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Induction of Early Growth Response-1 Gene Expression by Calmodulin Antagonist Trifluoperazine through the Activation of Elk-1 in Human Fibrosarcoma HT1080 Cells*

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The early growth response gene-1 (Egr-1) is a transcription factor that plays an important role in cell growth and differentiation. It has been known that *Egr-1* expression is down-regulated in many types of tumor tissues, including human fibrosarcoma HT1080 cells, and introduction of the *Egr-1* gene into HT1080 cells inhibits cell growth and tumorigenic potential. Trifluoperazine (TFP), a phenothiazine class calmodulin antagonist, is known to inhibit DNA synthesis and cell proliferation and potentially important in antitumor activities. To understand the regulatory mechanism of *Egr-1*, we investigated the effect of TFP on expression of *Egr-1* in HT1080 cells. Herein, we report that *Egr-1* expression was increased by TFP in synergy with serum at the transcriptional level. Both the Ca^{2+}/calmodulin-dependent protein kinase II inhibitor KN62 and the calcineurin inhibitor cyclosporin A enhanced TFP-dependent increase of Egr-1, suggesting that the Ca^{2+}/calmodulindependent pathway plays a role in regulation of *Egr-1* expression in HT1080 cells. The TFP-stimulated increase of the Egr-1 protein was preferentially inhibited by the MEK-specific inhibitor PD98059. In addition, activation of human *Egr-1* promoter and the transcriptional activation of the ternary complex factor Elk-1 induced by TFP were inhibited both by pretreatment of PD98059 and by expression of the dominant-negative RasN17. These results indicate that the Ras/MEK/Erk/Elk-1 pathway is necessary for TFP-induced *Egr-1* expression. We propose that the calmodulin antagonist TFP stimulates *Egr-1* gene expression by modulating Ras/MEK/Erk and activation of the Elk-1 pathway in human fibrosarcoma HT1080 cells.

Ca^{2+} is an important intracellular messenger in many biological processes. Calmodulin is a ubiquitous Ca^{2+}-binding protein that acts as a Ca^{2+} mediator (1). Ca^{2+}-bound calmodulin regulates the activities of a large number of enzymes, including calmodulin-dependent protein kinases (CaMK)[1] such as CaMK-II and CaMK-IV. Calmodulin is involved in the Ca^{2+}-mediated regulation of gene expression and in the regulation of cellular proliferation process, including DNA synthesis and cell cycle progression (2–6). It has been reported that the calmodulin level is elevated in several tumor cell lines (3, 7). Moreover, *in vivo* treatment with calmodulin antagonists has been shown to reduce tumor size (8), indicating that calmodulin plays an important role in control of cell growth. It is now known that calmodulin antagonists are cytotoxic against tumor cells and can restore sensitivity to drug-resistant cells, thus addressing the possibility that calmodulin antagonists may be valuable chemotherapeutic agents in the treatment of certain cancers (9, 10). However, little information is available regarding the mechanisms responsible for regulation of cell growth by calmodulin antagonists.

Trifluoperazine (TFP) is a phenothiazine derivative antipsychotic drug. TFP is a well known calmodulin antagonist that has been used for studying the function of calmodulin (11, 12). Previous reports have demonstrated that TFP inhibits DNA synthesis and cell proliferation and, thereby, is potentially an important antitumor agent, as well as having antipsychotic properties (13).

The product of the early growth response gene *Egr-1* (14), which is also known as NGFI-A (15), zif268 (16), krox24 (17), or Tis8 (18), is a transcription factor that has three Cys_2-His_2-type zinc finger-containing DNA binding domains in the C-terminal portion of the molecule. Egr-1 preferentially binds to GC-rich regulatory elements with the consensus sequence of GCGGGGGCG or TCCTCCTCCTCC (16, 19), leading to induction or repression of its target genes. It has been demonstrated that *Egr-1* is important in regulating cell growth, differentiation, and development (20). Expression of *Egr-1* is significantly

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[1] The abbreviations used are: CaMK, calmodulin-dependent protein kinase; TFP, trifluoperazine; TCF, ternary complex factor; DMEM, Dulbecco's modified Eagle's medium; MOPS, 4-morpholinepropanesulfonic acid; kb, kilobase(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; CMV, cytomegalovirus; PMA, phorbol 12-myristate 13-acetate; CPZ, chlorpromazine; SRE, serum response element; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; JNK, c-Jun N-terminal kinase; KSR, kinase suppressor of Ras.

reduced in a number of tumor cells (21, 22), and loss of expression is closely associated with tumor formation in mammalian cells and tissues (22). On the other hand, stable expression of *Egr-1* inhibited cell proliferation and soft agar growth in NIH3T3 cells transformed with v-*sis*, indicating that *Egr-1* functions as a tumor suppressor (23).

We examined the effect of TFP on expression of the tumor suppressor *Egr-1* in human fibrosarcoma HT1080 cells. Our results revealed that treatment of cells with serum and TFP increased levels of both mRNA and the protein of *Egr-1*. The increment was associated with the increase of *Egr-1* promoter activity through activation of the ternary complex factor (TCF) Elk-1.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and LipofectAMINE 2000 were purchased from Life Technologies, Inc. (Gaithersburg, MD). Cyclosporin A, KN62, and ionomycin were obtained from Calbiochem (San Diego, CA). Trifluoperazine, chlorpromazine, and GF109203X were products of RBI (Natick, MA). Antibodies against Egr-1 and ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). *Trans*-activator plasmid pFA2-Elk1 (amino acids 307–427), which contains the GAL4 DNA binding domain (amino acids 1–147), and the reporter plasmid pFR-Luc, which contains five repeats of GAL4 binding elements and the luciferase gene, were obtained from Stratagene (La Jolla, CA). The pCMV/β-gal plasmid and assay kits for luciferase and β-galactosidase activity were purchased from Promega (Madison, WI). The cDNA probe for *Egr-1* was provided by Dr. I. K. Lim of the Ajou University School of Medicine, South Korea.

Western Blot Analysis—Cells were lysed in 20 mM HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (24). Protein samples (20 μg of each) were separated by SDS-polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose filters. The blots were incubated with anti-Egr-1 antibodies and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). The same blot was stripped and reprobed with anti-ERK antibody for use as an internal control. In some experiments, the scanning densitometry was performed after exposure to x-ray film. The relative band intensities of Egr-1 and ERK1/2 in each lane were measured by quantitative scanning densitometer and image analysis software, Bio-1D version 97.04.

Northern Blot Analysis—Total RNA was isolated according to the methods described previously by Chomczymski and Sacchi (25). 10 μg of total RNA was separated on 1.2% agarose gel containing 6% formaldehyde in 0.02 M MOPS, pH 7.0, 8 mM sodium acetate, and 1 mM EDTA, then transferred to a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech) by the capillary method. Cross-linking was then performed by UV irradiation. The membrane was incubated overnight at 42 °C in Northern-Max hybridization solution (Ambion, Inc., Austin, TX) containing [α-^{32}P]dCTP-labeled probes. The probes used were *Egr-1* (the 1.4-kb-long *Eco*RI fragment purified from the pGEM/TIS8 plasmid) and GAPDH (the 0.5-kb *Xba*I-*Hin*dIII fragment from the pUC/GAPDH plasmid). The membranes were then washed with 2× SSC/0.1% SDS for 20 min at room temperature, 2× SSC/0.1% SDS at 42 °C for 30 min, and 0.5× SSC/0.1% SDS for 30 min at 65 °C. For rehybridization, the probes were stripped from the membrane by boiling in 0.1× SSC/0.5% SDS.

Human Egr-1 Promoter Construction—Human *Egr-1* promoter fragments (26) spanning from nucleotides −668 to +1 and −454 to +1 were synthesized by PCR in a reaction containing 0.5 μg of human *Egr-1* genomic clone TIS8 (obtained from ATCC) as a template with the following upstream primers: −668, 5′-CCCGCACTCCCggtaccCTCTCAC-3′, and −454, 5′-TCCCGGCTTggtaccAGGGAGGA-3′. A *Kpn*I restriction site is indicated by *lowercase letters*. A single downstream primer (5′-CTCTCGaagcttCCCGGATCCGC-3′) containing a *Hin*dIII site, which is indicated by *lowercase letters*, was used in each PCR amplification. The PCR fragments were then digested with the restriction enzymes *Kpn*I and *Hin*dIII. The fragments were extracted from the agarose gel and inserted into the *Kpn*I and *Hin*dIII sites of the pGL2-basic luciferase-encoding reporter vector (Promega, Madison, WI). Plasmid p-233egrLuc was constructed by digestion of p-688egrLuc with the restriction enzymes *Sac*I and *Hin*dIII, followed by ligation into the pGL2 basic vector digested by *Sac*I and *Hin*dIII. The resultant constructs were verified by DNA sequencing.

Cell Culture, Transient Transfection, and Reporter Gene Assay—HT1080 cells were grown in DMEM with 10% heat-inactivated fetal calf serum. For *Egr-1* promoter analysis, HEK293T cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum. One day after seeding cells into 35-mm dishes (6×10^5 cells), the cells were cotransfected with 0.5 μg of 5′-deletion constructs of the *Egr-1* promoter and 0.2 μg of pCMV/β-gal plasmid using LipofectAMINE 2000 reagents according to the manufacturer's instructions. Where indicated, mammalian expression vectors of dominant-active Ras61L, pZip/Ras61L, and dominant-negative RasN17, pMT/RasN17, (0.5 μg each) were included. For analysis of Elk-1 activity, pFA2-Elk1 (50 ng) and pFR-Luc (0.5 μg) were cotransfected into HEK293T cells. Plasmid pCMV/β-gal was included to monitor the transfection efficiency. The total amount of DNA was maintained at 1–2 μg with an empty vector pCDNA3.0. Twenty-four hours post-transfection, cells were treated with TFP. Cells were harvested after 6–12 h of TFP treatment, and protein extracts were prepared by three cycles of freezing and thawing. 1–5 μg of protein was assayed for luciferase and β-galactosidase activities. Luminescence was measured using a luminometer model TD 2020 (Berthold, Tubingen, Germany). Transfection efficiencies were normalized by a ratio of luciferase activity to β-galactosidase activity obtained from the same sample.

RESULTS

Egr-1 Expression by Serum Is Rescued in the Presence of TFP or CPZ in Human Fibrosarcoma HT1080 Cells—It has been reported that stable expression of *Egr-1* leads to decreased DNA synthesis and tumorigenesis in fibrosarcoma HT1080 cells (23). We investigated whether inhibition of DNA synthesis by calmodulin antagonists is related to a gain in *Egr-1* expression. Phorbol ester (phorbol 12-myristate 13-acetate (PMA)) was used as a positive control. When cells in the log phase were treated with TFP for 2 h, a slight increase in the amount of the Egr-1 protein was observed (Fig. 1*A*). Serum-starved cells showed a marginal increase in the Egr-1 protein level when the cells were treated with 20% serum. However, an increase was more evident in cells treated with 25 μM TFP. Costimulation with serum and TFP resulted in a dramatic increase in the level of Egr-1 expression. Another calmodulin antagonist chlorpromazine (CPZ) also increased the level of the Egr-1 protein (Fig. 1*B*). Reprobing the blots with anti-ERK1/2 antibodies revealed that similar amounts of proteins were present in all lanes. These results indicate that costimulation with serum and calmodulin antagonist has a synergistic effect on expression of Egr-1 in serum-starved HT1080 cells.

Kinetics of TFP-dependent Egr-1 Expression—To determine the effect of TFP on the increase in the Egr-1 expression level, HT1080 cells were serum-starved for 24 h, then treated with 20% serum in the presence of various concentrations of TFP. The Egr-1 protein was detected in cells treated with serum and 10 μM TFP. The protein level was markedly increased at a concentration of 50 μM TFP (Fig. 2*A*). When the effect of this concentration of TFP on cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, the cell survival was about 50% of untreated control (data not shown). Time course studies showed that an increase in the Egr-1 protein level due to TFP was detected at 2 h with a continual increase through 3 h after addition of TFP (Fig. 2*B*). Reprobing the blots with anti-ERK antibodies revealed that similar amounts of proteins were present in all lanes.

Northern blot analysis was performed to determine whether the increase in the expression level of the Egr-1 protein due to TFP occurs at the mRNA level. The GAPDH mRNA level was used as an internal control. Expression of *Egr-1* mRNA was markedly increased by treatment with 50 μM TFP (Fig. 3*A*). This result was consistent with results of Western blot analysis. Time course studies showed that the *Egr-1* mRNA level was increased at 0.5 h after addition of serum and TFP with a maximum increase at 1 h, followed by a slow decline (Fig. 3*B*).

Egr-1 Promoter Activity Is Stimulated by Serum and

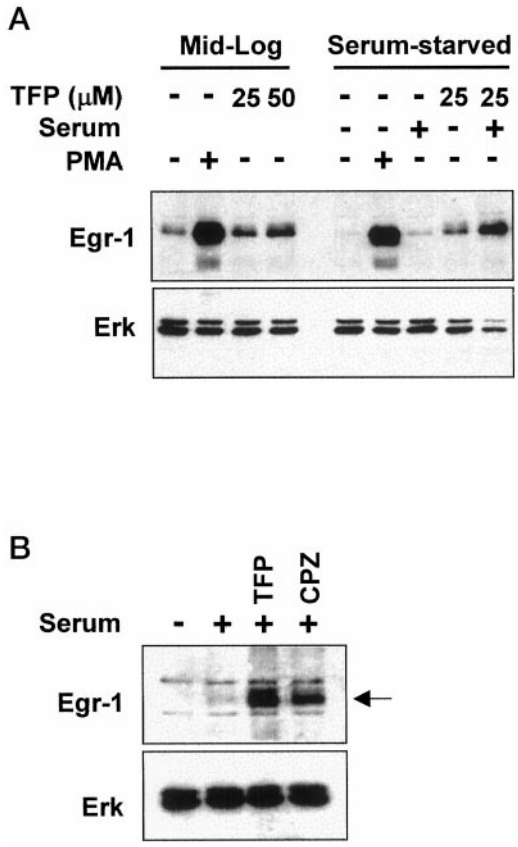

FIG. 1. **Effect of calmodulin antagonists on Egr-1 expression.** *A*, mid-log cultured or serum-starved (with 0.5% serum for 24 h) HT1080 cells were pretreated with the indicated concentrations of TFP for 30 min and treated with 20% serum for an additional 2 h. 50 nM PMA was used as a positive control. The Egr-1 level was detected in whole cell lysates (20 μg/lane) by Western blotting against rabbit anti-Egr-1 antibodies (1:1000). The *lower panel* shows the same blot stripped and reprobed with rabbit anti-ERK1/2 antibodies for an internal control of the protein contents per lane. *B*, serum-starved HT1080 cells treated with TFP (50 μM) or CPZ (50 μM) for 30 min before addition of 20% serum. After 2 h, whole cell lysates were prepared and Western blotting was performed as in *A*.

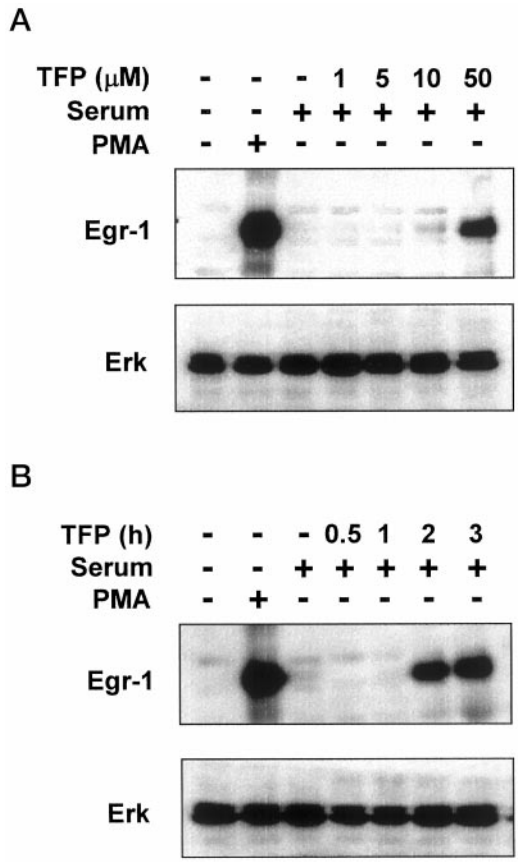

FIG. 2. **Dose- and time-dependent increase of the Egr-1 protein by TFP.** Serum-starved HT1080 cells (0.5% serum for 24 h) were treated with various concentrations of TFP for 2 h (*A*) or 50 μM TFP for the indicated times (*B*). PMA (50 nM) was used as a positive control. Whole cell lysates were analyzed by Western blotting against rabbit anti-Egr-1 antibodies (1:1000). The *lower panel* shows the same blot stripped and reprobed with rabbit anti-ERK1/2 antibodies (1:6000) as an internal control of the protein contents per lane.

TFP—To determine whether TFP causes an increase in *Egr-1* mRNA at the transcription level, we constructed a series of luciferase reporter plasmids containing serial deletions of 5′-flanking sequences and the TATA motif of the human *Egr-1* promoter (26). Each promoter-reporter construct was transfected with pCMV/β-gal for transfection efficiency into HEK293T cells. Twenty-four hours after transfection, cells were treated without or with 50 μM TFP (Fig. 4). Results were expressed as -fold increases in luciferase activity normalized for β-galactosidase activity. The −668 and −454 constructs exhibited 20- and 14-fold increased luciferase activity, respectively, due to TFP treatment. A further deletion between −454 to −233 (p-233egrLuc), which removed the SRE cluster, displayed 4.2-fold increased promoter activity, suggesting that SRE cluster region is important for the promoter activity increment. These results indicate that TFP stimulates *Egr-1* gene expression at the transcription level.

The Ca^{2+}/CaM-dependent Pathway Is Involved in Serum and TFP-stimulated Egr-1 Expression—Calmodulin modulates the action of various calmodulin-binding proteins, including CaMK-II and calcineurin. Therefore, we investigated whether the Ca^{2+}/CaM/CaMKII/calcineurin pathway is involved in TFP-dependent *Egr-1* induction. HT1080 cells were treated in both the presence and absence of TFP with the Ca^{2+} ionophore ionomycin and KN62, a selective inhibitor of CaMK-II. After exposure to x-ray film, the relative intensities of Egr-1 and ERK1/2 bands in each lane were determined by quantitative scanning densitometer. As shown in Fig. 5*A*, ionomycin alone had no effect on Egr-1 induction, whereas serum- and TFP-dependent Egr-1 expression was significantly attenuated by the addition of ionomycin. In addition, KN62 and cyclosporin A, a calcineurin inhibitor, enhanced TFP-dependent Egr-1 expression (Fig. 5, *B* and *C*). These results suggest that the Ca^{2+}/CaM/CaMK-II pathway negatively regulates Egr-1 expression.

PKC Is Not Required for Egr-1 Accumulation Induced by Serum and TFP—It has been reported that calmodulin antagonists modulate protein kinase C (PKC) activity (27) and that the PKC activator PMA stimulates *Egr-1* gene induction (Figs. 1–3). We investigated the effect of PKC inhibitors on TFP-dependent Egr-1 expression. Serum-starved HT1080 cells were incubated with specific inhibitors of PKC (rottlerin and GF109203X) before addition of serum and TFP. Results showed that PKC inhibitors did not have any significant effect on TFP-dependent Egr-1 expression (Fig. 6*A*). To further determine the role of PKC, the effect of down-regulation of PKCs by prolonged treatment with high concentrations of PMA was examined (Fig. 6*B*). Down-regulation of PKCs was determined by Western blotting using anti-PKCα antibody. After prolonged treatment with 1 μM PMA, PKCα was no longer detected. Because Egr-1 was not efficiently induced by retreatment with 50 nM PMA, full suppression of PKC activity is indicated. Under these conditions, Egr-1 remained inducible by stimulation with serum and TFP (compared with control cells). These

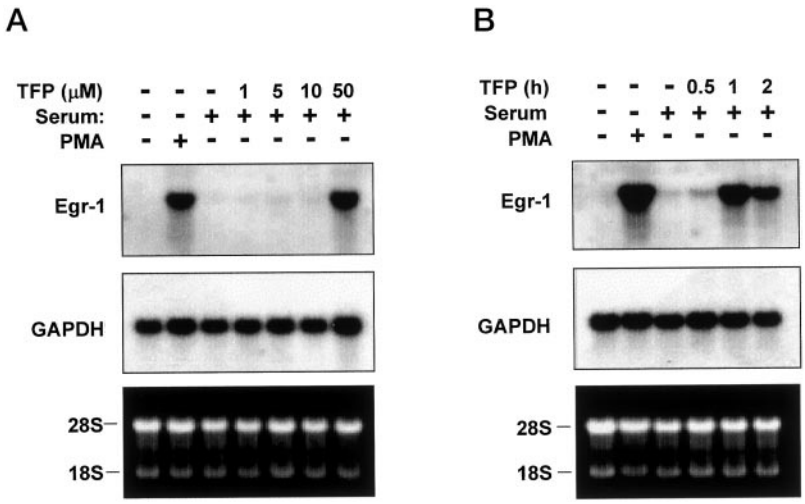

FIG. 3. **Dose- and time-dependent induction of *Egr-1* mRNA expression by TFP.** HT1080 cells were serum-starved, treated with various concentrations of TFP for 2 h (*A*) or 50 μM TFP for the indicated times (*B*). PMA (50 nM) was used as a positive control. Total RNA (10 μg) was isolated from cells, electrophoresed on 1% agarose-gel, and capillary transferred to a nylon filter. The blot was hybridized with the ^{32}P-labeled *Egr-1* probe. GAPDH mRNA was determined as a control to verify the amount of RNA in each lane. The *lower panel* shows ethidium bromide stained total RNA.

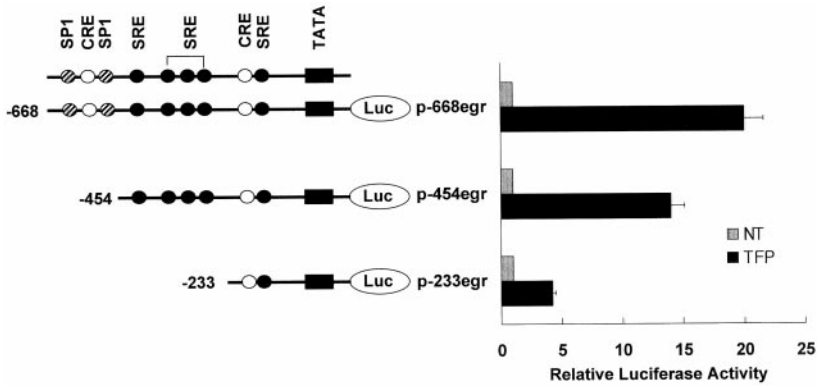

FIG. 4. **Effect of TFP on human *Egr-1* promoter activity.** The 5′ boundaries of plasmids containing various truncations of the *Egr-1* promoter fused to luciferase reporter genes are shown. The numeric designations of each construct refer to the 5′-deletion end points derived from the transcription start site at +1. The positions of putative regulatory elements (*SP1*, *CRE*, and *SRE*) are indicated by *circles*. *A*, human HEK293T cells cultured on 35-mm dishes were cotransfected with each construct of the *Egr-1* promoter (0.5 μg) and the pCMV/β-gal plasmid (0.2 μg). After 24 h of transfection, cells were treated without (*NT*) or with 50 μM TFP. *B*, human HEK293T cells cultured on 35-mm dishes were cotransfected with p-233egrLuc (0.5 μg) and the pCMV/β-gal plasmid (0.2 μg). After 24 h of transfection, cells were treated with the indicated concentrations of TFP. After incubation for an additional 12 h, cells were harvested and protein extracts were prepared by three cycles of freezing and thawing. 1 μg of protein was assayed for luciferase and β-galactosidase activities. Results are shown as the amount of induction after correcting for β-galactosidase activity. *Bars* represent the mean of a single experiment performed in triplicate ± S.D. Similar results were observed in three independent experiments.

results demonstrate that PKCs are not involved in TFP-dependent Egr-1 expression.

The MEK Pathway Is Necessary to Stimulate Egr-1 Expression by TFP—To determine the signal pathway involved in the TFP response, several kinase inhibitors were tested (Fig. 7*A*). Protein-tyrosine kinase inhibitor (genistein), phosphatidylinositol 3-kinase inhibitor (LY294002), and PLC inhibitor (U73122) had no effect on Egr-1 expression due to serum and TFP. In contrast, PD98059, a specific MEK inhibitor, efficiently inhibited TFP-stimulated Egr-1 expression. For confirmation, the effect of PD98059 concentration dependence was examined (Fig. 7*B*). Addition of increasing concentrations of PD98059 progressively lowered the level of Egr-1 expression due to serum and TFP, indicating that the MEK signaling cascade is required for TFP-dependent Egr-1 expression.

TFP-dependent Activation of the Egr-1 Promoter Is Inhibited by Dominant-negative Mutants of Ras—To demonstrate that the Ras/MEK pathway responds to TFP-stimulated activation of the *Egr-1* promoter, HEK293T cells were transiently cotransfected with a p-688egrLuc construct and either a dominant-negative mutant (pMT/RasN17) or a dominant-active mutant (pZip/Ras61L) of the Ha-Ras expression plasmid. As shown in Fig. 7*C*, TFP-stimulated *Egr-1* promoter activity was efficiently inhibited by dominant-negative RasN17 or by pretreatment with PD98059. However, the expression of dominant-active Ras61L continuously stimulated *Egr-1*. These data indicate that the Ras/MEK pathway plays a role in TFP-dependent *Egr-1* gene expression.

ERK1/2 Are Not Directly Activated by TFP—To investigate whether TFP stimulates ERK, a downstream target of MEK, we first examined the kinetics of ERK activation in response to serum stimulation in HT1080 cells using antibodies specific for phosphorylated forms of ERK1 and ERK2. In a control experiment for ERK1 and ERK2 phosphorylation, quiescent 3Y1 fibroblasts treated with serum were included. HT1080 cells were serum-starved for 24 h, then treated with 20% serum for various times. Phosphorylation of ERK was analyzed by Western blot analysis. As shown in Fig. 8*A*, increased phosphorylation of ERK1/2 was detected after 30 min of serum stimulation in 3Y1 fibroblasts. In contrast, a relatively high level of ERK1/2

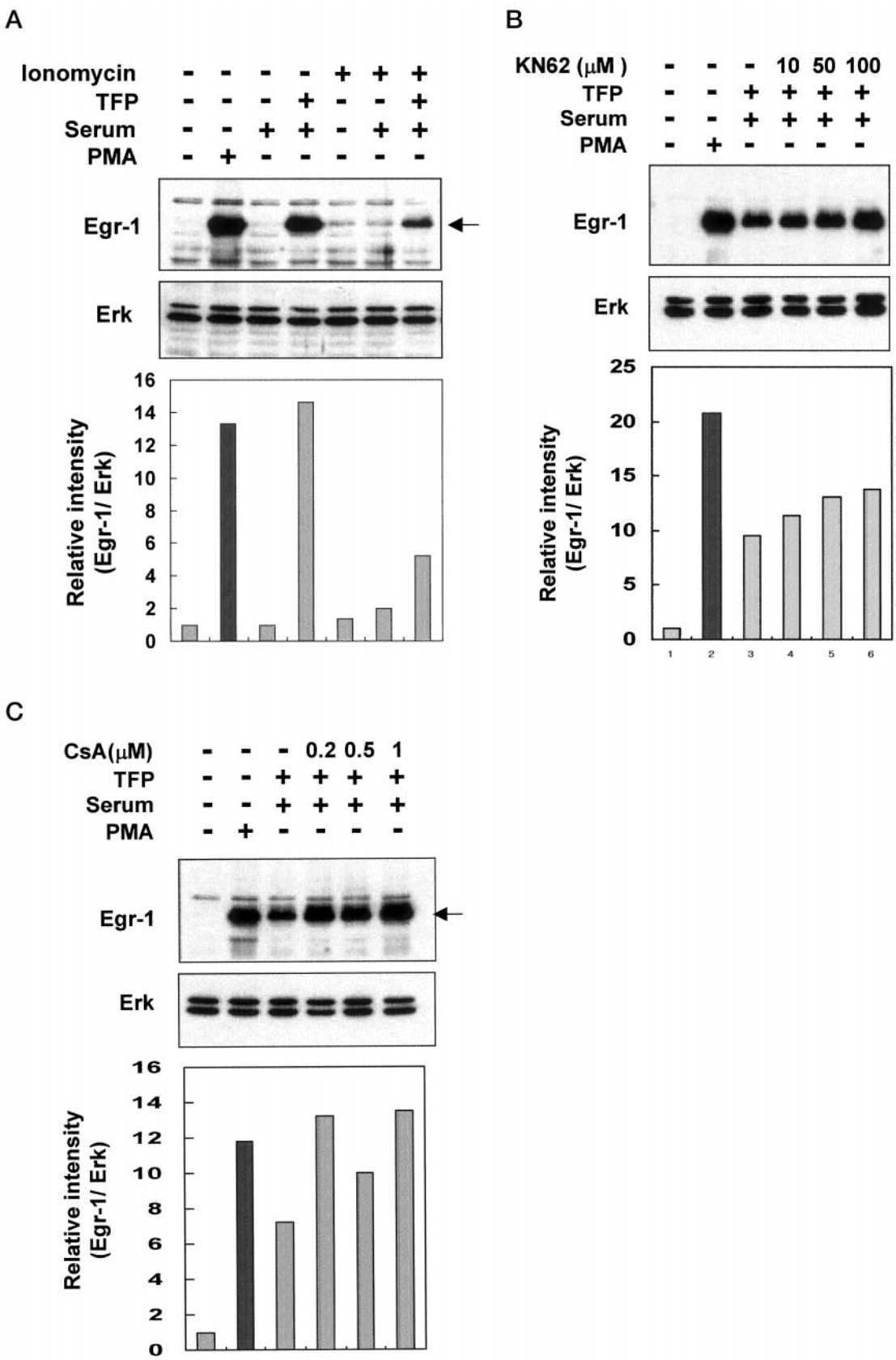

FIG. 5. **Effect of ionomycin, KN62, and CsA on TFP-mediated Egr-1 expression.** *A*, HT1080 cells were serum-starved, pretreated with TFP (50 μM) for 30 min, then exposed to 20% serum in the absence or presence of ionomycin (0.5 μM). *B* and *C*, serum-deprived HT1080 cells were pretreated with TFP (25 μM) for 30 min together with various concentrations of either KN62 (*B*) or CsA (*C*), then exposed to 20% serum. After an additional incubation for 2 h, cells were harvested and whole cell lysates were prepared for Western blot analysis against rabbit anti-Egr-1 antibodies (1:1000). The *middle panel* shows the same blot stripped and reprobed with rabbit anti-ERK1/2 antibodies (1:6000) as an internal control of the protein contents per lane. The Egr-1 band is indicated by an *arrow*. The *bottom panel* shows the relative intensity of the fluorographic data quantified by densitometry, and the ratio of Egr-1 to ERK1/2 was determined.

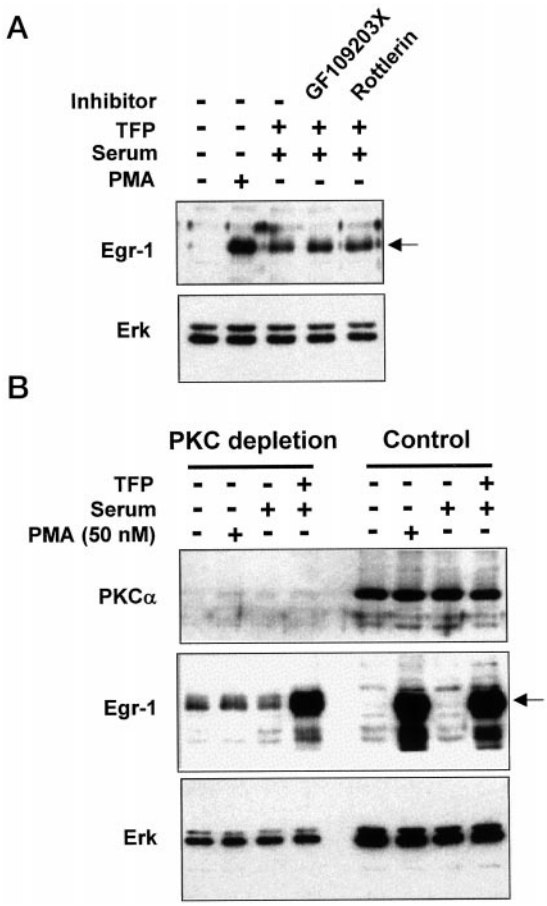

FIG. 6. **PKC is not involved in TFP-mediated Egr-1 induction.** *A*, HT1080 cells were serum-starved, pretreated with either GF109203X (2 μM) or rottlerin (20 μM) for 30 min, then treated with TFP (50 μM). After incubation for 30 min, cells were exposed to 20% serum. After an additional incubation for 2 h, cells were harvested and whole cell lysates were prepared for Western blot analysis against rabbit anti-Egr-1 antibodies (1:1000). The *lower panel* shows the same blot stripped and reprobed with rabbit anti-ERK1/2 antibodies (1:6000) as an internal control of the protein contents per lane. The Egr-1 band is indicated by an *arrow*. *B*, HT1080 cells were serum-starved in the presence (*PKC depletion*) or absence (*control*) of 1 μM PMA for 24 h. After incubation, cells were pretreated with TFP (50 μM) for 30 min, then exposed to 20% serum. PMA (50 nM) was used as a positive control. After an additional incubation for 2 h, cells were harvested and whole cell lysates were prepared for Western blot analysis against anti-PKCα (*upper panel*), anti-Egr-1 (*middle panel*), and anti-ERK1/2 (*lower panel*) antibodies. An *arrow* indicates the Egr-1 protein.

phosphorylation was detected in unstimulated HT1080 cells and a further increase of phosphorylation was not observed until 120 min after serum stimulation, demonstrating that ERK1/2 are continuously activated regardless of serum stimulation in HT1080 cells. These results are consistent with previous reports that HT1080 cells are classified as type III tumor cells with regard to the constitutive strong activation of 41- and 43-kDa MAPKs (28). Fig. 8*B* shows that serum and TFP had no effect on the increase of ERK1/2 phosphorylation, which was inhibited by pretreatment with PD98059. Based on these observations we hypothesize that downstream of ERK1/2 is involved in activation of the *Egr-1* promoter in response to TFP.

Elk-1 Is Involved in TFP-dependent Egr-1 Expression—Previous studies have demonstrated that Ca^{2+}/calmodulin-dependent phosphatase calcineurin negatively regulates the transcription activation of Elk-1 (29, 30). Serum-induced *Egr-1* transcription is controlled through a serum response element (SRE), which binds a complex of the serum response factor and a member of the ternary complex factor (TCF). The TCF family includes Elk-1 (31), Sap-1 (32), and Sap-2/Net/Erp (33–35). The classical Ras/MEK/ERK cascade is responsible for the phosphorylation of Elk-1, and Elk-1 phosphorylation by ERK correlates with increased transcription activation (36).

To examine whether Elk-1 is involved in TFP-dependent *Egr-1* expression, a pFA-Elk1 construct that encodes the fusion protein of the Gal4 DNA binding domain and the activation domain of Elk-1 (amino acids 307–428) was transfected into HEK293T cells. pFR-Luc, which contains five repeats of the Gal4 binding element upstream of the luciferase reporter gene, was also transfected. This assay system allows direct assessment of Elk-1-mediated transcription activation in response to stimuli. When cells were treated with serum and TFP, activation of Elk-1 was observed, which was inhibited in a dose-dependent manner by pretreatment with PD98059 (Fig. 9*A*). In addition, TFP-stimulated Elk-1 activation was inhibited by expression of dominant-negative RasN17 (Fig. 9*B*). Dominant-active Ras61L constitutively stimulated Elk-1 transactivation, which was partially inhibited by PD98059, confirming that MEK is necessary for TFP-stimulated Elk-1 activation. Together, these results support a model in which the calmodulin-dependent pathway stimulates *Egr-1* expression by modulating Ras/MEK/ERK/Elk-1 cascades.

DISCUSSION

It is well known that *Egr-1* is an immediate-early response transcription factor that is rapidly induced in many different cell types by a variety of growth factors, cytokines, and injurious stimuli (37). Although *Egr-1* is rapidly expressed due to mitogenic signals, evidence is accumulating that suggests that *Egr-1* negatively regulates cell growth. *Egr-1* is little or not expressed in a number of tumor cells, including breast carcinoma ZR-75-1, glioblastoma U251, osteosarcoma Saos-2, and fibrosarcoma HT1080 cells (23). Expression of antisense *Egr-1* mRNA in v-*sis*-transformed NIH3T3 cells leads to an enhanced transformed phenotype (38), indicating that loss of *Egr-1* gene expression is important for tumorigenic potential. On the contrary, introduction of *Egr-1* into HT1080 fibrosarcoma cells inhibits cell growth and tumorigenesis (21–23) by induction of transforming growth factor-β1, fibronectin, and the plasminogen activator inhibitor-1 (20, 39), supporting the notion that *Egr-1* does possess a growth suppressor activity.

TFP is a calmodulin antagonist that binds to calmodulin and inhibits Ca^{2+}/calmodulin-regulated enzyme activities. Calmodulin is a Ca^{2+}-binding protein present in all eukaryotic cells that plays a role as a Ca^{2+} mediator (1). Recent evidence suggests that calmodulin is involved in regulation of cellular proliferation, and calmodulin antagonists offer the potential for new therapeutic approaches in the treatment of certain cancers. However, the mechanisms responsible for the antitumor activities of calmodulin antagonists remain to be determined.

We found that the calmodulin antagonists TFP and CPZ stimulate *Egr-1* gene expression in human fibrosarcoma HT1080 cells. Stimulation of Egr-1 expression by TFP was more evident in serum-starved HT1080 cells than in mid-log phase cells (Fig. 1*A*). We also found that costimulation with serum and TFP synergistically enhances *Egr-1* expression by transcriptional events (Figs. 3 and 4). We hypothesize that serum-induced *Egr-1* transcription in HT1080 cells is suppressed by some unknown factors, and this repression can be reversed by treatment with calmodulin antagonists.

To investigate whether the calmodulin-dependent pathway is involved in suppression of serum-induced Egr-1, we used the inhibitors KN62 (inhibitor of CaMKII) and cyclosporin A (inhibitor of calcineurin). In the presence of serum, TFP-mediated Egr-1 expression was decreased by treatment with calcium ionophore and ionomycin, whereas expression was enhanced by

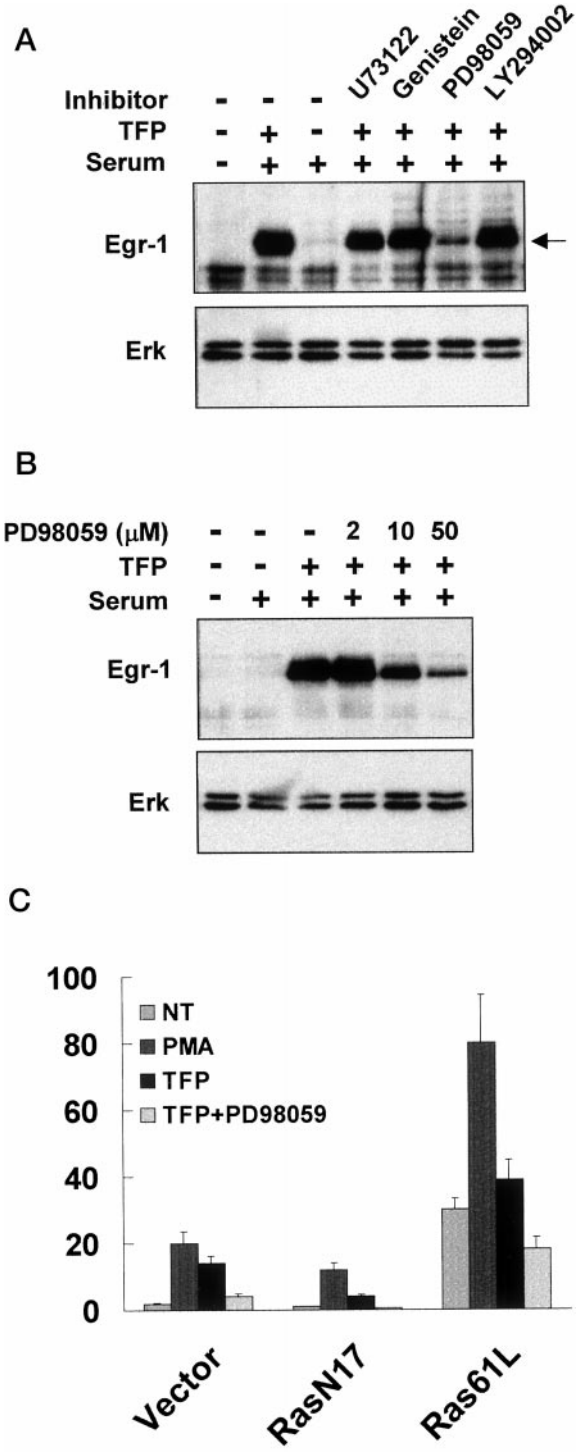

FIG. 7. **Effect of Ras/MEK cascades on TFP-mediated Egr-1 expression.** *A*, HT1080 cells were serum-starved for 24 h, preincubated with U73122 (10 μM), genistein (100 μM), PD98059 (50 μM), or Ly294002 (50 μM) for 30 min, then treated with TFP (50 μM). After incubation for 30 min, cells were exposed to 20% serum. After an additional incubation for 2 h, cells were harvested and whole cell lysates were prepared for Western blot analysis against rabbit anti-Egr-1 antibodies (1:1000). The *lower panel* shows the same blot stripped and reprobed with rabbit anti-ERK1/2 antibodies (1:6000) as an internal control of the protein contents per lane. The Egr-1 band is indicated by an *arrow*. *B*, HT1080 cells were serum-starved, pretreated with the indicated concentrations of PD98059 for 30 min, then treated with TFP (50 μM). After incubation for 30 min, cells were exposed to 20% serum. After an additional incubation for 2 h, cells were harvested and whole cell lysates were prepared for Western blot analysis against rabbit anti-Egr-1 antibodies (1:1000). The *lower panel* shows the same blot stripped and reprobed with rabbit anti-ERK1/2 antibodies (1:6000) as an internal control of the protein contents per lane. The Egr-1 band is indicated by an *arrow*. *C*, HEK293T cells were transfected with the p-688EgrLuc construct (0.5 μg) together with expression vectors for dominant-active Ras61L (0.5 μg), and dominant-negative RasN17 (0.5 μg). The pCMA/β-gal plasmid (0.2 μg) was included as an internal control for normalization of transfection efficiency. The total amount of DNA was maintained at 1.2 μg with the pcDNA3.0 plasmid. After transfection, cells were serum-starved and treated with either PMA (20 nM) or serum in the absence or presence of PD98059 (50 μM). After 12 h the luciferase and β-galactosidase activities were measured. Normalization for transfection efficiency was determined by β-galactosidase expression. Results are presented as the amount of induction considering the luciferase activity of the cells that were not treated (*NT*) transfected with the empty pCDNA3.0 vector. *Bars* represent the mean of a single experiment performed in triplicate $\pm$ S.D. Similar results were observed in three independent experiments.

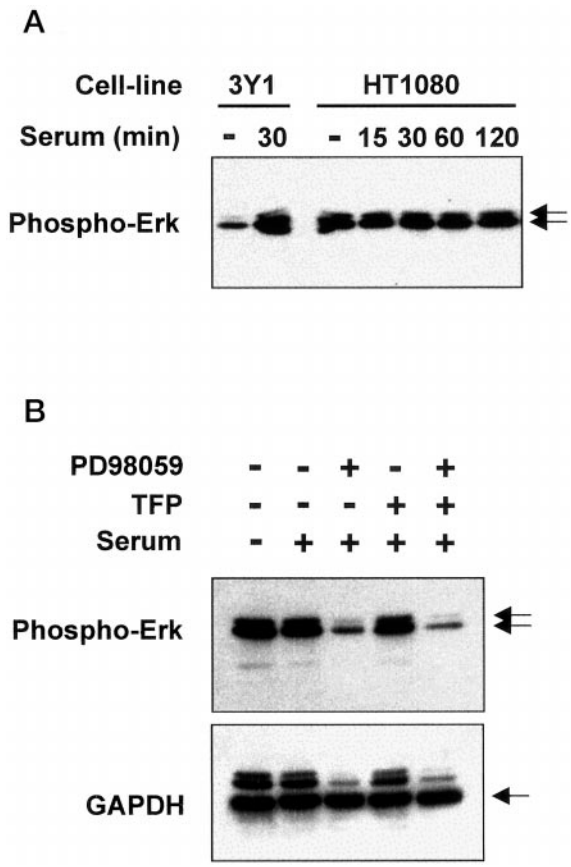

FIG. 8. **TFP has no effect on serum-stimulated ERK activation.** *A*, 3Y1 fibroblast and HT1080 cells were serum-starved for 24 h, then treated with 20% serum for the indicated times. The cells were then harvested and lysed. The protein samples (20 μg of each) were separated by 10% SDS-polyacrylamide gel electrophoresis. Activation of ERK was analyzed by Western blotting against antibodies (1:1000) specific for the ERK phosphorylated form (anti-phospho-ERK). *Arrows* indicate the phosphorylated forms of ERK1 and ERK2. *B*, HT1080 cells were serum-starved for 24 h, pretreated with the indicated concentrations of PD98059 for 30 min, then stimulated with TFP (50 μM). After 30 min of incubation, cells were added with 20% serum for an additional 30 min. After treatment, cells were lysed and protein extracts (20 μg of each) were separated by 10% SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting against anti-phospho-ERK antibodies (*upper panel*). The same blot was reprobed with anti-GAPDH antibodies without stripping as an internal control of the protein contents per lane (*lower panel*). *Arrows* indicate the phosphorylated forms of ERK1 and ERK2 in the *upper panel*, and GAPDH in the *lower panel*.

KN62 and cyclosporin A (Fig. 5). These data indicate that Ca^{2+}/calmodulin-dependent protein kinase II and calcineurin negatively regulate serum-stimulated Egr-1 expression in HT1080 cells. These findings are consistent with previous studies, which have shown that expressions of Ca^{2+}-induced *Egr-1* and c-*fos* are enhanced by inhibition of calcineurin with cyclos-

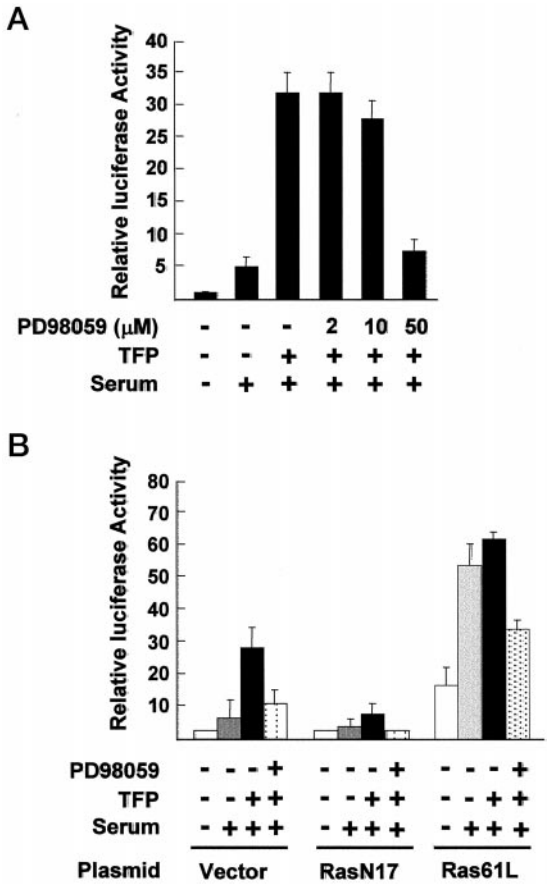

FIG. 9. **Effect of TFP on Elk-1-mediated transcriptional activation.** *A*, HEK293T cells were cotransfected with *trans*-activator pFA2-Elk1 (50 ng) and the pFR-Luc reporter plasmid (0.5 μg). After transfection, cells were pooled, split into 35-mm dishes, and serum-starved for 24 h. Cells were pretreated without and with the indicated concentrations of PD98059 for 30 min, then stimulated with TFP (50 μM). After an additional incubation for 30 min, cells were added with 20% serum. After 12 h, cells were harvested and the protein extracts were prepared by three cycles of freezing and thawing. 1 μg of protein was assayed for luciferase activity. *Bars* represent the mean of a single experiment performed in triplicate ± S.D. Similar results were observed in three independent experiments. *B*, HEK293T cells were transfected with the pFA2-Elk1 (50 ng) and the pFR-Luc reporter plasmids (0.5 μg) together with either expression vectors for dominant-active Ras61L (0.5 μg) or dominant-negative RasN17 (0.5 μg) as indicated. The pCMA/β-gal plasmid (0.2 μg) was included as an internal control for normalization of transfection efficiency. The total amount of DNA was maintained at 1.25 μg by addition of empty pcDNA3.0 plasmid. After transfection, cells were serum-starved and treated with 20% serum and 50 μM TFP in the absence or presence of PD98059 as indicated. After 12 h, the luciferase and β-galactosidase activities were measured. Results are presented as the amount of induction after normalization for transfection efficiency with β-galactosidase activity. *Bars* represent the mean of a single experiment performed in triplicate ± S.D. Similar results were observed in three independent experiments.

porin A in the PC12 and ELM-I-1 murine erythroleukemia cells types (40, 41). Calcineurin is a ubiquitously expressed serine/threonine protein phosphatase under the control of Ca^{2+}/calmodulin (42). Calcineurin is the main component of the calcium signaling pathway in T lymphocytes, regulating the translocation of transcription factor NF-AT by dephosphorylation (43). As it does in T cells, calcineurin may play an important role in the regulation of gene expression in other cell types. It has been reported that activation of calcineurin inhibits epidermal growth factor-induced stimulation of the Ets family of transcription factor Elk-1 and plays a negative role in induction of c-*fos* transcription (30). However, it remains to be established whether calmodulin/calcineurin constantly inhibits Elk-1 activity in HT1080 cells.

It has been reported that expression of the *Egr-1* gene in response to several mitogenic stimuli and the oncogene v-*src* and v-*raf* is mediated primarily through the serum response elements (SREs) in the 5′-flanking region of the gene (44–46). Once activated, the two transcription factors, serum response factor and ternary complex factor (TCF), form ternary complexes on the SRE and mediate transcription activation. Phosphorylation of TCF, including Elk-1, Sap-1, and Sap-2/Net/Erp, causes increased DNA binding and formation of ternary complexes and thereby plays a primary role in regulating early response gene expression, such as c-*fos* and *Egr-1* (47, 48). It is well known that mitogens, including serum and growth factors, rapidly activate signal transduction pathways involving the Ras/MEk/ERK pathway. The classic Ras/MEK/ERK cascade is responsible for phosphorylation of Elk-1, which is a well characterized TCF. Elk-1 phosphorylation correlates well with increased transcription activation of c-*fos* and *Egr-1* (36).

We found that serum- and TFP-induced Egr-1 expression was inhibited efficiently by PD98059, a specific MEK inhibitor, but not by the PLC inhibitor U73122, the phosphatidylinositol 3-kinase inhibitor LY294002, or the tyrosine kinase inhibitor genistein (Fig. 7, *A* and *B*). We also found that TFP-dependent activation of the *Egr-1* promoter was repressed by transient expression of the dominant-negative mutant of RasN17 and by pretreatment with PD98059 (Fig. 8*C*). These findings indicate that the Ras/MEK cascades are necessary to activate *Egr-1* expression due to serum and TFP. Elk-1 was activated by treatment with TFP, and TFP-stimulated Elk-1 *trans*-activation was inhibited by both PD98059 and the dominant-negative mutant RasN17 (Fig. 9). Because TFP did not directly activate ERK1/2 (Fig. 8*B*), TFP probably acts downstream of ERK1/2, at least at the level of Elk-1. There is evidence that Ca^{2+}/calmodulin-dependent calcineurin is a major phosphatase of Elk-1. Sugimoto *et al*. (29) and the Tian and Karin group (30) reported that constitutive activated expression of calcineurin or activation of endogenous calcineurin by Ca^{2+} ionophore decreased epidermal growth factor-induced phosphorylation of Elk-1 and that treatment with cyclosporin A prevented the effect of calcineurin on Elk-1 phosphorylation and Ca^{2+}-induced c-*fos* induction. These results are consistent with the idea that TFP acts upstream of calcineurin to participate in stimulation of Elk-1. Two other MAPKs, JNK and p38, are also reported to phosphorylate Elk-1 (26, 49). Although the present data did not preclude the possible involvement of JNK and p38 pathways in TFP-mediated Elk-1 activation, the efficient inhibition of TFP-mediated Elk-1 activation by the dominant-negative RasN17 and PD98059 (Fig. 9) indicates that calcineurin is the major target of TFP, because it is known that the MEK inhibitor PD98059 does not directly inhibit JNK or p38 activity. We suggest that the calmodulin antagonist TFP stimulates *Egr-1* gene induction via modulation of the Ras/MEK/ERK/Elk-1 cascade. Thus, the convergence of the Ca^{2+}/calmodulin-dependent pathway and the MEK/ERK/Elk-1 cascades coordinates suppression of *Egr-1* gene expression in HT1080 cells.

It has been reported that mutation of the *ras* oncogene is commonly found in a variety of human tumors, and it is now clear that mutated *ras* expression plays a pivotal role in controlling neoplastic transformation. Several lines of evidence suggest a role for the constitutive activation of the Ras/MEK/ERK pathway in a mutated Ras-mediated malignant transformation (28, 50). In human fibrosarcoma HT1080 cells, a point mutation at codon 61 of N-Ras that converts Gln^{61} to Lys^{61} has been found, which may be needed to transform these cells to transformation states (51). If loss of *Egr-1* expression plays a role in the transformed state of HT1080 cells (23) and the

transformation states (51). If loss of *Egr-1* expression plays a role in the transformed state of HT1080 cells (23) and the classic Ras/MEK/ERK pathway mediates the transcription activation of *Egr-1* via Elk-1, how *Egr-1* is down-regulated in oncogenic Ras-expressed HT1080 cells is still unknown. One intriguing possibility is negative feedback regulation of *Egr-1* expression by oncogenic Ras. In this regard, several studies have demonstrated that oncogenic Ras is involved in negative feedback suppression mechanisms as part of the regulation of redundant incoming signals (52–54). Chen *et al*. (55) reported that expression of activated Ras negatively regulates calcium-dependent c-*fos* and *Egr-1* gene induction in lymphocytes. They also found that the negative effect of Ras on *Egr-1* and c-*fos* gene induction by calcium is abolished by pretreatment with cyclosporin A, indicating involvement of calcineurin in oncogenic Ras-mediated negative feedback regulation. Recently, Sugimoto *et al*. (56) reported that a kinase suppressor of Ras (KSR) specifically blocks epidermal growth factor and Ras-induced transcription activation of all members of the TCF family, including Elk-1, Sap1a, and Sap2, without affecting the MAPK activity. Moreover, they found that the effect of KSR on the inhibition of Elk-1 transcription activation is mediated by accumulation of dephosphorylated Elk-1 via the action of calcineurin. Based on these reports, one can speculate that KSR plays a critical role in suppressing expression of tumor suppressor *Egr-1*, thereby promoting Ras-induced transformation in HT1080 cells. In future studies it will be of interest to examine the possible roles of KSR in the progression of Ras-induced transformation. We cannot, however, rule out the possibility that *Egr-1* transcription can be regulated by other proteins that are sensitive to oncogenic Ras expression.

Our study demonstrates that TFP as a calmodulin antagonist synergistically stimulates induction of tumor suppressor *Egr-1* gene expression in the presence of serum in HT1080 human fibrosarcoma cells. TFP probably plays a role in modulating Ras/MEK/ERK/Elk-1 cascades, presumably through inhibition of the Ca^{2+}/calmodulin-dependent pathway. It will be of interest to determine whether TFP-mediated Elk-1 activation of transcription through SRE is the only pathway by which TFP induces expression of SRE-regulated genes. The use of calmodulin antagonists may provide a useful approach to suppression of neoplastic growth.

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Induction of Early Growth Response-1 Gene Expression by Calmodulin Antagonist Trifluoperazine through the Activation of Elk-1 in Human Fibrosarcoma HT1080 Cells

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