The EMBO Journal vol.12 no.13 pp.5097-5104, 1993

Activation of ternary complex factor Elk-1 by MAP kinases

Ralf Janknecht, Wolfram H.Ernst, Vera Pingoud and Alfred Nordheim

Institute for Molecular Biology, Hannover Medical School, D-30623 Hannover, Germany

Communicated by A.Nordheim

Ternary complex factors (TCFs), one of which is Elk-1, have been implicated in mediation of c-*fos* induction. They have been shown to be phosphorylated by mitogen-activated protein kinases (MAPKs) *in vitro*. We demonstrate that recombinant Elk-1 is hyperphosphorylated *in vivo* upon joint overexpression of MAPKs and constitutively activated Raf-1 kinase, the latter serving as an indirect *in vivo* activator of MAPKs. This phosphorylation is accompanied by a conformational change and results in an elevated transactivation potential of Elk-1. Mutation of mapped *in vivo* phosphorylation sites, which are potential targets for MAPKs, reduced Elk-1-mediated transcription. Thus, MAPKs are very probably controlling Elk-1 activity by direct phosphorylation *in vivo*. Furthermore, Elk-1 was shown to stimulate transcription from both the c-*fos* serum response element and also from an Ets binding site. While binding of TCFs to the c-*fos* promoter is dependent on the serum response factor, TCFs can autonomously interact with Ets binding sites. This indicates that TCFs may participate in the transcriptional regulation of two different sets of genes.

Key words: c-*fos*/Ets proteins/phosphorylation/serum response factor/transactivation

Introduction

The Ets oncoproteins are characterized by a common ETS domain that mediates DNA binding to target sequences containing a $GGA^{A}/_{T}$ core (Karim *et al.*, 1990; Nye *et al.*, 1992; Woods *et al.*, 1992). A subclass of this oncoprotein family, defined structurally by the N-terminal localization of the ETS domain, comprises the Elk-1, SAP-1a, SAP-1b and SAP-2 proteins. All of them have been shown to be ternary complex factors (TCFs) targeting the serum response element (SRE) of the c-*fos* proto-oncogene (Hipskind *et al.*, 1991; Dalton and Treisman, 1992). TCFs themselves do not stably interact with the c-*fos* SRE but require recruitment by a dimer of SRE-bound serum response factor (SRF). Mutation of the TCF binding site within the c-*fos* SRE severely diminishes the transcriptional upregulation of the c-*fos* proto-oncogene upon stimulation of quiescent cells (Shaw *et al.*, 1989; Graham and Gilman, 1991). This strongly suggests that TCFs are important mediators of c-*fos* transcriptional activity, although their requirement for c-*fos* induction may be bypassed in some instances (Graham and Gilman, 1991; König, 1991).

Under conditions that lead to the activation of c-*fos*, TCFs are apparently phosphorylated (Gille *et al.*, 1992; Zinck *et al.*, 1993). Recently, some phosphorylation sites were identified in fusion proteins consisting of C-terminal Elk-1 amino acids and the GAL4 or LexA DNA binding domain (Hill *et al.*, 1993; Marais *et al.*, 1993). These phosphorylation sites are all potential targets for mitogen-activated protein kinases (MAPKs) and mutating them to alanine resulted in decreased transcriptional acitivity mediated by GAL4/LexA-Elk-1 fusion proteins. Since MAPKs could phosphorylate a biochemical preparation of TCFs or recombinant Elk-1 protein *in vitro* (Gille *et al.*, 1992; Marais *et al.*, 1993), phosphorylation of TCFs by MAPKs might be a prerequisite for transcriptional activation mediated by TCFs *in vivo*.

MAPKs are final effectors of signal transduction pathways initiated at the cell membrane. Several components of these pathways have been identified, including the proto-oncogenes *ras* and *raf*-1 (Marx, 1993). The Raf-1 kinase acts upstream of MAPKs and induces their activation via phosphorylation of MEKs (MAPK or ERK kinases) (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Wu *et al.*, 1993), while Raf-1 itself is apparently activated by Ras (Moodie *et al.*, 1993; Vojtek *et al.*, 1993; Zhang *et al.*, 1993). The Ras protein seems to be linked to growth factor receptors possessing a tyrosine kinase activity via two proteins, Grb2 and Sos (McCormick, 1993). Therefore, while one possible signal transduction pathway may have been completely unravelled, the nuclear targets are scarcely characterized. Since a plethora of agents activate MAPKs (Pelech and Sanghera, 1992) and thus very probably TCFs, these transcription factors might represent a pivotal link between protein kinase-dependent signal transduction pathways and enhanced gene transcription.

In this report we demonstrate that activated MAPKs could phosphorylate Elk-1 *in vivo*. Consistently, the transcriptional activity of this TCF was influenced by several potential MAPK phosphorylation sites. Furthermore, we show that Elk-1 does not function exclusively in a ternary complex with the SRF and c-*fos* SRE; it is equally effective when targeting an Ets binding site autonomously of SRF. This dual function of a TCF may allow the activation of two different sets of genes by MAPKs.

Results

Phosphorylation of Elk-1

A monomer of the Elk-1 protein can form complexes with several Ets target sequences, including the *Drosophila* E74 binding site (E74-BS), independent of the presence of SRF (Janknecht and Nordheim, 1992a; Rao and Reddy, 1992). Using purified hexahistidine-tagged Elk-1 produced in HeLa cells (N6His-Elk-1; Janknecht and Nordheim, 1992b) and the E74-BS in a gel retardation assay, two complexes were detected (Figure 1A, lane 1). A similar behaviour was displayed by ternary complexes, which consist of one

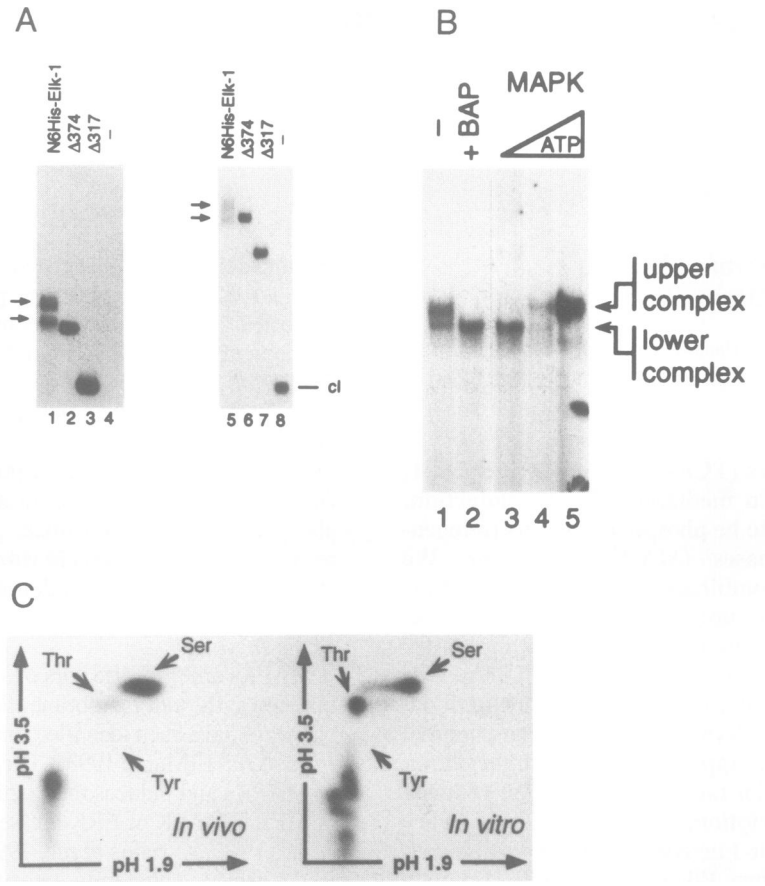

Fig. 1. Phosphorylation of Elk-1. (**A**) Gel retardation assays with purified N6His-Elk-1 or C-terminal truncations thereof (Δ374, Δ317). Both the interaction with an E74-BS (lanes 1–4) and with a preformed complex (cI) consisting of a $SRF_{132-245}$ dimer and the c-*fos* SRE (lanes 5–8) are shown. Arrows indicate the respective upper and lower complexes containing N6His-Elk-1. Free ^{32}P-labelled probes were run off the gel. (**B**) N6His-Elk-1 (lane 1) was treated with BAP (lane 2) and subsequently phosphorylated *in vitro* by MAPK in the presence of 0.1 (lane 3), 10 (lane 4) or 100 μM ATP (lane 5). Shown is the result of a gel retardation assay with a ^{32}P-labelled E74-BS. (**C**) Phosphoamino acid analysis of purified N6His-Elk-1 ^{32}P-labelled either *in vivo* or *in vitro* by $p44^{MAPK}$. Shown are autoradiograms after two-dimensional thin layer electrophoresis at pH 1.9 and 3.5. Coelectrophoresed phosphoamino acids (indicated by arrows) were visualized by ninhydrin staining.

molecule N6His-Elk-1, a dimer of a truncated version of SRF ($SRF_{132-245}$) and the c-*fos* SRE (Figure 1A, lane 5). Treatment with bacterial alkaline phosphatase (BAP) abrogated the appearance of the upper complex with the E74-BS (Figure 1B, lane 2) and also with the SRE–SRF complex (not shown). This indicates that the upper complexes contain hyperphosphorylated Elk-1 protein, while the lower complexes contain non- or hypophosphorylated Elk-1. C-terminal amino acids are apparently a main target for phosphorylation, because only one complex was detectable upon deleting 54 (Δ374) or 111 (Δ317) amino acids from the C-terminus of N6His-Elk-1 (Figure 1A). Since the C-terminal 120 amino acids of Elk-1 encompass nine potential MAPK phosphorylation sites, we assayed MAPKs for their ability to phosphorylate N6His-Elk-1 *in vitro*. Treatment of dephosphorylated N6His-Elk-1 with $p44^{MAPK}$ (Figure 1B) or $p42^{MAPK}$ (not shown) restored the upper complex in an ATP-dependent fashion indicating that Elk-1 is a substrate for MAPKs *in vitro*.

N6His-Elk-1 phosphorylated by $p44^{MAPK}$ *in vitro* contained phosphoserine and phosphothreonine residues (Figure 1C). However, the content of phosphothreonine was much higher than that observed for *in vivo* ^{32}P-labelled N6His-Elk-1 (Figure 1C). In addition, the pattern of both tryptic and chymotryptic phosphopeptides was only partially congruent between *in vitro* and *in vivo* phosphorylated N6His-Elk-1 (not shown). This could be due to a relaxed specificity of MAPKs *in vitro* or may indicate that *in vivo* additional kinases phosphorylate Elk-1.

To investigate whether MAPKs also phosphorylate Elk-1 *in vivo*, we coexpressed recombinant Elk-1 (Elk_{2-428}) with $p44^{MAPK}$ in RK13 cells. Since MAPK requires phosphorylation for activation, we also utilized a constitutively active form of Raf-1 kinase (BXB; Bruder *et al.*, 1992) as an indirect *in vivo* activator. Coexpression of BXB or $p44^{MAPK}$ alone with Elk_{2-428} led to small increases in the ratio of hyper- to hypophosphorylated Elk_{2-428}, as illustrated by the respective upper and lower complexes in gel retardation assays (Figure 2A). Conversely, joint overexpression of BXB and $p44^{MAPK}$ resulted in Elk_{2-428} shift activity mainly confined to the upper complexes; densitometric analysis revealed that the ratio of upper to lower complexes was enhanced by >6-fold. These results imply that activated MAPKs lead to hyperphosphorylation of Elk-1 *in vivo*, probably without further intermediate kinases.

Transcriptional activation mediated by Elk-1

To unravel the *in vivo* function of phosphorylation by MAPKs, transcriptional activity of a luciferase gene driven

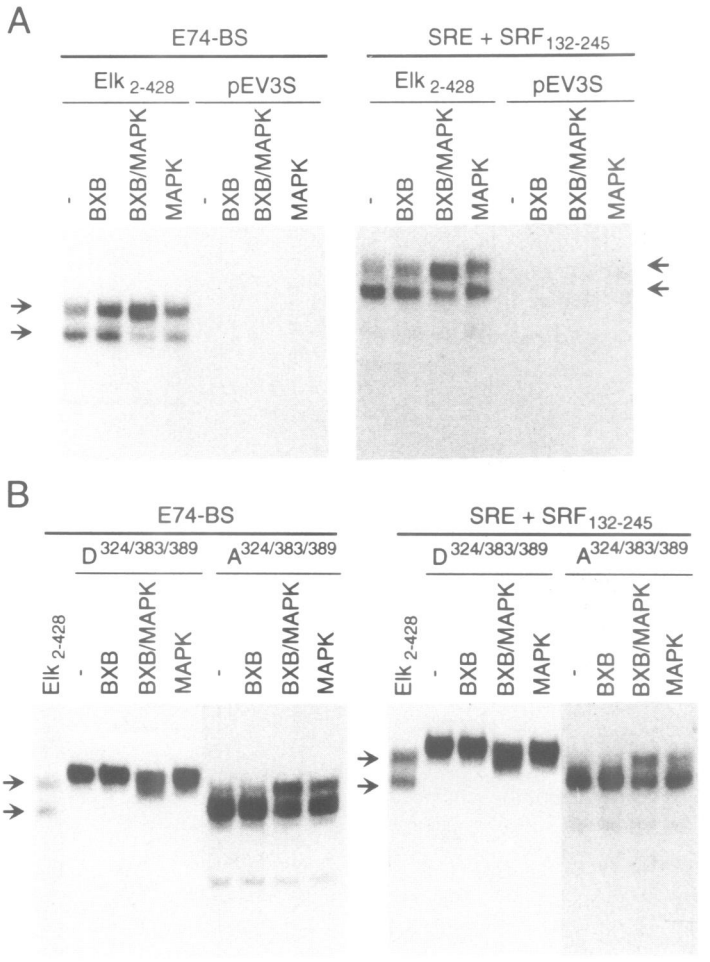

Fig. 2. Phosphorylation of Elk-1 by BXB and $p44^{MAPK}$ *in vivo*. (**A**) RK13 cells were transiently transfected with either 2 μg Elk_{2-428} expression plasmid or 2 μg of the empty expression plasmid pEV3S. Where indicated, 2 μg of BXB and/or $p44^{MAPK}$ expression plasmids were cotransfected. Shown are the results of gel retardation assays with protein extracts derived from transfected cells using either the ^{32}P-labelled E74-BS or a preformed complex consisting of ^{32}P-labelled c-*fos* SRE and a $SRF_{132-245}$ dimer. Arrows point to the upper and lower complexes containing Elk_{2-428}. (**B**) Analogous for Elk_{2-428} mutants $A^{324/383/389}$ and $D^{324/383/389}$.

by either three E74-BSs or two c-*fos* SREs was studied in transient transfection experiments. Overexpression of BXB alone led to small increases of relative luciferase activity (Vector Control, Figure 3A and B) mediated by endogenous transcription factors, possibly TCFs. These may be phosphorylated by endogenous MAPKs that are activated by BXB via endogenous MEKs. Similarly, overexpression of $p44^{MAPK}$ alone resulted in slightly elevated levels of relative luciferase activity, possibly due to activation of overexpressed $p44^{MAPK}$ by autophosphorylation or residual MEK activity. In contrast, high transcriptional stimulation was measured upon joint expression of BXB and $p44^{MAPK}$. Control experiments with a luciferase gene solely driven by a minimal promoter (tk80) did not result in significant transcriptional stimulation upon kinase overexpression, demonstrating that the observed effects were binding site specific (Vector Control, Figure 3C).

To investigate Elk-1 *in vivo*, Elk_{2-428} was overexpressed at such levels that endogenous TCFs were vastly outnumbered and thereby prevented from activating the reporter constructs (see below). Elk_{2-428} overexpression *per se* led to repression of E74-BS-driven transcription, while the SRE-driven reporter was nearly unaffected (black bars, Figure 3A and B). This repression could be due to titration of limiting basal transcription factors (squelching; Ptashne, 1988), or may be a consequence of the replacement of endogenous, transcriptionally more potent Ets proteins capable of interacting with the reporter constructs by Elk_{2-428}. This phenomenon seems to be correlated to the presence of the N-terminal DNA binding domain, since no repression was observed upon its deletion in Elk_{83-428}, while C-terminal truncations Elk_{2-374} and Elk_{2-317}, still capable of direct DNA binding and interaction with c-*fos* SRE−SRF (Janknecht and Nordheim, 1992a), even showed a trend towards higher repression. Again, these effects were binding site specific (Figure 3C).

Similar to endogenous TCFs, transcription mediated by Elk_{2-428} was coordinately induced by BXB and $p44^{MAPK}$ from both the E74-BS and c-*fos* SRE-driven reporters, but not from the minimal tk80 promoter (Figure 3A−C). This activation was more efficient than in the absence of overexpressed Elk_{2-428}. In contrast, C-terminal truncations Elk_{2-374} and Elk_{2-317} did not respond to kinase overexpression. This demonstrates the importance of C-terminal amino acids for transactivation. It also proves that under our experimental conditions endogenous transcription factors were prevented from activating the reporter constructs upon overexpression of Elk-1 variants capable of interaction with

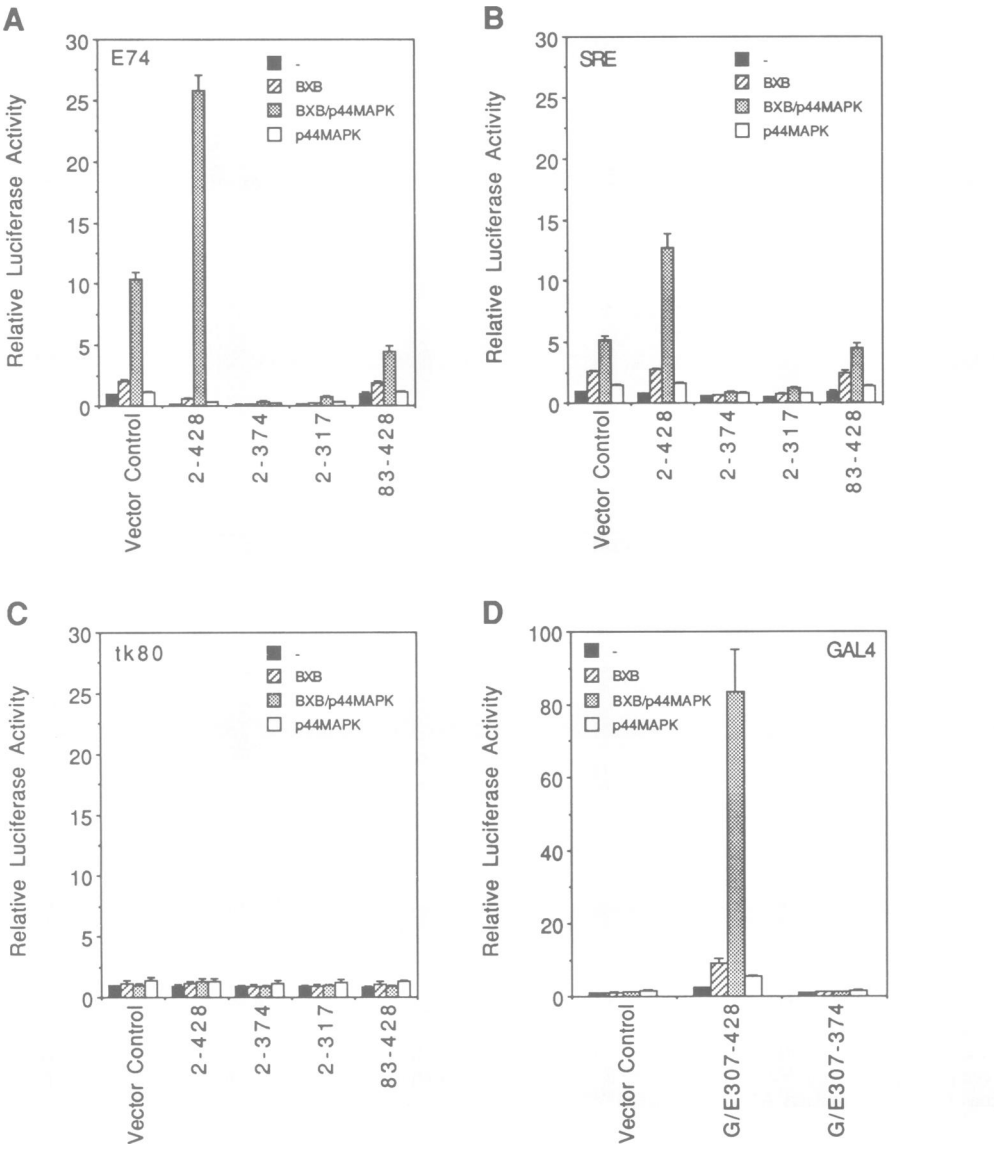

Fig. 3. Elk-1-mediated transactivation in RK13 cells. (**A**) Transactivation mediated by indicated Elk-1 variants was measured with a luciferase reporter construct driven by three E74-BSs in transient transfection assays. Vector Control denotes the empty expression plasmid pEV3S (Matthias *et al.*, 1989). Where indicated, BXB and/or $p44^{MAPK}$ expression plasmids were cotransfected. (**B**) Analogous with a luciferase reporter driven by two c-*fos* SREs. (**C**) Analogous with a luciferase reporter (tk80−luc) driven by the tk80 minimal promoter. (**D**) Transcriptional activity of fusion proteins between the GAL4 DNA binding domain and Elk-1 amino acids 307−428 (G/E307-428) or 307−374 (G/E307-374) was measured with a luciferase reporter construct driven by two GAL4 binding sites. Vector Control denotes the parental plasmid pAB-Gal-linker, which directs the expression of the GAL4 DNA binding domain.

the E74-BS or c-*fos* SRE. Although the DNA binding incompetent truncation Elk_{83-428} cannot prevent endogenous transcription factors from interacting with and thus activating reporter constructs, maximal transactivation by BXB and $p44^{MAPK}$ slightly decreased upon Elk_{83-428} overexpression. This may be due to competitive inhibition of phosphorylation of endogenous TCFs, since Elk_{83-428} may still act as a potent kinase substrate.

To prove transactivation potential inherent to the C-terminal region of Elk-1, amino acids 307−428 were fused to the GAL4 DNA binding domain (G/E307-428) and tested with a luciferase reporter driven by two GAL4 binding sites. Overexpression of G/E307-428 *per se* led to 2.4-fold activation compared with the GAL4 DNA binding domain alone (Figure 3D). This activation could be vastly enhanced by joint overexpression of BXB and $p44^{MAPK}$. In contrast, Elk-1 amino acids 307−374 fused to the GAL4 DNA binding domain (G/E307-374), or the GAL4 DNA binding domain alone (Vector Control), were not sufficient to increase basal transcription or to confer transcription activation by BXB and $p44^{MAPK}$, indicating the importance of the last 54 amino acids for transactivation. We note that the same 54 amino acids were also required for the generation of upper shifts in gel retardation assays (see Figure 1A).

Impact of phosphorylation on Elk-1 function

In an attempt to identify phosphorylation sites in the C-terminal 120 amino acids of Elk-1, we focused on all sites (S^{324} and S^{389}) conforming strictly to the MAPK consensus target ($PX^{S}/_{T}P$; Pelech and Sanghera, 1992). In addition, we studied S^{383}, since it is highly conserved between Elk-1

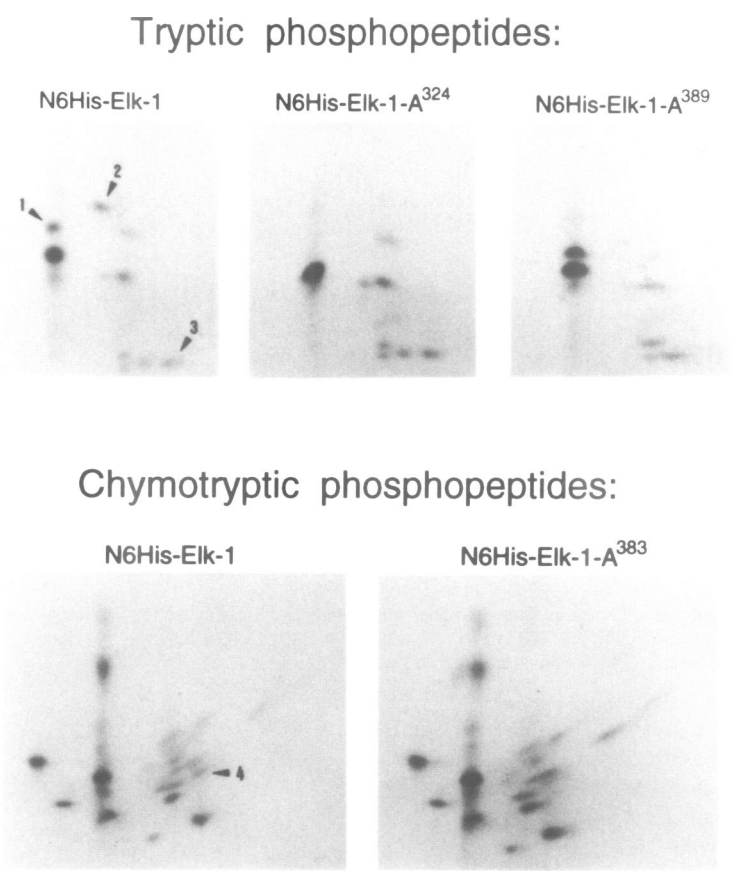

Fig. 4. Two-dimensional separation of Elk-1 phosphopeptides. *In vivo* ^{32}P-labelled N6His-Elk-1 and respective mutants A^{324}, A^{383} and A^{389} were digested with trypsin or chymotrypsin. Marked phosphopeptides vanished in the A^{324} (1, 2), A^{383} (4) or A^{389} (3) mutants. The presence of phosphopeptide 2 in the A^{389} mutant was clearly detectable in the original autoradiogram.

and SAP-1a, and is part of a less stringent MAPK phosphorylation site ($^{S}/_{T}P$). Mutating S^{324} or S^{389} to alanine caused the disappearance of specific *in vivo* ^{32}P-labelled tryptic peptides (peptides 1/2 or 3, respectively; Figure 4), while mutation of S^{383} did not (not shown), possibly because S^{383} is located within a tryptic peptide (amino acids 336–388) containing several potential phosphorylation sites. However, analysis of chymotryptic phosphopeptides revealed the disappearance of one peptide upon mutating S^{383} (peptide 4, Figure 4). Thus, S^{324}, S^{383} and S^{389} are all phosphorylated *in vivo*. In addition, peptides 3 and 4, but not peptides 1 and 2, were observable as major signals with N6His-Elk-1 phosphorylated by purified $p44^{MAPK}$ *in vitro* (not shown), demonstrating that at least S^{383} and S^{389} are direct targets of MAPK. The complexity of the phosphopeptide patterns implies additional phosphorylation to occur *in vivo* (see also below). This may involve T^{363}, T^{368}, T^{417} and S^{422}, which were identified as phosphorylation sites together with S^{383} and S^{389} in fusion proteins between the GAL4 DNA binding domain and Elk-1 amino acids 307–428 (Marais *et al.*, 1993).

Introducing the single alanine mutations A^{324}, A^{383} or A^{389} into $Elk_{2\text{-}428}$ revealed that both hyper- and hypophosphorylated forms were detectable for each individual mutant in gel retardation assays with extracts from transiently transfected RK13 cells (data not shown). In contrast, nearly no upper complex was observed upon overexpression of the triple alanine mutant $A^{324/383/389}$ (Figure 2B). However, coexpression of BXB and $p44^{MAPK}$ induced the appearance of upper complexes indicative of a phosphorylation event (Figure 2B). Thus, S^{324}, S^{383} and S^{389} are not the only *in vivo* phosphorylation sites in Elk-1. We next tried to mimic phosphorylation at these three serines by mutating them to negatively charged aspartic acid residues. This mutant $D^{324/383/389}$ displayed a more retarded complex than the upper, hyperphosphorylated complex of $Elk_{2\text{-}428}$ in gel retardation assays (Figure 2B). Coexpression of both BXB and $p44^{MAPK}$ with this mutant resulted in smearing of the observed complexes to less retarded positions equivalent to the upper complexes of $Elk_{2\text{-}428}$ (Figure 2B). This again indicates that additional phosphorylation sites exist besides S^{324}, S^{383} and S^{389}.

We next performed transactivation studies with the above mutants. Both with the E74-BS and the c-*fos* SRE-driven reporter constructs, maximal transactivation upon BXB/$p44^{MAPK}$ overexpression was reduced when mutating S^{383} or S^{389} to alanine (Figure 5A and B). The A^{383} mutant displayed the more severe reduction of transcriptional activity. On the other hand, the A^{324} mutant had only very little effect. Mutation of all three serines to alanine resulted in the lowest maximal transactivation. Consistently, in corresponding GAL4–Elk-1 fusion proteins the respective mutants displayed a similar phenotype: both basal and BXB/$p44^{MAPK}$ stimulated relative luciferase activity was reduced (Figure 5C). Taken together, these results imply that phosphorylation at S^{324}, S^{383} and S^{389} increases the transactivation potential of Elk-1 *in vivo*.

Interestingly, the $D^{324/383/389}$ mutant seems to be less

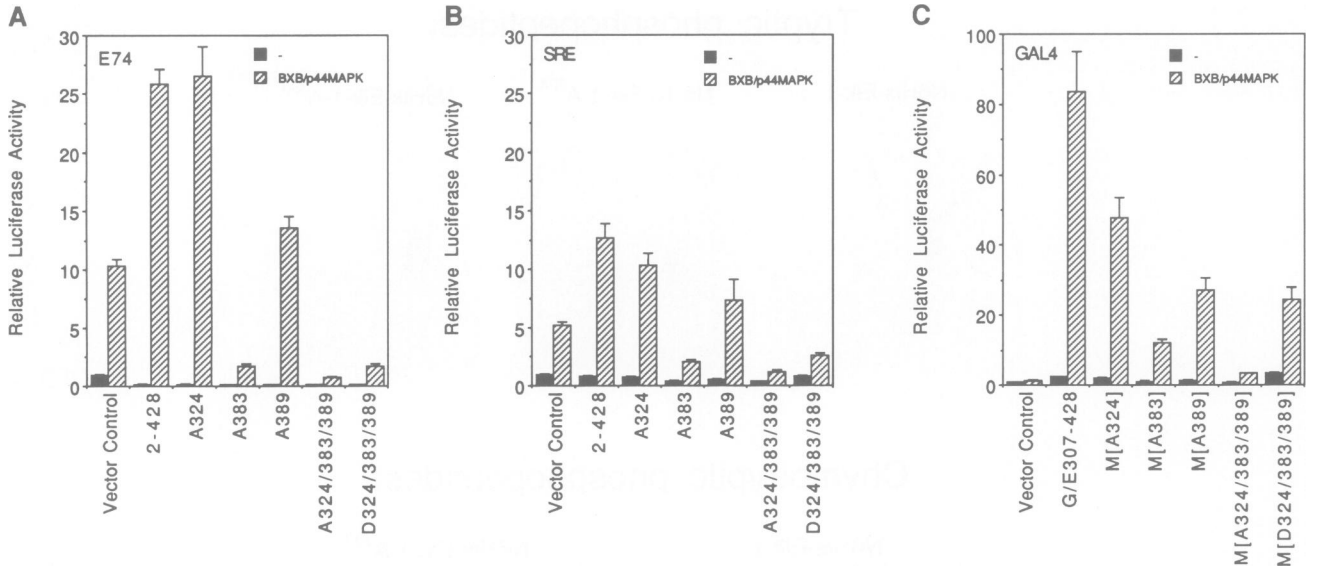

Fig. 5. Influence of Elk-1 phosphorylation sites on transactivation. (**A**) Transactivation mediated by indicated Elk_{2-428} variants measured with an E74-BS-driven reporter construct as in Figure 3A. (**B**) Analogous with a c-*fos* SRE-driven reporter. (**C**) Transcriptional activity of point mutants (M) of G/E307−428 was determined with a GAL4 binding site-driven reporter.

activated by BXB/$p44^{MAPK}$ than Elk_{2-428} (Figure 5A and B). In addition, no increased basal transcriptional activity was observed. However, in the corresponding G/E307-428 fusion protein, basal transcriptional activity was slightly enhanced from 2.4- to 3.5-fold, while BXB/$p44^{MAPK}$ stimulated transactivation was reduced (Figure 5C), albeit not as drastically as in the context of the Elk_{2-428} protein. These results demonstrate that fusions of Elk-1 C-terminal amino acids to a DNA binding domain do not in all instances faithfully reflect the behaviour of Elk-1.

Discussion

Phosphorylation of Elk-1 by MAPKs in vivo

MAPKs have been shown to phosphorylate TCFs or recombinant Elk-1 *in vitro* (Gille *et al.*, 1992; Marais *et al.*, 1993). Our results indicate that MAPKs do perform the same function *in vivo*, since (i) activated $p44^{MAPK}$ led to hyperphosphorylation of Elk_{2-428} *in vivo*, (ii) activated $p44^{MAPK}$ and also $p42^{MAPK}$ (not shown) enhanced transcriptional efficacy of Elk_{2-428}, and (iii) mutation of two potential MAPK *in vivo* phosphorylation sites (S^{383} and S^{389}), which were also targeted by $p44^{MAPK}$ *in vitro*, reduced Elk-1-mediated transactivation.

Phosphorylation seems to induce a conformational change in Elk-1. This becomes evident by the fact that hyperphosphorylation is correlated with a decreased mobility in gel retardation assays, although phosphorylation should increase the charge to mass ratio and thereby result in enhanced mobility. A similar conformational change could be elicited by introducing negative charge at S^{324}, S^{383} and S^{389} in the $D^{324/383/389}$ mutant. However, these negative charges *per se* were not sufficient to enhance transcriptional efficacy since it was impaired in the $D^{324/383/389}$ mutant. This is reminiscent of the CREB protein, where replacement of the phosphoacceptor S^{133} by alanine and also by aspartic or glutamic acid results in abolition of transactivation (Gonzalez and Montminy, 1989).

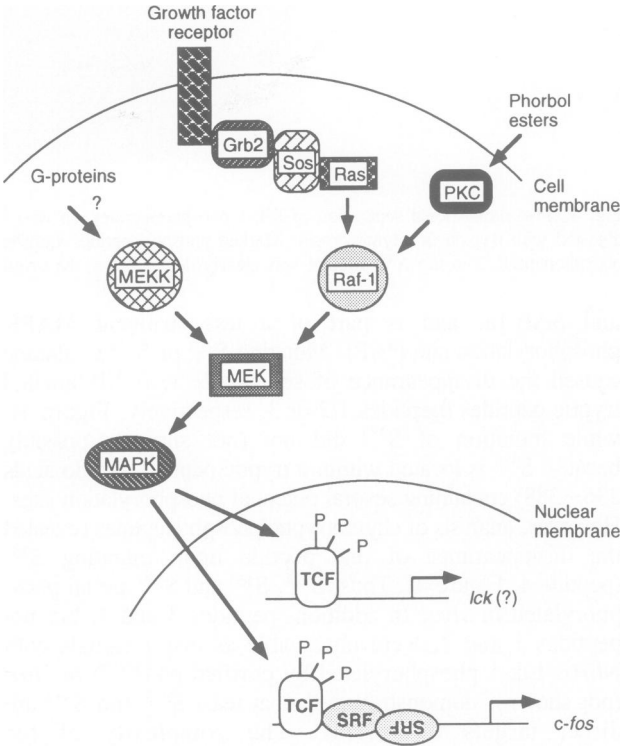

Fig. 6. Activation of TCFs by several signal transduction pathways.

Elk-1-mediated transactivation

Ternary complex factors are implicated in the regulation of the c-*fos* proto-oncogene via the SRE. We have shown for the first time that using the authentic c-*fos* SRE, the TCF Elk-1 can upregulate c-*fos* transcription when MAPKs are stimulated *in vivo*. These results are in agreement with the behaviour of LexA−Elk-1 fusions, which could stimulate transcription from an altered c-*fos* SRE upon stimulation of quiescent cells with serum (Hill *et al.*, 1993), since thereby MAPKs should also become activated (Pelech and Sanghera, 1992).

Disruption of the C-terminal transactivation domain of Elk-1 in Elk_{2-317} and Elk_{2-374} results in the complete suppression of c-*fos* SRE-mediated induction in response to MAPK stimulation. These C-terminal truncations act in a dominant negative fashion, since they prevent endogenous TCFs from interacting with the c-*fos* SREs in our reporter construct. Thus, endogenous SRF in a complex with Elk_{2-317} or Elk_{2-374} is not capable of stimulating c-*fos* transcription upon MAPK stimulation. Therefore, TCFs are likely the sole targets of signal transduction pathways funnelling through MAPKs and leading to the activation of c-*fos*. Although these results indicate that the main role of SRF in c-*fos* upregulation is that of a tether required to recruit TCFs to the SRE, SRF C-terminal amino acids may contribute to transcriptional stimulation (Hill *et al.*, 1993).

In addition to being a TCF, we have shown that Elk-1 possesses the capability to transactivate independent of SRF via an Ets binding site upon phosphorylation by MAPKs. This may allow TCFs to activate different sets of genes, such as those that are controlled by certain Ets binding sites and those that are regulated by SREs with juxtaposed TCF binding sites. Since the spacing and the relative orientation of the TCF binding site to the SRE might be very flexible (Treisman *et al.*, 1992), many of the SRE-regulated immediate early genes besides c-*fos* might be targets for TCFs, e.g. *egr*-1 (Rim *et al.*, 1992). On the other hand, the *lck* proto-oncogene, whose activity is dependent on a PEA3 element (Leung *et al.*, 1993) capable of direct interaction with Elk-1 (Rao and Reddy, 1992), might be a target for TCFs acting independent of SRF.

TCFs as end-points of signal transduction cascades

Our study has identified the TCF Elk-1 as a target for MAPKs. Preliminary data suggest the same for another TCF, SAP-1a (R.Janknecht and W.H.Ernst, unpublished data). Since MAPKs are activated by a plethora of agents (e.g. growth factors, phorbol esters), TCFs will nearly always be activated upon stimulation of quiescent cells. Several pathways lead to the activation of MAPKs (Figure 6). Binding of growth factors to their cognate receptors results in the activation of Ras via Grb2 and Sos (McCormick, 1993), which in turn may activate Raf-1 (Moodie *et al.*, 1993; Vojtek *et al.*, 1993; Zhang *et al.*, 1993). Thus activated Raf-1 phosphorylates MEKs, which then phosphorylate and thereby activate MAPKs. However, Raf-1 activation may not be exclusively dependent on Ras, since protein kinase C (PKC), which is activated by phorbol esters, may exert the same function (Kolch *et al.*, 1993). Furthermore, Raf-1 may be dispensable, since a MEK kinase (MEKK) could also activate MEKs and thereby MAPKs (Lange-Carter *et al.*, 1993). Thus, a variety of different signal transduction pathways funnel through MEKs and MAPKs and lead to the activation of TCFs, which in turn may activate many different genes. TCFs may therefore represent a pivotal link between signal transduction pathways and the induction of gene transcription.

Materials and methods

Eukaryotic expression vectors

The construction of expression vectors for Elk_{2-428} (identical to Elk-wt), Elk_{2-374}, Elk_{2-317} and Elk_{83-428} (identical to $Elk_{\Delta 1-82}$) has been described previously (Janknecht and Nordheim, 1992a). The Δ374 and Δ317 derivatives of N6His-Elk-1 (Janknecht and Nordheim, 1992b) were analogous to Elk_{2-374} and Elk_{2-317}, respectively. To obtain fusions of the first 147 GAL4 amino acids containing the GAL4 DNA binding domain with Elk-1 amino acids, respective fragments of *Elk*-1 cDNA were cloned into pAB-Gal-linker (Schmitz and Baeuerle, 1991). $p44^{MAPK}$ or BXB expression plasmids have been described by Meloche *et al.* (1992) or Bruder *et al.* (1992), respectively.

Gel retardation assays

Gel retardation assays and the preparation of utilized protein extracts were essentially as described (Janknecht and Nordheim, 1992a).

Analysis of ^{32}P-labelled proteins

The N6His-Elk-1 expression plasmid (or corresponding A^{324}, A^{383} and A^{389} mutants) was transiently transfected into HeLa cells that had been labelled with 0.5 mCi/ml $^{32}P_i$ for 5 h. Cell lysis and the subsequent Ni^{2+}-NTA affinity chromatography were performed as described by Janknecht and Nordheim (1992b). Further purification of ^{32}P-labelled N6His-Elk-1 by SDS−polyacrylamide gel electrophoresis, phosphoamino acid analysis, proteolytic cleavage and two-dimensional separation of phosphopeptides were performed according to standard procedures (van der Geer *et al.*, 1993). *In vitro* phosphorylation was performed with purified N6His-Elk-1, which had been treated with bacterial alkaline phosphatase before, in the presence of 30 mM β-glycerophosphate and 10 mM *p*-nitrophenyl phosphate. Used recombinant $p44^{MAPK}$ was produced in HeLa cells and then affinity-purified (R.Janknecht, in preparation).

Reporter constructs

tk80−luc was constructed by cloning a gene cassette consisting of HSV thymdine kinase sequences −80 to +52 in front of firefly luciferase cDNA, the SV40 small t intron and a polyadenylation site into pBluescript. Where indicated in the text, three E74-BSs, two c-*fos* SREs or two GAL4 binding sites were cloned in front of this gene cassette.

Transactivation assays

Rabbit kidney epithelial-like RK13 cells grown to 25% confluency on 6 cm dishes were transfected by the calcium phosphate coprecipitation method. Two micrograms of reporter construct, 1 μg of β-galactosidase expression plasmid pEQ176 containing a CMV enhancer, 50 ng of expression plasmids for Elk-1 variants or alternatively 500 ng of expression plasmids for GAL4 fusion proteins and pBluescript as carrier (up to a total of 10 μg) were employed. Where indicated, 1.5 μg of kinase expression plasmid were additionally used. Cells were kept in medium supplemented with 10% FCS for 36 h after transfection and luminescence and β-galactosidase activity were then determined (Janknecht *et al.*, 1993). Relative luciferase activity was obtained by normalizing luminescence to β-galactosidase activity and is presented as the mean ± SE of at least three independent experiments.

Acknowledgements

We thank U.Wiedemann and M.Petry for technical support, C.Marshall, J.Pouysségur and U.Rapp for providing $p42^{MAPK}$, $p44^{MAPK}$ and BXB expression plasmids, respectively, R. Treisman for providing *SAP*-1a cDNA, E.-M.Mandelkow for purified $p42^{MAPK}$, H.Althöfer for help in the construction of the Elk-1 $D^{324/383/389}$ mutant and B.Lüscher for advice and discussion. This work was supported by grants from the DFG (No. 120/7-1) and the Mildred-Scheel-Stiftung (W37/92/No1).

References

Bruder,J.T., Heidecker,G. and Rapp,U.R. (1992) *Genes Dev.*, **6**, 545−556.
Dalton,S. and Treisman,R. (1992) *Cell*, **68**, 597−612.
Dent,P., Haser,W., Haystead,T.A.J., Vincent,L.A., Roberts,T.M. and Sturgill,T.W. (1992) *Science*, **257**, 1404−1407.
Gille,H., Sharrocks,A.D. and Shaw,P.E. (1992) *Nature*, **358**, 414−417.
Gonzalez,G.A. and Montminy,M.R. (1989) *Cell*, **59**, 675−680.
Graham,R. and Gilman,M. (1991) *Science*, **251**, 189−192.
Hill,C.S., Marais,R., John,S., Wynne,J., Dalton,S. and Treisman,R. (1993) *Cell*, **73**, 395−406.
Hipskind,R.A., Rao,V.N., Mueller,C.G.F., Reddy,E.S.P. and Nordheim,A. (1991) *Nature*, **354**, 531−534.
Howe,L.R., Leevers,S.J., Gómez,N., Nakielny,S., Cohen,P. and Marshall,C.J. (1992) *Cell*, **71**, 335−342.
Janknecht,R. and Nordheim,A. (1992a) *Nucleic Acids Res.*, **20**, 3317−3324.
Janknecht,R. and Nordheim,A. (1992b) *Gene*, **121**, 321−324.
Janknecht,R., Ernst,W.H., Houthaeve,T. and Nordheim,A. (1993) *Eur. J. Biochem.*, **216**, 469−475.

Karim,F.D. *et al.* (1990) *Genes Dev.*, **4**, 1451–1453.
Kolch,W., Heidecker,G., Kochs,G., Hummel,R., Vahidi,H., Mischak,H., Finkenzeller,G., Marmé,D. and Rapp,U.R. (1993) *Nature*, **364**, 249–252.
König,H. (1991) *Nucleic Acids Res.*, **19**, 3607–3611.
Kyriakis,J.M., App,H., Zhang,X.-F., Banerjee,P., Brautigan,D.L., Rapp,U.R. and Avruch,J. (1992) *Nature*, **358**, 417–421.
Lange-Carter,C.A., Pleiman,C.M., Gardner,A.M., Blumer,K.J. and Johnson,G.L. (1993) *Science*, **260**, 315–319.
Leung,S., McCracken,S., Ghysdael,J. and Miyamoto,N.G. (1993) *Oncogene*, **8**, 989–997.
McCormick,F. (1993) *Nature*, **363**, 15–16.
Marais,R., Wynne,J. and Treisman,R. (1993) *Cell*, **73**, 381–393.
Marx,J. (1993) *Science*, **260**, 1588–1590.
Matthias,P., Müller,M.M., Schreiber,E., Rusconi,S. and Schaffner,W. (1989) *Nucleic Acids Res.*, **17**, 6418.
Meloche,S., Pagès,G. and Pouysségur,J. (1992) *Mol. Biol. Cell*, **3**, 63–71.
Moodie,S.A., Willumsen,B.M., Weber,M.J. and Wolfman,A. (1993) *Science*, **260**, 1658–1661.
Nye,J.A., Petersen,J.M., Gunther,C.V., Jonsen,M.D. and Graves,B.J. (1992) *Genes Dev.*, **6**, 975–990.
Pelech,S.L. and Sanghera,J.S. (1992) *Trends Biochem. Sci.*, **17**, 233–238.
Ptashne,M. (1988) *Nature*, **335**, 683–689.
Rao,V.N. and Reddy,E.S.P. (1992) *Oncogene*, **7**, 65–70.
Rim,M., Qureshi,S.A., Gius,D., Nho,J., Sukhatme,V.P. and Foster,D.A. (1992) *Oncogene*, **7**, 2065–2068.
Schmitz,M.L. and Baeuerle,P.A. (1991) *EMBO J.*, **10**, 3805–3817.
Shaw,P.E., Schröter,H. and Nordheim,A. (1989) *Cell*, **56**, 563–572.
Treisman,R., Marais,R. and Wynne,J. (1992) *EMBO J.*, **11**, 4631–4640.
van der Geer,P., Luo,K., Sefton,B.M. and Hunter,T. (1993) In Hardie,D.G. (ed.), *Protein Phosphorylation: A Practical Approach.* IRL Press, Oxford, pp. 31–59.
Vojtek,A.B., Hollenberg,S.M. and Cooper,J.A. (1993) *Cell*, **74**, 205–214.
Woods,D.B., Ghysdael,J. and Owen,M.J. (1992) *Nucleic Acids Res.*, **20**, 699–704.
Wu,J., Harrison,J.K., Dent,P., Lynch,K.R., Weber,M.J. and Sturgill,T.W. (1993) *Mol. Cell. Biol.*, **13**, 4539–4548.
Zhang,X.-F., Settleman,J., Kyriakis,J.M., Takeuchi-Suzuki,E., Elledge,S.J., Marshall,M.S., Bruder,J.T., Rapp,U.R. and Avruch,J. (1993) *Nature*, **364**, 308–313.
Zinck,R., Hipskind,R.A., Pingoud,V. and Nordheim,A. (1993) *EMBO J.*, **12**, 2377–2387.

Received on August 26, 1993; revised on September 29, 1993