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Simian Virus 40 Small t Antigen Cooperates with Mitogen-Activated Kinases To Stimulate AP-1 Activity

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The simian virus 40 small tumor antigen (small t) specifically interacts with protein phosphatase type 2A (PP2A) in vivo and alters its catalytic activity in vitro. Among the substrates for PP2A in vitro are the activated forms of MEK and ERK kinases. Dephosphorylation of the activating phosphorylation sites on MEK and ERKs by PP2A in vitro results in a decrease in their respective kinase activities. Recently, it has been shown that overexpression of small t in CV-1 cells results in an inhibition of PP2A activity toward MEK and ERK2 and a constitutive upregulation of MEK and ERK2 activity. Previously, we have observed that overexpression of either ERK1, MEK1, or a constitutively active truncated form of c-Raf-1 (BXB) is insufficient to activate AP-1 in REF52 fibroblasts. We therefore examined whether overexpression of small t either alone or in conjunction with ERK1, MEK1, or BXB could activate AP-1. We found that coexpression of small t and either ERK1, MEK1, or BXB resulted in an increase in AP-1 activity, whereas expression of either small t or any of the kinases alone did not have any effect. Similarly, coexpression of small t and ERK1 activated serum response element-regulated promoters. Coexpression of kinase-deficient mutants of ERK1 and ERK2 inhibited the activation of AP-1 caused by expression of small t and either MEK1 or BXB. Coexpression of an interfering MEK, which inhibited AP-1 activation by small t and BXB, did not inhibit the activation of AP-1 caused by small t and ERK1. In contrast to REF52 cells, we observed that overexpression of either small t or ERK1 alone in CV-1 cells was sufficient to stimulate AP-1 activity and that this stimulation was not enhanced by expression of small t and ERK1 together. These results show that the effects of small t on immediate-early gene expression depend on the cell type examined and suggest that the mitogen-activated protein kinase activation pathway is distinctly regulated in different cell types.

The stimulation of quiescent cells with growth factors induces widespread changes in gene expression, including the activation of promoters controlled by the transcription factor AP-1 (30). Following the binding of growth factors to cell surface receptors, a protein kinase cascade is activated, which generally results in the activation of two kinases termed ERK1 and ERK2. ERK1 and ERK2 belong to a family of kinases known as extracellular signal-regulated kinases (ERKs) or mitogen-activated protein (MAP) kinases (12). Both ERK1 and ERK2 are activated by phosphorylation on specific threonine and tyrosine residues (44). This phosphorylation is catalyzed by a threonine/tyrosine kinase known as MEK (MAP and ERK kinase) (15, 16, 53, 54, 57). MEK itself is activated by phosphorylation on uncharacterized serine and/or threonine residues by both c-Raf-1 or MEK kinase (17, 28, 31, 32, 58). c-Raf-1 appears to couple this cascade to Ras (39, 51, 52, 56), while MEK kinase may be activated by a distinct mechanism (32).

ERK1 and ERK2, as well as MEK1, can be deactivated in vitro by treatment with the serine/threonine specific phosphatase type 2A (PP2A), which dephosphorylates the activating threonine in ERK1 and ERK2, and the activating serines and threonines in MEK1 (1, 4, 7, 22, 23, 27, 28, 31). It is not entirely clear, however, how PP2A impacts this cascade in vivo. Indirect evidence suggesting a role for PP2A in the regulation of ERKs has been obtained by treatment of adipocyte cells with okadaic acid, which is a specific inhibitor of PP2A and PP1 (27). Exposure of these cells to concentrations of okadaic acid which preferentially inhibit PP2A results in an increase in cellular MAP kinase activity. This effect seems to be specific for adipocyte cells, however, since no such effect is observed when fibroblasts are treated with okadaic acid.

Recently, it has been shown that the simian virus 40 (SV40) small tumor antigen (small t) binds to PP2A and inhibits its ability to dephosphorylate a variety of phosphoproteins in vitro, including ERK2 and MEK1 (40, 42, 43, 47, 55). It has also been shown that expression of small t in the SV40-permissive cell line CV-1 leads to an increase in the activity of ERK2 and MEK1 and ultimately causes these cells to proliferate (47). These effects appear to be dependent on the ability of small t to bind to PP2A, since deletion of the portion of small t that interacts with PP2A abolishes its ability to stimulate ERK2 and MEK1 activity as well as promote cell growth. Since the only known biochemical function of small t is to bind PP2A and thereby alter its substrate specificity, these results suggest that PP2A may serve to downregulate cellular ERK and MEK activity.

Previously, we have shown that ERK activity is required for the activation of AP-1 in REF52 fibroblasts by wild-type H-Ras, tetradecanoyl phorbol acetate (TPA), and serum (20).

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However, overexpression of either ERK1 or ERK2 in quiescent REF52 cells does not result in an increase in AP-1 activity. This finding suggests either that in quiescent REF52 cells endogenous MEK is inactive or that an inhibitor of ERK activity is present. Similarly, overexpression of a constitutively active mutant of c-Raf-1 (BXB) (9) does not have any effect in these cells, suggesting that this signaling pathway is strongly downregulated when these cells are in a quiescent state. A likely candidate enzyme to downregulate one or more of the enzymes in this activation pathway is PP2A. To investigate this possibility, we have expressed small t either alone or in combination with ERK1, MEK1, or BXB and monitored the cells for resultant changes in cellular AP-1 activity. The cells were also monitored for changes in *fos* promoter and serum response element (SRE)-dependent promoter activity. In addition, we have tested the effects of overexpression of these proteins in CV-1 cells. We find that in REF52 cells, coexpression of small t and either ERK1, MEK1, or BXB results in an increase in AP-1 activity that is not observed when any one of these genes is expressed alone. Coexpression of small t and ERK1 also increases SRE-dependent reporter activity. This is distinct, however, from what occurs in CV-1 cells, in which the individual expression of small t or ERK1 alone strongly induces AP-1 activity. Furthermore, this activation is not significantly increased by coexpression of small t and ERK1. Since the only known biochemical activity of small t is to alter PP2A activity, these results suggest that the activity of this signaling pathway is inhibited by PP2A and that the level of PP2A activity in these two cell types differs in the quiescent state. These results may have implications for the role of small t in promoting cellular transformation by the SV40 large tumor t antigen (large T).

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MATERIALS AND METHODS

Cell culture. Both REF52 and CV-1 cells were grown in Dulbecco's modified Eagle medium (DMEM; Fisher) supplemented with 10% fetal bovine serum (FBS; Gemini), 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin sulfate per ml. For microinjection assays, cells were plated on glass coverslips (Fisher) in DMEM–10% FBS. At 30 to 50% confluence, the cells were rendered quiescent by serum deprivation for 48 h in DMEM–0.05% FBS.

Eukaryotic expression vectors. Wild-type small t was subcloned into the *Hin*dIII-*Bam*HI sites of pCEP4 (Invitrogen) after excision of the *Hin*dIII-*Bam*HI fragment released from pCMV5/Smt (47) and was designated pCEP4/Smt. Small t mutant 3 (mut 3) was subcloned into pCEP4 by first excising the 340-bp fragment from pCMV5/Smt (mut 3) (50) with *Eco*RI-*Bam*HI. Smt (mut 3) was then subcloned from pBS (KS+)/Smt (mut 3) by digestion with *Hin*dIII-*Bam*HI, and this fragment was inserted into pCEP4 cut with *Hin*dIII-*Bam*HI. This plasmid was designated pCEP4/Smt (mut 3). pCEP4/ERK1, pCEP4/K71R ERK1, and pCEP4/K52R ERK2 were made as described previously (20). pCEP4/MEK1 and pCEP4/MEK3 were subcloned from pBS/MEK1 and pBS/MEK3 by excising the ~2.1-kb cDNA fragments with *Not*I-*Xho*I and inserting them into pCEP4 cut first with *Not*I and then with *Xho*I. Activated c-Raf-1 (BXB) was contained in Rc/CMV (Invitrogen) and was provided by M. Karin.

Microinjection and analysis of reporter plasmid expression. For TPA response element (TRE-*lacZ*) and cyclic AMP (CAMP) response element (CRE-*lacZ*) studies, cells were injected with TRE-*lacZ* or CRE-*lacZ* reporter plasmid (100 to 150 μg/ml), the appropriate expression plasmids, and rat immunoglobulin G (IgG; Sigma) (5 μg/ml). The expression plasmids were injected at the following concentrations: pCEP4/Smt and pCEP4/Smt (mut 3), 60 μg/ml; pCEP4/ERK1 and BXB, 30 to 60 μg/ml; pCEP4/K71R ERK1 and pCEP4/K52R ERK2, 30 μg/ml; and pCEP4/MEK1 and pCEP4/MEK3, 100 μg/ml. Cells expressing β-galactosidase (β-Gal) were detected by staining with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Boehringer Mannheim) as previously described (34). Cells were stained for injected rat IgG by using a rhodamine-conjugated donkey anti-rat antibody (Jackson) as described previously (3, 20). For the *fos*-chloramphenicol acetyltransferase (CAT) and TRE-*lacZ* expression studies, both the *fos*-CAT and TRE-*lacZ* plasmids were injected at 200 μg/ml. CAT and *lacZ* expression were detected by indirect immunofluorescence as described previously (3). Injected cells were detected by indirect immunofluorescent staining for a coinjected guinea pig IgG (5 mg/ml; Sigma), using an AMCA-conjugated donkey anti-guinea pig IgG (Jackson) as described previously (3). All cells were analyzed and photographed with a Zeiss Axiophot epifluorescence microscope as previously described (3).

RESULTS

We have used short-term transcription assays (20) to determine whether expression of small t in REF52 cells is sufficient to activate AP-1 on its own or in conjunction with various growth-associated kinases. Accordingly, we have microinjected eukaryotic expression constructs encoding small t, ERK1, MEK1, and a constitutively active form of c-Raf-1 (BXB) (9) into the nuclei of quiescent REF52 fibroblasts along with an AP-1-responsive reporter plasmid (TRE-*lacZ*). Previous work has shown that injection of eukaryotic expression plasmids into the nuclei of these cells results in the detectable expression of the encoded protein by direct immunofluorescence within 30 min of injection in approximately 80% of the injected cells and that expression is maintained for at least 24 h after injection (3). Furthermore, expression from reporter constructs such as the TRE-*lacZ* plasmid can be reliably detected 2 to 3 h after injection by staining with the chromogenic substrate X-Gal (20). It should be noted that intense β-Gal staining with X-Gal often interferes with the indirect immunofluorescent staining for a coinjected marker antibody used to detect injected cells. Since REF52 cells have no endogenous β-Gal activity, however, darkly staining cells must have been injected and are treated as such in this study.

In quiescent REF52 cells, injection of the TRE-*lacZ* plasmid typically results in β-Gal expression in 10 to 15% of the injected cells. This background activity is not significantly enhanced by expression of any level of small t (Fig. 1A). Similarly, expression of ERK1, MEK1, or BXB either alone or in any combination tested does not activate AP-1 (data not shown). However, coexpression of small t and ERK1, small t and MEK1, or small t and BXB results in a strong induction of AP-1 activity within 3 h of injection (Fig. 1C, E, and G). This effect is greatest when small t and ERK1 are coexpressed, in which case approximately 80% of the injected cells stain positive for β-Gal (Fig. 2A). This probably represents maximal reporter expression in this system, since injection of TRE-*lacZ* alone followed by stimulation with TPA, 20% fetal bovine serum, or purified growth factors does not result in greater than 80% of the cells producing β-Gal within 3 h (data not shown). The ability of small t and either ERK1, MEK1, or

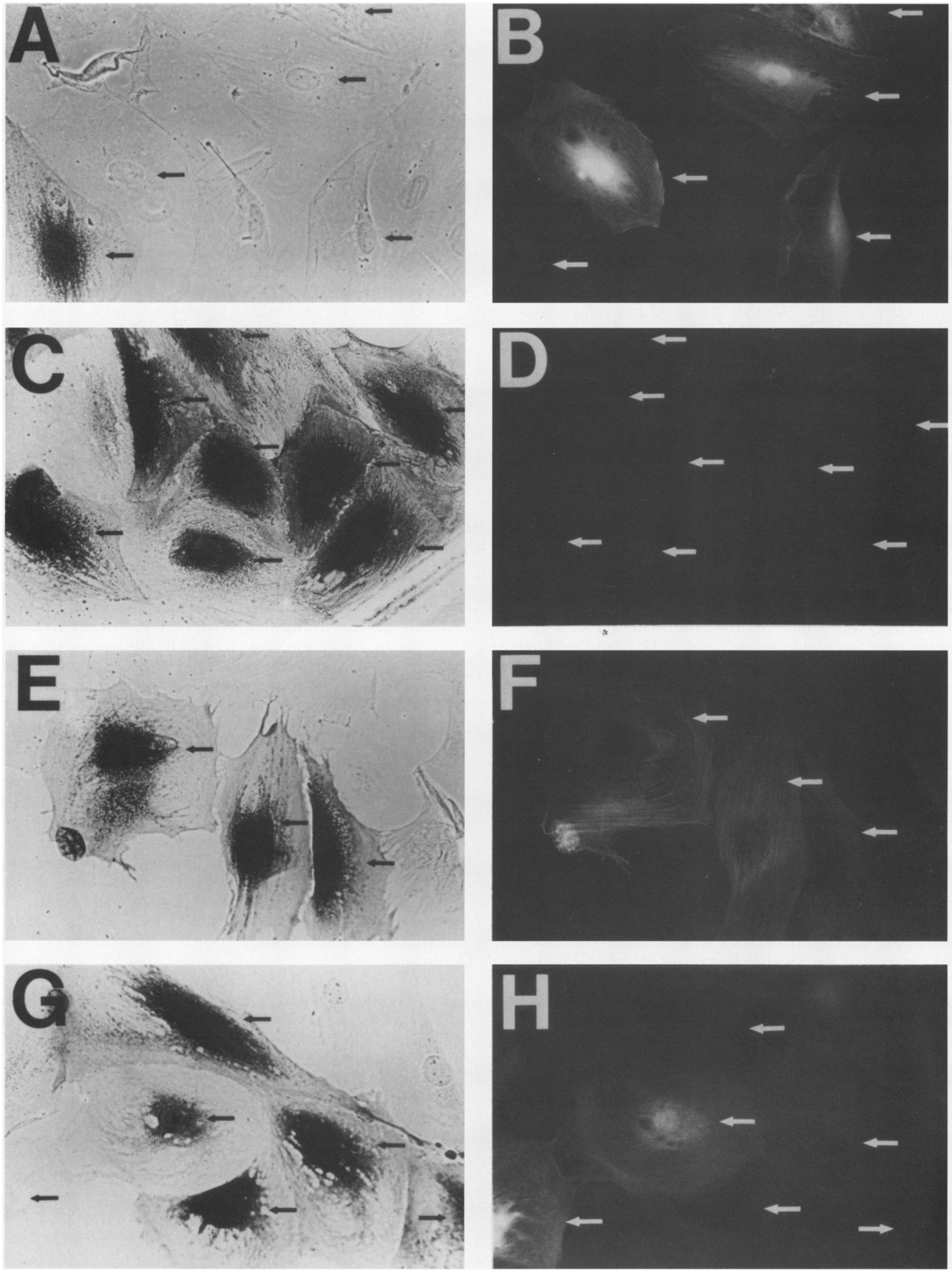
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FIG. 1. Coexpression of small t and growth-associated kinases activates a TRE-*lacZ* reporter construct injected into quiescent REF52 fibroblasts. (A, C, E, and G) Phase-contrast micrographs of cells stained for the presence of β-Gal with X-Gal. Cells expressing β-Gal are darkly stained. (B, D, F, and H) Indirect immunofluorescent staining for a coinjected rat IgG used as a marker for injected cells. Note that intense β-Gal staining blocks the fluorescent signal. Arrows denote injected cells in all panels. The following expression plasmids were injected along with TRE-*lacZ* (150 μg/ml) and rat IgG (5 mg/ml): (A and B) pCEP4/Smt (60 μg/ml); (C and D) pCEP4/Smt (60 μg/ml) and pCEP4/ERK1 (60 μg/ml); (E and F) pCEP4/Smt (60 μg/ml) and pCEP4/MEK1 (100 μg/ml); (G and H) pCEP4/Smt (60 μg/ml) and pBXB (30 μg/ml).

BXB to activate AP-1 is dependent on the presence of the PP2A binding domain of small t since expression of a small t mutant lacking this domain (mut 3) (50) either alone or in combination with these kinases does not increase AP-1 activity (Fig. 2A to C). This finding suggests that small t must interact with PP2A in order to affect AP-1 activity.

To test whether this effect was specific for the AP-1-responsive construct, we coinjected small t and either ERK1, MEK1, or BXB with a *lacZ* reporter plasmid which is controlled by a multimer of the CRE (CRE-*lacZ*) (25, 26, 38). The CRE-*lacZ* reporter plasmid is strongly induced by exposure of cells to 8-bromo-cAMP and the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) (0.25 mM each) but is unresponsive to AP-1-activating agents such as TPA or serum growth factors. We observed that coinjection of small t and either ERK1, MEK1, or BXB with the CRE-*lacZ* plasmid did not induce β-Gal expression, suggesting that the effects of these proteins was specific for TRE-regulated promoters (data not shown).

It has recently been shown that MEK1 directly phosphorylates and activates ERK1 and ERK2 in vitro and that MEK1 can be activated by phosphorylation by the c-Raf-1 kinase. Furthermore, genetic experiments have demonstrated that the budding yeast homologs of the ERKs (FUS3 and KSS1) are directly activated by the MEK homolog STE7, thus constituting part of a kinase cascade that is activated in response to pheromones (18, 33, 59). We examined whether the ability of small t and either BXB, MEK1, or ERK1 to activate AP-1 in these cells was dependent on the functionality of this cascade by coexpressing dominant interfering, kinase-deficient forms of ERK1 and ERK2 (K71R ERK1 and K52R ERK2, respectively). Previously we have shown that coexpression of K71R ERK1 and K52R ERK2 inhibits the activation of AP-1 by TPA, serum, or wild-type H-*ras* (20). When K71R ERK1 and

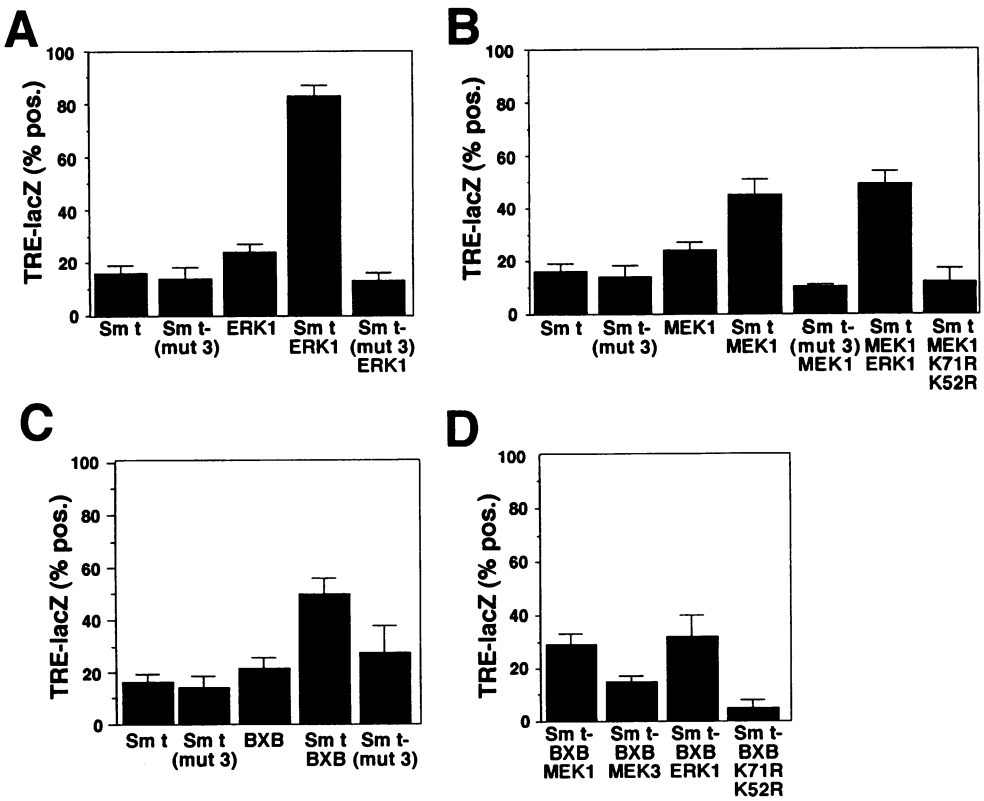


FIG. 2. AP-1 activation in REF52 fibroblasts by expression of small t and growth-associated kinases requires the PP2A binding domain of small t and also may require the integrity of the MAP kinase cascade. Cells were injected with the denoted expression vectors at the concentrations described in Materials and Methods, along with the TRE-*lacZ* reporter vector (150 μg/ml) and a rat IgG (5 mg/ml), which was used as a marker for injected cells. K71R and K52R refer to pCEP4/K71R ERK1 and pCEP4/K52R ERK2, respectively. TRE-lacZ (% pos.) refers to the average percentage of injected cells expressing β-Gal, as determined from at least three independent experiments. The total number of injected cells per experiment was determined as the sum of β-Gal-positive cells and cells not expressing β-Gal that stain for injected marker antibody. Error bars represent the standard error of the mean.

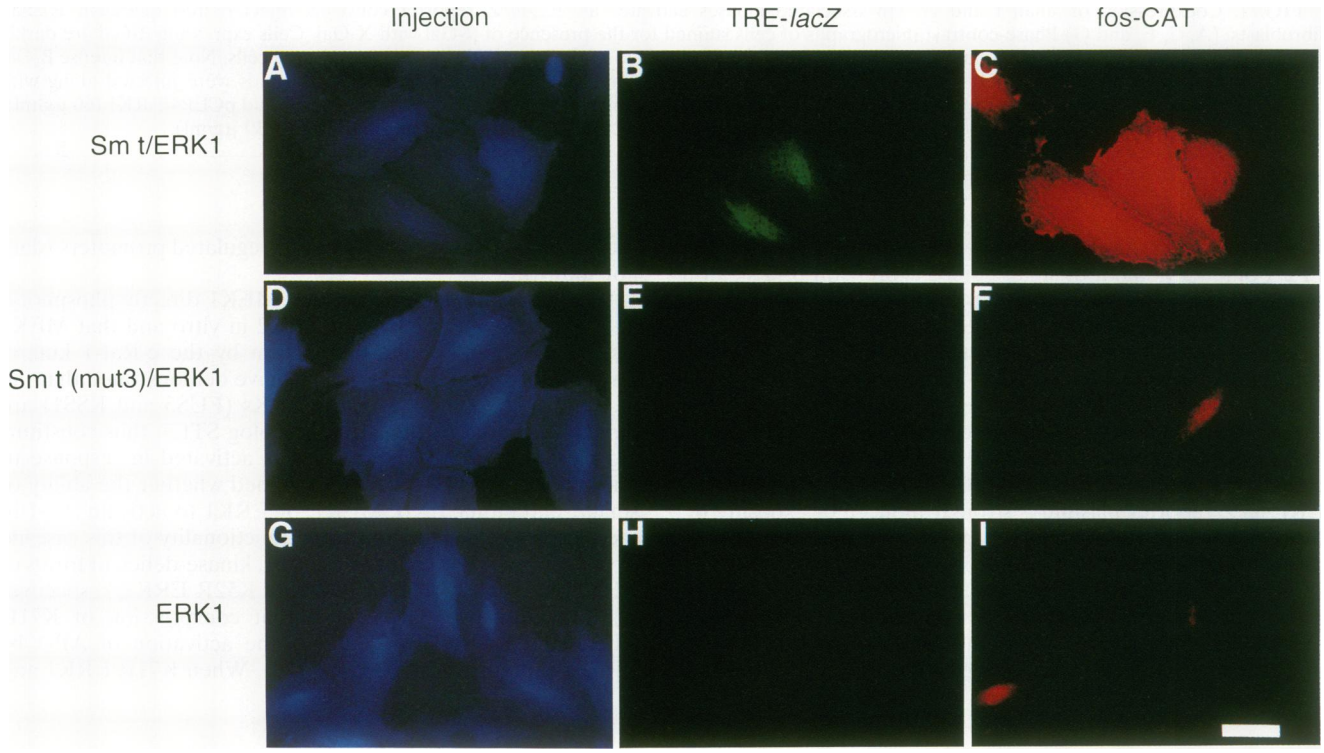
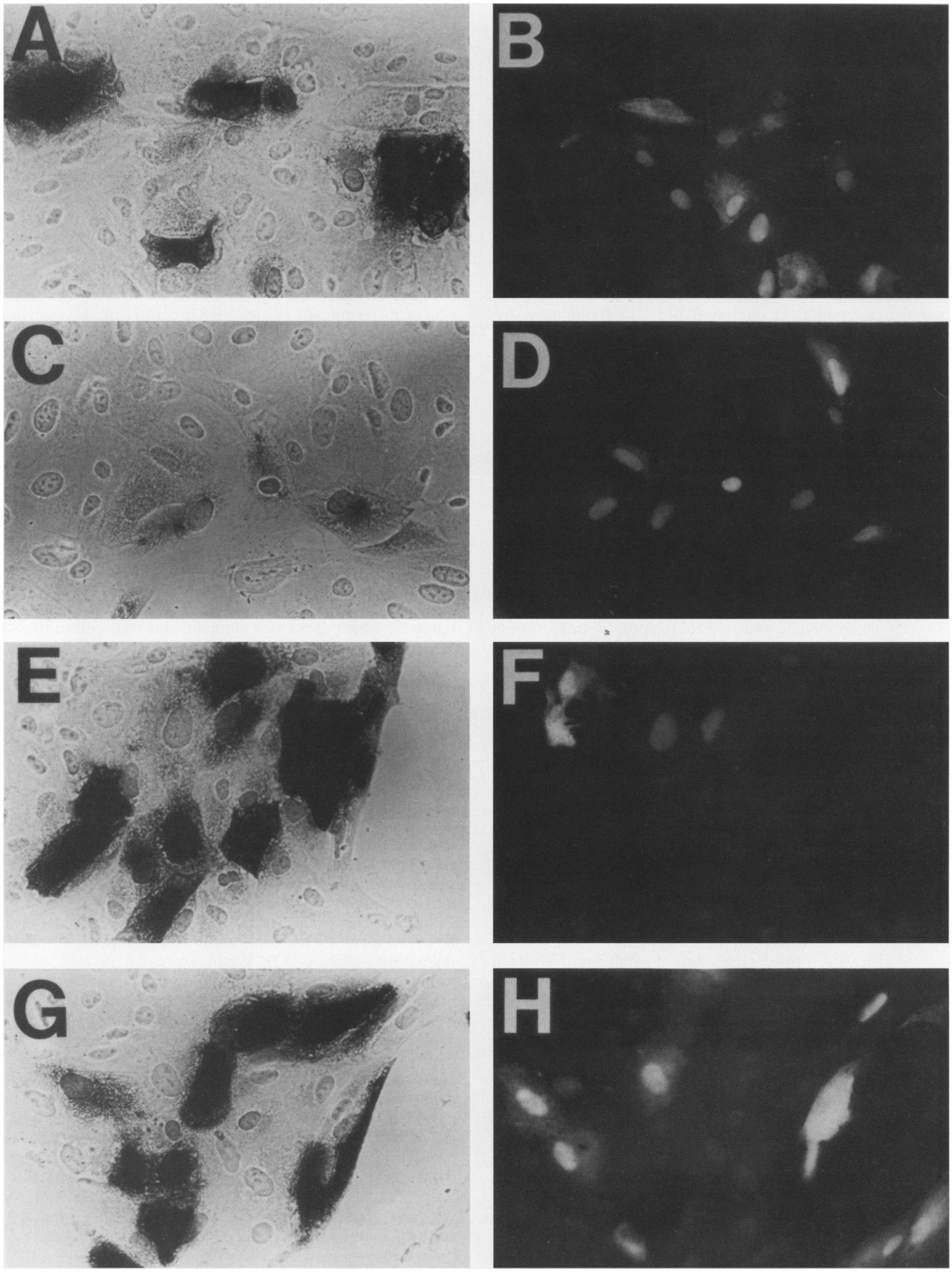


FIG. 3. Coexpression of small t and ERK1 in REF52 cells activates a *fos*-CAT reporter plasmid as well as a coinjected TRE-*lacZ* reporter plasmid. All cells were injected with guinea pig IgG (5 mg/ml), TRE-*lacZ* (200 μg/ml), and *fos*-CAT (200 μg/ml). The cells in panels A to C were injected with pCEP4/Smt (60 μg/ml) and pCEP4/ERK1 (60 μg/ml). The cells in panels D to F were injected with pCEP4/Smt (mut 3) (60 μg/ml), which codes for a small t protein incapable of binding to PP2A, and pCEP4/ERK1 (60 μg/ml). The cells in panels G to I were injected with pCEP4/ERK1 (60 μg/ml) alone. (A, D, and G) Indirect immunofluorescent staining for a coinjected guinea pig IgG used as a marker for injected cells. (B, E, and H) Indirect immunofluorescent staining for expressed β-Gal, detected as described in Materials and Methods. (C, F, and I) Indirect immunofluorescent staining for expressed CAT protein, detected as described in Materials and Methods.

K52R ERK2 were coexpressed with small t and BXB, AP-1 activation by these constructs was abolished. Coexpression of wild-type ERK1 (as a control) with small t and BXB only slightly inhibited AP-1 activation (Fig. 2D). Similarly, the induction of AP-1 activity by coexpression of small t and MEK1 was inhibited by coexpression of K71R ERK1 and K52R ERK2 but not by coinjection of an equivalent amount of wild-type ERK1 vector (Fig. 2B). Thus, these experiments suggest that the activation of AP-1 by either small t and BXB or small t and MEK1 depended on the activity of endogenous ERKs.

To test whether the activation of AP-1 by small t and BXB depended on the activity of endogenous MEKs, we coexpressed either wild-type MEK1 or a kinase-deficient splice variant of MEK1 (MEK3) with small t and BXB (57). AP-1 activation caused by small t and BXB was abolished by coexpression of MEK3 but only partially inhibited by coexpression of MEK1 (Fig. 2D). No inhibition was observed when the pCEP4 expression vector lacking MEK cDNAs was coinjected. Though it is not clear why coexpression of wild-type MEK1 partially inhibited small t-BXB-mediated AP-1 activation, the complete inhibition observed with coexpressed MEK3 suggests that endogenous MEK activity is required for the increase in AP-1 activity. The inhibition of small t-BXB-mediated AP-1 activation by coexpression of K71R ERK1 and K52R ERK2 together, or MEK3 alone, was also not due to a nonspecific inhibition of *lacZ* expression, since expression of these kinase-deficient constructs had no effect on the activation of the CRE-*lacZ* reporter plasmid stimulated by 8-bromo-cAMP and IBMX (data not shown). The ability of small t and ERK1 to activate AP-1 was not affected by coexpression of MEK3 (data not shown). Thus, in the context of small t-BXB-mediated AP-1 activation, these experiments show that small t is not likely to function downstream of the ERKs. Also, since the BXB construct lacks the N-terminal regulatory region of c-Raf-1 and should be constitutively active, these results suggest that the activity of small t is confined to relieving an inhibitory influence on expressed ERK1 and MEK1, most likely caused by endogenous PP2A activity. This follows since coexpression of the small t mutant which cannot bind PP2A (mut 3) plus ERK1, MEK1, or BXB did not lead to increased AP-1 activity. These findings also suggest that BXB functions

FIG. 4. Expression of either small t or ERK1 alone in CV-1 cells induces AP-1 activity. (A, C, E, and G) Phase-contrast micrographs of cells stained for the presence of β-Gal with X-Gal. Cells expressing β-Gal are darkly stained. (B, D, F, and H) Indirect immunofluorescent staining for a coinjected rat IgG used as a marker for injected cells. Note that intense β-Gal staining blocks the fluorescent signal. The following expression plasmids were injected along with TRE-*lacZ* (150 μg/ml) and rat IgG (5 mg/ml): (A and B) pCEP4/Smt (60 μg/ml); (C and D) pCEP4/Smt (mut 3) (60 μg/ml); (E and F) pCEP4/ERK1 (60 μg/ml); (G and H) pCEP4/Smt (60 μg/ml) and pCEP4/ERK1 (60 μg/ml).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upstream of the ERKs and MEKs and that MEK1 functions upstream of the ERKs, consistent with the cellular hierarchy believed to exist for these kinases.

It has recently been shown that the activation of c-*fos* transcription by exposure of quiescent cells to serum is dependent on the phosphorylation of the $p62^{TCF}$ protein by ERK kinases (30, 37). $p62^{TCF}$, or Elk-1, is a member of the ternary complex which binds to the SRE in the c-*fos* promoter and allows for the activation of c-*fos* transcription by serum and individual growth factors (50). We examined whether coexpression of small t and ERK1 could activate the c-*fos* promoter by coinjecting a *fos*-CAT reporter plasmid along with the small t and ERK1 expression vectors. This *fos*-CAT construct contains the first 711 bp of the human c-*fos* promoter, including the SRE, and has been shown to recapitulate cellular c-*fos* expression in vivo (20, 54). Activation of the *fos*-CAT plasmid was detected by indirect immunofluorescent staining for expressed CAT protein. As an internal control in these same cells, we coinjected the TRE-*lacZ* reporter plasmid, which is strongly induced by coexpression of small t and ERK1. β-Gal expression was assayed in these experiments by indirect immunofluorescent staining for β-Gal protein. Expression of ERK1 alone did not significantly activate the *fos*-CAT or TRE-*lacZ* reporter plasmids (Fig. 3G to I). However, coexpression of small t and ERK1 led to a strong activation of the *fos*-CAT construct (Fig. 3C). Staining for *lacZ* showed that the TRE-*lacZ* construct was also strongly induced in these same cells (Fig. 3B). Similarly, small t and ERK1 synergized to activate an SRE-*lacZ* reporter plasmid (data not shown). On the other hand, coexpression of small t mut 3 with ERK1 did not significantly induce either the *fos*-CAT or TRE-*lacZ* reporter plasmid (Fig. 3D to F). Since ERK1 has been shown to directly phosphorylate and activate the $p62^{TCF}$ transcription factor, these data imply that coexpression of small t and ERK1 leads to an activation of ERK1 kinase activity. Furthermore, since small t mut 3 was unable to synergize with ERK1 to induce the c-*fos* promoter, this activation appears to be dependent on the ability of small t to interact with PP2A. These experiments also indicate that the activation of ERK1 by small t can result in the enhancement of SRE- as well as TRE-regulated promoters.

Sontag et al. have recently shown that the introduction of small t alone into the SV40-permissive cell line CV-1 stimulates endogenous MEK and ERK activity and also promotes cell growth (47). Given these results, we decided to test whether expression of small t alone was sufficient to activate AP-1 in these cells. As shown in Fig. 4A, expression of wild-type small t alone led to the induction of AP-1 activity in these cells. This induction required the small t domain which interacts with PP2A, since expression of small t mut 3, which does not interact with PP2A, did not significantly activate AP-1 (Fig. 4B). Interestingly, expression of wild-type ERK1 alone in these cells strongly activates the TRE-*lacZ* construct (Fig. 4C). Moreover, this activation was not significantly enhanced by the coexpression of small t with ERK1 (Fig. 4D). Thus, these results suggest that the balance of the regulatory components of this kinase cascade is different in CV-1 cells than REF52 cells. Because small t was capable of inducing AP-1 on its own, we were able to test if coexpression of the dominant interfering ERKs with small t would inhibit AP-1 activation. If this were the case, it would suggest that the effects of small t are limited to the indirect activation of MEKs in these cells. We observed, however, that coexpression of K71R ERK1 and K52R ERK2 with small t only partially inhibited AP-1 activation (data not shown). Thus, small t may directly affect the regulation of the ERKs as well as the MEKs in CV-1 cells. However, it is also possible that the kinase-deficient ERK mutants are not complete inhibitors of endogenous ERK activities in these cells (47).

DISCUSSION

We have shown that coexpression of SV40 small t with either ERK1, MEK1, or a constitutively active form of c-Raf-1 (BXB) leads to an activation of AP-1 activity in REF52 cells, whereas expression of any one of these genes alone is inactive in this regard. We have also shown that the ability of small t and BXB to activate AP-1 requires the activity of endogenous MEKs and ERKs, since coexpression of dominant negative constructs either of ERK1 and ERK2 or of MEK1 blocks AP-1 induction. Similarly, the ability of small t and MEK1 to activate AP-1 depends on ERK activity, since this is inhibited by coexpression of the dominant negative ERK constructs. The activation of AP-1 by coexpression of small t and ERK1, however, may be independent of the function of Raf, as coexpression of dominant interfering MEK3 did not inhibit the AP-1 activation caused by small t and ERK1. However, we cannot conclude this with certainty, as cells contain several forms of MEK, and not all may be interfered with by the overexpression of the MEK3 construct used here.

In agreement with other studies, the present experiments indicate that activated Raf, MEK1, and ERK1 function in a pathway in which the stimulation of AP-1 by Raf is dependent on MEK and ERK function and the stimulation of AP-1 activity by MEK1 is dependent upon ERK function. We have also found that the ability of small t to synergize with these kinases for AP-1 activation required the presence of the small t domain which interacts with PP2A, since expression of small t mut 3, which lacks this domain, does not promote AP-1 activity. This observation suggests that PP2A may negatively regulate the activity of these kinases in vivo. The finding that small t inhibits the ability of PP2A to dephosphorylate activated ERK2 and MEK1 in vitro and in vivo supports this model.

We had previously shown that overexpression of the catalytic subunit of PP2A ($PP2A_C$) can activate AP-1 in quiescent cells (2). Because PP2A efficiently dephosphorylated the inhibitory C-terminal phosphorylation sites of c-Jun, it was hypothesized that overexpression of $PP2A_C$ might lead to the dephosphorylation of c-Jun and its subsequent activation. This could occur even if $PP2A_C$ inhibited the activation of the ERK pathway, since the dephosphorylated form of c-Jun is likely to be transcriptionally active (34). It is not clear if the ability of $PP2A_C$ to activate AP-1 accurately reflects what occurs in vivo, however, since PP2A is normally complexed with two other regulatory subunits (13). When expressed in cells, the free catalytic subunit may directly dephosphorylate the inhibitory phosphorylation sites of c-Jun while the holoenzymes do not.

The induction of c-*fos* transcription following the exposure of quiescent cells to serum growth factors has been shown to depend on the activity of a transcription factor complex which binds to the SRE within the c-*fos* promoter (21, 23, 49). The ability of this complex to transactivate the c-*fos* gene, in turn, has been shown to depend on the phosphorylation of $p62^{TCF}$ (Elk-1) by ERKs (35). Thus, the activation of the c-*fos* promoter following small t and ERK1 coexpression demonstrates that the effects of small t on transcription are not limited to AP-1-responsive promoters and also include SRE-regulated genes. Thus, these data suggest that other genes whose promoter activity is dependent on ERK activity may be activated by coexpression of small t and ERK1.

Because others have observed that expression of small t

alone in the SV40-permissive cell line CV-1 is sufficient to induce ERK2 and MEK1 activity (47), we examined whether its expression could activate AP-1 in these cells. Our results show that expression of either small t or ERK1 alone in quiescent CV-1 cells strongly induces AP-1 and that this induction cannot be significantly enhanced by coexpression of these two genes. This was not the case in REF52 cells, wherein coexpression of small t and either ERK1, MEK1, or BXB was required for AP-1 activation. Since PP2A exists as a heterotrimeric complex in vivo, and the overall levels of $PP2A_C$ do not appear to differ between these two cell types (data not shown), these findings may reflect differences in the levels of the PP2A regulatory subunits between these two cell types. Biochemical characterization of the PP2A isoforms which regulate AP-1 activity will be necessary to explain differences between small t activity in CV-1 and REF52 cells.

Previous work has demonstrated that expression of SV40 large T is sufficient to transform cycling but not resting cells (5, 6, 8, 11, 14, 19, 36, 37, 41, 45, 46). Transformation can occur in resting cells, however, if either small t is coexpressed or the cells are treated with phorbol esters. One interpretation of this data is that small t functions by stimulating cell growth, thereby promoting stable transformation by large T. The observations described here suggest that small t accomplishes this by inhibiting the ability of PP2A to downregulate the activity of serum-responsive kinases such as MEK and ERK (47), thereby stimulating gene expression from TRE- and SRE-regulated promoters.

Several groups have cloned isoforms of a dual-specificity phosphatase from mammalian cells that dephosphorylate phosphotyrosine as well as phosphoserine- and phosphothreonine-containing substrates (10, 24, 29, 48). Among these is the protein encoded by the immediate-early gene 3CH134, or MKP-1 (48). The finding that activated ERK2 is an excellent substrate for MKP-1 as well as the observation that constitutive expression of MKP-1 inhibits ERK activation strongly suggests that MKP-1 acts in vivo as an ERK phosphatase. In light of this finding, it is tempting to speculate that PP2A isoforms function in vivo as MEK phosphatases. We are currently investigating this possibility.

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