The EMBO Journal vol.14 no.5 pp.951-962, 1995

ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation

Hendrik Gille, Monika Kortenjann, Oliver Thomae, Carolyn Moomaw[1], Clive Slaughter[1], Melanie H.Cobb[2] and Peter E.Shaw[3]

Max-Planck-Institut für Immunbiologie, Spemann Laboratories, Stübeweg 51, 79108 Freiburg, Germany and [1]Howard Hughes Medical Institute and [2]Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75235, USA

[3]Corresponding author

Communicated by R.Kemler

Induction of the human c-*fos* proto-oncogene by mitogens depends on the formation of a ternary complex by p62^{TCF} with the serum response factor (SRF) and the serum response element (SRE). We demonstrate that Elk-1, a protein closely related to p62^{TCF} in function, is a nuclear target of two members of the MAP kinase family, ERK1 and ERK2. Phosphorylation of Elk-1 increases the yield of ternary complex *in vitro*. At least five residues in the C-terminal domain of Elk-1 are phosphorylated upon growth factor stimulation of NIH3T3 cells. These residues are also phosphorylated by purified ERK1 *in vitro*, as determined by a combination of phosphopeptide sequencing and 2-D peptide mapping. Conversion of two of these phospho-acceptor sites to alanine impairs the formation of ternary complexes by the resulting Elk-1 proteins. Removal of these serine residues also drastically diminishes activation of the c-*fos* promoter in epidermal growth factor-treated cells. Analogous mutations at other sites impair activation to a lesser extent without affecting ternary complex formation *in vitro*. Our results indicate that phosphorylation regulates ternary complex formation by Elk-1, which is a prerequisite for the manifestation of its transactivation potential at the c-*fos* SRE.

Key words: c-*fos*/Elk-1/ERK/p62^{TCF}/phosphorylation

Introduction

An increasing body of evidence links the triggering of membrane-bound growth factor receptors to the activation of numerous intracellular proto-oncogene products. A paradigm for such a signalling network is the induction of the immediate early gene c-*fos* in response to the growth factor stimulation of quiescent cells. We have embarked on an analysis of the signalling mechanism leading to the activation of the human c-*fos* gene through the serum response element (SRE).

The SRE is sufficient to confer inducibility to serum, TPA and epidermal growth factor (EGF) upon the c-*fos* gene (Treisman, 1985; Fisch *et al.*, 1987). This response is mediated by the serum response factor (SRF; Treisman, 1992) and p62^{TCF} (Shaw *et al.*, 1989b) which are known to bind to the SRE both *in vitro* and *in vivo* (Herrera *et al.*, 1989). A dimer of the SRF occupies the central part of the SRE (Treisman, 1992). However, SRE responsiveness to many stimuli depends on the ternary complex factor p62^{TCF}, which has no intrinsic affinity for the SRE (Shaw *et al.*, 1989b; Schröter *et al.*, 1990; Graham and Gilman, 1991). p62^{TCF} requires the presence of bound SRF for its interaction with the left arm of this c-*fos* promoter element. In the nucleoprotein complex, p62^{TCF} interacts with an extended region of the SRF core domain and contacts a limited region of the left part of the SRE (Mueller and Nordheim, 1991; Shaw, 1992).

MAP kinases phosphorylate p62^{TCF} *in vitro*. Phosphorylation of p62^{TCF} leads to an increase in its capacity to form a ternary complex (Gille *et al.*, 1992). Nuclear extracts prepared from cells cultivated under conditions that activate the MAP kinases ERK1 and ERK2 yield more ternary complex activity, arguing for the modification of p62^{TCF} *in vivo* in response to growth factor stimulation (Malik *et al.*, 1991; Gille *et al.*, 1992).

ERK activation is the result of a series of events initiated by the binding of growth factors to their cognate receptors at the cell surface (Boulton *et al.*, 1991b). The ensuing intracellular receptor autophosphorylation on tyrosine residues creates recognition sites for SH2-containing proteins (Anderson *et al.*, 1990; Lowenstein *et al.*, 1992). Thus, enzymatic activities are brought to the inner surface of the cell membrane, resulting in the activation of p21^{Ras} by guanine nucleotide exchange factors (Rozakis-Adock *et al.*, 1992, 1993; Buday and Downward, 1993; Egan *et al.*, 1993). By an as yet unknown mechanism involving direct interaction between Raf and Ras (Warne *et al.*, 1993), Raf kinase activity is induced, leading to the phosphorylation and activation of MEKs, the dual specificity kinases that are capable of activating ERKs by phosphorylation of threonine and tyrosine residues (Alessandrini *et al.*, 1992; Nakielny *et al.*, 1992; Rossomando *et al.*, 1992).

A yeast screen of a HeLa cell cDNA library led to the isolation of a p62^{TCF} clone, SRF accessory protein (SAP) 1a (Dalton and Treisman, 1992). SAP1a, an *ets*-family transcription factor, shares biochemical properties with p62^{TCF}. It interacts with the SRE only when SRF is present, and extends the footprint produced by SRF on the SRE in a manner that is highly reminiscent of p62^{TCF}. Its closest known relative, the product of the *Elk-1* gene, has similar properties and is antigenically and structurally related to p62^{TCF}, making it a second candidate for p62^{TCF} (Hipskind *et al.*, 1991).

Apart from their *ets* DNA binding motifs, SAP1a and Elk-1 share two regions of homology. The Elk-1 B domain has been shown to be required for the interaction with SRF (Janknecht and Nordheim, 1992). The C-terminal

© Oxford University Press

homology region has been implicated as a target for the growth factor-mediated regulation of p62^{TCF}/Elk-1 (Gille *et al.*, 1992; Marais *et al.*, 1993). Elk-1 has been shown recently to be phosphorylated by a MAP kinase preparation, resulting in altered ternary complex mobility and increased transcriptional activation of various reporter constructs (Janknecht *et al.*, 1993; Marais *et al.*, 1993).

This report shows that ERK1 and ERK2 are the nuclear Elk-1 kinases that are activated in cells within minutes of growth factor stimulation. We have determined the Elk-1 phosphorylation sites both *in vitro* and *in vivo* by a combination of peptide sequencing and phosphopeptide mapping. Furthermore, consistent with our previous results (Gille *et al.*, 1992), mutations in the individual phosphoacceptor sites have allowed us to demonstrate that phosphorylation of Elk-1 enhances ternary complex formation with SRF and the c-*fos* SRE. The removal of either of two single phospho-acceptor sites impairs ternary complex factor (TCF) activity. Mutation of these sites concomitantly impairs induction of the c-*fos* promoter following EGF stimulation of NIH3T3 cells. The fact that analogous mutations at other sites also impair induction, albeit to a lesser extent, argues for two different steps in TCF/Elk-1 activation that are positively affected by phosphorylation.

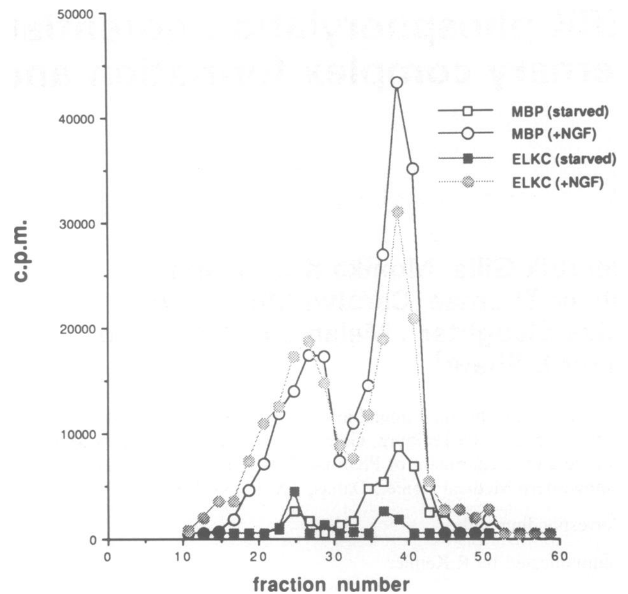

Fig. 1. Elk kinase activities in PC12 cells. Nuclear extracts from quiescent PC12 cells treated with or without NGF for 8 min were fractionated by chromatography over a Mono Q column. Alternate fractions were analysed in parallel for their ability to phosphorylate MBP and ElkC.

Results

The MAP kinases ERK1 and ERK2 are the major TCF/Elk-1 kinases

ERK1 and ERK2 have been reported to translocate rapidly to the nucleus following growth factor stimulation of cells (Chen *et al.*, 1992; Lenormand *et al.*, 1993), making them candidate enzymes for the phosphorylation and activation of transcription factors. TCF/Elk-1 has been reported to be a nuclear target for ERKs (Gille *et al.*, 1992; Marais *et al.*, 1993; Zinck *et al.*, 1993). Prior to studying the phosphorylation of p62^{TCF}/Elk-1 in further detail, we sought to identify clearly the nuclear protein kinases that are able to phosphorylate the ternary complex factor Elk-1.

To establish the identity of relevant growth factor-inducible kinases, PC12 cells were serum-starved and stimulated briefly with nerve growth factor (NGF). Nuclear extracts were prepared from quiescent and induced cells and fractionated by Mono Q chromatography. The resulting fractions were assayed in parallel for their ability to phosphorylate the recombinant C-terminal region of the Elk-1 protein (amino acids 213–428) or the MAP kinase substrate myelin basic protein (MBP; Figure 1). Two activities that could phosphorylate MBP were induced ~5-fold by NGF treatment of PC12 cells. These activities corresponded to the MAP kinases ERK2 and ERK1, as demonstrated previously (Boulton *et al.*, 1991b), and were confirmed by immunoblotting (data not shown). Both ERK2 and ERK1 also phosphorylated the recombinant Elk-1 protein. MBP and Elk-1 kinase activities co-elute from the Mono Q column and show comparable activation ratios, indicating that ERK2 and ERK1 are the major Elk-1 kinases in nuclei of growth factor-stimulated cells. We could not detect any preference of either MAP kinase for Elk-1 compared with their respective activities towards MBP. No other ElkC kinase activities were detected.

Similar results were obtained in rat1HIRc B cells treated with insulin. While more active ERK1 could be recovered from NGF-treated PC12 cells, ERK2 activity was slightly more abundant in rat1HIRc B cells. Again, no other Elk-1 kinase activity was detected. Although the resolution offered by a single Mono Q column is not sufficiently high to conclude that ERK1 and ERK2 are the only Elk-1 kinases, these results indicate that they are the major nuclear kinases that phosphorylate Elk-1 in PC12 and rat1HIRc B cells.

Analysis of Elk-1 phosphorylation in vitro

Preliminary experiments revealed that all ERK1 phosphorylation sites in Elk-1 reside in a C-terminal CNBr fragment of the protein (data not shown). We therefore sought to determine the phosphorylation sites by direct phosphopeptide sequencing. ERK1 and ERK2 have been shown to phosphorylate preferentially PX(S/T)P motifs, where X represents a basic or neutral amino acid. However, the first proline is not absolutely required (Haycock *et al.*, 1992; Cheng *et al.*, 1993). Thus, the several (S/T)P motifs contained in the C-terminal region of the protein all constitute potential MAP kinase phosphorylation sites, most of which are conserved between Elk-1 and SAP1a (Figure 2).

A C-terminal region containing the potential phosphorylation sites (ElkC; amino acids 213–428) was produced by expression as a hexahistidine fusion in *Escherichia coli* and purified by affinity chromatography. ERK1, purified to homogeneity from rat1HIRc B cells treated with insulin (Boulton *et al.*, 1991a), was used to phosphorylate ElkC *in vitro*. This substrate could be phosphorylated to a stoichiometry of 3.3 mol of phosphate by incubation with homogeneous ERK1, consistent with the presence of at least four phosphorylation sites.

The recombinant ElkC protein was re-purified by preparative SDS–PAGE and digested with trypsin and/or

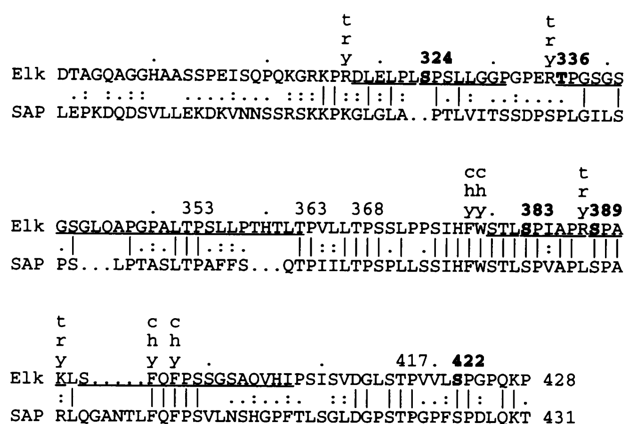

Fig. 2. Alignment of Elk-1 and SAP1a. The alignment was generated using the Bestfit program from the Genetics Computer Group, WI. Elk-1 sequences determined by peptide sequencing are underlined and the relevant protease sites are given. All potential ERK phosphorylation sites are indicated above the Elk-1 sequence. Residues shown to be phosphorylated *in vivo* are indicated in bold.

chymotrypsin. The resulting peptides were purified by two consecutive reversed-phase (RP) HPLC runs. Several phosphorylated peptides could be isolated by this approach and subsequently subjected to automated Edman degradation in an Applied Biosystems sequencer (Table I and Figure 2). The region encompassing serines 383 and 389 yielded overlapping serine-phosphorylated peptides (peptides A–C). This may be due to incomplete trypsin digestion at R388 when S389 is phosphorylated (see below). Peptide B contained only one serine, thus pinpointing the phosphorylation site S389. Likewise, peptide C appeared to contain only one serine, thus strongly suggesting that S383 is also a site.

S324 and S422 are inferred as the phosphorylated sites in peptides D and E, respectively. These peptides were highly purified and each contained exclusively serine phosphate. Peptide D contained a single SP motif; assuming that peptide E terminated at the predicted tryptic cleavage site, it also contained a single SP motif. Phosphorylation of both serine residues was confirmed by 2-D phosphopeptide mapping (see below). The 29 amino acid peptide F contained exclusively phosphothreonine (data not shown). However, this peptide would be expected to encompass 43 amino acids and contain four TP motifs (amino acids 336–378), thus precluding the identification of individual phospho-acceptor sites.

To identify phosphothreonines in this region, to confirm the other assignments and to allow functional studies, we mutated potential phospho-acceptor sites in the Elk-1 C-terminus to alanine. Recombinant proteins were produced by expression in *E.coli* and purified. After phosphorylation with ERK1 the proteins were digested with trypsin, chymotrypsin or both. The ElkC protein appeared to be extremely resistant to proteolytic cleavage. Prolonged exposure to chymotrypsin leads to cleavage at L, M, A, D and E residues, in addition to Y, P and W. It also results in the occasional appearance of additional phosphopeptides

Table I. TCF/Elk-1 phosphopeptide sequences[a]

Cycle	Phosphopeptides A	B	C	D	E	F
1	S	X	S	D	X	T
2	T	P	T	L	S	P
3	L	A	L	E	F	G
4	S	K	S	L	Q	S
5	P	-	P	P	F	G
6	I		I	L	P	S
7	A		A,I	S	S	G
8	P		P	P	S	S
9	R		R	S	X	G
10	X		-	L	X	L
11	P			L	A	Q
12	A			X	Q	A
13	K			X	V	P
14	-			P	X	G
15					I	P
16					P	A
17						L
18						T
19						P
20						S
21						L
22						L
23						P
24						T
25						H
26						T
27						L
28						T
29						P

[a]The amino acid identified in each cycle is indicated by single-letter code. Positions at which unambiguous identification of the amino acid was not possible are indicated with X. Hyphens (-) indicate terminated sequences. The phosphopeptides A, D, E and F are underlined in Figure 2. Peptides B and C are derived from peptide A by tryptic cleavage at R388.

in the upper region of the chromatogram (see, for example, the wild-type chymotryptic map in Figure 3). Thus, in some cases mixing experiments were performed to confirm assignments. The phosphopeptide maps shown in Figure 3 verify the phosphorylation of serines 324, 383, 389 and 422 by ERK1. The results are summarized in Figure 4c. The mutation of S324 leads to the loss of a tryptic phosphopeptide (peptide 5). The mutation of either S383 or S389 causes the disappearance of multiple phosphopeptides (2, 4, 8 and 9, and 1–4, respectively). The region containing these two serines is flanked by two closely spaced chymotrypsin sites on either side (Figure 2). In addition, trypsin does not cleave quantitatively after R388, as demonstrated by the recovery of a phosphopeptide encompassing both residues after RP-HPLC. Thus, this region gives rise to several overlapping peptides. The phosphopeptide maps also demonstrate phosphorylation of T336, not identified previously by peptide sequencing. We could not detect the phosphorylation of any of the other (S/T)P motifs at residues 353, 363, 368 and 417 (Figure 3). Thus, ERK1 phosphorylates five sites in the C-terminal region of Elk-1 *in vitro*.

Phosphorylation of Elk-1 in vivo

Elk-1 was transiently expressed in NIH3T3 cells. Serum-starved cells were metabolically labelled with [^{32}P]orthophosphate and stimulated with EGF or serum. Elk-1 was

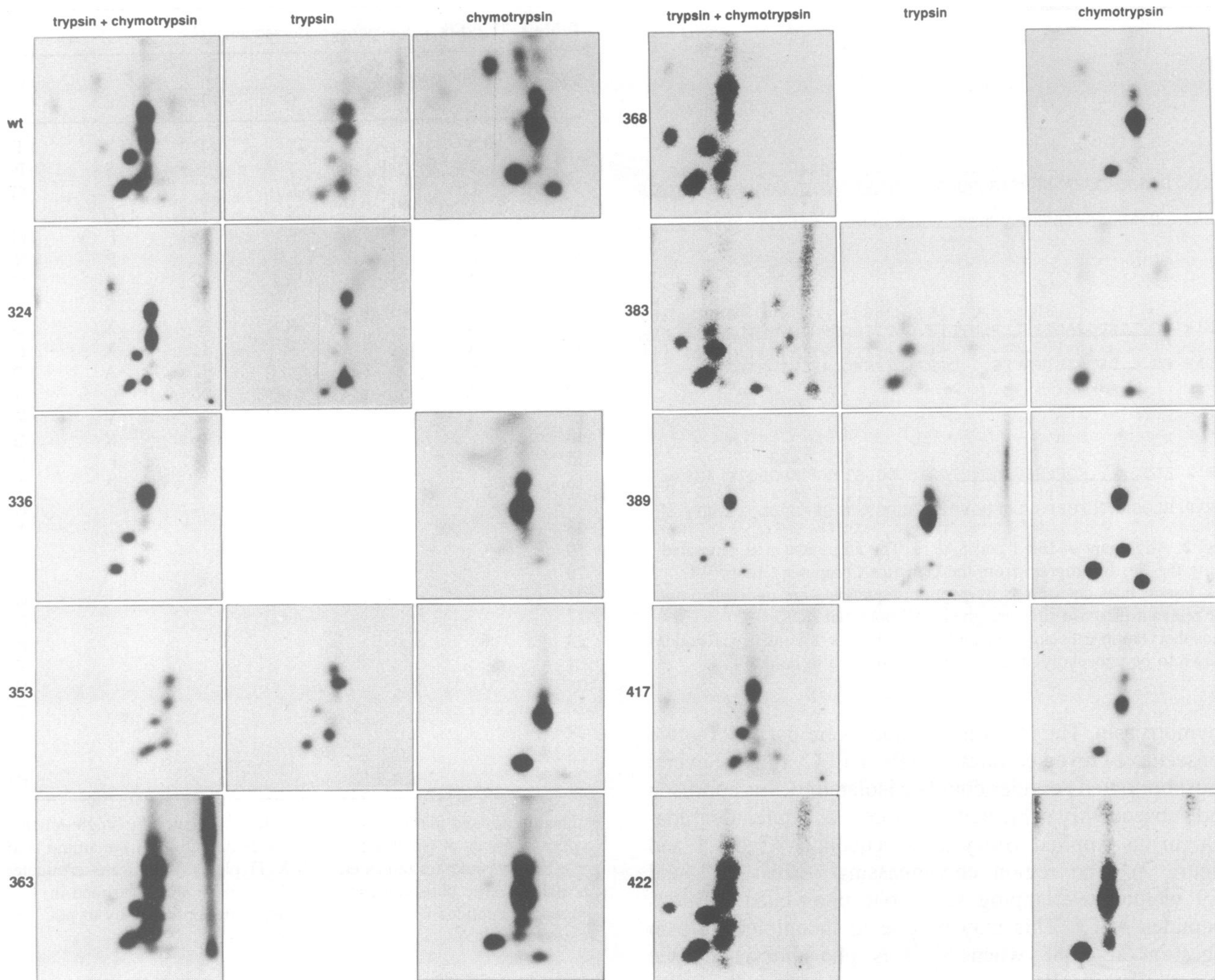

Fig. 3. Analysis of Elk-1 phosphorylation *in vitro*. 2-D phosphopeptide maps generated from recombinant ElkC and derivatives in which individual phospho-acceptor sites were mutated to alanine. The mutations are indicated to the left of the double digests. Separation was achieved as indicated in Figure 4b. The proteins were phosphorylated with ERK1 and cleaved with the proteases given below each panel. In some cases, over-digestion with chymotrypsin led to the appearance of additional phosphopeptides with a high mobility in the second dimension (see wild-type chymotrypsin pattern), which are not reproducibly observed.

recovered from the cells and visualized by autoradiography after SDS–PAGE (Figure 4a). Treatment of the cells with EGF or serum induces the rapid phosphorylation of an ~60 kDa protein that is not detected in untransfected or quiescent cells. The protein was recovered from the gel and digested with proteases. The resulting phosphopeptides were analysed by 2-D phosphopeptide mapping (Figure 4b) and the maps were compared with those obtained from the *in vitro* mapping experiments (Figure 4c). Several Elk-1 phosphopeptides can be detected that are also phosphorylated by purified ERK1 *in vitro*. Most of the radioactivity is contained in the set of partially overlapping peptides encompassing serines 383 and 389 (peptides 1–4, 8 and 9).

In contrast to the other phosphorylated residues, T336, S324 and S422 appear to be substoichiometrically labelled *in vivo* (peptides 5–7 and 10). Two unidentified peptides (x and y) are faintly visible. It is not clear whether these represent additional phosphorylated peptides in Elk-1 or stem from a contaminating phosphoprotein(s). However, they do not represent major sites as the majority of the radioactivity is contained in the assigned peptides, thus showing that the relevant *in vivo* sites have been identified.

Effect of phosphorylation on ternary complex formation

Phosphorylation of $p62^{TCF}$ from HeLa cells by ERK2 *in vitro* has been shown to regulate its capacity to form a ternary complex with the SRF and SRE (Gille *et al.*, 1992). In contrast, a concomitant change in conformation upon phosphorylation has been proposed for Elk-1, manifested by a reduced mobility in gel retardation analyses (Janknecht *et al.*, 1993; Marais *et al.*, 1993). To clarify the observed differences in ternary complex formation, Elk-1, unphosphorylated or phosphorylated with ERK1, was tested for TCF activity with $core^{SRF}$ under different binding conditions. A striking difference in the ability of the unphosphorylated protein to form the ternary complex is observed, depending on the stringency of the binding reaction (Figure 5a). In the presence of high amounts of double-stranded, non-specific, competitor DNA, unphosphorylated Elk-1 is unable to form a ternary complex.

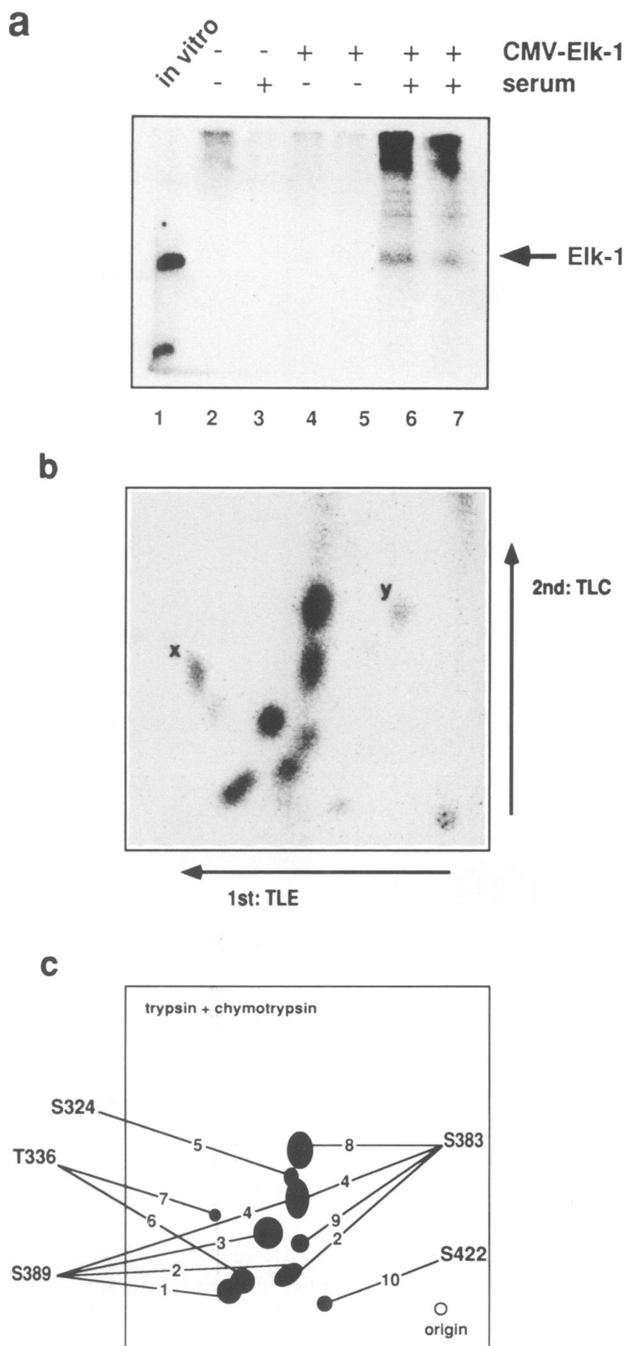

Fig. 4. Elk-1 phosphorylation *in vivo*. (**a**) Ni^{2+}-agarose-purified Elk-1 was isolated from ^{32}P-labelled cells transfected with pCMV-Elk-1 and visualized by SDS–PAGE. (**b**) 2-D phosphopeptide map of Elk-1 isolated from the gel shown in (a). (**c**) Diagrammatic representation of the phosphopeptide map generated from ElkC labelled *in vitro* and from Elk-1 labelled *in vivo*.

When the competitor DNA concentration is lowered, however, binding of the unphosphorylated protein is apparent as a faster migrating complex (cII*).

These experiments do not elucidate the basis of the increase in ternary complex formation following phosphorylation. TCF/Elk-1 interacts not only with the DNA but also makes extensive contacts with the SRF. Thus, the observed increase in binding under stringent conditions could be the effect of an increase in affinity for DNA. On the other hand, the interaction with SRF could also be promoted by phosphorylation of Elk-1. To distinguish between these possibilities, the *ets* DNA binding domain of Elk-1 was replaced by that of the Gal4 protein (Sadowski and Ptashne, 1989). If the unphosphorylated growth factor-regulated domain of Elk-1 were to interfere with ternary complex formation by precluding DNA binding by the *ets* domain, this effect should be relieved by the insertion of a heterologous DNA binding domain.

Recombinant Gal-Elk was phosphorylated and tested for DNA binding with an SRE-derived binding site in which the *ets* motif was replaced by a weak Gal4 binding site (GalRE). Analogous to Elk-1, the fusion protein was incapable of interacting with this Gal4 site in the absence of SRF (Figure 5b and c). Upon addition of full-length SRF, phosphorylated but not unphosphorylated Gal-Elk was able to form a ternary complex with SRF and the GalRE binding site. Unfortunately, Elk-1 also binds to the GalRE in an SRF- and phosphorylation-dependent manner (results not shown).

Gal-Elk was also tested for DNA binding with the mutant elSRE binding site. Elk-1 is unable to form a ternary complex with the elSRE site (Figure 6b, lane 7; Hipskind *et al.*, 1991), but Gal-Elk interacts with this mutant SRE in a phosphorylation- and SRF-dependent manner (Figure 5b and c). The mobility observed for the Gal-Elk ternary complex is slower than that formed by Elk-1 with the wild-type SRE sequence. Furthermore, deletion of the Gal4 dimerization domain impairs the formation of a ternary complex by Gal-Elk. These results suggest that Gal-Elk binds as a dimer. Thus, although the regulation of DNA binding cannot be ruled out, this observation stresses the importance of protein–protein interactions in the formation of ternary complexes with SRF.

Contribution of individual phospho-acceptor sites to Elk-1 binding

To investigate the contribution of individual phospho-acceptor sites to the observed increase in ternary complex formation, recombinant full-length Elk-1 proteins bearing single phosphorylation site mutations were generated (Figure 6a). The proteins were either phosphorylated with purified ERK1 or mock-treated prior to analysis for ternary complex formation under stringent binding conditions (Figure 6b).

The binding assays were performed numerous times with different preparations of each Elk-1 mutant. The experiment shown in Figure 6b was performed with the protein preparations shown in Figure 6a. With the exception of the S353 mutant (Figure 6b, lane 15), this single experiment most closely reproduces the relative behaviour of the Elk-1 mutants consistently observed. In this assay, the mutation of serines 383 or 389 to alanine reduces the ability of the phosphorylated protein to form a ternary complex (17 and 34%, respectively). The generation of the double mutant 383/389 does not lead to a further reduction in ternary complex activity (data not shown). The replacement of serine 422 with alanine has a less severe effect. The removal of other phosphorylation sites has no reproducible effect. Thus mutations at the two sites phosphorylated at high stoichiometry *in vivo* upon mitogen treatment of cells impair ternary complex formation with

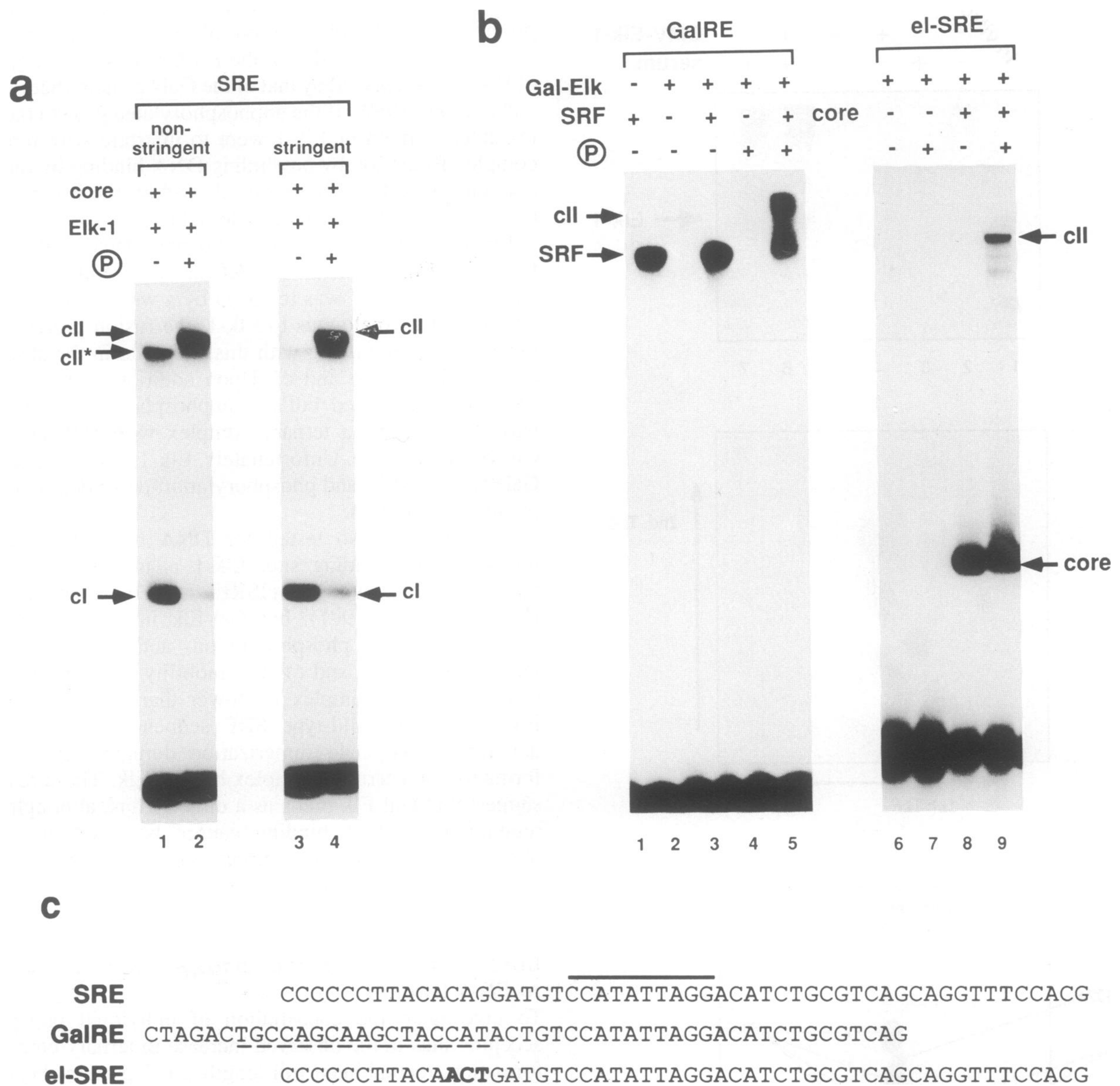

Fig. 5. Ternary complex formation by Elk-1 and Gal-Elk. (**a**) Gel retardation analysis of unphosphorylated or phosphorylated Elk-1 under different binding conditions. Binding reactions were set up either as specified in Materials and methods, or with 1.2 μg poly(dI-dC) and 3.25 μg herring sperm DNA as non-specific competitor DNA for reduced stringency. (**b**) Gal-Elk ternary complexes were formed with vaccinia-expressed full-length SRF, $core^{SRF}$ and either the GalRE oligonucleotide (left panel) or the el derivative of the SRE (right). Binding reactions with Gal-Elk lacked poly(dI-dC). (**c**) Sequences of the DNA binding probes (upper strands only). The SRF binding site is overlined, the weak Gal4 site is underlined and the el mutation is in bold type.

SRF by Elk-1. These results argue strongly for the regulation of ternary complex formation by Elk-1 due to phosphorylation of serines 383 and 389 by MAP kinases.

In addition to testing the ternary complex formation by bacterially expressed Gal-Elk *in vitro*, the behaviour of Gal-Elk and mutant derivatives expressed in eukaryotic cells was examined. The analysis of cell extracts from transiently transfected NIH3T3 cells by gel retardation in the absence of poly(dI-dC), which as mentioned above inhibits ternary complex formation by Gal-Elk, led to the formation of several unspecific complexes that obscured the Gal-Elk–DNA complexes (results not shown). To overcome this problem, Gal-Elk and several mutant derivatives were recloned into a cytomegalovirus (CMV)-based expression vector and overexpressed in COS cells.

As observed in Figure 7, under these conditions the treatment of COS cells with serum stimulates the formation of a ternary complex containing Gal-Elk (lanes 1 and 2); this is confirmed by the disruption of the complex by a polyclonal antibody raised against the DNA binding domain of Gal4 (lane 3). Thus, Gal-Elk behaves similarly to $p62^{TCF}$ in so far as ternary complex formation with SRF is stimulated upon treatment of cells with mitogens (Malik *et al.*, 1991; Gille *et al.*, 1992). Furthermore, the mutations S383A and S422A, both of which impair ternary complex formation by Elk-1 *in vitro* (Figure 6b), similarly

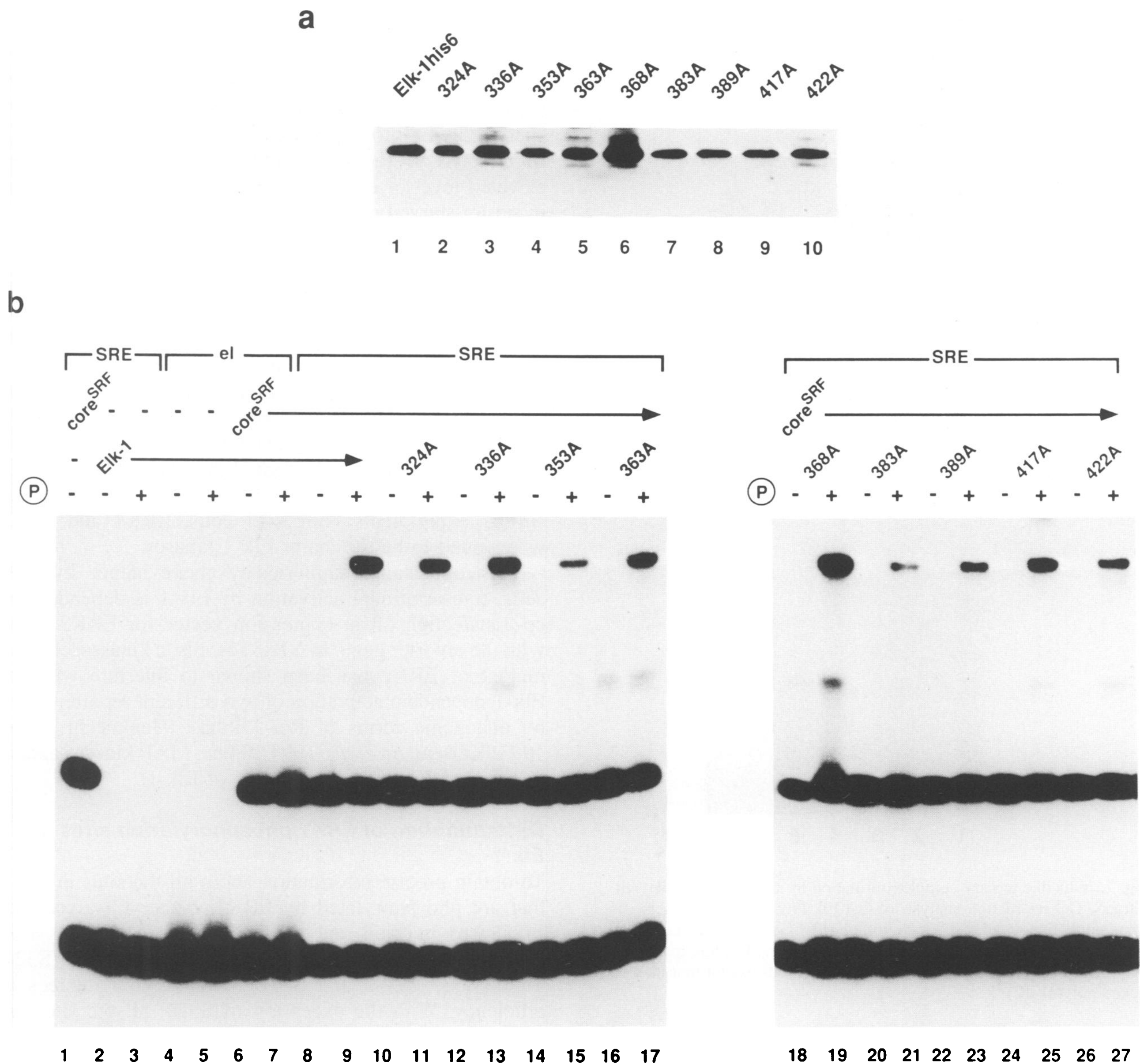

Fig. 6. Influence of individual phospho-acceptor site mutations on ternary complex formation. (**a**) Immunoblot of the recombinant Elk-1 proteins used for gel retardation analysis. (**b**) Ternary complexes formed by wild-type Elk-1 protein and derivatives bearing mutations at individual phospho-acceptor sites as indicated. Elk-1 derivatives were either unphosphorylated (−) or phosphorylated with ERK1 (+). In this experiment the concentration of herring sperm competitor DNA was raised to 13 μg per 10 μl reaction. The decrease in ternary complex formation by Elk 353A seen in this experiment was not reproducibly observed.

reduce complex formation by Gal-Elk (Figure 7, lanes 4–7).

TCF/Elk-1 transactivation potential as a function of individual phospho-acceptor site mutants

The effect of removing individual phosphorylation sites on the transactivation potential of Elk-1 was investigated in the context of the full c-*fos* promoter. As demonstrated in Figures 5b and 7, Gal-Elk is capable of interacting with a mutant variant of the c-*fos* SRE (elSRE) that does not allow SRF to recruit TCF/Elk-1 or endogenous TCF activities to the SRE (Shaw *et al.*, 1989b; Hipskind *et al.*, 1991).

In NIH3T3HERc cells treated with EGF, activation of the corresponding reporter construct (el2CAT) is dependent on co-transfection of Gal-Elk. The inducible expression closely parallels that of the wild-type c-*fos* promoter. Deletion of the Elk-1 kinase-regulated domain from Gal-Elk (Gal-ElkΔA) reduces expression to control levels (Figure 8a, compare lanes 5 and 6 with 1 and 2, and c). The mutations at individual phospho-acceptor sites of Elk-1 were introduced into the Gal-Elk eukaryotic expression vector and tested for their ability to impair expression from the c-*fos* promoter in this assay system. In contrast to their effect on ternary complex formation (Figure 6b), all of the individual mutants, with the exception of T336A, reduced the level of EGF-induced expression by at least 50% (Figure 8b and c). The mutation of

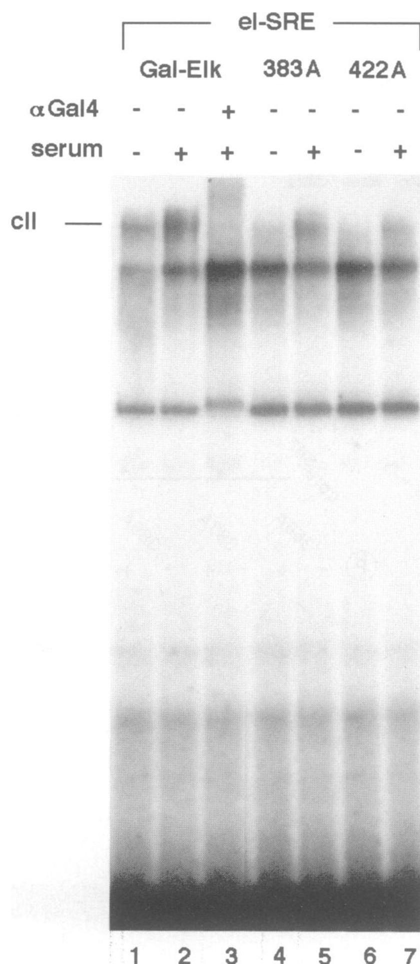

Fig. 7. Inducible ternary complex formation by Gal-Elk in whole-cell extracts. Gel retardation analysis of Gal-Elk in whole-cell extracts prepared from starved and serum-stimulated COS cells transfected with the corresponding Gal-Elk expression vectors. Binding reactions were set up as described in Materials and methods except that they lacked poly(dI-dC).

serines 383 or 389 has the most severe effect, reducing the EGF-induced level of expression to <20% of Gal-Elk. The mutant T336A has no effect on the level of induced expression, but consistently gives rise to a 20% elevation in the level of expression from the reporter in uninduced cells (Figure 8b, lane 3, and c). Thus, the two mutations that impair ternary complex formation *in vitro* are also the most effective in reducing transcriptional activation *in vivo*.

Discussion

The results described above demonstrate that in PC12 and rat1HIRcB cells, two nuclear kinases that are activated by mitogenic stimuli and phosphorylate the C-terminal region of Elk-1 correspond to the MAP kinases ERK1 and ERK2. Five of the nine potential MAP kinase phosphorylation sites in Elk-1 are phosphorylated *in vivo*, as confirmed by sequencing and 2-D mapping of Elk-1 phosphopeptides derived from proteins labelled *in vitro* and *in vivo*. The mutation of two phospho-acceptor sites to alanine results in the severe impairment of SRF-dependent ternary complex formation by Elk-1 *in vitro*. The same mutations virtually abolish EGF-induced expression from a c-*fos* reporter gene *in vivo*. Mutations at other phosphorylation sites have a less detrimental effect on EGF-induced expression. However, mutation at only one of the phosphorylation sites, T336, fails to impair EGF-induced expression *in vivo*. In contrast to the other mutations, T336A causes an elevated level of expression from the c-*fos* reporter gene in serum-starved cells.

ERK1 and ERK2 are nuclear Elk-1 kinases

Several reports show that ERK2 is able to phosphorylate Elk-1 *in vitro* (Marais *et al.*, 1993; Zinck *et al.*, 1993). The separation of serine/threonine kinases by Mono Q chromatography is a well-established biochemical procedure. Following the protocol used previously to separate MAP kinases, we obtained two peaks of mitogen-induced Elk-1 kinase activity from the nuclei of NGF-treated PC12 cells. Immunoblotting confirmed that these Elk-1 kinases corresponded to ERK1 and ERK2. Thus, in these and similar experiments with Rat1 cells, ERK1 and ERK2 were found to be the major Elk-1 kinases.

This conclusion is supported by other evidence. In RK13 cells, transcriptional activation by Elk-1 is dependent on co-transfection of an expression vector for ERK2 along with the reporter gene. In NIH3T3 cells, a kinase-defective mutant of ERK2 has been shown to interfere with the Elk-1-dependent activation of two different reporter genes by oncogenic forms of Raf-1 kinase (Janknecht *et al.*, 1993; Kortenjann *et al.*, 1994). Thus, MAP kinases appear to be essential for the regulation of Elk-1.

Determination of ERK1 phosphorylation sites in Elk-1

To obtain precise information about all the sites in Elk-1 that are phosphorylated by ERK1, bacterially expressed ElkC was purified and incubated with ERK1 *in vitro*. ERK1 phosphorylates five C-terminal sites in Elk-1 (S324, T336, S383, S389 and S422) with varying degrees of efficiency. With the exception of S324, all the sites are conserved between Elk-1 and SAP1a. The yield of multiple overlapping peptides containing serines 383 and 389 phosphorylated to a high stoichiometry suggests that they are the preferred substrates in Elk-1 for ERK1. They lie within the region most highly conserved between Elk-1 and SAP1a. Additional residues were recently found to be phosphorylated by a MAP kinase preparation containing both enzymes (Marais *et al.*, 1993). The *in vitro* phosphorylation experiments described here were performed with a purified preparation of ERK1.

The phosphopeptides derived from Elk-1 phosphorylated *in vivo* upon stimulation of NIH3T3 cells with serum generate a well-resolved map upon 2-D resolution. No incorporation of ^{32}P could be detected in serum-starved cells, indicating that the kinases responsible are mitogen-dependent. This observation is consistent with the results from our fractionation data. The individual peptides were identified by comparison with maps generated from wild-type Elk-1 and mutant derivatives lacking individual phosphorylation sites, phosphorylated by purified ERK1 *in vitro*. Our comprehensive phosphopeptide mapping analysis of each individual potential Elk-1 phosphorylation site allowed the unequivocal assignment of each

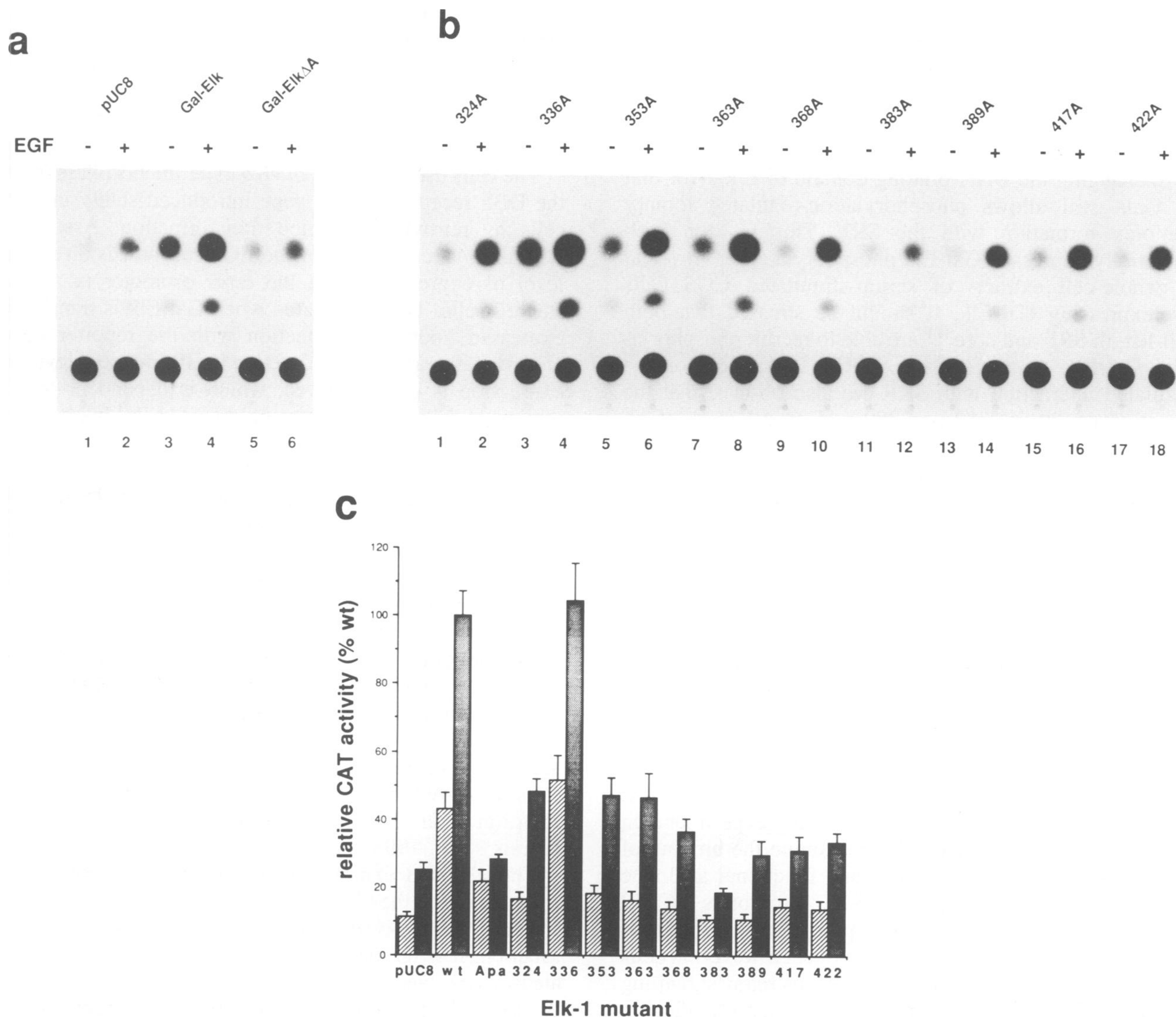

Fig. 8. Transactivation potential of individual Gal-Elk mutants. (**a**) Effect of removing the C-terminal Elk-1 domain (Gal-ElkΔA) on EGF-inducible expression from el2CAT. (**b**) Influence of mutations at individual phospho-acceptor sites. (**c**) Quantification of EGF-inducible expression from el2CAT. Hatched bars are uninduced values; solid bars represent induced values. The data given represent averages from three experiments and are expressed relative to Gal-Elk (wt, induced).

phosphopeptide to the phosphorylation of one or two sites in Elk-1. This approach allowed the identification of a fifth site, T336, as a target for phosphorylation *in vivo*, which was not identified by phosphopeptide sequence analysis. These results provide the first comprehensive analysis of Elk-1 phosphorylation by a purified MAP kinase.

Phosphorylation as a regulator of ternary complex formation

The influence of phosphorylation on the SRF-dependent ternary complex formation by TCF/Elk-1 is a matter of some controversy. Concurrent with the first description of $p62^{TCF}$, models were proposed involving the regulated SRF-dependent interaction of $p62^{TCF}$ with the SRE (Shaw *et al.*, 1989a). The demonstration that in A431 cells both SRF and $p62^{TCF}$ were bound to the c-*fos* promoter prior to and during induction of the c-*fos* gene by EGF provided evidence against the regulated binding of TCF (Herrera *et al.*, 1989). Nevertheless, inducible ternary complex activity at the c-*fos* SRE has been reported (Malik *et al.*, 1991). Results consistent with the existence of a dynamic complex between TCF/Elk-1 and SRF bound to the SRE have also been forthcoming (Janknecht and Nordheim, 1992). A rapid exchange of TCFs would remain undetected *in vivo* as their *ets* DNA binding domains presumably make identical DNA contacts.

Recently it was demonstrated that MAP kinases stimulate ternary complex formation by phosphorylating TCF/Elk-1 (Gille *et al.*, 1992). On the other hand, the results of others have been interpreted as showing that phosphorylation induces a conformational change in TCF/Elk-1 and at the same time confirming that SRF-dependent DNA binding by TCF/Elk-1 is constitutive (Zinck *et al.*, 1993). Here it is shown that the effect of phosphorylating Elk-1 on ternary complex formation *in vitro* depends on the binding conditions. Low non-specific competitor DNA concentrations allow unphosphorylated TCF/Elk-1 to form

a complex with SRF and the SRE, whereas only phosphorylated TCF/Elk-1 forms a ternary complex under stringent conditions. Regulation of the DNA binding activity of *ets* proteins by phosphorylation has also been observed for direct DNA binding by Ets-1 and Ets-2 (Pognonec *et al.*, 1989).

Exchanging the DNA binding domain of Elk-1 for that of Gal4 still allows phosphorylation-regulated ternary complex formation with the SRF. This is seen with bacterially expressed Gal-Elk phosphorylated *in vitro* and in whole-cell extracts of serum-stimulated COS cells overexpressing Gal-Elk. It should be stressed that both full-length SRF and core^{SRF} are able to recruit phosphorylated Gal-Elk to the mutant elSRE. In support of the regulated interaction with SRF, we also observe that the mutation of either S383 or S389 and, to a lesser extent, S422 in TCF/Elk-1 impairs SRF-dependent ternary complex formation. It is worth noting that S383 and S389 are the two sites most readily phosphorylated by ERK1 *in vitro* and which contain the most labelled phosphate in our *in vivo* phosphopeptide analyses. Thus, the most efficient phosphorylation sites in TCF/Elk-1 appear to regulate its capacity to interact with SRF at the SRE.

It is unclear whether upon phosphorylation TCF/Elk-1 associates with uncomplexed SRF or displaces a factor bound previously. The *in vivo* footprint of the c-*fos* promoter would suggest that the latter situation occurs in cells. However, as explained below, this observation may have been due to the background activity of the Ras–Raf signalling pathway in A431 cells. Consistent with this view are the results of *in vivo* footprinting experiments in *Saccharomyces cerevisiae*, which examine the binding of STE12, a MAP kinase substrate and functional analogue of TCF/Elk-1, to the FUS1 and STE2 promoters. STE12 is constitutively bound to these promoters in wild-type cells, even in the absence of pheromone. However, in cells harbouring mutations that block the upstream signalling cascade, STE12 binding cannot be detected (H.Winkler, M.Primig and G.Ammerer, personal communication).

Conversion of several C-terminal phospho-acceptor sites in Elk-1 to alanine impairs the transcriptional activation of the c-*fos* promoter by Gal-Elk while leaving the ternary complex activity *in vitro* unaffected. This observation suggests that while ternary complex formation is a prerequisite for the c-*fos* transcriptional response to EGF under these conditions, a second aspect is involved in the activation of TCF/Elk-1. For example, the phosphorylation of additional sites may elevate the efficacy of transcriptional activation, perhaps by facilitating the interaction with downstream components of the transcriptional machinery.

Functional analysis of Elk-1 phosphorylation *in vivo*

The observation that Gal-Elk binds to elSRE in a phosphorylation- and SRF-dependent manner provided a means of analysing the functional importance of each individual Elk-1 phosphorylation site in the context of the c-*fos* promoter. It was shown previously that the el mutation in the SRE prevents the interaction of p62^{TCF} with SRF *in vitro* and impairs the induction of c-*fos* reporter genes by mitogens (Shaw *et al.*, 1989b; Graham and Gilman, 1991). Gal-Elk overcomes this promoter mutation as it binds to the elSRE and rescues EGF-inducible expression *in vivo* from a c-*fos* reporter gene containing the elSRE mutation. The EGF response is mediated exclusively by the SRE in this reporter because the interferon-stimulated gene factor (ISGF) binding site is deleted. All other known c-*fos* promoter elements are intact.

The cells used for these *in vivo* experiments overexpress the EGF receptor from a gene introduced stably into the cells by retrovirus-mediated transformation. Associated with the overexpression of the EGF receptor is a residual level of expression from the c-*fos* promoter in serum-starved cells. This is elevated when Gal-Elk is transiently expressed upon co-transfection with the reporter gene. Nonetheless, an increase in the EGF-induced level of expression is also observed, which is dependent on the region of Elk-1 encompassing the MAP kinase phosphorylation sites.

The Gal-Elk point mutants show three distinct phenotypes *in vivo*. The mutation of serines 383 and 389 severely impairs EGF-inducible c-*fos* promoter activity. As their removal also reduces Elk-1 ternary complex activity *in vitro*, this result implicates the regulation of ternary complex formation in the mechanism of c-*fos* activation. Serines 383 and 389 are the preferred sites detected upon phosphorylation by ERK1 *in vitro* and after growth factor stimulation *in vivo*, and so a low level of MAP kinase activity would be expected to target them first. Thus, a low basal activity of the Ras–Raf signalling pathway may account for the residual Elk-1 phosphorylation in quiescent cells observed by others, as well as the constitutive footprint over the SRE *in vivo* (Herrera *et al.*, 1989; Marais *et al.*, 1993).

The elevated expression in uninduced, serum-starved cells seen with the Gal-Elk mutant T336 suggests that this site may be involved in the negative regulation of Elk-1 function. It is the only threonine found to be phosphorylated *in vivo*, and although it is phosphorylated by ERK1 *in vitro*, albeit at a low stoichiometry, we cannot rule out that it is a more efficient substrate for a different kinase. For example, a novel S/T kinase family related to MAP kinases has been described with a substrate specificity distinguishable from that of ERK1 and ERK2 (Hibi *et al.*, 1993; Derijard *et al.*, 1994).

All other mutations at phospho-acceptor sites have a detrimental effect on the level of expression from the c-*fos* reporter. This suggests that, in contrast to the phosphorylation of serines 383 and 389, the positive effect on transcriptional activation brought about by phosphorylation of the C-terminal region of Elk-1 at multiple sites may be pleiotropic in nature.

In conclusion, our results suggest that the phosphorylation of Elk-1 by MAP kinases, in particular on serines 383 and 389, is a prerequisite for SRF-dependent binding to the c-*fos* SRE. Ternary complex formation by TCF/Elk-1, in turn, is necessary for the stimulation of the c-*fos* gene by growth factors.

Materials and methods

Cell culture and extract preparation

PC12, rat1HIRc B (McClain *et al.*, 1987), COS and NIH3T3 cells were grown in DMEM supplemented with 10% fetal calf serum (FCS). Cells were transfected by the standard DNA–calcium phosphate co-

precipitation procedure and processed as described (Kortenjann *et al.*, 1994). Cells were transfected with 4 μg of the el2CAT reporter and 4 μg of pSG Gal-Elk or the corresponding expression vectors for the Gal-Elk mutants (Kortenjann *et al.*, 1994). Precipitates were left on the cells for 15–20 h, after which cells were washed with medium without serum, starved for another 22 h and subsequently induced with 5 nM EGF. At 8 h after induction extracts were prepared by sonifying cells in 0.25 M Tris–HCl, pH 7.8. Transfection efficiency was monitored by measuring the β-galactosidase activity resulting from co-transfection of 1 μg of pCH110 (Pharmacia) per plate; the quantity of extract for each point in the CAT assay was normalized according to the relative transfection efficiency.

Whole-cell extracts were prepared from COS cells transfected with 10 μg of the corresponding pCMV5 Gal-Elk expression vectors. Starved cells were either untreated or stimulated with serum for 10 min. The cells were washed with ice-cold PBS and scraped into 0.5 ml lysis buffer containing 10 mM Tris–HCl, pH 7.2, 50 mM $Na_4P_2O_7$, 50 mM NaCl, 50 mM NaFl, 0.5 mM Na_3VO_4, 1% Triton X-100, 1 mM dithiothreitol, 5 mM *p*-nitrophenylphosphate, 1 mM benzamidine and 0.2 mM phenylmethylsulfonyl fluoride. The lysates were vortexed vigorously and centrifuged at 4°C for 15 min. The supernatants were frozen in liquid nitrogen and stored at −80°C.

Plasmids

pBSElk-1 contains the Elk-1 cDNA inserted between the *Bgl*II and *Xba*I sites of pBluescript KS$^+$ (Kortenjann *et al.*, 1994). pQElkC was constructed by ligating an Elk-1 PCR fragment from pBSElk-1 generated with the primers 3X-3 and 16QE-2 with the *Nco*I–*Bgl*I 5′ acceptor from pQE6 and the *Hin*dIII–*Bgl*I 3′ acceptor from pQE16 (Qiagen). Thus, pQElkC expresses amino acids 213 (A)–428 of Elk-1, followed by a C-terminal hexahistidine tag. pQElk-1 was derived by ligating the *Bgl*I–*Nco*I fragment of pQE6 with the N-terminal *Nco*I–*Sma*I Elk-1 fragment from pBSElk-1 and the *Bgl*I–*Sma*I C-terminal fragment from pQElkC.

pGNElk was generated by the insertion of a 615 bp *Nco*I–*Stu*I PCR fragment from pBSElk-1 into the *Nco*I–*Sac*I sites of pGEX-KG (Guan and Dixon, 1991). The resulting plasmid drives the expression of a glutathione-S-transferase (GST) fusion containing the N-terminal 205 residues of Elk-1.

pCMV-Elk was generated by the insertion of an *Eco*RI–*Hin*dIII fragment bearing the hexahistidine-tagged Elk-1 from pQElk-1 into the pCMV5 vector (Andersson *et al.*, 1989).

pQGal-Elk was derived from the ligation of the pQE6 *Nco*I–*Bgl*I fragment with the *Nco*I–*Bgl*I fragment from pQElkC and an *Nco*I–*Sma*I fragment from pSG Gal-Elk (Kortenjann *et al.*, 1994). The reporter plasmid el2CAT contains a tandem repeat of the elSRE sequence (Shaw *et al.*, 1989b) cloned into a deletion derivative of pF711CAT (Treisman, 1985) lacking the *c-fos* promoter sequence from −353 to 275, which includes the ISGF element and the SRE.

pCMV5Gal-Elk and its derivatives were constructed by inserting the *Hin*dIII–*Xba*I fragments of the corresponding pSGGal-Elk plasmids into the polylinker of pCMV5.

PCR primers

The following PCR primers were used: 3X-3, GCCTGGATCCAGGC-TGCCATGGCCGGCTTGCC; 16QE-2, GTGGTAGGGATCCTGGCTT-CTGGGG; e-5′, GCACGGATCCACCATGGACCCATCTGTGAGGC; and e-mid, GGATGACATCGATAGGCAAGCCGGCC.

In vitro labelling of elk-1 and peptide mapping

Approximately 1 μg recombinant ElkC protein was incubated with purified ERK1 in the presence of 20 μCi [γ-^{32}P]ATP for 30 min at 37°C. For HPLC purification of proteolytic peptides, 50 μg ElkC were phosphorylated with ERK1. The phosphorylated ElkC was re-purified by preparative SDS-PAGE, TCA-precipitated and digested with protease in 50 mM ammonium bicarbonate, pH 8.3, as described (Boyle *et al.*, 1991). Labelled peptides were lyophilized, spotted onto cellulose plates (Kodak) and analysed by 2-D peptide mapping on HTLE-7000 apparatus (CBS Scientific). Electrophoresis was carried out at pH 8.9 in ammonium carbonate for 50 min at 1000 V. Ascending chromatography was performed in *n*-butanol/pyridine/acetic acid/water in a ratio of 6.5:5:1:4. Phosphorylated peptides were visualized by autoradiography or on a Fujix BAS 1000 bioimaging analyser. 1-D phospho-amino acid analysis was carried out as described (Boulton *et al.*, 1991b).

Immunoblotting

A GST fusion protein containing the N-terminal 205 residues of Elk-1 was isolated from bacterial lysates by affinity purification on GST–agarose beads. The Elk-1 moiety was cleaved off the beads by digestion with thrombin. The resulting NElk protein was used for immunization. After separation on a 10% acrylamide–SDS gel, the proteins were transferred to nitrocellulose. The blot was probed with the polyclonal anti-NElk serum, followed by a peroxidase-coupled secondary antibody (Dianova). Immunoblotted, recombinant Elk-1 proteins were visualized using the enhanced chemiluminescence system (Amersham); the exposed film was scanned for quantification. Gal-Elk proteins were identified as described (Kortenjann *et al.*, 1994).

Gel retardation analysis

Identical amounts of each Elk-1 protein were phosphorylated with ERK1 and aliquots were tested for their ability to form the ternary complex with core^{SRF} and an SRE oligonucleotide, as described (Gille *et al.*, 1992). Unless otherwise stated, 1.2 μg of poly(dI-dC) and 9.75 μg herring sperm DNA were used as non-specific competitor DNA in each 10 μl binding reaction. Gels were dried and ternary complex formation was quantified with the aid of a Fujix BAS 1000 bioimaging analyser. The resulting intensities were corrected for slight differences in the Elk-1 protein amounts from the scanned immunoblot image.

Complexes containing Gal-Elk proteins were analysed with the elSRE probe as described, except that poly(dI-dC) was omitted from the binding reactions. Comparable levels of Gal-Elk in the whole-cell extracts were confirmed by the determination of protein concentration and immunoblotting. Ternary complex formation by Gal-Elk was facilitated by the addition of exogenous, recombinant vaccinia–SRF; complexes containing Gal-Elk were confirmed with a polyclonal serum raised against the N-terminal 147 amino acids of Gal4.

In vivo labelling

NIH3T3 cells transfected with pCMV5Elk-1 were starved for 20 h and transferred to phosphate-free medium for 90 min. Cells were labelled for an additional 60 min at 1 mCi/ml and stimulated by the addition of FCS to 10%. After 10 min the cells were washed in ice-cold PBS and lysed in 1 ml of lysis buffer by passage through a 0.45 mm syringe needle. The lysate was clarified by centrifugation and the supernatant was bound to Ni^{2+}-agarose. The agarose beads were washed extensively in 50 mM imidazole and the Elk-1 protein was eluted in 400 mM imidazole, pH 7.0. The eluted material was purified further by preparative SDS–PAGE.

Chromatography

Nuclear extracts from PC12 cells were prepared from subconfluent 150 mm dishes bearing quiescent or NGF-treated cells. The cells were washed with PBS and scraped off in buffer A containing 0.1% NP-40 (Ostrowski *et al.*, 1991). After swelling for 10 min on ice, the cells were lysed by vortexing and nuclei were harvested by centrifugation. The nuclei were washed in buffer A without NP-40 and resuspended in 500 μl buffer C containing protease and phosphatase inhibitors as described (Gille *et al.*, 1992). The nuclei were lysed by passing the suspension rapidly through a 0.45 mm hypodermic needle. The nuclei were extracted by incubation on ice and vigorous mixing. The nuclear lysate was cleared by centrifugation for 10 min at 4°C. The lysates were loaded onto a Mono Q FPLC column and eluted at a low flow rate of 0.2 ml/min with a shallow NaCl gradient (0–300 mM) exactly as described (Boulton *et al.*, 1991b).

Recombinant Elk-1 proteins were purified from *E.coli* SG13009 pRep4 (Gottesmann *et al.*, 1981). Briefly, 2 l of a culture in LB medium were induced with 1 mM IPTG for 4 h. The cells were washed in PBS and lysed in a French press. The lysate was clarified by centrifugation and the supernatant was applied to a 1 ml Ni^{2+}-agarose column. The column was washed with 30 ml 50 mM Na-phosphate, pH 8.0, 0.5 M NaCl, followed by 200 ml 10 mM imidazole, pH 7.0. The column was eluted with a linear 10–300 mM imidazole gradient (40 ml).

Elk-1 peptides were purified by two consecutive HPLC runs as described (Rowles *et al.*, 1991). The proteolytic digest was applied to a reverse-phase (RP) HPLC column (RP-300, Applied Biosystems), equilibrated in 0.1% TFA and eluted with a 0–70% acetonitrile gradient. Phosphopeptide-containing fractions were identified by Cerenkov counting and applied to the same column equilibrated in 0.1% ammonium acetate. The column was eluted as above and fractions were collected onto polyethylene discs for sequence analysis which was carried out on an Applied Biosystems Inc. sequencer (model 477A). Peptide B was purified from the RP-HPLC flow-through fraction by conventional chromatography on a DEAE column.

Acknowledgements

We thank A.Ullrich for providing NIH3T3HERc cells, R.Hipskind for the gift of vaccinia SRF, D.Ebert for provision of homogeneous ERK1 and H.Winkler, M.Primig and G.Ammerer for communicating data prior to publication. We thank Annette Lenzner, Karin Wilker and Clark Garcia for excellent technical assistance and K.H.Klempnauer and M.Reth for critical comments on the manuscript. We are indebted to Lore Lay for photographic work. This work was supported by the Max-Planck-Gesellschaft and by grants to P.E.S. from the Deutsche Forschungsgemeinschaft (Sh 29/1-2) and the Dr Mildred Scheel Stiftung für Krebsforschung (W 40/92/Sh 1). H.G. was partially supported by a DAAD (HSPII) exchange fellowship.

References

Alessandrini,A., Crews,C.M. and Erikson,R.L. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 8200–8204.
Anderson,D., Koch,C.A., Grey,L., Ellis,C., Moran,M.F. and Pawson,T. (1990) *Science*, **250**, 979–982.
Andersson,S., Davis,D.L., Dahlbäck,H., Jörnvall,H. and Russell,D.W. (1989) *J. Biol. Chem.*, **264**, 8222–8229.
Boulton,T.G., Gregory,J.S. and Cobb,M.H. (1991a) *Biochemistry*, **30**, 278–286.
Boulton,T.G. *et al.* (1991b) *Cell*, **65**, 663–675.
Boyle,W.J., van der Geer,P. and Hunter,T. (1991) *Methods Enzymol.*, **201**, 110–149.
Buday,L. and Downward,J. (1993) *Cell*, **73**, 611–620.
Chen,R.-H., Sarnecki,C. and Blenis,J. (1992) *Mol. Cell. Biol.*, **12**, 915–927.
Cheng,J.-T., Cobb,M.H. and Baer,R. (1993) *Mol. Cell. Biol.*, **13**, 801–808.
Dalton,S. and Treisman,R. (1992) *Cell*, **68**, 597–612.
Derijard,B., Hibi,M., Wu,I.-H., Barrett,T., Su,B., Deng,T., Karin,M. and Davis,R.J. (1994) *Cell*, **76**, 1025–1037.
Egan,S.E., Giddings,B.W., Brooks,M.W., Buday,L., Sizeland,A.M. and Weinberg,R.A. (1993) *Nature*, **363**, 45–51.
Fisch,T., Prywes,R. and Roeder,R.G. (1987) *Mol. Cell. Biol.*, **7**, 3490–3502.
Gille,H., Sharrocks,A.D. and Shaw,P.E. (1992) *Nature*, **358**, 414–417.
Gottesmann,S., Halpern,E. and Trisler,P. (1981) *J. Bacteriol.*, **148**, 265–273.
Graham,R. and Gilman,M. (1991) *Science*, **251**, 189–192.
Guan,K. and Dixon,J.E. (1991) *Anal. Biochem.*, **192**, 262–267.
Haycock,J.W., Ahn,N.G., Cobb,M.H. and Krebs,E.G. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 2365–2369.
Herrera,R.E., Shaw,P.E. and Nordheim,A. (1989) *Nature*, **340**, 68–70.
Hibi,M., Lin,A., Smeal,T., Minden,A. and Karin,M. (1993) *Genes Dev.*, **7**, 2135–2148.
Hipskind,R.A., Rao,V.N., Mueller,C.G.F., Reddy,E.S.P. and Nordheim,A. (1991) *Nature*, **354**, 531–534.
Janknecht,R. and Nordheim,A. (1992) *Nucleic Acids Res.*, **20**, 3317–3324.
Janknecht,R., Ernst,W.H., Pingoud,V. and Nordheim,A. (1993) *EMBO J.*, **12**, 5097–5104.
Kortenjann,M., Thomae,O. and Shaw,P.E. (1994) *Mol. Cell. Biol.*, **14**, 4815–4824.
Lenormand,P., Sardet,C., Pagès,G., L'Allemain,G., Brunet,A. and Pouysségur,J. (1993) *J. Cell Biol.*, **122**, 1079–1088.
Lowenstein,E.J, Daly,R.J., Batzer,A.G., Li,W., Margolis,B., Lammers,R., Ullrich,A. and Schlessinger,J. (1992) *Cell*, **70**, 431–442.
Malik,R.K., Roe,M.W. and Blackshear,P.J. (1991) *J. Biol. Chem.*, **266**, 8576–8582.
Marais,R., Wynne,J. and Treisman,R. (1993) *Cell*, **73**, 381–393.
McClain,D.A., Maegawa,H., Lee,J., Dull,T.J., Ulrich,A. and Olefsky,J.M. (1987) *J. Biol. Chem.*, **262**, 14663–14671.
Mueller,C.G.F. and Nordheim,A. (1991) *EMBO J.*, **10**, 4219–4229.
Nakielny,S., Cohen,P., Wu,J. and Sturgill,T.W. (1992) *EMBO J.*, **11**, 2123–2129.
Ostrowski,J., Sims,J.E., Sibley,C.H., Valentine,M.A., Dower,S.K., Meier,K.E. and Bomsztyk,K. (1991) *J. Biol. Chem.*, **266**, 12722–12733.
Pognonec,P., Boulukos,K.E. and Ghysdael,J. (1989) *Oncogene*, **4**, 691–697.
Rossomando,A., Wu,J., Weber,M.J. and Sturgill,T.W. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 5221–5225.
Rowles,J., Slaughter,C., Moomaw,C., Hsu,J. and Cobb,M.H. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9548–9552.
Rozakis-Adock,M. *et al.* (1992) *Nature*, **260**, 689–692.
Rozakis-Adock,M., Fernley,R., Wade,J., Pawson,T. and Bowtell,D. (1993) *Nature*, **363**, 83–85.
Sadowski,I. and Ptashne,M. (1989) *Nucleic Acids Res.*, **17**, 7539.
Schröter,H., Mueller,C.G.F., Meese,K. and Nordheim,A. (1990) *EMBO J.*, **9**, 1123–1130.
Shaw,P.E. (1992) *EMBO J.*, **11**, 3011–3019.
Shaw,P.E., Hipskind,R.A., Schröter,H. and Nordheim,A. (1989a) *Nucleic Acids and Molecular Biology*. Springer-Verlag, Berlin, Germany, pp. 120–132.
Shaw,P.E., Schröter,H. and Nordheim,A. (1989b) *Cell*, **56**, 563–572.
Treisman,R. (1985) *Cell*, **42**, 889–902.
Treisman,R. (1992) *Trends Biochem. Sci.*, **17**, 423–426.
Warne,P.H., Viciana,P.R. and Downward,J. (1993) *Nature*, **364**, 352–355.
Zinck,R., Hipskind,R.A., Pingoud,V. and Nordheim,A. (1993) *EMBO J.*, **12**, 2377–2387.

Received on May 16, 1994; revised on November 29, 1994