MOLECULAR AND CELLULAR BIOLOGY, July 1994, p. 4815–4824
0270-7306/94/$04.00+0

Vol. 14, No. 7

Inhibition of v-*raf*-Dependent c-*fos* Expression and Transformation by a Kinase-Defective Mutant of the Mitogen-Activated Protein Kinase Erk2

MONIKA KORTENJANN, OLIVER THOMAE, AND PETER E. SHAW*

Max-Planck-Institut für Immunbiologie, Spemann Laboratories, 79108 Freiburg, Germany

Received 27 December 1993/Returned for modification 7 February 1994/Accepted 1 April 1994

Receptor-bound growth factors elicit intracellular signals that lead to the phosphorylation and activation of numerous intracellular kinases and transcription factors with consequent changes in patterns of gene expression. Several oncogene products are able to mimic these signals, resulting in cell transformation and proliferation. For example, the introduction of oncogenic forms of Raf-1 kinase into fibroblasts induces transformation and leads to the constitutive expression of, among others, the c-*fos* proto-oncogene. Here it is shown that the elevation of c-*fos* promoter activity brought about by v-*raf* is mediated by TCF/Elk-1, which forms a ternary complex with SRF at the serum response element and is a substrate for mitogen-activating protein kinases in vitro. In NIH 3T3 fibroblasts, v-*raf* activates Erk2, and overexpression of an interfering mutant of Erk2 both blocks the ability of v-*raf* to activate the c-*fos* promoter and suppresses transformation. Mutation of individual mitogen-activating protein kinase phosphoacceptor sites in TCF/Elk-1 also compromises v-*raf*-activated expression of a Gal-Elk/Gal-chloramphenicol acetyltransferase reporter system. However, in at least one instance the introduction of glutamate, but not aspartate, at a phosphoacceptor site is compatible with activation. These results provide compelling evidence that phosphorylation of TCF/Elk-1 by Erk2 is a major link in the Raf-1 kinase-dependent signal transduction pathway that activates c-*fos* expression.

The introduction of transforming oncogenes into eukaryotic cells leads to a series of changes in the genetic program of the cells, which stems from alterations in gene expression and culminates in rapid and uncontrolled cell proliferation (5). One of the specific changes brought about by numerous oncogenes is the constitutive elevation of c-*fos* expression, which may represent a causal event in the transformation process.

The c-*fos* gene was first identified as its transforming viral counterpart in murine osteosarcoma viruses (for a review, see reference 10). Consistent with this finding, proto-oncogene c-*fos* is expressed in a restricted fashion in osteo- and chondroblastic tissue during ontogeny (15). Its overexpression in transgenic mice causes defects in bone development and tooth eruption (63), as does its loss in homozygous null mutant mice (32, 77). However, in many other cell types c-*fos* expression can be induced, rapidly and transiently, by a wide variety of growth factors and other exogenous stimuli (26, 37, 49, 52). The gene product is a nuclear phosphoprotein and constitutes a component of the transcriptional regulator protein AP-1 (11). Short-term expression of c-*fos* and concomitant changes in the activation potential of AP-1 have been proposed to trigger long-term changes in the expression of other genes (10).

The rapid and transient inducibility of expression is conferred upon the c-*fos* gene by a promoter sequence referred to as the serum response element (SRE) (for a review, see reference 75). It is bound by at least two transcription factors, SRF (55) and TCF/Elk-1 (13, 29), which form a ternary complex with the SRE (66, 69). TCF/Elk-1 is able to bind to the SRE only in the presence of SRF and makes both direct DNA contacts and specific interactions with SRF (51, 68, 69).

The receptors for epidermal growth factor and platelet-derived growth factor, which both activate c-*fos* expression (18, 76), are membrane proteins with associated tyrosine kinase activity. When bound by their cognate growth factors, the intracellular tyrosine kinase domains of the receptors are activated, resulting in receptor autophosphorylation and subsequent phosphorylation of other intracellular substrates (8, 57, 65). The autophosphorylated receptor tyrosine residues present specific binding sites for intracellular molecules harboring *src* homology 2 (SH2) domains, such as phospholipase Cγ (45, 47), GRB2 (42), and SHC (60), thereby recruiting them and proteins complexed with them to the inner surface of the cell membrane (57, 58). In this way, the Ras guanine nucleotide-releasing protein is brought within the vicinity of $p21^{ras}$ by the SH2-SH3 adaptor protein GRB2, thereby coupling receptor tyrosine kinases to Ras activation (7, 9, 40).

A further effect of growth factor stimulation is the successive phosphorylation and activation of multiple, cytoplasmic serine/threonine protein kinases. One such family of kinases involved in this phosphorylation cascade is that of the mitogen-activated protein (MAP) kinases or extracellular signal-regulated kinases (Erks) (6, 54, 59). They are activated by phosphorylation on both tyrosine and threonine residues by dual-specificity kinases, referred to as MAP/Erk kinases (MEKs) (2, 36, 46, 53). MEKs, in turn, appear to be activated by either of two kinases, Raf-1 kinase (14, 20, 30, 38), the cellular homolog of the transforming oncogene v-*raf*, or the recently isolated MEK kinase (39). Furthermore, Raf-1 kinase is known to be activated both by oncogenic forms of Ras (48, 72, 78, 80, 81) and by Ca^{2+}-dependent protein kinase C (34, 70). Thus, a conceptual link has been established for the transduction of signals from membrane-bound growth factor receptors to nuclear protein kinases.

The transcription factor TCF/Elk-1 has been implicated as a nuclear target of MAP kinases. Treatment of Swiss 3T3 cells with epidermal growth factor activates MAP kinases and induces c-*fos* expression. In contrast, insulin treatment acti-

* Corresponding author. Mailing address: Max-Planck-Institut für Immunbiologie, Spemann Laboratories, Stübeweg 51, 79108 Freiburg, Germany. Phone: 761 5108 378. Fax: 761 5108 221.

vates $p70^{s6k}$ kinase in these cells, but neither MAP kinases nor c-*fos* expression (21). Furthermore, TCF/Elk-1 has been shown to be an efficient MAP kinase substrate in vitro (21, 43).

This study examines the relationship among Raf-1 kinase, MAP kinases, and TCF/Elk-1 in the activation of the c-*fos* promoter. In NIH 3T3 cells, an oncogenic form of Raf-1 kinase activates and overexpression of an interfering mutant of Erk2 efficiently blocks both induction of the c-*fos* promoter and transformation by v-*raf*. In a similar way, mutation of discrete phosphoacceptor sites in TCF/Elk-1, which are phosphorylated in vivo upon growth factor stimulation and in vitro by MAP kinases, impairs the ability of oncogenic Raf-1 kinase to activate expression of our reporter system, although the introduction of a negatively charged glutamate residue can mitigate this effect. These results provide direct evidence that activation of Erk2 plays a role in cellular transformation by v-*raf*. Furthermore, the results imply that by phosphorylating TCF/Elk-1, Erk2 is intimately involved in the activation of c-*fos* expression by growth factors.

MATERIALS AND METHODS

Cell culture, CAT assays, and transformation assay. NIH 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Cells were transfected by the standard DNA-calcium phosphate coprecipitation procedure. Precipitates were left on the cells for 15 to 20 h. Afterwards, cells were washed with medium without serum and serum starved for another 30 h before extract preparation. Unless otherwise stated, cells were transfected with 4 μg of the *fos*-chloramphenicol acetyltransferase (CAT) reporters or 2 μg of the Gal-CAT reporter per 10-cm-diameter dish, along with 4 μg of the Raf expression vectors and 2 μg of the expression vectors for Erk2, Gal-Elk, and their derivatives.

For CAT assays, cells were lysed by sonication in 0.25 M Tris-HCl (pH 7.8). Transfection efficiency was monitored by measuring the β-galactosidase activity resulting from cotransfection of 1 μg of pCH110 (Pharmacia) per plate, and the quantity of extract for each point in the CAT assay was normalized according to the relative transfection efficiency. The relative CAT activities given represent the averages of four separate experiments and were quantified with the aid of a Fujix BAS 1000 bioimaging analyzer.

For the transformation assays, NIH 3T3 cells were seeded in 4-cm-diameter plates, and duplicates were transfected in parallel with either 1 or 2 μg of the plasmids pMNC and 3611MSV alone or in combination with 1 or 4 μg of pCMV5.Erk2K>R. Eighteen hours posttransfection, the cells were reseeded onto 10-cm-diameter plates, and after 2 weeks of subsequent growth, they were washed in phosphate-buffered saline (PBS) and stained with Giemsa's solution, and foci were scored.

Purification of His-tagged Erks from transfected cells. For the analysis of protein expression and MAP kinase activation, cells transfected with 4 μg of the expression vectors for Erk2 and v-*raf* per 10-cm-diameter dish were washed twice with PBS, collected in 1 ml of buffer A (5 mM benzamidine and 1 mM Na_3VO_4 in PBS), pelleted, and resuspended in a solution of 20 mM Tris-HCl (pH 8.0), 50 mM NaF, 40 mM $Na_4P_2O_7$, 5 mM $MgCl_2$, 100 μM Na_3VO_4, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 40 μg of leupeptin per ml, 3 mM phenylmethylsulfonyl fluoride (30), 5 mM benzamidine, and 1 μM okadaic acid. After incubation for 10 min at 4°C, the lysates were centrifuged at 15,000 × *g* for 5 min, and the supernatants were stored at −80°C. Extracts from several plates were pooled and incubated for 1 to 2 h with 200 μl of nickel-nitrilotriacetic acid-agarose pretreated with 10 mM imidazole in buffer A. The nickel-nitrilotriacetic acid-agarose was then transferred to a small column and washed with 6 ml of 20 mM imidazole (pH 7.0). His-tagged Erk2 was eluted with 400 μl of 250 mM imidazole (pH 7.0).

Antibodies and immunoblotting. After electrophoresis in 10% acrylamide-SDS gels, proteins were transferred to nitrocellulose that was subsequently blocked with 5% nonfat dry milk and 0.05% Tween 20 in Tris-buffered saline. Blots were probed with antibody 691, which detects Erk2; an anti-Raf-1 antibody, which detects a C-terminal domain common to v-*raf*; or anti-Gal4 monoclonal antibodies, which detect the DNA-binding domain of Gal4. For visualization of antibody binding, peroxidase-coupled secondary antibodies (Dianova) and the Amersham ECL detection system were used.

In-gel kinase assay. The in-gel kinase assays were performed as published elsewhere (24, 33), with myelin basic protein as the substrate. Equal amounts of His-tagged Erk2, eluted in 250 mM imidazole, were separated in SDS–10% polyacrylamide gels containing 0.5 mg of myelin basic protein per ml. After electrophoresis, SDS was removed by a washing with 20% isopropanol in 50 mM Tris-HCl (pH 8.0) and then 5 mM β-mercaptoethanol in 50 mM Tris-HCl (pH 8.0) at room temperature. The proteins were denatured in 6 M guanidinium-HCl for 1 h at room temperature and renaturated by being washed with seven or eight changes of a solution of 0.04% Tween 40, 5 mM β-mercaptoethanol, and 50 mM Tris-HCl (pH 8.0) overnight at 4°C. After equilibration in the assay buffer (40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 8.0], 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $MgCl_2$) for 30 min at room temperature, the gel was transferred into fresh assay buffer containing 40 to 50 μCi of [γ-^{32}P]ATP for 1 h. The reaction was stopped, and unincorporated radioactivity was removed by extensive washing in 1% sodium PP_i and 5% (wt/vol) trichloroacetic acid. The dried gel was exposed to X-ray film.

Plasmid constructs. pF711CAT contains the c-*fos* promoter from −711 to +42 fused to the CAT gene (73). PS4wt-CAT and el-CAT are derivatives of PS4wt and PS4EL, respectively (69), in which the simian virus 40 early gene was replaced by the CAT gene. The Gal-CAT reporter used (G_5 E1B CAT) contains five copies of a Gal4 binding site placed in front of the adenovirus E1B basal promoter (41).

The eukaryotic expression vectors encoding His-tagged rat Erk2 and Erk2K>R under the control of the cytomegalovirus promoter were cloned into pCMV5 (4) from their respective NpT7-5 clones (62).

The expression vectors for v-*raf* (3611MSV) (61), c-*raf* (pMNC c-raf), and the dominant negative form of c-*raf* (p301-1) and the empty expression vector (pMNC) have been described (35).

BlueScript-Elk-1 contains the coding sequences of Elk-1, and QE-ElkC contains Elk-1 sequences encoding amino acids 213 to 428 with a C-terminal histidine tag, which were amplified by PCR from a clone of the Elk-1 cDNA, provided by V. N. Rao. pSG Gal-Elk was constructed by fusing Gal4 amino acids 1 to 147, present in pSG424 (64), to Elk-1 amino acids 83 to 428 by ligating an *Acc*I (blunt)-*Xba*I fragment from BS-Elk-1 into the *Sma*I-*Xba*I sites of pSG424. The histidine tag was added by replacing the C terminus with that of QE-ElkC as an *Xma*I-*Hin*dIII fragment.

pSG Gal-ElkΔA was produced by deleting the *Apa*I fragment from pSG Gal-Elk, which removes Elk-1 amino acids 254 to 424.

The Gal-Elk mutants 324-422A, 383D, and 383E were produced as follows: PCR-based, site-directed mutagenesis

was used as described elsewhere (67) to introduce the mutations into pQE-ElkC; they were subsequently transferred as *Apa*I fragments into pSG Gal-ElkΔA. The following mutagenic primers were used: 324A, 5′-GCGCCCCGTGCCCC GGCCAAGC; 336A, 5′-GAACGGGCCCCAGGATCGGG; 353A, 5′-CTGGCCCCATCCCTGCTTCC; 363A, 5′-CATAC ATTGGCCCCGGTGC; 368A, 5′-CTGGCACCCAGCTCG CTGCC; 383A, 5′-CTGGCTCCCATTGCGCCCCG; 389A, 5′-GAGCTTCCACTCGCCCCGAGCCTGC; 417A, 5′-CTC TCGGCCCCCGTGGTGC; 422A, 5′-GTGCTCGCCCCA GGGCCCCAG; and 383D/E, 5′-CTGGAt/aCCCATTGCG CCCCG.

All plasmids were constructed with standard recombinant DNA procedures. Sequences derived by PCR amplification were subsequently verified by dideoxy sequencing. Further details of the plasmids, if required, may be obtained upon request.

RESULTS

TCF/Elk-1 mediates transactivation of c-*fos* expression by v-*raf*. Elevation of c-*fos* expression by activated mutants of Raf-1 kinase such as v-*raf* has been described previously (31, 50). The effects of various forms of Raf-1 kinase on expression from a CAT reporter driven by the human c-*fos* promoter (*fos*-CAT) are shown in Fig. 1. In serum-starved cells, no effect on CAT expression is observed upon coexpression of either Raf-1 kinase (c-*raf*) or the kinase-defective mutant 301-1 with the reporter plasmid, whereas coexpression of v-*raf* causes a 14-fold increase in CAT activity (Fig. 1, lanes 3 to 5). An analogous reporter plasmid, from which sequences between −358 and −259 are deleted, responds only weakly to expression of v-*raf* (result not shown). This mutation deletes the v-*sis*-inducible element (SIE) (27), the SRE, and the c-*fos* AP-1 (FAP) site (19) from the promoter. Replacement of the SRE and FAP site without the SIE (PS4wt-CAT) rescues activation by v-*raf* (Fig. 1, lane 8).

The SRE is essential for the transcriptional response of the c-*fos* promoter to a wide variety of mitogens (18, 22, 25, 74). It is bound by two transcription factors, SRF and TCF/Elk-1 (12, 13, 29, 55). The interaction of TCF/Elk-1 with the SRE in vitro can be abolished specifically by mutations at the 5′ end of the SRE without affecting SRF binding. Such a mutation (el) was previously shown to reduce the serum inducibility of the c-*fos* promoter in vivo to about 20% of that of the wild type (69). This result implicated TCF/Elk-1 in the c-*fos* serum response. When a c-*fos* reporter plasmid harboring this mutation (el-CAT) was transfected in place of PS4wt-CAT, expression of v-*raf* elicited a weak stimulation of CAT activity reminiscent of the degree of residual serum inducibility previously observed with this specific mutation (Fig. 1, lane 13). These results demonstrate that activation of the c-*fos* promoter by v-*raf* and, by implication, other activated mutants of Raf-1 kinase involves primarily TCF/Elk-1 bound at the SRE.

Activation of Erk2 by v-*raf*. TCF/Elk-1 has been proposed to be a nuclear target for MAP kinases (21, 43). Furthermore, MAP kinases have been shown to be activated by MEKs (1, 3, 36, 46, 53), which can be directly phosphorylated and activated by Raf-1 kinase (14, 20, 30, 38). As a consequence, MAP kinases would be expected to be active in v-*raf*-transfected cells. To verify this, NIH 3T3 cells were transfected with an expression vector for Erk2 bearing an amino-terminal histidine tag, and either v-*raf* or the empty expression vector (pMNC). After serum starvation for 24 h, extracts were prepared from the cells and expression of v-*raf* was ascertained by immunoblotting with a polyclonal antibody (Fig. 2). Exogenous Erk2 was isolated from the extracts by affinity purification with

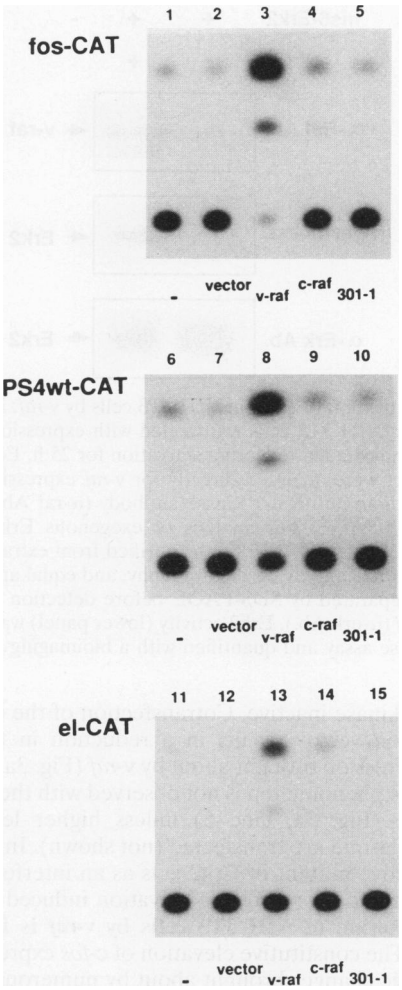

FIG. 1. v-*raf* induced c-*fos* expression is mediated by TCF/Elk-1. NIH 3T3 cells were transfected with equal amounts of the reporter constructs *fos*-CAT (lanes 1 to 5), PS4wt-CAT (lanes 6 to 10), or el-CAT (lanes 11 to 15) and equal amounts of the empty expression vector pMNC (lanes 2, 7, and 12) or vectors encoding v-*raf* (3611MSV [lanes 3, 8, and 13]), c-*raf* (lanes 4, 9, and 14), or the dominant-negative mutant 301-1 (lanes 5, 10, and 15). Cells were harvested for CAT analysis after serum starvation for 30 h.

nickel-agarose, and its expression was also confirmed by immunoblotting. The activity of the purified Erk2 was assayed by an in-gel kinase assay. The exogenous Erk2 was activated over sixfold in cells expressing v-*raf*. Thus, in NIH 3T3 cells, Erk2 appears to be stimulated by the expression of v-*raf*.

A kinase-defective mutant of Erk2 interferes with activation of c-*fos* by v-*raf*. The above evidence implicates Erk2 in the Raf-1 kinase-dependent signalling pathway that induces c-*fos* expression. Erk2 is activated by Raf-1 kinase via an intermediate kinase, referred to as MEK (14, 20, 30, 38). However, although TCF/Elk-1 is efficiently phosphorylated by Erk2 in vitro (21, 43), which results in stimulation of ternary complex formation with SRF and the SRE (21), direct evidence for its function in vivo is lacking. As a more direct test for the involvement of Erk2 in the mechanism of transactivation by v-*raf*, the effect of a kinase-defective, interfering mutant of Erk2 on the v-*raf*-dependent activation of the *fos*-CAT reporter was analyzed.

Erk2K>R contains a point mutation at lysine 52 (62), which

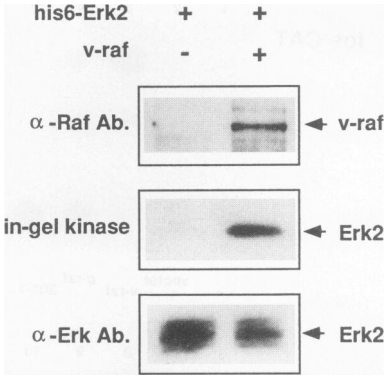

FIG. 2. Activation of Erk2 in NIH 3T3 cells by v-*raf*. Extracts were prepared from NIH 3T3 cells transfected with expression vectors for the proteins indicated, after serum starvation for 25 h. Equal amounts of each extract were analyzed directly for v-*raf* expression by immunoblotting with an anti-Raf-1 kinase antibody (α-raf Ab.) after separation by SDS-PAGE. For analysis of exogenous Erk2 expression (middle panel), His-tagged Erk2 was purified from extracts by nickel-nitrilotriacetic acid affinity chromatography, and equal amounts of the eluate were separated by SDS-PAGE before detection with an anti-Erk2 antibody (α-erk Ab.). Erk2 activity (lower panel) was detected by an in-gel kinase assay and quantified with a bioimaging analyzer.

renders the kinase inactive. Cotransfection of the corresponding expression vector results in a reduction in the level of *fos*-CAT expression brought about by v-*raf* (Fig. 3a, lane 4, and Fig. 3b). This phenomenon is not observed with the unmutated Erk2 kinase (Fig. 3a, lane 5) unless higher levels of the expression plasmid are transfected (not shown). In summary, a kinase-defective mutant of Erk2 acts as an interfering mutant to suppress the c-*fos* promoter activation induced by v-*raf*.

Transformation of NIH 3T3 cells by v-*raf* is inhibited by Erk2K>R. The constitutive elevation of c-*fos* expression is one of the specific changes brought about by numerous oncogenes and, as such, may represent a contributory event in the transformation process. Thus, it was also of interest to test whether the interfering mutant of Erk2 has an inhibitory effect on transformation by v-*raf*.

A convenient method for measuring transformation efficiency is a focus-formation assay. Accordingly, NIH 3T3 cells were transfected with the v-*raf* expression vector 3611MSV alone or together with the expression vector for Erk2K>R. After replating and subsequent growth for 2 weeks, the numbers of emergent foci were scored. As expected, NIH 3T3 cells transfected with 3611MSV gave rise to numerous foci with a characteristic morphology. Cotransfection of the expression vector for Erk2K>R reduced the number of foci by over 50% (Table 1). Thus, in NIH 3T3 cells the interfering mutant of Erk2 also suppresses transformation by v-*raf*.

These results are consistent with the notion that v-*raf* activates Erk2 in NIH 3T3 cells and that this event is critical both for transformation of these cells and induction of c-*fos* expression by v-*raf*.

Gal4-Elk fusion proteins mediate activation of a Gal4 reporter gene by v-*raf*. The above results implicate phosphorylation of TCF/Elk-1 by Erk2 in the v-*raf*-dependent activation of c-*fos* expression. To explore this possibility further, a derivative of TCF/Elk-1 in which the *ets* box DNA-binding domain was replaced by that of the yeast transcription factor Gal4 (64) was constructed (Fig. 4a). The resultant hybrid protein, referred to as Gal-Elk, when expressed by cell-free transcription and translation, bound with high affinity to a canonical Gal4 DNA-binding site (results not shown).

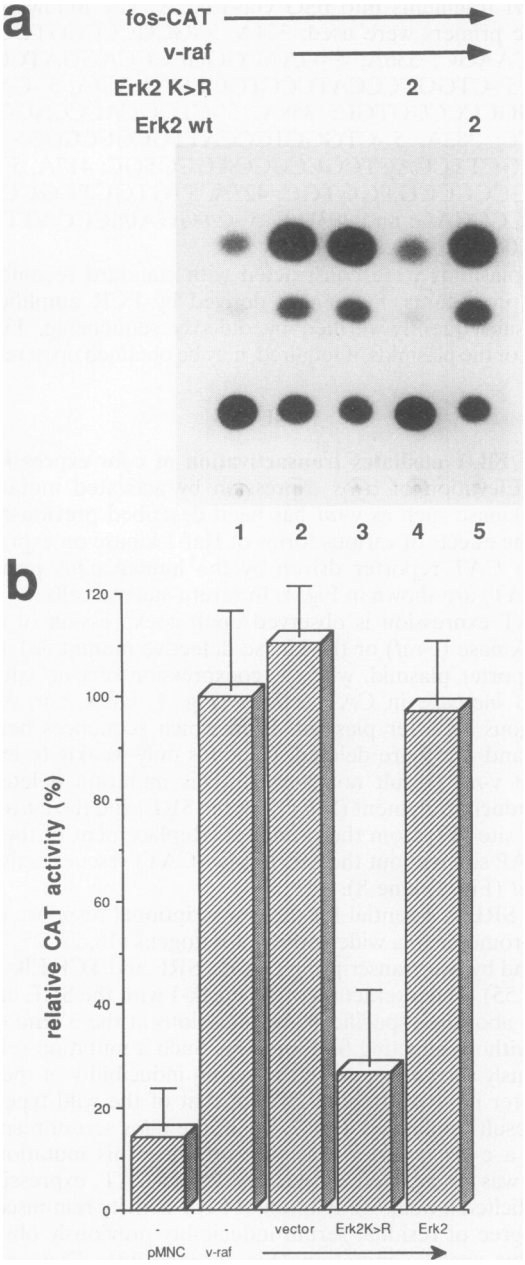

FIG. 3. Interference with v-*raf* activation of c-*fos* expression by a kinase-defective mutant of Erk2. (a) NIH 3T3 cells were transfected with the *fos*-CAT reporter plasmid and either the empty expression vector for v-*raf* alone (lane 1), the v-*raf* expression vector alone (lane 2), the v-*raf* expression vector with the empty expression vector for Erk2 (lane 3), the v-*raf* expression vector and 2 μg of the Erk2K>R expression vector (lanes 4), or the v-*raf* expression vector and 2 μg of the Erk2 expression vector (lanes 5). (b) Quantification of the results shown in panel a. Each point represents the mean of four independent experiments.

Transfection of the expression vector for v-*raf* along with a Gal-CAT reporter did not lead to an increase in CAT expression in serum-starved cells, in contrast to its effect on a *fos*-CAT reporter. However, cotransfection of an expression vector for Gal-Elk led to a dramatic activation of the Gal-CAT

TABLE 1. Suppression of v-*raf*-induced focus formation by an interfering mutant of Erk2

NIH 3T3 cells transfected with:	No. of expts	Mean no. of foci		% v-*raf* foci
		Per plate	Per μg of v-*raf*	
pUC8/pMNC	4	0	0	0
v-*raf*	4	43.7	22.9	100
Erk2wt	1	0	0	0
Erk2K>R	4	0	0	0
v-*raf* + Erk2wt	2	43	23.6	91.3
v-*raf* + Erk2K>R	4	19.3	10.1	46.6

reporter gene (Fig. 4b, lane 3). This effect was dependent on the integrity of the TCF/Elk-1 moiety of Gal-Elk, as neither an internal deletion mutant of Gal-Elk lacking the MAP kinase phosphorylation sites, Gal-ElkΔA, nor the DNA-binding and dimerization domain of Gal4 alone permitted activation of the Gal-CAT reporter by v-*raf* (Fig. 4b, lanes 4 and 5). Thus, the region of TCF/Elk-1 that harbors phosphorylation sites for MAP kinases is indispensable for activation by v-*raf*.

If activation of the Gal-CAT reporter by v-*raf* is mediated in the same way as activation of the *fos*-CAT reporter, it would be expected to be inhibited likewise by the interfering mutant of Erk2. Indeed, as in the case of the *fos*-CAT reporter, the Erk2K>R mutant reduces expression from the Gal-CAT reporter to uninduced levels (Fig. 4c, lane 4, and Fig. 4d).

Abrogation of v-*raf* activation by mutation of individual MAP kinase phosphorylation sites in TCF/Elk-1. The sites at which TCF/Elk-1 is phosphorylated upon growth factor stimulation in vivo and by MAP kinases in vitro have been mapped (43). In order to probe the relevance of phosphorylation of these sites for the v-*raf*-dependent activation of c-*fos* expression, individual serine and threonine phosphoacceptor sites in TCF/Elk-1 were changed to alanine residues by site-directed

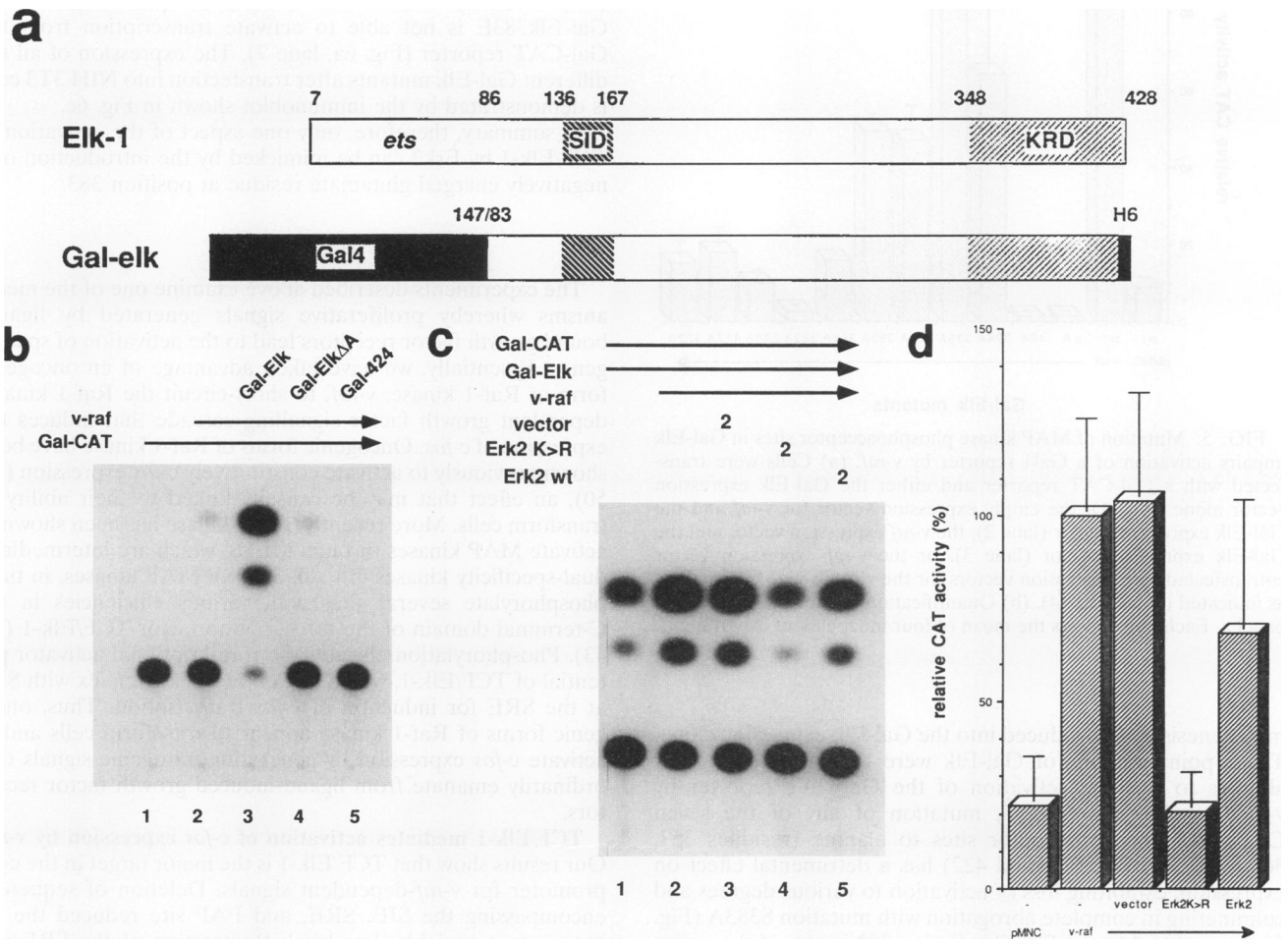

FIG. 4. Gal-Elk fusion proteins mediate activation of a Gal4 reporter by v-*raf*. (a) Diagrammatic representation of Elk-1 and the Gal-Elk fusion protein used. The three stippled boxes in Elk-1 indicate the *ets* box DNA-binding domain (*ets*), the SRF interaction domain (SRF), and the region of homology to the related proteins SAP-1 and SAP-2 (SAP). Amino acid residues are indicated above the diagrams; H6 refers to the C-terminal histidine tag in Gal-Elk. (b) Cells were transfected with a Gal-CAT reporter and either the empty expression vector for v-*raf* (lane 1) or the v-*raf* expression vector alone (lane 2) or cotransfected with expression vectors for Gal-Elk (lane 3), Gal-ElkΔA (lane 4), or pSG424, which encodes Gal4 amino acids 1 to 147 (lane 5). (c) NIH 3T3 cells were transfected with the Gal-CAT reporter plasmid and the Gal-Elk expression vector and the empty expression vector for v-*raf* alone (lane 1), the v-*raf* expression vector alone (lane 2), the v-*raf* expression vector with the empty expression vector for Erk2 (lane 3), the v-*raf* expression vector and 2 μg of the Erk2K>R expression vector (lane 4), or the v-*raf* expression vector and 2 μg of the Erk2 expression vector (lane 5). (d) Quantification of the results shown in panel c. Each point represents the mean of two independent experiments.

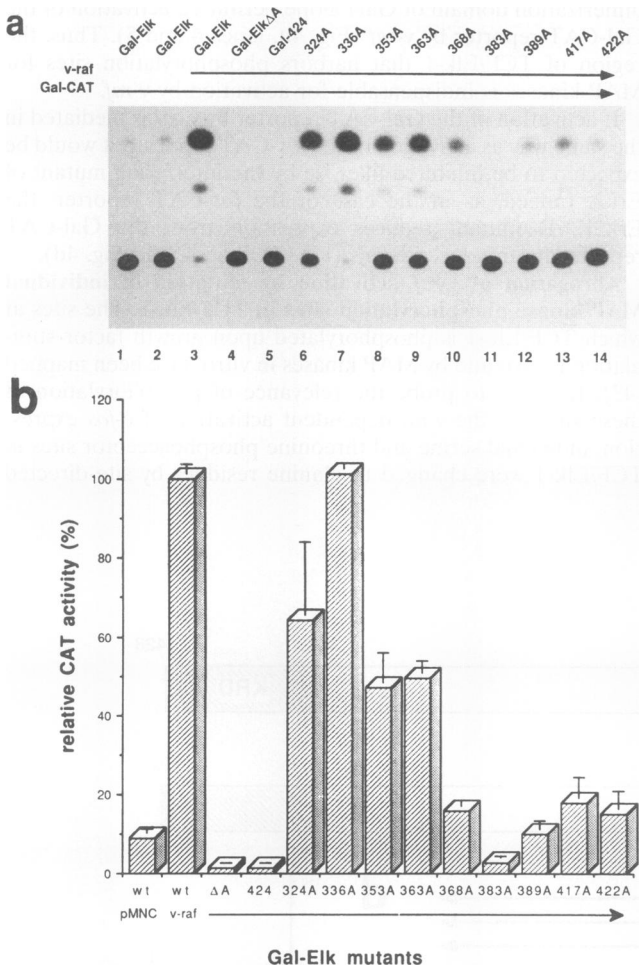

FIG. 5. Mutation of MAP kinase phosphoacceptor sites in Gal-Elk impairs activation of a Gal4 reporter by v-*raf*. (a) Cells were transfected with a Gal-CAT reporter and either the Gal-Elk expression vector alone (lane 1), the empty expression vector for v-*raf* and the Gal-Elk expression vector (lane 2), the v-*raf* expression vector and the Gal-Elk expression vector (lane 3), or the v-*raf* expression vector cotransfected with expression vectors for the various Gal-Elk mutants as indicated (lanes 4 to 14). (b) Quantification of the results shown in panel a. Each point shows the mean of four independent experiments.

mutagenesis and introduced into the Gal-Elk expression clone. These point mutants of Gal-Elk were then tested for their abilities to mediate activation of the Gal-CAT reporter by v-*raf*. As shown in Fig. 5, mutation of any of the seven C-terminal phosphoacceptor sites to alanine (residues 353, 363, 368, 383, 389, 417, and 422) has a detrimental effect on expression, exhibiting loss of activation to various degrees and culminating in complete abrogation with mutation S383A (Fig. 5a, lanes 8 to 14, and Fig. 5b). Serine 383 is one of the major sites of TCF/Elk-1 phosphorylation by MAP kinases in vitro and is phosphorylated in vivo upon growth factor stimulation (reference 43 and our unpublished results).

The dual mutation of sites 417 and 422 also ablates the activation by v-*raf* (not shown), whereas the individual mutations have less effect. In addition, the dual mutation of sites 383 and 389 abolishes detectable CAT activity (not shown). Thus, in NIH 3T3 cells, the v-*raf*-dependent activation of c-*fos* expression mediated by the SRE appears to require phosphorylation of TCF/Elk-1 at multiple C-terminal MAP kinase phosphorylation sites.

Glutamate can functionally substitute for phosphoserine at residue 383 in Gal-Elk. Negatively charged residues can replace serine and threonine in some proteins without loss of function (16, 17, 44, 56). It has been proposed that phosphorylation of TCF/Elk-1 enhances its transcriptional activator potential by inducing a conformational change in the protein (43). Furthermore, phosphorylation of TCF/Elk-1 is reported to stimulate ternary complex formation with SRF at the SRE, which also implies a regulated conformational change (21). To test the ability of a negatively charged residue to substitute for a phosphoacceptor site in TCF/Elk-1, serine 383 in TCF/Elk-1 was changed to either aspartate or glutamate by site-directed mutagenesis and introduced into the Gal-Elk expression gene. As shown in Fig. 6, Gal-Elk383D is not activated by coexpression of v-*raf*, its activity being comparable to that of Gal-Elk in the absence of v-*raf* (Fig. 6a, compare lanes 1 and 4, and Fig. 6b). In contrast, Gal-Elk383E supports a level of v-*raf*-induced CAT expression nearly 70% that of Gal-Elk (Fig. 6a, compare lanes 2 and 5). In the absence of v-*raf* expression, however, Gal-Elk383E is not able to activate transcription from the Gal-CAT reporter (Fig. 6a, lane 7). The expression of all the different Gal-Elk mutants after transfection into NIH 3T3 cells is demonstrated by the immunoblot shown in Fig. 6c.

In summary, therefore, only one aspect of the activation of TCF/Elk-1 by Erk2 can be mimicked by the introduction of a negatively charged glutamate residue at position 383.

DISCUSSION

The experiments described above examine one of the mechanisms whereby proliferative signals generated by ligand-bound growth factor receptors lead to the activation of specific genes. Essentially, we have taken advantage of an oncogenic form of Raf-1 kinase, v-*raf*, to short-circuit the Raf-1 kinase-dependent growth factor signalling cascade that induces the expression of c-*fos*. Oncogenic forms of Raf-1 kinase have been shown previously to activate constitutively c-*fos* expression (31, 50), an effect that may be causally linked to their ability to transform cells. More recently, Raf-1 kinase has been shown to activate MAP kinases through MEKs, which are intermediate, dual-specificity kinases (14, 20, 30, 38). MAP kinases, in turn, phosphorylate several sites with various efficiencies in the C-terminal domain of the transcription factor TCF/Elk-1 (21, 43). Phosphorylation elevates the transcriptional activator potential of TCF/Elk-1, which is required in a complex with SRF at the SRE for induction of c-*fos* transcription. Thus, oncogenic forms of Raf-1 kinase appear to transform cells and to activate c-*fos* expression by generating mitogenic signals that ordinarily emanate from ligand-induced growth factor receptors.

TCF/Elk-1 mediates activation of c-*fos* expression by v-*raf*. Our results show that TCF/Elk-1 is the major target in the c-*fos* promoter for v-*raf*-dependent signals. Deletion of sequences encompassing the SIE, SRE, and FAP site reduced the response to a variable, low level. Reinsertion of the SRE and FAP site rescued the response, ruling out a role for the SIE. That TCF/Elk-1 is the active component was demonstrated by the specific mutation of its recognition sequence in the SRE, which severely impaired activation. A direct comparison shows that the v-*raf*-dependent expression by el-CAT is less than 20% that of *fos*-CAT. However, weak elevation of c-*fos* promoter activity due to v-*raf* was consistently observed with el-CAT, and we are unable to distinguish between the possibilities that TCF/Elk-1 still interacts weakly with the mutated SRE in

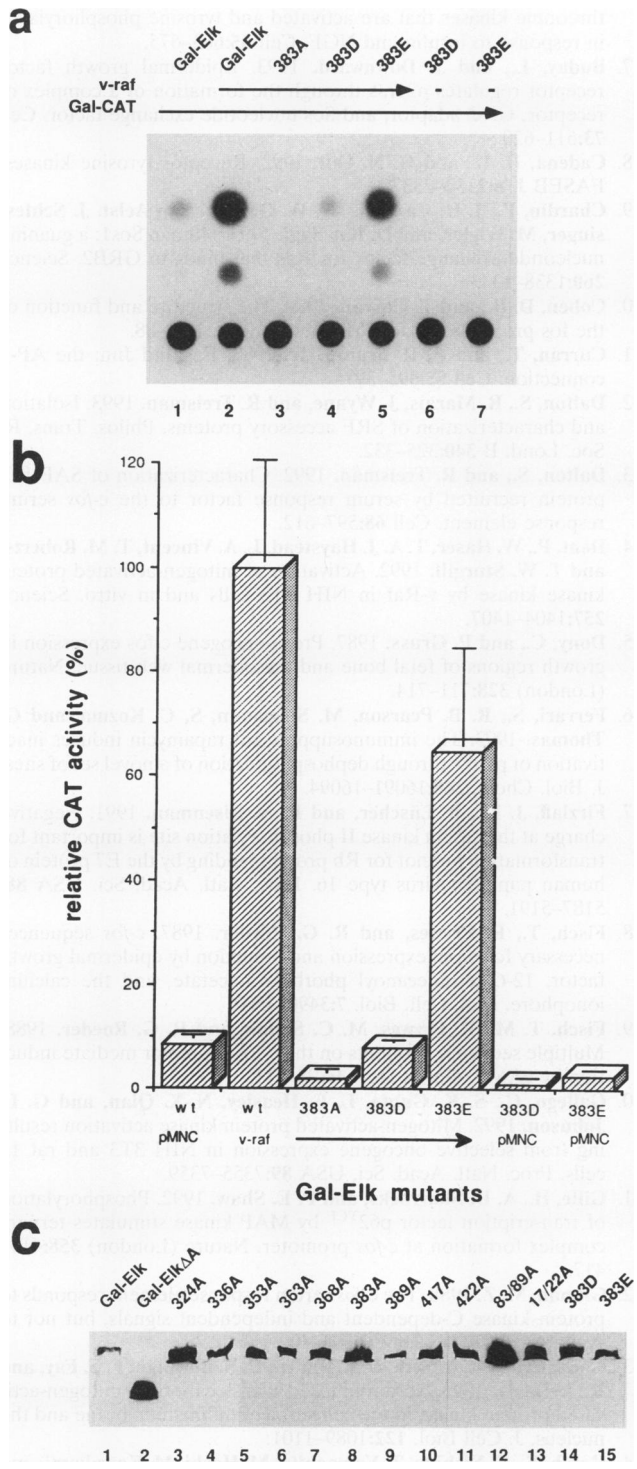

FIG. 6. Substitution of phosphoserine at residue 383 in Gal-Elk with negatively charged residues. (a) Cells were transfected with a Gal-CAT reporter along with either the empty expression vector for v-*raf* and the Gal-Elk expression vector (lane 1), the v-*raf* expression vector and the Gal-Elk expression vector (lane 2), the v-*raf* expression vector cotransfected with expression vectors for Gal-Elk mutants as indicated (lanes 3 to 5), or expression vectors for the Gal-Elk mutants alone as indicated. (b) Quantification of the results shown in panel a. Each point shows the mean of three independent experiments. (c) Immunoblot of extracts from NIH 3T3 cells transfected with the Gal-Elk expression vector as indicated. The blot was probed with a monoclonal antibody against the DNA-binding domain of Gal4.

el-CAT or that another element in the c-*fos* promoter responds, albeit poorly, to the v-*raf*-dependent signal.

Activation of Erk2 by Raf-1 kinase-dependent signals. Our results directly show that in NIH 3T3 cells, Raf-1 kinase-dependent mitogenic signals activate Erk2. In transient-transfection experiments in which v-*raf* and a histidine-tagged form of Erk2 were overexpressed, Erk2 could be recovered from the cells in an active form. Overexpression of v-*raf* along with a kinase-defective mutant of Erk2 in NIH 3T3 cells shows the same impairing effect on expression from the *fos*-CAT reporter and the Gal-Elk/Gal-CAT reporter system. These results support the conclusion that Raf-1 kinase activates a form of MEK that in turn activates Erk2. As both the MEK phosphorylation sites in Erk2K>R are still intact, the mutant could compete with wild-type Erk2 for phosphorylation by MEK. However, such kinase-negative Erks are seen to translocate to the nucleus (23). Thus, Erk2K>R could also compete with the active Erk2 for its nuclear substrates. We have no way of distinguishing between these mechanisms at present.

We also observe that transformation of NIH 3T3 cells by v-*raf* is suppressed by the interfering mutant of Erk2. Taken together, these results indicate that both the induction of c-*fos* expression in NIH 3T3 cells and their transformation by v-*raf* are mediated by Erk2 and imply that the mitogenic signals transmitted by Raf-1 kinase activate Erk2.

Phosphorylation of TCF/Elk-1 by Erk2 activates c-*fos* expression. As a consequence of exchanging the DNA-binding domain of TCF/Elk-1 for that of the yeast transcription factor Gal4, it was possible to use a Gal4 reporter construct to analyze the effect on v-*raf*-activated expression of mutations at each of the sites in the C-terminal domain of TCF/Elk-1 that are phosphorylated by MAP kinases (43). Mutation of a single serine residue at position 383 to alanine ablated the ability of Gal-Elk to mediate the v-*raf*-dependent activation of transcription. Serine 383 is one of the major sites in Elk-1 phosphorylated by MAP kinases in vitro and upon growth factor stimulation of NIH 3T3 cells in vivo (43). Other individual mutations at neighboring phosphorylation sites also impair v-*raf* activation, though none so severely as 383A. Indeed, removal of any of the seven C-terminal phosphorylation sites reduces activation by v-*raf* by over 50%. A double mutation (417A/422A) also ablated activation by v-*raf*, indicating that a cumulative, negative effect is achieved by removing phosphoacceptor sites from Elk-1.

A detrimental effect upon the serum inducibility of reporter genes mediated by the C-terminal domain of Elk-1 fused to the DNA-binding domains of both Gal4 and LexA has previously been ascribed to mutations at sites phosphorylated in vivo in serum-stimulated NIH 3T3 cells and by MAP kinases in vitro (28, 43). Our observations concur with these results and extend them by demonstrating the specific involvement of both Erk2 and Raf-1 kinase in the signal transduction mechanism impinging on TCF/Elk-1. However, as serum contains a complex mixture of growth factors, it cannot be ruled out that additional signalling mechanisms amplify the serum-derived signal that reaches TCF/Elk-1.

It has been proposed that phosphorylation of Elk-1 by MAP kinases may induce conformational changes in the C-terminal domain of the protein by the disruption of so-called ASX turns (71, 79), thus allowing its activation domain to become functional (43). We now show that the introduction of a glutamate residue in place of serine at position 383 allows Gal-Elk to respond to activation by v-*raf*, whereas the introduction of a similarly charged aspartate residue at the same position, which differs from glutamate in that its side chain lacks a methylene group, has no such mitigating effect. Thus, there is not simply

a requirement for a negatively charged residue, and the phosphate group at serine 383 in Elk-1 may indeed be influencing the protein structure, an effect that the smaller aspartate cannot emulate. However, the pleiotropic effect of introducing negative charge per se into the C-terminal domain of Elk-1 may play an important role in the activation mechanism. Certainly Gal-Elk383E requires phosphorylation at other sites to become active, because in the absence of activation by v-*raf* there is no expression from the Gal-CAT reporter. It may therefore be more appropriate to test the effect of negative charge with mutations at other sites in the protein.

Constitutive c-*fos* expression as a corollary of v-*raf* transformation. We have shown that v-*raf* activates Erk2 in NIH 3T3 cells and that both cell transformation and the induction of the c-*fos* promoter ensue. Thus, the correlation between transformation by v-*raf* and the activation of c-*fos* expression is extended to include the activation of Erk2 in both instances. However, these results do not go so far as to demonstrate a causal role for the constitutive expression of c-*fos* in the transformation of NIH 3T3 cells by v-*raf*. The definitive experiment will be to test the ability of v-*raf* to transform 3T3 cells that lack the c-*fos* gene (32, 77).

In conclusion, we have shown that the constitutive activation of c-*fos* expression by an oncogenic form of Raf-1 kinase introduced into NIH 3T3 cells involves the activation of Erk2. TCF/Elk-1 is phosphorylated at multiple C-terminal MAP kinase phosphorylation sites, which results in activation of its transcriptional potential. Activation can be blocked by overexpression of an interfering mutant of Erk2. It is also impaired by the site-specific mutation of MAP kinase phosphoacceptor sites in TCF/Elk-1, but the introduction of a negatively charged glutamate residue can alleviate this defect. We are presently engaged in trying to understand how the phosphorylation of TCF/Elk-1 leads to the activation of transcription from the c-*fos* promoter.

ACKNOWLEDGMENTS

We thank Annette Lenzner and Karin Wilker for excellent technical assistance, W. Kölch for providing the Raf-1 kinase constructs and the anti-Raf-1 kinase antibody, M. Cobb for providing vectors for Erk2 and Erk2k>R and an antibody against MAP kinases, M. Ptashne for pSG424 and pG5E1BCAT, and P. Chambon for monoclonal antibodies against Gal4. We are grateful to E. Ruscher for the photographic work and to H. Gille and M. Reth for comments on the manuscript.

This work was supported by a grant from the Dr. Mildred Scheel Stiftung für Krebsforschung (W 40/92/Sh 1) and by the Max-Planck-Gesellschaft.

REFERENCES

1. **Adams, P. D., and P. J. Parker.** 1992. Activation of mitogen-activated protein (MAP) kinase by a MAP kinase-kinase. J. Biol. Chem. **267:**13135–13137.
2. **Ahn, N. G., R. Seger, and E. G. Krebs.** 1992. The mitogen-activated protein kinase activator. Curr. Opin. Cell Biol. **4:**992–999.
3. **Alessandrini, A., C. M. Crews, and R. L. Erikson.** 1992. Phorbol ester stimulates a protein-tyrosine/threonine kinase that phosphorylates and activates the *Erk-1* gene product. Proc. Natl. Acad. Sci. USA **89:**8200–8204.
4. **Andersson, S., D. L. Davis, H. Dahlbäck, H. Jörnvall, and D. W. Russell.** 1989. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydoxylase, a bile acid biosynthetic enzyme. J. Biol. Chem. **264:**8222–8229.
5. **Bishop, J. M.** 1991. Molecular themes in oncogenesis. Cell **64:**235–248.
6. **Boulton, T. G., S. H. Nye, D. J. Robbins, N. Y. Ip, E. Radziejewska, S. D. Morgenbesser, R. A. DePinho, N. Panayotatos, M. H. Cobb, and G. D. Yancopoulos.** 1991. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell **65:**663–675.
7. **Buday, L., and J. Downward.** 1993. Epidermal growth factor receptor regulates p21ras through the formation of a complex of receptor, Grb2 adaptor, and Sos nucleotide exchange factor. Cell **73:**611–620.
8. **Cadena, D. L., and G. N. Gill.** 1992. Receptor tyrosine kinases. FASEB J. **6:**2332–2337.
9. **Chardin, P., J. H. Camonis, N. W. Gale, L. Van Aelst, J. Schlessinger, M. Wigler, and D. Bar-Sagi.** 1993. Human Sos1: a guanine nucleotide exchange factor for Ras that binds to GRB2. Science **260:**1338–1343.
10. **Cohen, D. R., and T. Curran.** 1989. The structure and function of the fos proto-oncogene. Crit. Rev. Oncog. **1:**65–88.
11. **Curran, T., and B. R. Franza, Jr.** 1988. Fos and Jun: the AP-1 connection. Cell **55:**395–397.
12. **Dalton, S., R. Marais, J. Wynne, and R. Treisman.** 1993. Isolation and characterization of SRF accessory proteins. Philos. Trans. R. Soc. Lond. B **340:**325–332.
13. **Dalton, S., and R. Treisman.** 1992. Characterization of SAP-1, a protein recruited by serum response factor to the c-*fos* serum response element. Cell **68:**597–612.
14. **Dent, P., W. Haser, T. A. J. Haystead, L. A. Vincent, T. M. Roberts, and T. W. Sturgill.** 1992. Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. Science **257:**1404–1407.
15. **Dony, C., and P. Gruss.** 1987. Proto-oncogene c-fos expression in growth regions of fetal bone and mesodermal web tissue. Nature (London) **328:**711–714.
16. **Ferrari, S., R. B. Pearson, M. Siegmann, S. C. Kozma, and G. Thomas.** 1993. The immunosuppressant rapamycin induces inactivation of $p70^{s6k}$ through dephosphorylation of a novel set of sites. J. Biol. Chem. **268:**16091–16094.
17. **Firzlaff, J. M., B. Lüscher, and R. N. Eisenman.** 1991. Negative charge at the casein kinase II phosphorylation site is important for transformation but not for Rb protein binding by the E7 protein of human papillomavirus type 16. Proc. Natl. Acad. Sci. USA **88:**5187–5191.
18. **Fisch, T., R. Prywes, and R. G. Roeder.** 1987. c-*fos* sequences necessary for basal expression and induction by epidermal growth factor, 12-*O*-tetradecanoyl phorbol-13-acetate, and the calcium ionophore. Mol. Cell. Biol. **7:**3490–3502.
19. **Fisch, T. M., R. Prywes, M. C. Simon, and R. G. Roeder.** 1988. Multiple sequence elements on the c-fos promoter mediate induction by cAMP. Genes Dev. **3:**198–211.
20. **Gallego, C., S. K. Gupta, L. E. Heasley, N.-X. Qian, and G. L. Johnson.** 1992. Mitogen-activated protein kinase activation resulting from selective oncogene expression in NIH 3T3 and rat 1a cells. Proc. Natl. Acad. Sci. USA **89:**7355–7359.
21. **Gille, H., A. D. Sharrocks, and P. E. Shaw.** 1992. Phosphorylation of transcription factor $p62^{TCF}$ by MAP kinase stimulates ternary complex formation at c-*fos* promoter. Nature (London) **358:**414–417.
22. **Gilman, M. Z.** 1988. The c-*fos* serum response element responds to protein kinase C-dependent and independent signals, but not to cyclic AMP. Genes Dev. **2:**394–402.
23. **Gonzalez, F. A., A. Seth, D. L. Raden, D. S. Bowman, F. S. Fay, and R. J. Davis.** 1993. Serum-induced translocation of mitogen-activated protein kinase to the cell surface ruffling membrane and the nucleus. J. Cell Biol. **122:**1089–1101.
24. **Gotoh, Y., E. Nishida, T. Yamashita, M. Hoshi, M. Kawakami, and H. Sakai.** 1990. Microtubule-associated-protein (MAP) kinase activated by nerve growth factor and epidermal growth factor in PC12 cells. Eur. J. Biochem. **193:**661–669.
25. **Greenberg, M. E., Z. Siegfried, and E. B. Ziff.** 1987. Mutation of the c-*fos* dyad symmetry element inhibits inducibility in vivo and the nuclear regulatory factor binding in vitro. Mol. Cell. Biol. **7:**1217–1225.
26. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. Nature (London) **311:**433–438.
27. **Hayes, T. E., A. M. Kitchen, and B. H. Cochran.** 1987. Inducible binding of a factor to the c-fos regulatory region. Proc. Natl. Acad.

Sci. USA **84**:1272–1276.
28. **Hill, C. S., R. Marais, S. John, J. Wynne, S. Dalton, and R. Treisman.** 1993. Functional analysis of a growth factor-responsive transcription factor complex. Cell **73**:395–406.
29. **Hipskind, R. A., V. N. Rao, C. G. F. Mueller, E. S. P. Reddy, and A. Nordheim.** 1991. Ets-related protein Elk-1 is homologous to the c-*fos* regulatory factor $p62^{TCF}$. Nature (London) **354**:531–534.
30. **Howe, L. R., S. J. Leevers, N. Gómez, S. Nakielny, P. Cohen, and C. J. Marshall.** 1992. Activation of the MAP kinase pathway by the protein kinase raf. Cell **71**:335–342.
31. **Jamal, S., and E. B. Ziff.** 1990. Transactivation of c-*fos* and beta-actin genes by *raf* as a step in early response to transmembrane signals. Nature (London) **344**:463–466.
32. **Johnson, R. S., B. M. Spiegelman, and V. Papaioannou.** 1992. Pleiotropic effects of a null mutation in the c-*fos* proto-oncogene. Cell **71**:577–586.
33. **Kameshita, I., and H. Fujisawa.** 1989. A sensitive method for detection of calmodulin-dependent protein kinase II activity in sodium dodecyl sulphate-polyacrylamide gel. Anal. Biochem. **183**:139–143.
34. **Kölch, W., G. Heidecker, G. Kochs, R. Hummel, H. Vahidi, H. Mischak, G. Finkenzeller, D. Marmé, and U. R. Rapp.** 1993. Protein kinase Ca activates RAF-1 by direct phosphorylation. Nature (London) **364**:249–252.
35. **Kölch, W., G. Heidecker, P. Lloyd, and U. R. Rapp.** 1991. Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature (London) **349**:426–428.
36. **Kosako, H., Y. Gotoh, S. Matsuda, M. Ishikawa, and E. Nishida.** 1992. *Xenopus* MAP kinase activator is a serine/threonine/tyrosine kinase activated by threonine phosphorylation. EMBO J. **11**:2903–2908.
37. **Kruijer, W., J. A. Cooper, T. Hunter, and I. Verma.** 1984. Platelet-derived growth factor induces rapid but transient expression of the c-*fos* gene and protein. Nature (London) **312**:711–716.
38. **Kyriakis, J. M., H. App, X. Zhang, P. Banerjee, D. L. Brautigan, U. R. Rapp, and J. Avruch.** 1992. Raf-1 activates MAP kinase-kinase. Nature (London) **358**:417–421.
39. **Lange-Carter, C. A., C. M. Pleiman, A. M. Gardner, K. J. Blumer, and G. L. Johnson.** 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science **260**:315–319.
40. **Li, N., A. Batzer, R. Daly, V. Yajnik, E. Skolnik, P. Chardin, D. Bar-Sagi, B. Margolis, and J. Schlessinger.** 1993. Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature (London) **363**:85–88.
41. **Lillie, J. W., and M. R. Green.** 1989. Transcription activation by the adenovirus E1a protein. Nature (London) **338**:39–44.
42. **Lowenstein, E. J., R. J. Daly, A. G. Batzer, W. Li, B. Margolis, R. Lammers, A. Ullrich, E. Y. Skolnik, D. Bar-Sagi, and J. Schlessinger.** 1992. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to *ras* signaling. Cell **70**:431–442.
43. **Marais, R., J. Wynne, and R. Treisman.** 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell **73**:381–393.
44. **Marcus, F., J. Rittenhouse, L. Moberly, I. Edelstein, E. Hiller, and D. T. Rogers.** 1988. Yeast (*Saccharomyces cerevisiae*) fructose-1,6-bisphosphatase: properties of phospho and dephospho forms and of two mutants in which serine 11 has been changed by site-directed mutagenesis. J. Biol. Chem. **263**:6058–6062.
45. **Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger.** 1989. EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signalling. Cell **57**:1101–1107.
46. **Matsuda, S., H. Kosako, K. Takenaka, K. Moriyama, H. Sakai, T. Akiyama, Y. Gotoh, and E. Nishida.** 1992. *Xenopus* MAP kinase activator: identification and function as a key intermediate in the phosphorylation cascade. EMBO J. **11**:973–982.
47. **Meisenhelder, J., P. G. Suh, S. G. Rhee, and T. Hunter.** 1989. Phospholipase C-g is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vitro and in vivo. Cell **57**:1109–1122.
48. **Moodie, S. A., B. M. Willumsen, M. J. Weber, and A. Wolfman.** 1993. Complexes of Ras · GTP with Raf-1 and mitogen-activated protein kinase kinase. Science **260**:1658–1661.
49. **Morgan, J. I., and T. Curran.** 1986. Role of ion flux in the control of c-fos expression. Nature (London) **322**:552–555.
50. **Morrison, D. K., D. R. Kaplan, J. A. Escobedo, U. R. Rapp, T. M. Roberts, and L. T. Williams.** 1989. Direct activation of the serine/threonine kinase activity of Raf-1 through tyrosine phosphorylation by the PDGF b-receptor. Cell **58**:649–657.
51. **Mueller, C. G. F., and A. Nordheim.** 1991. A protein domain conserved between yeast MCM1 and human SRF directs ternary complex formation. EMBO J. **10**:4219–4229.
52. **Müller, R., R. Bravo, J. Burckhardt, and T. Curran.** 1984. Induction of *c-fos* gene and protein by growth factors precedes activation of c-myc. Nature (London) **312**:716–720.
53. **Nakielny, S., P. Cohen, J. Wu, and T. W. Sturgill.** 1992. MAP kinase activator from insulin-stimulated skeletal muscle is a protein threonine/tyrosine kinase. EMBO J. **11**:2123–2129.
54. **Nishida, E., and Y. Gotoh.** 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. Trends Biochem. Sci. **18**:128–131.
55. **Norman, C., M. Runswick, R. Pollock, and R. Treisman.** 1988. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds the c-fos serum response element. Cell **55**:989–1003.
56. **Ofir, R., V. Dwarki, D. Rashid, and I. M. Verma.** 1990. Phosphorylation of the C terminus of Fos protein is required for transcriptional transrepression of the *c-fos* promoter. Nature (London) **348**:80–82.
57. **Panayotou, G., and M. D. Waterfield.** 1993. The assembly of signalling complexes by receptor tyrosine kinases. Bioessays **15**: 171–177.
58. **Pawson, T., and J. Schlessinger.** 1993. SH2 and SH3 domains. Curr. Biol. **3**:434–442.
59. **Pelech, S. L., and J. S. Sanghera.** 1992. Mitogen-activated protein kinases: versatile transducers for cell signalling. Trends Biochem. **17**:233–238.
60. **Pelicci, G., L. Lanfrancone, F. Grignani, J. McGlade, F. Cavallo, G. Forni, I. Nicoletti, F. Grignani, T. Pawson, and P. G. Pelicci.** 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. Cell **70**:93–104.
61. **Rapp, U. R., M. D. Goldsborough, G. E. Mark, T. I. Bonner, J. Groffen, F. H. Reynolds, Jr., and J. R. Stephenson.** 1983. Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. Proc. Natl. Acad. Sci. USA **80**:4218–4222.
62. **Robbins, D. J., E. Zhen, H. Owaki, C. A. Vanderbilt, D. Ebert, T. D. Geppert, and M. H. Cobb.** 1993. Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 *in vitro*. J. Biol. Chem. **268**:5097–5106.
63. **Rüther, U., C. Garber, D. Komitowski, R. Müller, and E. F. Wagner.** 1987. Deregulated c-fos expression interferes with the normal bone development in transgenic mice. Nature (London) **325**:412–416.
64. **Sadowski, I., and M. Ptashne.** 1989. A vector for expressing Gal4 (1-147) fusions in mammalian cells. Nucleic Acids Res. **17**:7539.
65. **Schlessinger, J., and A. Ullrich.** 1992. Growth factor signaling by receptor tyrosine kinases. Neuron **9**:383–391.
66. **Schröter, H., C. G. F. Mueller, K. Meese, and A. Nordheim.** 1990. Synergism in ternary complex formation between the dimeric glycoprotein $p67^{SRF}$, polypeptide $p62^{TCF}$ and the c-fos serum response element. EMBO J. **9**:1123–1130.
67. **Sharrocks, A. D., and P. E. Shaw.** 1992. Improved primer design for PCR-based, site-directed mutagenesis. Nucleic Acids Res. **20**:1147.
68. **Shaw, P. E.** 1992. Ternary complex formation over the c-*fos* serum response element: $p62^{TCF}$ exhibits dual component specificity with contacts to DNA and an extended structure in the DNA-binding domain of $p67^{SRF}$. EMBO J. **11**:3011–3019.
69. **Shaw, P. E., H. Schröter, and A. Nordheim.** 1989. The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter. Cell **56**:563–572.
70. **Sözeri, O., K. Vollmer, M. Liyanage, D. Frith, G. Kour, G. E. Mark III, and S. Stabel.** 1992. Activation of the c-Raf protein kinase by protein kinase C phosphorylation. Oncogene **7**:2259–2262.
71. **Suzuki, M.** 1989. SPXX, a frequent sequence motif noted in gene regulatory proteins. J. Mol. Biol. **207**:61–84.
72. **Thomas, S. M., M. DeMarco, G. D'Arcangelo, S. Halegoua, and J. S. Brugge.** 1992. Ras is essential for nerve growth factor- and

phorbol ester-induced tyrosine phosphorylation of MAP kinases. Cell **68:**1031–1040.

73. **Treisman, R.** 1985. Transient accumulation of *c-fos* RNA following serum stimulation requires a conserved 5′ element and *c-fos* 3′ sequences. Cell **42:**889–902.
74. **Treisman, R.** 1986. Identification of a protein-binding site that mediates transcriptional response of the *c-fos* gene to serum factors. Cell **48:**567–574.
75. **Treisman, R.** 1990. The SRE: a growth factor responsive transcriptional regulator. Cancer Biol. **1:**47–58.
76. **Wagner, B. J., T. E. Hayes, C. J. Hoban, and B. H. Cochran.** 1990. The SIF binding element confers sis/PDGF inducibility onto the c-fos promoter. EMBO J. **9:**4477–4484.
77. **Wang, Z.-Q., C. Ovitt, A. E. Grigoriadis, U. Möhle-Steinlein, U. Rüther, and E. F. Wagner.** 1992. Bone and haematopoietic defects in mice lacking c-*fos*. Nature (London) **360:**741–745.
78. **Warne, P. H., P. R. Viciana, and J. Downward.** 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 *in vitro*. Nature (London) **364:**352–355.
79. **Wilmot, C. M., and J. M. Thornton.** 1988. Analysis and prediction of the different types of beta-turn in proteins. J. Mol. Biol. **203:**221–232.
80. **Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis.** 1992. *ras* mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell **68:**1041–1050.
81. **Zhang, X., J. Settleman, J. M. Kyriakis, E. Takeuchi-Suzuki, S. J. Elledge, M. S. Marshall, J. T. Bruder, U. R. Rapp, and J. Avruch.** 1993. Normal and oncogenic $p21^{ras}$ proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature (London) **364:**308–313.