Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3844–3848, April 1994
Cell Biology

A requirement for extracellular signal-regulated kinase (ERK) function in the activation of AP-1 by Ha-Ras, phorbol 12-myristate 13-acetate, and serum

JEFFREY A. FROST*, THOMAS D. GEPPERT†, MELANIE H. COBB‡, AND JAMES R. FERAMISCO*

*Departments of Medicine and Pharmacology, Cancer Center, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0636; and †Department of Internal Medicine and Harold Simmons Arthritis Research Center, and ‡Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75235-9041

Communicated by Edwin G. Krebs, November 1, 1993

ABSTRACT **The role of ERK-1 and ERK-2 in wild-type (wt) Ha-Ras, phorbol 12-myristate 13-acetate (PMA), and serum-induced AP-1 activity was studied. Microinjection of ERK-specific substrate peptides inhibited the induction of AP-1 activity by all three stimuli, whereas a control peptide had no effect. By using eukaryotic expression constructs encoding wt ERK-1 and kinase-deficient mutants of ERKs 1 and 2, it was found that ERK-1 and ERK-2 activities are required for AP-1 activation stimulated by either wt Ha-Ras, PMA, or serum. Overexpression of ERK-1 augmented wt Ha-Ras stimulation of AP-1, while having no effect upon PMA or serum stimulation. Overexpression of either kinase-deficient ERK-1 or kinase-deficient ERK-2 partially inhibited AP-1 activation by wt Ha-Ras but had no effect on PMA or serum-induced activation. Coexpression of both interfering mutants abolished AP-1 induction by wt Ha-Ras, PMA, or serum. We conclude that ERKs are necessary components in the pathway leading to the activation of AP-1 stimulated by these agents.**

The stimulation of quiescent cells through the expression of wild-type (wt) or activated mutants of the *ras* gene, as well as through exposure to purified growth factors or the tumor promoter phorbol 12-myristate 13-acetate (PMA), results in altered gene transcription (1, 2). Recently, serine/threonine protein kinases have been discovered that could serve, at least in part, as mediators of this information transfer. These kinases, termed *m*itogen-*a*ctivated *p*rotein kinases (MAP kinases) or *e*xtracellular signal-*r*egulated *k*inases (ERKs) are activated in response to Ras expression or exposure to serum growth factors and tumor promoters (for reviews, see refs. 3–5). *In vitro* ERKs have been shown to phosphorylate proteins likely to be involved in Ras-stimulated gene regulation, including c-Jun, c-Myc, and $p62^{TCF}$ (6–9). In the case of c-Myc and $p62^{TCF}$, mutagenesis of the sites phosphorylated by ERKs *in vitro* inhibits their ability to transactivate their respective target genes (10–14). For c-Jun the situation is not as clear because ERKs can phosphorylate both activating and inhibitory sites *in vitro* (6, 7, 9). Still, the phosphorylation of these factors is evidently an important mode of transcriptional control. Whether the ERKs are required for the cellular mediation of Ras-dependent and -independent transcriptional activation has not been determined.

Therefore, we have used short-term (2–4 hr) Ras-responsive transcription assays in living fibroblasts (15, 16). By microinjecting either peptide inhibitors of ERKs or expression constructs encoding various forms of ERK-1 and -2 together with an AP-1-regulated transcriptional reporter construct (TRE/lacZ), we have shown that both ERK-1 and ERK-2 participate in a signaling pathway initiated by wt Ha-Ras, PMA, or serum and leading to the activation of AP-1 transcriptional activity.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Cell Culture. REF-52 cells were cultured as described (17). For microinjection assays the cells were plated on acid-washed glass coverslips (Fisher) in Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum. At ≈30% confluency the cells were placed in starvation medium (DMEM/0.05% fetal bovine serum) for ≈48 hr before injection, with one change of medium ≈24 hr before injection. At injection the cells were typically ≈60% confluent.

Construction of ERK-1, K71R ERK-1, and K52R ERK-2 Eukaryotic Expression Vectors. wt ERK-1 cDNA was tagged with sequences encoding the hemagglutinin peptide (YPYDVPDYASL) as well as 5′ *Xba* I and *Mlu* I sites and 3′ *Not* I and *Bam*HI sites using PCR. *Xba* I and *Bam*HI-digested product was ligated into pBluescript (Stratagene) cut with *Xba* I and *Bam*HI. ERK-1 cDNA was then excised by *Not* I digestion and ligated into *Not* I-digested pCEP4 (Invitrogen). The entire insert was sequenced. pCEP4/K71R ERK-1 was made by digesting pT7.5/K71R ERK-1 with *Bal* I and *Bam*HI and ligating the ≈1-kb fragment into pCEP4/wt ERK1 cut with *Bal* I and *Bam*HI. pCEP4/K52R ERK-2 was made by excising the K52R ERK-2 cDNA from pT7.5/K52R ERK-2 with *Xba* I followed by an *Eco*RI partial digest. The ≈1.2-kb cDNA was then ligated into pBluescript digested with *Eco*RI and *Xba* I. K52R ERK-2 cDNA was then excised by *Kpn* I and *Not* I digestion and ligated into pCEP4 cut with *Kpn* I and *Not* I.

Peptides. The following peptides were used: J14 was derived from human tyrosine hydroxylase (EIAMSPRFK) (18); S49 was derived from human c-MYC (KKFELLPTPPLSPSRR) (6); D peptide corresponded to an hsc70 low-affinity ligand (ENQFGDCHY) (19). All peptides were synthesized on an Applied Biosystems 431A peptide synthesizer by using fluorenylmethoxycarbonyl chemistry. J14 and S49 peptides were purified by reverse-phase HPLC using a C_{18} column. D peptide was purified on a G10 gel-filtration column.

Peptide Competition of c-Jun Phosphorylation by Purified ERK-1. Various concentrations of J14, S49, or D peptides were used to compete with purified, recombinant human c-Jun protein (20) for phosphorylation by active ERK-1 (21) as described (16).

Microinjection and Analysis of TRE/LacZ or CRE/LacZ Expression. For peptide studies, starved cells were injected with 2 mM J14, S49, or D peptide along with either TRE/lacZ or CRE/lacZ plasmid at 0.1 mg/ml (16) and rat IgG (Sigma) at 5 or 10 mg/ml. For wt Ha-Ras stimulation, bacterially expressed, recombinant wt Ha-Ras protein was coinjected at 0.5 mg/ml (22). For stimulation by PMA (200 ng/ml) (Sigma)

Abbreviations: ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; wt, wild type; βGal, β-galactosidase; MEK, MAP kinase/ERK kinase; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

or serum (20% fetal bovine serum/DMEM), cells were injected as described and immediately stimulated. Two hours after stimulation the cells were fixed for 5 min at room temperature in 3.7% formaldehyde (vol/vol)/phosphate-buffered saline (PBS). They were stained for β-galactosidase (βgal) with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (Boehringer Mannheim), as described (6). Injected cells were visualized by indirect immunofluorescence using a rhodamine-conjugated donkey anti-rat antibody (The Jackson Laboratory), as described (6).

For studies involving eukaryotic expression vectors, starved cells were coinjected with either pCEP4, pCEP4/ERK1, pCEP4/K71R ERK-1 or pCEP4/K52R ERK-2 at 30 μg/ml and either TRE/lacZ or CRE/lacZ at 0.1 mg/ml, and rat IgG at 5 mg/ml. When K71R ERK-1 and K52R ERK-2 were coexpressed, each plasmid was used at 10–20 μg/ml. For wt Ha-Ras-stimulation, cells were coinjected with pHras at 100 μg/ml. Three hours later they were formaldehyde fixed, stained overnight for βGal with X-Gal, and then stained for injected cells by using the rhodamine donkey anti-rat antibody. For PMA or serum stimulation the cells were injected with the reporter plasmid, rat IgG, and the appropriate expression vector(s). Two hours later they were stimulated for 2 hr with PMA (200 ng/ml) or serum (DMEM/20% fetal bovine serum). After 2 hr the cells were formaldehyde fixed, stained overnight with X-Gal, and then stained for rat IgG by using the rhodamine donkey anti-rat antibody.

RESULTS

ERK-Specific Substrate Peptides Block AP-1 Activation *in Vivo*. REF-52 cells (a continuous line of rat embryo fibroblasts) contain both ERK-1 (43 kDa) and ERK-2 (42 kDa), as judged by immunoblotting of whole-cell extracts using an ERK-specific antibody (data not shown) (21). Peptide substrates or pseudosubstrates have been shown to selectively inhibit protein kinases *in vivo* (16, 23). We used this approach to study the role of ERK-1 and ERK-2 in AP-1-regulated gene expression. Three peptides were synthesized. Two corresponded to sequences surrounding ERK phosphorylation sites known to occur *in vivo*—one within human tyrosine hydroxylase and the other within human c-MYC (J14 and S49, respectively) (6–9, 18). The third peptide was a non-phosphorylatable control peptide (D peptide) (16). To demonstrate that these peptides could competitively inhibit the phosphorylation of a protein substrate, they were added to *in vitro* kinase reactions using bacterially expressed, recombinant c-Jun protein as a substrate and ERK-1 purified from insulin-stimulated rat 1 HIRc B cells. The concentrations of the J14 and S49 peptides that inhibited phosphorylation 50% were 0.8 mM and 1.1 mM, respectively. The D peptide did not inhibit the kinase reaction at any concentration tested (up to 1 mM), nor was it a substrate for ERK-1. Both the J14 and S49 peptides were phosphorylated by ERK-1. As anticipated, neither peptide was a substrate for cAMP-dependent protein kinase A, Ca^{2+}/calmodulin-dependent kinase II, or casein kinase II, further suggesting a specificity for ERKs (data not shown). Thus, the J14 and S49 peptides may serve as competitive inhibitors of ERKs in living cells. Because of the overlapping substrate specificities of ERK-1 and -2 and the likelihood that there are other protein kinases closely related to them (24–26), the J14 and S49 peptides may be recognized by several other members of the ERK family.

To test whether the ERK-specific peptide substrates could inhibit the activation of AP-1 by wt Ha-Ras protein, they were individually coinjected into quiescent REF-52 cells along with an AP-1 reporter construct (TRE/lacZ), purified wt Ha-Ras protein, and an IgG protein used as a marker for injected cells. The expression of βGal from the TRE/lacZ reporter depends upon the presence of the upstream TRE enhancer elements because the reporter vector lacking these elements does not produce βGal in response to any stimuli (27). Two hours after injection the cells were fixed, and the expression of βGal was assessed by staining with the chromogenic substrate X-Gal; this results in a dark-blue staining of the cells expressing βGal. Injected cells were identified by indirect immunofluorescent staining for the coinjected IgG. Thirty-three percent of the cells injected with either wt Ha-Ras protein alone or wt Ha-Ras with control peptide D expressed βGal (Fig. 1*c*). However, coinjection of the S49 peptide reduced wt Ha-Ras-stimulated reporter expression to 11% of the injected cells (Fig. 1*a*), which was equal to background expression in cells coinjected with the reporter construct and IgG alone (10%) (Fig. 2). Similarly, coinjection of the J14 peptide also inhibited wt Ha-Ras-stimulated TRE/lacZ expression, reducing the percentage of injected cells expressing βGal to 6% (Fig. 2). The peptides were injected at a concentration of 2 mM in the needle, which was determined empirically to be the lowest concentration of injected peptide that would cause a maximal, reproducible inhibition of AP-1 activation. Injection of similar concentrations of peptides inhibitory to casein kinase II but not ERKs has been shown to result in the apparent inhibition of cellular casein kinase II activity (16).

The inhibition of wt Ha-Ras-stimulated AP-1 activity by injection of the synthetic ERK peptide substrates suggested that the activation of AP-1 by wt Ha-Ras required ERK

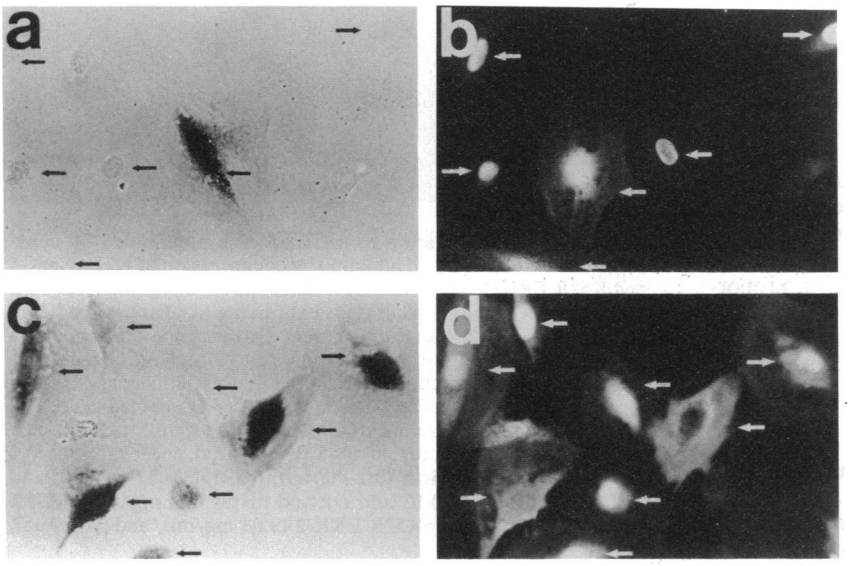

FIG. 1. Activation of AP-1 by injection of wt Ha-Ras protein is inhibited by coinjection of the S49 ERK-1 substrate peptide, but not by the control peptide, D. (*a* and *c*) Phase-contrast micrographs of cells stained for the presence of βGal with X-Gal. Cells expressing βGal are stained dark blue. (*b* and *d*) Indirect immunofluorescence staining for a coinjected rat IgG used as a marker for injected cells. Note that intense βGal staining sometimes blocks the fluorescent signal expected for the coinjected marker IgG. Arrows denote cells injected as follows: purified wt Ha-ras protein (0.5 mg/ml), S49 peptide (2 mM), rat IgG (5 mg/ml), and TRE/lacZ (0.1 mg/ml) (*a* and *b*); purified wt Ha-Ras protein (0.5 mg/ml), D peptide (2 mM), rat IgG (5 mg/ml), and TRE/lacZ (0.1 mg/ml) (*c* and *d*). Cells were photographed as described elsewhere (17).

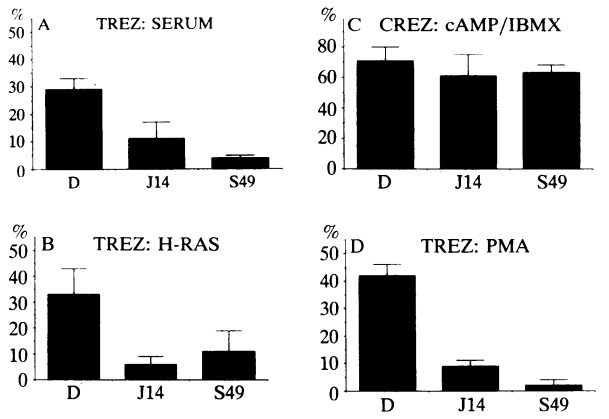

FIG. 2. Microinjection of the J14 and S49 ERK-1 substrate peptides, but not the control peptide D, will block AP-1 activation by wt Ha-Ras (*B*), PMA (*D*), and serum (*A*). Cells were injected with J14, S49, or D peptide (2 mM), rat IgG (5 mg/ml), and either the TRE/lacZ (TREZ, *A* and *B*) or CRE/lacZ (CREZ, *C*) reporter plasmids (0.1 mg/ml). For wt Ha-Ras stimulation of TRE/lacZ, purified, recombinant, bacterially expressed wt Ha-Ras protein (0.5 mg/ml) was coinjected. For PMA stimulation of TRE/lacZ, quiescent cells were injected and then immediately stimulated with 0.25 mM 8-Br-cAMP and isobutylmethylxanthine each (*C*). The numbers on the *y* axis refer to the percentage of cells staining positive for βGal compared with the total number of cells injected. An average of 300 cells was analyzed for each bar. Values represent the average of at least three experiments; error bars represent the SEM.

activity. To test whether a similar requirement existed for other activators of AP-1, we examined the effect of the ERK substrate peptides on induction of the TRE/lacZ reporter by the tumor promoter PMA and serum. Accordingly, quiescent cells were injected with peptide and TRE/lacZ plasmid and then immediately stimulated with either PMA or serum. Reporter gene expression was then determined 2 hr later. None of the peptides altered background reporter expression in the absence of PMA or serum. When the D peptide was injected, addition of PMA or serum to the cells increased reporter expression to 42% or 29% of the injected cells, respectively (Fig. 2). This was equal to the TRE/lacZ induction by either agent in the absence of injected D peptide. On the other hand, both the J14 and S49 peptides significantly inhibited TRE/lacZ induction by either PMA (reduced 9% and 2%) or serum (reduced to 11% and 4%) (Fig. 2). Thus, ERK peptide substrates inhibited the induction of AP-1 activity by wt Ha-Ras protein, PMA, and serum. These data suggest that ERK activity was necessary for the stimulation of AP-1 activity by these factors.

To determine whether the peptides influenced transcription from an unrelated reporter construct, their effects on transcription from a reporter containing cAMP-regulated enhancer elements (CRE/lacZ) were examined. This reporter construct is stimulated by intracellular cAMP, but not by serum or wt Ha-Ras (unpublished results; refs. 15 and 27). Quiescent cells were coinjected with the CRE/lacZ reporter construct and each of the peptides and then stimulated with 0.25 mM 8-Br-cAMP and isobutylmethylxanthine. These two agents cause a rapid increase in intracellular cAMP and a proportionate increase in expression of the CRE/lacZ reporter (15, 27). None of the coinjected peptides altered cAMP-stimulated reporter expression, suggesting that the J14 and S49 peptides were not simply inhibiting protein kinase-dependent signaling pathways nonselectively (Fig. 2).

Wild-Type ERK-1 Potentiates and Kinase-Deficient Forms of ERK-1 and ERK-2 Inhibit wt Ha-Ras-Stimulated AP-1 Activity. To further define the role of ERKs in AP-1 activation, we overexpressed either wt ERK-1 or kinase-deficient forms of both ERK-1 and ERK-2 (K71R ERK-1 and K52R ERK-2, respectively). This was accomplished by microinjecting into the nuclei of quiescent REF-52 cells eukaryotic expression constructs encoding either wt ERK-1, K71R ERK-1, or K52R ERK-2 driven by a cytomegalovirus promoter. Previous work has shown that expression from a cytomegalovirus promoter expression construct injected into the nucleus of a fibroblast is detectable 30 to 60 min after injection and can be detected for up to 2 days after injection (17). In this case, the overproduction of either wt or mutant ERK-1 protein was detected by indirect immunofluorescent staining and was seen in ≈70% of the injected cells within 2 hr (data not shown). Overexpression of K52R ERK-2 could also be detected, although the staining was less intense. When the presence of ERK-1 or ERK-2 was assayed by indirect immunofluorescent staining at later times, the percentage of cells staining positive for ERKs did not decrease. Therefore, ERKs were expressed for the duration of the transcription assays reported here (4 hr).

In control experiments, βGal was detected in 10–13% of quiescent REF-52 cells injected with the TRE/lacZ reporter construct alone (data not shown). This response, which represented background activity of the reporter in quiescent cells, was equivalent to that from cells coinjected with reporter and the parental pCEP4 vector lacking ERK cDNAs, and to that from cells coinjected with reporter and either ERK-1, K71R ERK-1, or K52R ERK-2. The lack of effect on AP-1 activity is likely due to the fact that ERK-1 and -2 require phosphorylation on threonine and tyrosine to be active, events that are undetectable in quiescent cells (28). On the other hand, injection of a wt Ha-Ras expression construct increased expression of the reporter (Figs. 3*A* and 4). This result was

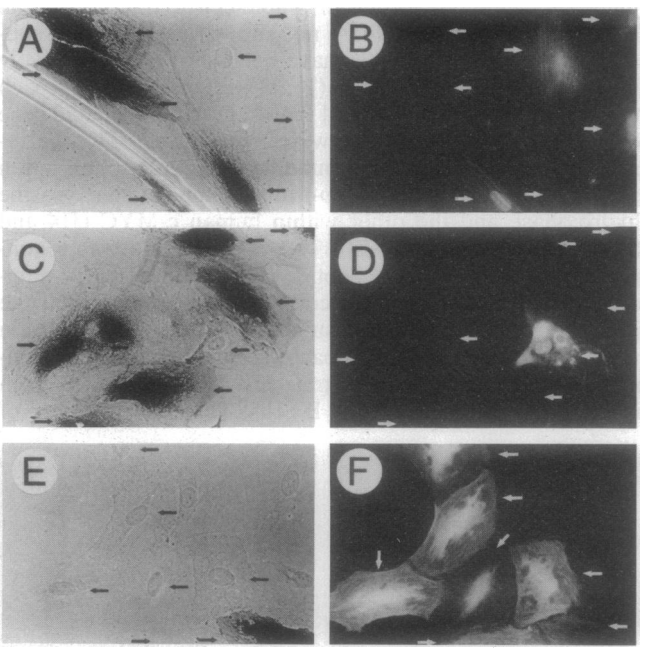

FIG. 3. AP-1 activation by wt Ha-Ras is potentiated by coexpression of wt ERK-1 and inhibited by coexpression of K71R ERK-1 and K52R ERK-2. (*A*, *C*, and *E*) Phase-contrast micrographs of cells stained for βGal with X-Gal. Cells expressing βGal are stained dark blue. (*B*, *D*, and *F*) Indirect immunofluorescence staining for a coinjected rat IgG used to detect injected cells. Note that intense staining for βGal sometimes blocks the fluorescent signal expected for the marker IgG staining. The arrows refer to injected cells. The following plasmids were injected: pHras (0.1 mg/ml), pCEP4 (0.03 mg/ml) (expression vector lacking ERK cDNAs), and TRE/lacZ (0.1 mg/ml) (*A* and *B*); pHras (0.1 mg/ml), pERK1 (0.03 mg/ml), and TRE/lacZ (0.1 mg/ml) (*C* and *D*); and pHras (0.1 mg/ml), pK71R ERK-1 (0.03 mg/ml), pK52R ERK-2 (0.03 mg/ml), and TRE/lacZ (0.1 mg/ml) (*E* and *F*).

expected because overexpression of wt Ha-Ras protein can induce cellular phenotypes associated with transformation (29, 30). This induction of AP-1 activity was enhanced when a combination of wt ERK-1 and wt-Ha-Ras was injected together (Fig. 3*C*), suggesting a positive relationship between these two signaling molecules in the regulation of AP-1.

Kinase-deficient mutants of protein kinases have been successfully used by others for interference with endogenous protein kinase activities. For example, expression of kinase-deficient c-Raf and Fyn has been found to create phenotypes in cells consistent with an inhibition of the respective endogenous kinase (31, 32). Thus, the effect on wt Ha-Ras-induced AP-1 activity was evaluated by using kinase-deficient ERK-1 (K71R ERK-1) and ERK-2 (K52R ERK-2) constructs. Previous work using bacterially expressed K71R ERK-1 and K52R ERK-2 phosphorylated on activating sites has shown that these mutants possess <1% and 5% wt kinase activity *in vitro*, respectively (33). Hence, when expressed *in vivo* they might be expected to interfere with endogenous ERK-1 and ERK-2 activity. Thus, the wt Ha-Ras expression plasmid was coinjected with the K71R ERK-1 or K52R ERK-2 expression constructs separately, or with a combination of the two. Individually, each interfering ERK construct was found to partially inhibit wt Ha-Ras-induced AP-1 activity (Fig. 4). Expression of both mutants together with wt Ha-Ras completely inhibited AP-1 activation, reducing apparent AP-1 activity below background (Figs. 3*E* and 4). This result suggests that wt Ha-Ras utilizes both ERK-1 and ERK-2 to activate AP-1. This result also suggests that the inhibition of wt Ha-Ras-induced TRE/lacZ activation by coinjection of ERK-specific substrate peptides was probably due to the inhibition of endogenous ERK-1 and ERK-2 activities.

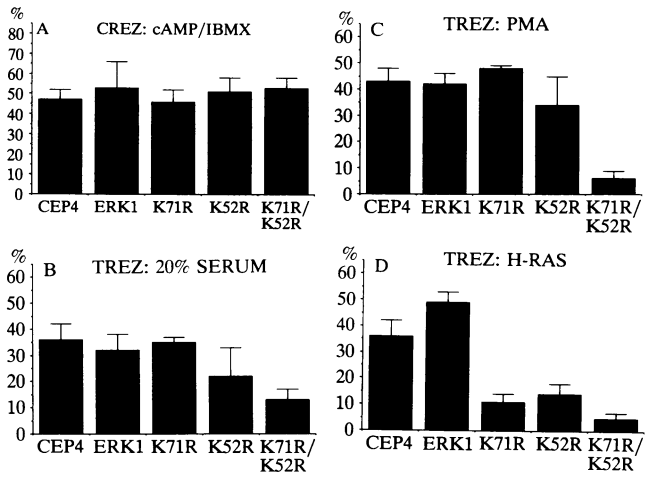

FIG. 4. Coexpression of K71R ERK-1 and K52R ERK-2 blocks AP-1 activation by wild-type Ha-Ras (*D*), PMA (*C*), and serum (*B*). Cells were injected with the following expression plasmids, rat IgG (5 mg/ml), and either the TRE/lacZ (0.1 mg/ml) or CRE/lacZ (0.1 mg/ml) reporter plasmids: pCEP4 (0.03 mg/ml) (expression vector lacking ERK cDNAs), pERK1 (0.03 mg/ml), pK71R ERK-1 (0.03 mg/ml), pK52R ERK-1 (0.03 mg/ml), or K71R ERK-1 and K52R ERK-2 together (0.02 mg/ml each). For wt Ha-Ras stimulation of TRE/lacZ, the cells were coinjected with the pH-Ras expression vector. For PMA (200 ng/ml) or serum (20% fetal bovine serum) stimulation of TRE/lacZ, the cells were injected and then stimulated 2 hr later. For experiments with the CRE/lacZ reporter plasmid, cells were injected and then stimulated 2 hr later with 0.25 mM 8-Br-cAMP and isobutylmethylxanthine each (*A*). Numbers on the *y* axis refer to the percentage of cells staining positive for βGal of the total number of cells injected. Each bar represents the average of at least three independent experiments, except for the inhibition of wt Ha-Ras-stimulated TRE/lacZ activity by coexpressed K71R ERK-1 and K52R ERK-2, which was done twice. An average of 300 cells was analyzed for each bar; error bars represent the SEM.

Coexpression of Kinase-Defective Forms of ERK-1 and ERK-2 Inhibits PMA and Serum-Induced AP-1 Activity. To determine whether ERK-1 and -2 are also required for AP-1 activation by other stimuli, the effect of expression of the K71R ERK-1 and K52R ERK-2 proteins on TRE/lacZ reporter induction by other stimuli was examined. Thus, PMA and serum were used to activate the TRE/lacZ construct in quiescent cells preinjected with the reporter plus the K71R ERK-1 and K52R ERK-2 constructs, either separately or together. In contrast to wt Ha-Ras stimulation, expression of either interfering mutant alone had no effect on AP-1 induction by either PMA or serum (Fig. 4), nor was overexpression of wt ERK-1 able to potentiate TRE/lacZ activation by these agents. This was true even with stimulation by submaximal concentrations of PMA, suggesting that the lack of inhibition by individually expressed mutants, or lack of potentiation by wt ERK-1 overexpression, was not due simply to a high degree of stimulation by PMA (data not shown). It was found, however, that coexpression of K71R ERK-1 and K52R ERK-2 inhibited the activation of the reporter, reducing lacZ expression in the presence of either PMA or serum to 6% and 13%, respectively (Fig. 4). This result suggests that, as found for wt Ha-Ras, ERK-1 and ERK-2 may be used and required for AP-1 regulation by PMA and serum.

To determine whether either the wt or interfering ERK mutants would inhibit other reporter constructs, the effects on cAMP-induced transcription from the CRE/lacZ reporter were examined. As expected, expression of the ERK constructs had no effect on cAMP-stimulated expression of this reporter (Fig. 4).

DISCUSSION

Three lines of evidence presented here suggest that ERK-1 and ERK-2 can act as mediators of AP-1 activation by wt Ha-Ras, PMA, and serum in REF-52 fibroblasts. (*i*) Microinjection of peptide substrates specific for ERK-1 and -2 *in vitro* inhibited the stimulation of AP-1 activity by wt Ha-Ras, PMA, and serum *in vivo*. (*ii*) Expression of a combination of kinase-deficient ERK-1 and -2 led to an inhibition of AP-1 normally stimulated by these agents. (*iii*) Coexpression of wt ERK-1 with wt Ha-Ras increased AP-1 activation stimulated by wt Ha-Ras. Thus, ERK-1 and ERK-2 appear necessary components in the signaling pathways leading to AP-1 activation by these agents in these cells.

The use of kinase-deficient mutants of ERK-1 and ERK-2 provided what appeared to be an effective means of interfering with endogenous ERK function, as judged by their ability to block AP-1 induction when coexpressed. In addition, the use of ERK-1 or ERK-2 mutants separately gave an indication of possible differences in the mode of activation of ERKs by different stimuli. AP-1 activation brought about by serum stimulation is likely to occur through both Ras-dependent and Ras-independent pathways, due to the diversity of growth factors in serum; this may be reflected, for example, in the inability of an interfering Ras mutant to block serum-induced c-Fos expression (34, 35). We have observed that expression of either K71R ERK-1 or K52R ERK-2 alone led to a partial inhibition of wt Ha-Ras-stimulated AP-1 activity, whereas coexpression of both mutants was necessary for the inhibition of AP-1 stimulation by serum or PMA. Even when submaximal concentrations of PMA were used to stimulate the cells, expression of either interfering mutant alone was not sufficient to inhibit AP-1 activation. Thus, these findings may reflect differences between these stimuli in the signaling pathways leading to ERK activation.

The mechanism of interference by the ERK-1 and ERK-2 mutants is unclear. Most likely they function either by binding to ERK substrates and thus preventing their phosphorylation by active, endogenous ERKs, or by serving as alternate substrates for cellular *M*AP kinase/*E*RK *k*inases

(MEKs), thus preventing ERK activation. If the mutant ERK-1 and ERK-2 proteins interfere with signaling by preventing the activation of endogenous ERKs, this might explain the differential inhibition of wt Ha-Ras, PMA, and serum-induced AP-1 activation observed here. The purification and cloning of distinct MAP kinase activators, or MEKs (28, 36–39), raise the possibility that wt Ha-Ras, PMA, and serum may activate different MEKs, either singly or in combination. This multiplicity of MEKs and ERKs may create a complex network of signal transduction in which different MEKs preferentially regulate different ERKs. Indeed, distinct, active MEKs purified from mammalian cells display a marked difference in their specificities for ERK-1, -2, and -3 *in vitro* (E. Zhen, M. Cheng, D. Robbins, and M.H.C., unpublished results). Thus, if the MEKs activated by wt Ha-Ras similarly recognize and regulate both ERK-1 and ERK-2, they may be inhibited by expression of a single interfering mutant. If, on the other hand, PMA and serum activate distinct combinations of MEKs that differentially regulate ERK-1 and ERK-2, expression of both K71R ERK-1 and K52R ERK-2 would be necessary to efficiently block signaling through these MEKs. Further work will be necessary to test this hypothesis.

AP-1-regulated gene expression is a critical feature of growth regulation by Ras-mediated pathways. It has been shown that serum-induced mitogenesis requires Ras, c-Jun, and c-Fos activity (40–43). These findings have led to the suggestion that AP-1 represents a downstream, nuclear target for Ras-dependent signaling (44). We do not as yet know if activated ERKs are directly responsible for the observed increase in AP-1 activity or if they require other downstream mediators. Published observations that ERK-1 and ERK-2 phosphorylate c-Jun on either activating or inhibitory sites *in vitro* reflect a potential lack of specificity within these experiments. These findings and the partial purification of UV- and serum-inducible kinases that selectively phosphorylate the N-terminal, activating sites on c-Jun suggest that other factors may be necessary for AP-1 activation, some of which may lie between the ERKs and AP-1 (45, 46). The absence of ERK activation following UV stimulation also suggests that distinct mechanisms of AP-1 activation not requiring ERK-1 and ERK-2 exist. Further analysis of ERK substrates will be necessary to resolve this issue.

Note. While this paper was in review, Pages *et al.* (47) published a report showing that overexpression of kinase-deficient ERK-1 mutants distinct from those used here, as well as expression of ERK-1 antisense constructs, inhibited serum-stimulated proliferation of PS120 cells.

We thank Mike Wigler for the wt Ha-Ras eukaryotic expression plasmid. We also thank Andrew Thorburn, Ron Sosnowski, and Art Alberts for helpful advice, as well as Judy Meinkoth, Dave Rose, Michael Karin, and Tony Hunter for critical review of the text. Also, special thanks to Audrey Majors and Kristin Martin for excellent help with manuscript preparation. This work was supported by grants from the National Institutes of Health, the Council for Tobacco Research, and the Juvenile Diabetes Foundation.

1. Marsh, L., Neiman, A. M. & Herskowitz, I. (1991) *Annu. Rev. Cell Biol.* **7**, 699–728.
2. Rozengurt, E. (1992) *Curr. Opin. Cell Biol.* **4**, 161–165.
3. Thomas, G. (1992) *Cell* **68**, 3–6.
4. Cobb, M. H., Boulton, T. G. & Robbins, D. J. (1991) *Cell Regul.* **2**, 965–978.
5. Levin, D. E. & Errede, B. (1993) *J. Natl. Inst. Health Res.* **5**, 49–52.
6. Alvarez, E., Northwood, I. C., Gonzalez, F. A., Latour, D. A., Seith, A., Abate, C., Curren, T. & Davis, R. J. (1991) *J. Biol. Chem.* **266**, 15277–15285.
7. Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E. & Woodgett, J. R. (1991) *Nature (London)* **353**, 670–674.
8. Gille, H., Sharrocks, A. D. & Shaw, P. E. (1992) *Nature (London)* **358**, 414–417.
9. Chou, S.-Y., Bichwal, V. & Ferrell, J. E., Jr. (1992) *Mol. Biol. Cell* **3**, 1117–1130.
10. Marais, R., Wynne, J. & Treisman, R. (1993) *Cell* **73**, 381–393.
11. Hill, C. S., Marais, R., John, S., Wynne, J., Dalton, S. & Treisman, R. (1993) *Cell* **73**, 395–406.
12. Seth, A., Alvarez, E., Gupta, S. & Davis, R. J. (1991) *J. Biol. Chem.* **266**, 23521–23524.
13. Seth, A., Gonzalez, F. A., Gupta, S., Raden, D. L. & Davis, R. J. (1992) *J. Biol. Chem.* **267**, 24796–24804.
14. Gupta, S., Seth, A. & Davis, R. J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3216–3220.
15. Hagiwara, M., Brindle, P., Alberts, A., Meinkoth, J., Feramisco, J., Deng, T., Karin, M. & Montminy, M. (1992) *Cell* **69**, 105–113.
16. Lin, A., Frost, J. A., Al-Alawi, N., Deng, T., Hunter, T. & Karin, M. (1992) *Cell* **70**, 777–789.
17. Alberts, A. S., Frost, J. A. & Thorburn, A. M. (1993) *DNA Cell Biol.* **12**, 935–943.
18. Haycock, J. W., Ahn, N. G., Cobb, M. H. & Krebs, E. G. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2365–2369.
19. Flynn, G. C., Chappell, T. G. & Rothman, J. E. (1989) *Science* **245**, 385–390.
20. Deng, T. & Karin, M. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8572–8576.
21. Boulton, I. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H. & Yancopoulos, G. D. (1991) *Cell* **65**, 663–675.
22. Gross, M., Sweet, R. W., Sathe, G., Yokoyama, S., Fasano, O., Goldfarb, M., Wigler, M. & Rosenberg, M. (1985) *Mol. Cell. Biol.* **5**, 1015–1024.
23. Fantozzi, D. A., Taylor, S. S., Howard, P. W., Maurer, R. A., Feramisco, J. R. & Meinkoth, J. L. (1992) *J. Biol. Chem.* **267**, 16824–16828.
24. Boulton, T. G. & Cobb, M. H. (1991) *Cell Regul.* **2**, 357–371.
25. Wang, H. C. & Erikson, R. L. (1992) *Mol. Biol. Cell* **3**, 1329–1337.
26. Gonzalez, F. A., Raden, D. L., Rigby, M. R. & Davis, R. J. (1992) *FEBS Lett.* **304**, 170–178.
27. Meinkoth, J., Alberts, A. & Feramisco, J. (1990) *CIBA Found. Symp.* **150**, 47–56.
28. Seger, R., Ahn, N. G., Posada, J., Munar, E. S., Jensen, A. M., Cooper, J. A., Cobb, M. H. & Krebs, E. G. (1992) *J. Biol. Chem.* **267**, 14373–14381.
29. Bar-Sagi, D. & Feramisco, J. R. (1986) *Science* **233**, 1061–1068.
30. Stacey, D. W. & Kung, H. F. (1984) *Nature (London)* **310**, 508–511.
31. Kolch, W., Heidecker, G., Lloyd, P. & Rapp, U. R. (1991) *Nature (London)* **349**, 426–428.
32. Cooke, M. P., Abraham, K. M., Forbush, K. A. & Perlmutter, R. M. (1991) *Cell* **65**, 281–291.
33. Robbins, D. J., Zhen, E., Owaki, H., Vanderbilt, C. A., Ebert, D., Geppert, T. D. & Cobb, M. H. (1993) *J. Biol. Chem.* **268**, 5097–5106.
34. Cai, H., Szeberenyi, J. & Cooper, G. M. (1990) *Mol. Cell. Biol.* **10**, 5314–5323.
35. Sosnowski, R. G., Feldman, S. T. & Feramisco, J. R. (1993) *J. Cell Biol.* **121**, 113–119.
36. Crews, C. M. & Erikson, R. L. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8205–8209.
37. Wu, J., Harrison, J. K., Vincent, L. A., Haystead, C., Haystead, T. A., Michel, H., Hunt, D. F., Lynch, K. R. & Sturgill, T. W. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 173–177.
38. Zheng, C. F. & Guan, K. L. (1993) *J. Biol. Chem.* **268**, 11435–11439.
39. Crews, C. M., Alessandrini, A. & Erikson, R. L. (1992) *Science* **258**, 478–480.
40. Riabowol, K. T., Vosatka, R. J., Ziff, E. B., Lamb, N. J. & Feramisco, J. R. (1988) *Mol. Cell. Biol.* **8**, 1670–1676.
41. Kovary, K. & Bravo, R. (1991) *Mol. Cell. Biol.* **11**, 4466–4472.
42. Holt, J. T., Venkat-Gopal, T., Moulton, A. D. & Nienhuis, A. W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4794–4798.
43. Nishikura, K. & Murray, J. M. (1987) *Mol. Cell. Biol.* **7**, 639–649.
44. Angel, P. & Karin, M. (1991) *Biochim. Biophys. Acta* **1072**, 129–157.
45. Adler, V., Polotskaya, A., Wagner, F. & Kraft, A. S. (1992) *J. Biol. Chem.* **267**, 17001–17005.
46. Hibi, M., Lin, A., Smeal, T., Minden, A. & Karin, M. (1993) *Genes Dev.* **7**, 2135–2148.
47. Pages, G., Lenormand, P., L'Allemain, G., Chambard, J.-C., Meloche, S. & Pouyssegur, J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8319–8323.