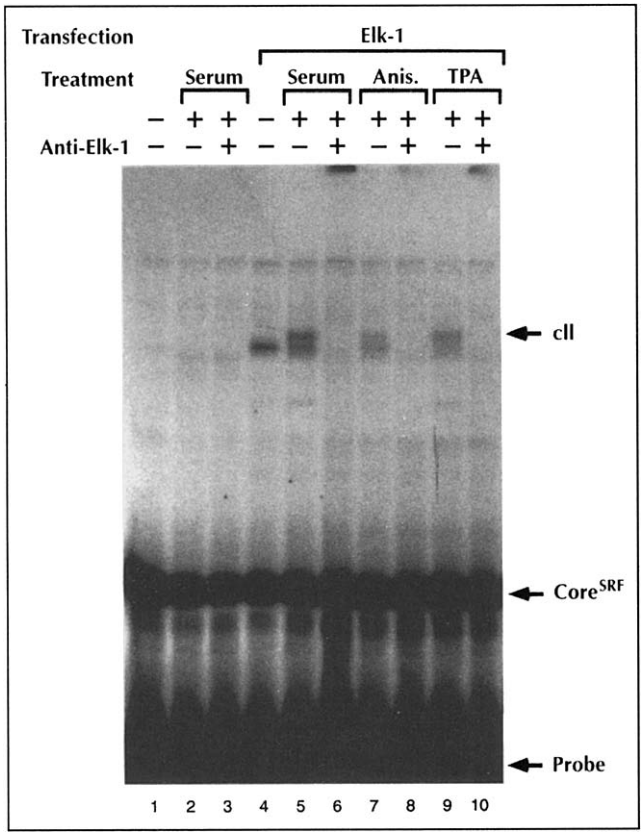

Fig. 2. Anisomycin stimulates ternary complex formation by Elk-1. Untransfected NIH3T3 cells or cells over-expressing Elk-1 were serum-starved or starved and treated with 15 % FCS (serum), 100 ng ml^{-1} TPA or 1000 ng ml^{-1} anisomycin (anis.) as indicated. After 30 min, lysates were prepared and tested for ternary complex activity by mobility shift assays with an SRE probe and $core^{SRF}$. The ternary complex with $core^{SRF}$ and Elk-1 is indicated as cII. The identity of the complexes was confirmed by including a polyclonal antibody against Elk-1 in the binding reactions (lanes 6,8,10).

Elk-1 was tested in immune-complex kinase assays. As shown in Figure 3a, Elk-1 is phosphorylated by both kinases, each of which is strongly stimulated by pre-treatment of cells with ultraviolet light. This is consistent with the observation that GST–ElkC fusion protein is phosphorylated by the endogenous SAPKs present in NIH3T3 cells (Fig. 1c).

Bacterially expressed Elk-1, phosphorylated with either $p46^{SAPK}$ or $p54^{SAPK}$, was analyzed for its ability to form ternary complexes with the SRE and $core^{SRF}$. Figure 3b shows that activated SAPKs isolated from cells stimulate the formation of a ternary complex by Elk-1 (Fig. 3b, lanes 8 and 10). Again, addition of a polyclonal antiserum raised against Elk-1 disrupted the complex formed after $p46^{SAPK}$ treatment of Elk-1 (Fig. 3b, lane 12). The additional bands seen in lanes 6–10 of Figure 3b, below the ternary complex, reflect complexes formed by truncated forms of bacterially expressed Elk-1. The inducible ternary complex activity that is observed upon anisomycin stimulation of cells can therefore be reproduced by phosphorylating recombinant Elk-1 with SAPKs *in vitro*. The observation that Elk-1 is phosphorylated directly and activated by $p46^{SAPK}$ and $p54^{SAPK}$ further implicates it in the stress-response induction of the c-*fos* gene.

Determination of SAPK phosphorylation sites in Elk-1

SAPKs are proline-directed serine/threonine kinases, as are their ERK relatives. However, except for sites in the amino-terminal transactivation domains of cJun [2] and ATF2 [15,21,22], little is known of their potential substrates. We have previously undertaken a comprehensive phosphopeptide mapping analysis of Elk-1 proteins that have been mutated at individual phosphorylation sites and phosphorylated by ERK1 [8]. On the basis of this information, which is summarized in schematic form in Figure 4e, we sought to determine whether $p46^{SAPK}$ or $p54^{SAPK}$ phosphorylates known sites in the regulatory domain of Elk-1. An amino-terminally truncated form of Elk-1 (ElkC) was radiolabelled by incubation with γ-[^{32}P]ATP and $p46^{SAPK}$ or $p54^{SAPK}$. For comparison, ElkC was also phosphorylated using recombinant ERK1 that had been activated by phosphorylation with a constitutively active mutant of the MAPK/ERK kinase MKK1 [23]. Figure 4 displays the phosphopeptide maps generated after phosphorylation of ElkC by the three kinases.

The phosphopeptide pattern generated after phosphorylation of ElkC with recombinant ERK1 (expressed in bacteria) is identical to that obtained with a homogeneous preparation of ERK1 from Rat1 cells [8]. This demonstrates that activated ERK1 generated from recombinant, purified components exhibits identical

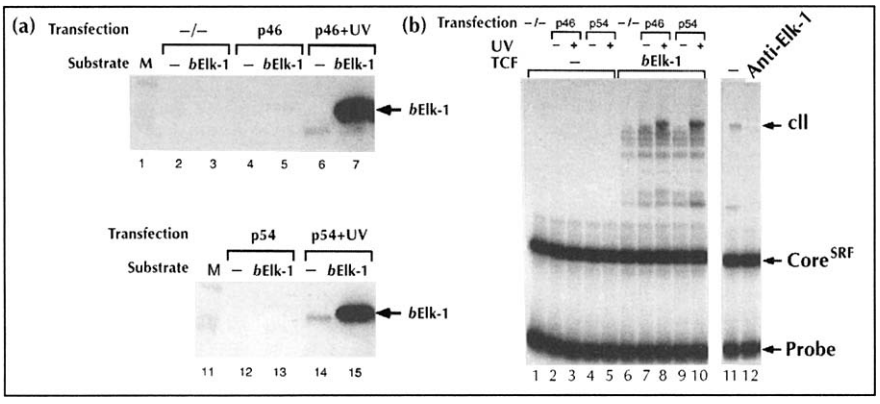

Fig. 3. Phosphorylation and activation of Elk-1 by SAPKs. **(a)** Mock-immune complexes or complexes containing either inactive or activated (+UV) $p46^{SAPK}$ or $p54^{SAPK}$ were incubated with bacterially expressed Elk-1 (bElk-1) or without further addition. Reaction products were separated on 10 % SDS–polyacrylamide gels. **(b)** Gel-retardation analysis of Elk-1 after phosphorylation with active or UV-activated $p46^{SAPK}$ or $p54^{SAPK}$ (or nothing, –) as indicated. The identity of the ternary complex was confirmed by including a polyclonal antibody against Elk-1 in the binding reaction (lane 12).

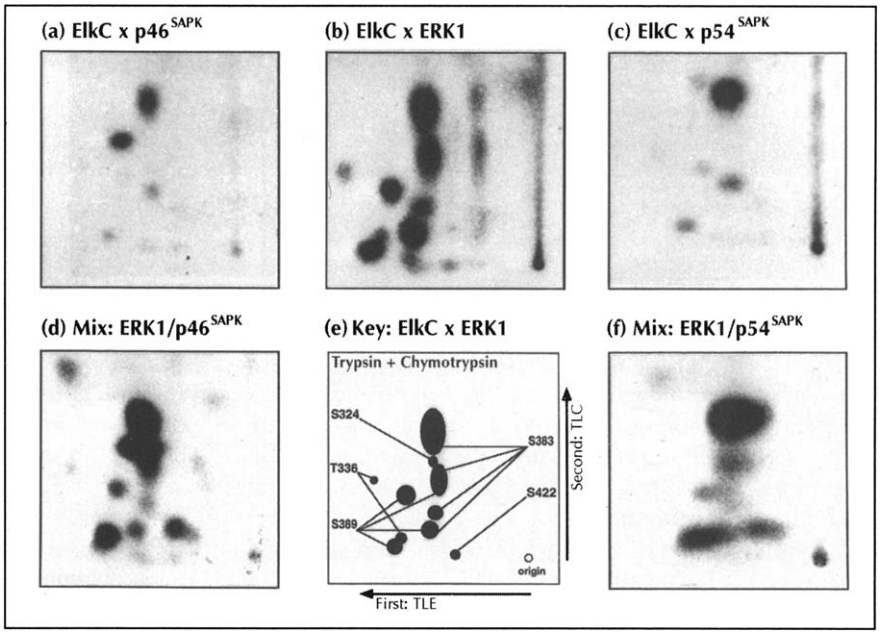

Fig. 4. Phosphopeptide analysis of amino-terminally truncated Elk-1 (ElkC) phosphorylated by ERK1 or SAPKs. Top row: ElkC phosphorylated with either **(a)** $p46^{SAPK}$, **(b)** ERK1, or **(c)** $p54^{SAPK}$. Bottom row: ElkC phosphorylated with either **(d)** $p46^{SAPK}$ or **(f)** $p54^{SAPK}$, mixed with ERK1-phosphorylated ElkC; and **(e)** a schematic representation of the previously identified peptides generated from ERK1-phosphorylated ElkC [8].

substrate specificity to the endogenous enzyme. The substrate specificities of $p46^{SAPK}$ and $p54^{SAPK}$ are more restricted than that of ERK1, as fewer ElkC phosphopeptides are recovered after phosphorylation with these enzymes. The major phosphopeptide detected with $p46^{SAPK}$ and with $p54^{SAPK}$ is similar in mobility to a major phosphopeptide in the ERK1 map (Fig. 4b). This peptide was previously demonstrated to contain phosphoserine at position 383 [8]. An additional phosphopeptide is generated by incubation of ElkC with $p46^{SAPK}$.

To confirm the identity of these peptides, mixing experiments were performed with ElkC phosphorylated by ERK1 and $p46^{SAPK}$ or $p54^{SAPK}$ (Fig. 4d,f). The major peptides derived from ElkC phosphorylated with $p46^{SAPK}$ and $p54^{SAPK}$ comigrate with a major phosphoserine 383-containing peptide from ERK1-phosphorylated ElkC, demonstrating that these are identical peptides. The additional peptide obtained with $p46^{SAPK}$ comigrates with a peptide containing Ser324, which is less strongly phosphorylated by ERK1 *in vitro*. Ternary complex formation by Elk-1 is increased after treatment with either of the SAPKs, suggesting that phosphorylation of Ser324 is not a prerequisite for ternary complex formation *in vitro*. This is entirely consistent with our previous observations [8].

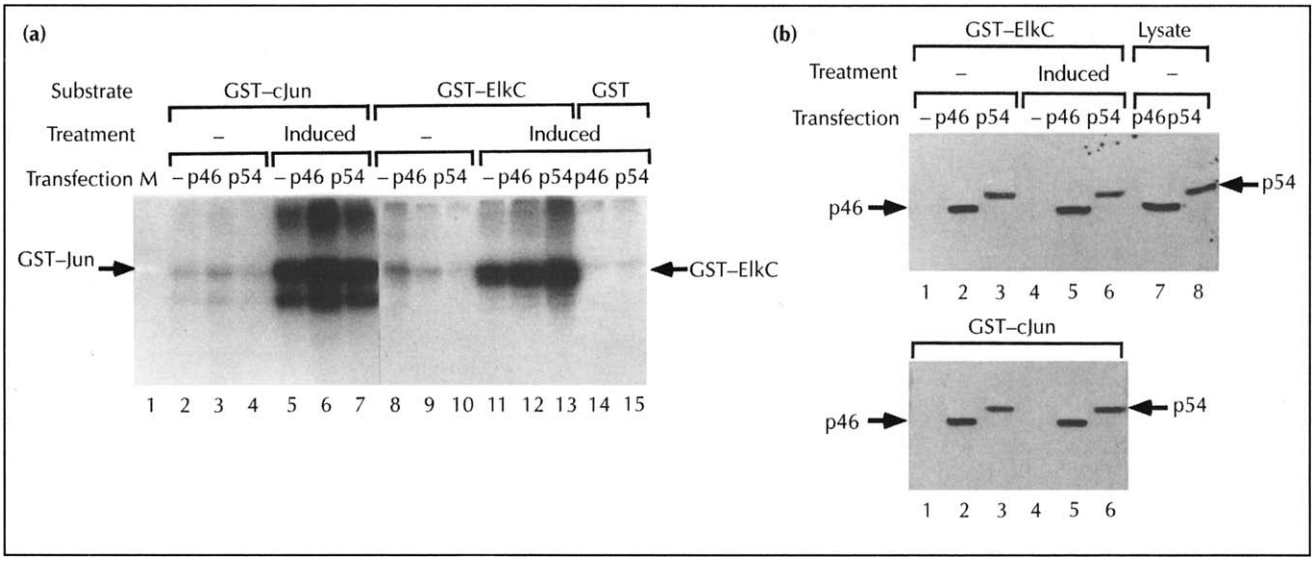

Fig. 5. SAPKs form complexes with Elk-1. **(a)** Lysates from COS cells transfected and treated as indicated were first pre-cleared with GST bound to glutathione–agarose beads. Subsequently, 6.5 μg of GST–cJun or GST–ElkC bound to beads were incubated in aliquots of the lysates. Beads were washed as described for the immunoprecipitations and resuspended in kinase buffer containing 5 μCi γ-[^{32}P]ATP. After 1 h, reactions were stopped and subjected to SDS–PAGE. Phosphorylation of GST–cJun or GST–ElkC was dependent on ultraviolet treatment of the cells (lanes 5–7 and 11–13). **(b)** As for (a), except that complexes were denatured directly and subjected to western immunoblot analysis with a monoclonal antibody directed against the haemagglutinin tag.

SAPKs form complexes with Elk-1 *in vitro*

The SAPKs have been shown to form stable complexes with their substrates cJun and ATF2 *in vitro* [2,15,21,22,24]. We therefore investigated whether SAPKs interact physically with Elk-1. Lysates from untransfected COS cells, or from cells transfected with epitope-tagged $p46^{SAPK}$ or $p54^{SAPK}$, were incubated with fusion proteins made up of either GST and cJun (residues 1–223) or GST and ElkC (residues 215–428 of Elk-1) pre-bound to glutathione–agarose beads. The beads were washed extensively, resuspended in kinase buffer and assayed for the presence of kinase activity. As seen in Figure 5a, both cJun and ElkC were phosphorylated after pre-incubation in lysates of cells treated with ultraviolet light but not in lysates of untreated cells. This is consistent with stress-inducible kinases that are present in the lysates of untransfected (Fig. 5a, lanes 5 and 11) and transfected (Fig. 5a, lanes 6, 7, 12 and 13) cells binding to GST–cJun and GST–ElkC.

To confirm that $p46^{SAPK}$ and $p54^{SAPK}$ had complexed with cJun and ElkC, GST–cJun and GST–ElkC beads were collected after incubation with the lysates, washed extensively and bound proteins were subjected to western immunoblot analysis with a monoclonal antibody directed against the epitope tag. Figure 5b shows that the $p46^{SAPK}$ and $p54^{SAPK}$ present in lysates of both uninduced and induced cells bound with comparable efficiency to cJun and ElkC. The binding of inactive SAPKs to cJun has been observed previously in U937 cells and appears to be a constitutive property of these enzymes [25]. Thus, as with other known substrates, SAPKs form complexes with Elk-1.

Elk-1 mediates transcriptional activation in response to stress signals

Phosphorylation by ERKs is known to stimulate the transactivation potential of Elk-1. To probe the involvement of Elk-1 in the transcriptional response of c-*fos* to stress signals, we used chimeric Gal–Elk fusion proteins. These have previously been shown to mediate the stimulation of *GAL4* reporter gene expression by mitogenic stimuli [6,8,9]. In NIH3T3 cells, co-transfection of an expression vector for Gal–Elk mediates expression from a transfected *GAL–CAT* reporter gene, which, as shown in Figure 6a,b, is potentiated upon treatment of serum-starved cells with anisomycin. Replacement of the major SAPK phosphorylation site in Gal–Elk, Ser383,

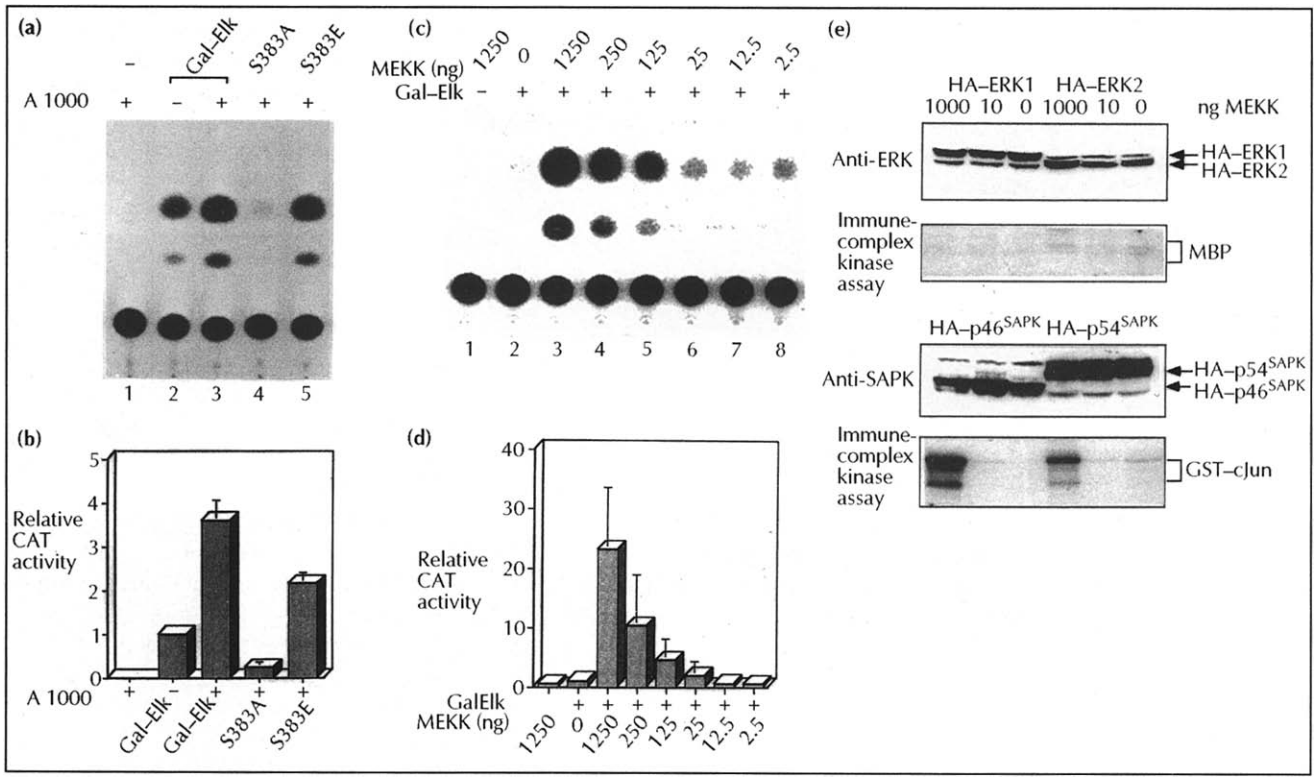

Fig. 6. (a) Activation of Gal–Elk-dependent gene expression by anisomycin. Cells were transfected with a Gal–Elk chimeric protein and treated with anisomycin as indicated. **(b)** Quantification of three experiments similar to the one shown in (a). Values are given as percentage CAT conversion relative to untreated cells expressing Gal–Elk; error bars give the standard deviation. **(c)** Activation of Gal–Elk-dependent transcription by MEKK1. The transfected amounts of MEKK1 expression vector are shown. **(d)** Quantification (as in b) of three experiments similar to the one shown in (b). Values are given as percentage CAT conversion relative to cells expressing Gal–Elk alone. **(e)** Selective activation of SAPKs by MEKK expression. Over-expression of epitope-tagged (HA-) ERK1 and ERK2 was monitored by western immunoblot analysis with a polyclonal anti-ERK antibody and MBP kinase activity by immune complex kinase assays (upper panels); over-expression of epitope-tagged $p46^{SAPK}$ and $p54^{SAPK}$ was monitored by western immunoblot analysis with a polyclonal anti-SAPK antibody and GST–cJun kinase activity by immune complex kinase assays (lower panels). The haemagglutinin-tagged kinases migrate more slowly than their endogenous counterparts.

with alanine (Gal–Elk^{S383A}) abrogates induction of reporter gene expression. In contrast, when Ser383 is replaced with glutamate (Gal–Elk^{S383E}) Gal–Elk retains 60 % of its activation potential, in agreement with previous analyses of Elk-1 activation by ERKs. These results show that phosphorylation at Ser383 is a prerequisite for transcriptional activation by Elk-1 in response to treatment of cells with anisomycin.

SAPK activation is a consequence of phosphorylation by upstream activators like SEK1, which in turn is activated by MEKK1 [26,27]. As a further test for the activation of Gal–Elk by SAPKs, cells were co-transfected with expression vectors encoding MEKK1 [28] Gal–Elk and the *GAL–CAT* reporter construct. Co-transfection of 1.25 μg MEKK1 expression vector elicited a 24-fold activation of Gal–Elk-dependent reporter expression in serum-starved NIH3T3 cells (Fig. 6c,d). The stepwise reduction of MEKK1 expression vector input to 2.5 ng led to the gradual loss of detectable reporter activity.

To confirm that MEKK1 expression selectively activates SAPKs and not ERKs under these conditions, epitope-tagged ERKs and SAPKs were over-expressed together with MEKK1 in serum-starved NIH3T3 cells, and their activities were assayed in immune-complex kinase assays, as before. As shown in Figure 6e, expression of MEKK1 fails to activate either ERK1 or ERK2, whereas both p46^{SAPK} and p54^{SAPK} are activated at expression levels that result in reporter gene expression. This result indicates that, at the levels of expression achieved, MEKK1 selectively activates SAPKs and that this is sufficient to induce Gal–Elk-dependent gene expression.

Discussion

Transcription of c-*fos* induced by mitogens or stress results from activation of distinct MAP kinase sub-groups

Several biological agonists, such as ultraviolet light, pro-inflammatory cytokines and anisomycin have been reported to stimulate the SAPK sub-group of MAP kinases with varying degrees of specificity. In order to study the relationship of SAPKs to the induction of c-*fos* transcription, we chose to use anisomycin because it allows a clear distinction to be made between mitogen-induced and stress-induced pathways. At concentrations that do not affect protein synthesis, anisomycin stimulates the activity of endogenous SAPKs and induces c-*fos* transcription in NIH3T3 cells without concomitant ERK activation. The level of c-*fos* induction achieved by anisomycin treatment is comparable to the response of cells to treatment with TPA. However, the latter is mediated by the stimulation of ERKs without detectable SAPK activation. The induction of c-*fos* expression resulting from exposure of cells to noxious agents can be distinguished from the induction in response to mitogens by its delayed kinetics. The observed time course of c-*fos* induction by anisomycin may thus reflect the prolonged activation kinetics exhibited by SAPKs (our unpublished observations). We also observe that the selective activation of endogenous SAPKs and ERKs by anisomycin and TPA, respectively, is faithfully reproduced by exogenously expressed, epitope-tagged versions of the kinases.

Elk-1 activation as a result of phosphorylation by SAPK

Efficient formation of a ternary complex between Elk-1, SRF and the c-*fos* SRE requires phosphorylation of Elk-1 on regulatory sites in its carboxy-terminal domain. We observe that ternary complex formation by Elk-1 is induced in serum-starved NIH3T3 cells treated with anisomycin. The level of ternary complex formation is comparable to that induced by TPA. However, whereas TPA stimulates ERKs selectively, anisomycin was found to stimulate only SAPKs. This result strongly suggests that SAPKs phosphorylate Elk-1 *in vivo*. Consistent with this is the observation that Elk-1 is phosphorylated directly by SAPKs *in vitro*, whereupon its ability to form a ternary complex with SRF is enhanced. Although we have observed that the ternary complex factor Sap1a is also phosphorylated by SAPKs *in vitro*, this does not appear to stimulate its capacity to form a ternary complex with SRF (our unpublished observations).

Phosphopeptide mapping shows that SAPKs phosphorylate Elk-1 predominantly at Ser383, the major carboxy-terminal site in Elk-1 phosphorylated in intact cells in response to serum, EGF, and expression of oncogenic forms of Raf-1 kinase [6,8,9,29]. Phosphorylation of Elk-1 by SAPKs therefore occurs at a physiologically relevant site. However, at least one other site in Elk-1 — Ser324 — is phosphorylated with similar efficiency by p46^{SAPK}, which appears to be the major anisomycin-induced SAPK in NIH3T3 cells. Comparison of SAPK phosphorylation sites in cJun, ATF2 and Elk-1 shows that the SAPKs may have a less stringent requirement for a basic residue before the phosphorylated residue than do the ERKs [30]. Otherwise, the sequences are remarkably similar. The specificity determinant may therefore be in the substrate domain that mediates interaction with the SAPKs.

SAPK interactions with Elk-1

As seen with other SAPK substrates such as cJun, Elk-1 forms stable complexes with both inactive and active forms of the stress-inducible kinases, including epitope-tagged p46^{SAPK} and p54^{SAPK}. The interaction of SAPKs with Elk-1 was demonstrated in two ways. Firstly, the kinases were found to remain complexed through several washes to immobilized GST–ElkC, and subsequently to phosphorylate the substrate to which they had bound. Secondly, the presence of SAPKs in complexes with GST–ElkC was confirmed by immunoreactivity. This approach revealed the interaction of inactive SAPKs with Elk-1. Inactive SAPKs have been reported previously to interact with their substrate cJun, implicating them in a mechanism of AP-1 repression in resting cells [25].

Although our data implicate SAPKs in phosphorylation of Elk-1 in response to stress signals, we cannot rule out the possibility that anisomycin-induced kinases other

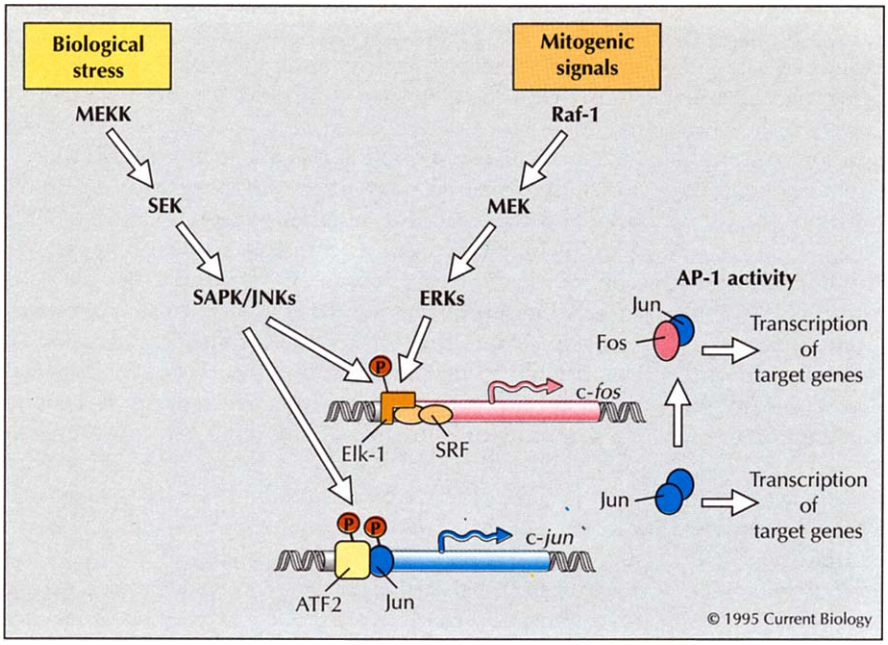

Fig. 7. Regulation of AP-1 activity by multiple mechanisms. Mitogenic signals lead to activation of the ERKs and phosphorylation of Elk-1, which results in induction of c-*fos* expression [5,6]. Alternatively, biological stress leads to the upregulation of AP-1 activity by several mechanism. SAPKs phosphorylate ATF2, resulting in increased expression of c-*jun* [21]. Phosphorylation of Elk-1 by SAPKs results in an increase in ternary complex activity, leading to an induction of c-*fos* expression. As the level of Fos in resting cells is extremely low, induction of its expression shifts the balance from cJun homodimers to Fos–cJun heterodimers, because cJun preferentially forms heterodimers with Fos. SAPKs also directly phosphorylate cJun, thereby increasing its transcriptional activity (see [3] and references therein).

than SAPKs may be involved in Elk-1 regulation. In this respect, a novel sub-group of mammalian MAP kinases was identified recently, whose members, exemplified by p38, are homologues of the yeast HOG1 kinase and can be activated by osmotic stress and arsenite treatment of mammalian cells [31,32]. This sub-group of MAP kinases appears to share some substrates with the SAPKs [33]. Although our in-gel kinase assays, performed with lysates from untransfected anisomycin-treated cells indicate that SAPKs are the major anisomycin-activated GST–ElkC kinases in NIH3T3 cells, we cannot rule out the possibility that other MAP kinases, such as p38/HOG1, may contribute to Elk-1 phosphorylation under some circumstances. The existence of other mammalian MAP kinases and the possibility that they activate Elk-1 in response to distinct stimuli is of great interest and will be the subject of future studies. Here, we have shown that, in addition to ERKs, members of a second group of MAP kinases, $p46^{SAPK}$ and $p54^{SAPK}$, phosphorylate and activate Elk-1.

Transcriptional regulation of Elk-1 by SAPKs

Although the phosphorylation of Ser383 is a prerequisite for Elk-1 function, the mutation of other potential phosphorylation sites in Elk-1 to alanine also impairs transcriptional activation [6–8], suggesting that phosphorylation of Ser383 alone is not sufficient for full transcriptional activation. Mutation of Ser383 to alanine in Gal–Elk abolishes the ability of the fusion protein to respond to mitogenic stimuli [6,8,9] and anisomycin (Fig. 6) in a *GAL4* reporter assay, whereas the introduction of a negative charge at residue 383 (Gal–Elk^{S383E}) allows 60 % activation compared to native Gal–Elk. However, expression of this construct does not lead to constitutive activation in the absence of a stimulus [8,9]. This also implies that other phosphorylation sites contribute to the full activity of Elk-1. In the case of anisomycin stimulation of NIH3T3 cells, $p46^{SAPK}$ is the predominant endogenous SAPK. This enzyme phosphorylates Ser324 in addition to Ser383 (Fig. 4). Therefore, Ser324 phosphorylation may contribute to the anisomycin-induced activation of Elk-1.

Activation of SAPKs is thought to occur upon sequential activation of MEKK1 and SEK1, which then phosphorylates the SAPKs [27,34]. Consistent with this model, the transient expression of MEKK1 in NIH3T3 cells led to high levels of transcription from the Gal–Elk-dependent reporter. However, overexpression of MEKK1 has been shown to activate both SAPKs and ERKs indiscriminately in some instances. Therefore it was important to monitor the activation of ERKs and SAPKs at various levels of MEKK1 expression under our experimental conditions. In contrast to published observations, we found that expression of MEKK1 failed to activate ERKs but did activate SAPKs and stimulated Gal–Elk-dependent transcription. We have only been able to detect the stimulation of ERKs by over-expressing a MEKK1 derivative consisting exclusively of the catalytic domain (our unpublished observations).

AP-1 regulation in response to biological stress

AP-1 activity is subject to upregulation in response to SAPK activation at several levels (Fig. 7). The SAPKs are implicated in the elevation of AP-1 activity through phosphorylation of two amino-terminal sites in cJun; they also form complexes with, and phosphorylate, ATF2, a component of AP-1 which is involved in the upregulation of c-*jun* transcription. Here, we have identified a third substrate of SAPKs, highlighting an additional mechanism of AP-1 upregulation in response to stress-induced signals. By phosphorylating and activating Elk-1, which acts through the SRE to activate c-*fos* transcription, SAPKs induce the production of Fos, a further component of AP-1.

The influence of phosphorylation on the DNA binding properties of Elk-1 is reminiscent of the regulation of

ATF2 DNA-binding and transcriptional activation by ERK2 and $p54^{SAPK}$ [15,35]. Thus, the production of two AP-1 components, cJun and Fos, is induced in response to stress signals. The increased availability of Fos protein is thought to shift the balance from cJun homodimers to Jun–Fos heterodimers, thereby changing the pattern of AP-1-dependent gene expression [36].

Conclusion

Activation of SAPKs involves a kinase cascade distinct from the signalling pathway involving Raf-1 kinase, MEKs and ERKs. In response to biological stress, MEKK1 and SEK1 appear to act sequentially to activate $p46^{SAPK}$ and $p54^{SAPK}$ [27], which then phosphorylate and activate cJun, ATF2 and Elk-1. AP-1 thus constitutes an integration point for multiple targets activated by cellular stress. Our results identify Elk-1 as a target for the pathway leading to induction of c-*fos* gene expression in response to biological stress. We conclude that both the mitogenic and the stress signalling pathways converge on a single ternary complex factor, Elk-1, at the c-*fos* SRE.

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Materials and methods

Cell culture and transfections

NIH3T3 and COS cells were grown in DMEM supplemented with 10 % FCS. Cells were transfected with DNA–calcium phosphate co-precipitates according to standard procedures. For CAT reporter analysis, cells were routinely starved for 20 h before treatment with anisomycin (1 $\mu g\ ml^{-1}$) and harvested 8 h later. Transfection efficiency was monitored by β-galactosidase activity in extracts expressed from co-transfected pCM110 (Pharmacia). CAT assays were performed as described [9]. Expression of Gal–Elk was monitored by western analysis with monoclonal antibodies against Gal4 (amino acids 1–147) [9]. For over-expression and isolation of epitope-tagged SAPKs and ERKs in NIH3T3 cells, 5 μg haemagglutinin-tagged $p46^{SAPK}$ and $p54^{SAPK}$ constructs [26] or 6 μg haemagglutinin-tagged ERK1 and ERK2 constructs were transfected. Cells were treated with serum, TPA or anisomycin as described in the figure legends. After 10 min (ERKs) or 30 min (SAPKs), cells were harvested.

Gel-retardation analysis

For mobility-shift analysis of Elk-1 ternary complex activity, lysates were prepared as described [8] from serum-starved cells or cells treated with serum, TPA or anisomycin as indicated in the Figure 2 legend. Elk-1 expression was monitored by western immunoblot analysis. Equal amounts of each extract were used for gel-retardation analysis with $core^{SRF}$ and an SRE oligonucleotide probe as described [8]. Polyclonal antibody (0.5 μl) specific for Elk-1 was added to DNA-binding reactions where indicated.

Immune-complex kinase assays

Cells were washed in ice cold PBS and lysed in RIPA buffer [37]. Lysates were clarified by centrifugation at 4 °C. Preswollen protein A–Sepharose was incubated 30 min in PBS with 1 % BSA to block non-specific protein binding. Beads were washed three times with WPTX (0.05 % Triton X-100, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 1 $mg\ ml^{-1}$ ovalbumin), and coupled to mouse monoclonal anti-haemagglutinin antibody (12CA5; Boehringer) by incubation in WPTX for 1 h. The beads were washed three times in the same buffer and aliquots were added to the lysates. Mock precipitations were performed with lysates from cells transfected with pUC8. Immunoprecipitations were carried out at 4 °C for 2–3 h. The beads were washed twice in WPTX, once in WPTX with 400 mM NaCl and again in WPTX. The final wash was carried out in ERK buffer (25 mM Tris–HCl pH 7.2, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EGTA, 20 mM p-nitrophenylphosphate) or SAPK buffer (20 mM HEPES pH 7.9, 20 mM $MgCl_2$, 10 mM 2-glycerophosphate, 0.5 mM Na_3VO_4, 10 mM p-nitrophenylphosphate and 2 mM DTT). The beads were used directly for kinase assays with MBP (ERKs) or GST–cJun (SAPKs) in the presence of 5 μCi γ-[^{32}P]ATP and 40 μM ATP. Reactions were terminated after one hour at 37 °C by addition of SDS sample buffer.

To obtain highly active SAPKs, COS cells were transfected with 8 μg haemagglutinin-tagged $p46\beta^{SAPK}$ and $p54\beta^{SAPK}$ constructs [26]; 24 h after transfection, cells were transferred to 0.5 % serum for 10–12 h. Cells were subjected to 254 nm UV radiation for 15 s, then the medium was returned to the cells. After 30 min, cells were lysed and SAPKs were immunoprecipitated as above. The protein A beads were used for kinase assays as described above. For subsequent gel-retardation analysis, kinase reactions were set up in SAPK buffer, containing 250 μM ATP. Reactions contained ~200 ng ternary complex factor Elk-1 or no further protein and were carried out for 1 h at 37 °C. Aliquots of the reactions were used for gel-retardation analysis.

In-gel kinase assays

ERK activity was assayed after renaturation in polyacrylamide gels as described [9]. Gels for SAPK assays contained ~10 $\mu g\ ml^{-1}$ GST–ElkC and 0.2 $\mu g\ ml^{-1}$ polyGlu–Tyr, which serves to suppress some of the nonspecific bands that are also present when gels were cast without the GST–ElkC substrate [12].

Phosphopeptide mapping

Kinase reactions contained 1 μg ElkC (carboxy-terminal amino acids 213–428), 20 μM ATP and 80–100 μCi γ-[^{32}P]ATP. The ElkC protein was repurified by SDS–polyacrylamide electrophoresis and cleaved in solution with a 10:1 mixture of trypsin and chymotrypsin following a previously established protocol to allow comparison with the two-dimensional phosphopeptide maps obtained with various mutants of Elk-1 [8]. For direct comparison, phosphopeptide maps from ERK1-phosphorylated ElkC were generated in parallel. R4F is a constitutively active mutant of MAP kinase kinase 1 (MKK1), which contains negatively charged residues at both activating phosphorylation sites and an internal amino-terminal

deletion [23]. ERK1 (400 ng) was activated by incubation with 24 ng R4F.MKK1 in 50 μl reactions containing 25 mM Tris–HCl pH 7.2, 10 mM $MgCl_2$, 500 nM okadaic acid, 5 mM p-nitrophenylphosphate, 1 mM DTT, 0.1 mM EGTA, 250 μM ATP for one hour at 37 °C. Mock reactions lacking ERK1 were also performed. Subsequently, 5–10 μl recombinant kinases were used to phosphorylate ElkC in the presence of 50μCi γ-[^{32}P]ATP without further ATP addition. No incorporation of radioactivity into ElkC was observed with R4F.MKK1 from the mock activation reactions.

DNA constructs

Expression vectors for haemagglutinin-tagged SAPKs [38] and for haemagglutinin-tagged ERKs [39] have been described. A MEKK cDNA was cloned from mouse spleen total cDNA by PCR and sequenced. The pCMV5-based eukaryotic vector [40] derived from the cDNA clone expresses a MEKK protein encoded by nucleotides 477–2500 of the published sequence [28] with a heptapeptide leader and a carboxy-terminal histidine tag.

The bacterial expression vectors for Elk-1 and ElkC (pQE.Elk-1 and pQE.ElkC) have been described [8], as has the vector for GST–Jun(1–223) [41]. The eukaryotic expression vectors for Elk-1, Gal–Elk, Gal–Elk^{S383A} and Gal–Elk^{S383E} (pCMV5.Elk-1, pSG.GalElk/383A/383E) and the reporter plasmid GAL–CAT have also been described previously [8,9].

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