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Extracellular Signal-Regulated Kinase Binds to TFII-I and Regulates Its Activation of the c-*fos* Promoter

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We have previously shown that TFII-I enhances transcriptional activation of the c-*fos* promoter through interactions with upstream elements in a signal-dependent manner. Here we demonstrate that activated Ras and RhoA synergize with TFII-I for c-*fos* promoter activation, whereas dominant-negative Ras and RhoA inhibit these effects of TFII-I. The Mek1 inhibitor, PD98059 abrogates the enhancement of the c-*fos* promoter by TFII-I, indicating that TFII-I function is dependent on an active mitogen-activated protein (MAP) kinase pathway. Analysis of the TFII-I protein sequence revealed that TFII-I contains a consensus MAP kinase interaction domain (D box). Consistent with this, we have found that TFII-I forms an in vivo complex with extracellular signal-related kinase (ERK). Point mutations within the consensus MAP kinase binding motif of TFII-I inhibit its ability to bind ERK and its ability to enhance the c-*fos* promoter. Therefore, the D box of TFII-I is required for its activity on the c-*fos* promoter. Moreover, the interaction between TFII-I and ERK can be regulated. Serum stimulation enhances complex formation between TFII-I and ERK, and dominant-negative Ras abrogates this interaction. In addition, TFII-I can be phosphorylated in vitro by ERK and mutation of consensus MAP kinase substrate sites at serines 627 and 633 impairs the phosphorylation of TFII-I by ERK and its activity on the c-*fos* promoter. These results suggest that ERK regulates the activity of TFII-I by direct phosphorylation.

TFII-I is a multifunctional protein that appears to have functions in both transcription and signal transduction. It was initially identified for its role in Inr-dependent transcription (27, 44, 45) and has been also implicated in E-box-dependent transcription (42, 43). Deletions of TFII-I are tightly linked with Williams-Beuren syndrome, which is a neurodevelopmental disease in humans (35). Recently, we and others reported that TFII-I can bind to the serum response element (SRE) and the c-sis/PDGF-inducible factor element (SIE) of c-*fos* promoter and can also interact with serum response factor (SRF) (13, 24). We have also demonstrated that TFII-I can enhance the c-*fos* promoter in vivo in a manner that is dependent on the upstream elements, including the SIE, the SRE, and also its own binding sites, which overlap with the c-*fos* SIE and SRE (24). Interestingly, TFII-I (BAP135) has been identified as a factor which can form a complex with the Bruton's tyrosine kinase (Btk) in B cells, and tyrosine phosphorylation of TFII-I can be regulated by Btk (54). In addition, we have previously shown that growth factor stimulation can enhance the tyrosine phosphorylation of TFII-I and potentiate its enhancement of c-*fos* promoter activation (24). These observations suggest that TFII-I plays a role in signal transduction as well as in transcriptional activation of the c-*fos* promoter.

The c-*fos* promoter is a very well studied immediate-early gene promoter and responds rapidly and transiently to a variety of extracellular stimuli (5, 12, 25, 31). Mechanisms for responses to calcium, cyclic AMP, tyrosine kinases, mitogen-activated protein (MAP) kinases have been identified (4). Despite the fact that the c-*fos* promoter has been extensively characterized, a number of important issues remain to be understood. For instance, in order to respond to various MAP kinase cascades, SRF can form a ternary complex at the c-*fos* SRE with various ternary complex factors (TCFs), such as Elk-1. The TCF proteins are substrates for MAP kinases and become transcriptionally active upon phosphorylation (6, 19, 48, 50). However, there is substantial evidence suggesting an alternative pathway which operates through SRF independently of TCF (10, 21, 39). This pathway is serum responsive and is mediated in part through the activation of the small G protein RhoA (18). However, the mechanism of activation of SRF by the Rho pathway has not been clearly established (46), although there has been some evidence suggesting that Rho kinase and the NF-κB and C/EBP transcription factors may be involved (3, 30).

Moreover, there is a substantial synergism between elements of the c-*fos* promoter (41). TFII-I interacts with both STAT proteins and SRF, in addition to binding to the promoter at positions that overlap with these sites (24). Thus, TFII-I is well positioned to mediate synergism between these elements of the promoter, although the mechanism of this synergism is not well understood.

TFII-I has only limited homology to other proteins, but it is characterized by six helix-loop-helix repeats. Analysis of the primary sequence of TFII-I also reveals a region of striking homology to the consensus D box of Elk-1 (see Fig. 5A). The D box is a MAP kinase interaction domain that was originally identified in the extracellular signal-related kinase (ERK) substrate and c-*fos* transcription factor Elk-1 (52, 53). The same or similar (δ domain) motifs can be found as MAP kinase docking sites in several MAP kinase interacting transcription factors (7, 20, 22, 23, 51). Moreover, TFII-I also contains putative MAP kinase phosphorylation sites which are very similar to those found in Elk-1 as shown in Fig. 10A (16, 29). This suggests that TFII-I, like Elk-1, may also interact with ERK and be phosphorylated by it.

To understand the regulation of TFII-I by signal transduction pathways, we report here that TFII-I can be regulated by Ras and Rho pathways and that it requires a functioning ERK pathway for the activity. We also demonstrate that TFII-I can

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interact with the MAP kinase ERK1 through its consensus MAP kinase binding domain (D box) and that this interaction is required for its activity on the c-*fos* promoter. Moreover, the interaction between TFII-I and ERK can be regulated by signal transduction pathways. In addition, we show here that TFII-I can be a direct substrate for ERK and that the consensus ERK phosphorylation sites of TFII-I are required for its activity. These results suggest that TFII-I functions as a regulated signaling molecule for the activation of the c-*fos* promoter.

MATERIALS AND METHODS

Plasmids and antibodies. For transient overexpression of human TFII-I, pEBG–TFII-I construct was used as previously described (2, 24). The wild-type c-*fos*–luciferase construct and pRL-TK luciferase construct for reporter assays were as previously described (24). Activated or dominant-negative Ras, Rac1, Cdc42h, or RhoA expression plasmids were as previously described (8, 9, 32, 36, 37). BXB-Raf and HA-ERK1 expression plasmids were also as previously described (11, 40). Mek1/EE expression plasmid was purchased from Stratagene, and pCDNA3 empty vector was obtained from Invitrogen. Anti-TFII-I polyclonal rabbit antisera was made against the purified recombinant glutathione *S*-transferase (GST) fusion protein containing C terminal 389 amino acids of human TFII-I. Anti-ERK1 antibody was obtained from Larry Feig. The anti-GST antibody was obtained from Sigma and the anti-hemagglutinin (HA) antibody was obtained from Boehringer Mannheim. Anti-phosphoERK antibody was obtained from New England Biolabs.

Cell culture and transfections. Murine NIH 3T3 fibroblasts were grown in Dulbecco modified Eagle medium (DMEM) with 10% calf serum (CS). COS-1 cells were cultured in DMEM with 10% fetal calf serum (FCS). For transient transfection, the calcium phosphate transfection kit from 5 Prime 3 Prime Co. was used. For reporter assays, NIH 3T3 cells were maintained for 30 to 40 h in medium containing 0.5% CS following transfection and stimulated with 10% FCS where indicated for 3 to 4 h before harvest. One hundred nanograms of reporter construct, 250 ng of pEBG–TFII-I (or pEBG) expression plasmid, and 25 ng of pRL-TK normalization plasmid were used per single well of a 12-well plate. Twenty-five nanograms of various small G proteins, BXB-Raf, or Mek1/EE expression plasmid was included where indicated. The total amount of DNA per well was kept at 400 ng by using pCDNA3 to adjust total DNA amounts where necessary. Dual luciferase assay was carried out as previously described (24). All transfection experiments were performed in duplicate, and results were normalized to the expression of the renilla luciferase transfection control. For inhibitor treatment, transfected NIH 3T3 cells were incubated with 100 nM PP1 (15), 100 nM wortmannin (34), or 25 μM PD98059 (1) for 1 h prior to 10% FCS stimulation, and the inhibitors were also included during serum stimulation. For Fig. 9, 25 μM PD98059 (1) or SB202190 (26) was added to the transfected COS-1 cells 1 h before harvest. PP1, wortmannin, PD98059, and SB202190 were obtained from Calbiochem. For protein-protein interaction assays, COS-1 cells were maintained before harvest for 36 h in medium containing 10% FCS following transfection. Five micrograms of pEBG, pEBG–TFII-I, pEBG–L289A–TFII-I, or HA-ERK, along with 2.5 μg of N17-Ras, N17-Rac, or N19-Rho construct, was used per 100-mm plate. The total amount of DNA was kept at 12.5 μg per 100-mm dish by using pCDNA3 to adjust total DNA amounts where necessary. For Fig. 7, NIH 3T3 fibroblasts were serum starved for 36 h with 0.5% CS and stimulated with 15% FCS for 10 min before harvest.

GST pulldown, immunoprecipitation, and Western blot analysis. Cell lysates were prepared in buffer containing 20 mM Tris (pH 7.8), 200 mM KCl, 10% glycerol, 0.4% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg each of leupeptin, antipain, pepstatin, and chymostatin per ml except in Fig. 10B. The lysates were then centrifuged for 10 min at 10,000 × *g* at 4°C, and the supernatants were used for GST pulldown or immunoprecipitation assays as previously described (24). Western blot analyses were carried out under standard conditions.

In vitro mutagenesis and isolation of overexpressed TFII-I. L289A-TFII-I, S627A–TFII-I, or S633A–TFII-I mutant was generated according to the manufacturer's protocol provided with the QuickChange Site-Directed Mutagenesis Kit from Stratagene. The following complementary oligonucleotides were used to synthesize mutated DNA strands: L289A (+), CCGTCTAAGAGACCAAA GGCCgcTGAGgcACCGCAGCCACCAGTCCCG; L289A (−), CGGGACTG GTGGCTGCGGTgcCTCAgcGGCCTTTGGTCTCTTAGACGG; S627A (+), GGCTAGTAAAATAAACACTAAAGCTTTGCAGgCtCCCAAAAGACCAC GAAGTCC; S627A (−), GGACTTCGTGGTCTTTTGGGaGcCTGCAAAGC TTTAGTGTTTATTTTACTAGCC; S633A (+), GCAGTCCCCCAAAAGAC CACGAgcTCCTGGGAGTAATTCAAAGGTTCC; and S633A (−), GGAAC CTTTGAATTACTCCCAGGAgcTCGTGGTCTTTTGGGGGACTGC. The wild-type or various mutant TFII-I proteins were purified from transfected COS-1 cells as previously described (24), and an equal amount of each protein was used for in vitro kinase assays.

In vitro kinase assay. For Fig. 10B, 10 μg of HA-ERK expression construct per 100-mm plate was transfected into COS-1 cells, and the cells were maintained for 36 h in medium containing 10% FCS and then stimulated with 25 ng of epidermal growth factor (EGF) per ml for 10 min before harvest. Cell lysates were prepared in the buffer containing 25 mM Tris (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 0.2 mM Na_3VO_4, 10% glycerol, 1% Triton X-100, 0.5 mM PMSF, and 1 μg each of leupeptin, antipain, pepstatin, and chymostatin per ml. The lysates were then centrifuged for 10 min at 10,000 × *g* at 4°C, and the supernatants were used for anti-HA antibody immunoprecipitation. Immunoprecipitated ERK containing beads were washed with kinase buffer (25 mM Tris [pH 7.5], 100 mM NaCl, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 0.2 mM Na_3VO_4). The immune complex kinase assay was performed essentially as described previously (47). Briefly, immunoprecipitates were incubated with 20 μCi (6,000 Ci/mmol) of [γ-^{32}P]ATP either in the presence or the absence of substrate proteins for 30 min at room temperature. The labeled proteins were then separated on by sodium dodecyl sulfate (SDS)–8% polyacrylamide gel electrophoresis (PAGE), fixed, dried, and visualized by autoradiography.

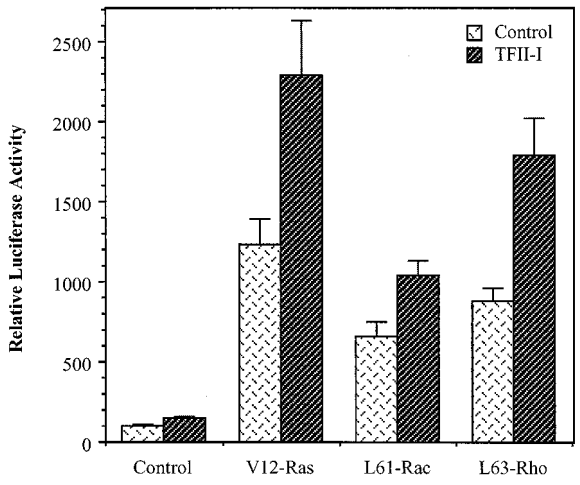

FIG. 1. V12-Ras and L63-Rho synergize with TFII-I for the c-*fos* promoter activation. The V12-Ras, L61-Rac, or L63-Rho expression plasmid was transfected into NIH 3T3 fibroblasts with the c-*fos*–luciferase reporter construct in the absence or presence of TFII-I, and the cells were maintained for 40 h in 0.5% CS before harvest. Cell extracts were then processed for luciferase activity. The relative luciferase activities shown here are from a representative experiment, and similar results were obtained from three further independent experiments. All transfections were performed in duplicate for each experiment, and the values between the duplicates were within 10% of the mean.

RESULTS

Ras and Rho pathways can regulate the activity of TFII-I. To investigate the functional relationship between various small G protein signal transduction pathways and TFII-I, the effects of Ras, Rac1, and RhoA on the activity of TFII-I were examined. To do this, constitutively activated V12-Ras, L61-Rac, or L63-Rho were transfected into NIH 3T3 fibroblasts with the c-*fos*–luciferase reporter in the absence or presence of TFII-I, and luciferase activity was measured. The cells were maintained in low-serum medium after transfection. As shown in Fig. 1, without serum or growth factor stimulation, the activated V12-Ras and L63-Rho efficiently synergized with TFII-I for c-*fos* promoter activation, whereas activated L61-Rac showed only weak effects. These results suggest that the Ras and Rho pathways are more important for the effects of TFII-I on the c-*fos* promoter than is the Rac pathway.

To further evaluate the functional cooperation between various small G protein pathways and TFII-I, we transfected dominant-negative N17-Ras, N17-Rac1, N17-Cdc42, or N19-RhoA into NIH 3T3 fibroblasts with the c-*fos*–luciferase reporter in the absence or presence of TFII-I, and the luciferase activity was measured after serum stimulation. As can be seen in Fig. 2, all of the dominant-negatives decreased overall activity of the c-*fos* promoter but to different degrees. However, only the

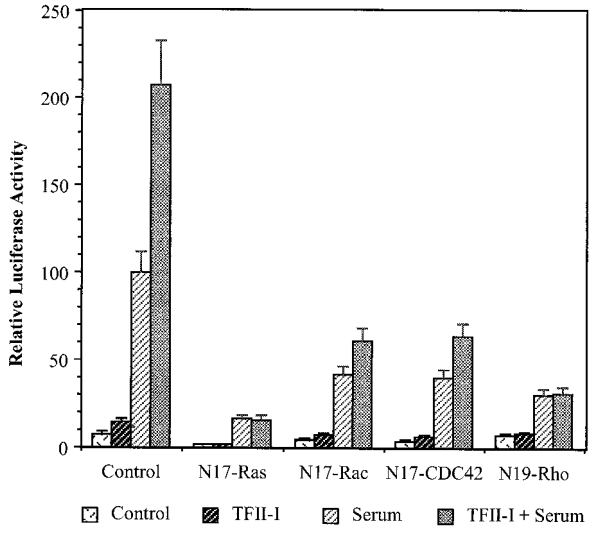

FIG. 2. N17-Ras and N19-Rho inhibit TFII-I enhancement of the c-*fos* promoter. NIH 3T3 cells were transfected with the c-*fos*–luciferase reporter construct and pEBG-TFII-I (or pEBG) plasmid in the presence of pCDNA3, N17-Ras, N17-Rac, N17-Cdc42, or N19-Rho plasmid. Cells were serum starved for 36 h in 0.5% CS and stimulated for 4 h with 10% FCS. Cell extracts were then processed for luciferase activity. Relative luciferase activities shown here are from a representative experiment, and similar results were obtained from three further independent experiments. All transfections were performed in duplicate for each experiment, and the values between the duplicates were within 10% of the mean.

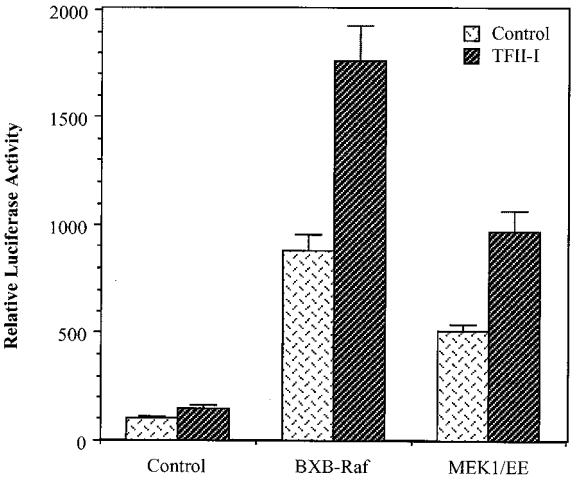

FIG. 3. BXB-Raf and Mek1/EE cooperate with TFII-I for c-*fos* promoter enhancement. BXB-Raf or Mek1/EE expression plasmids were transfected into NIH 3T3 fibroblasts with the c-*fos*–luciferase reporter construct in the absence or presence of TFII-I, and cells were maintained for 40 h in 0.5% CS before harvest. Cell extracts were then processed for luciferase activity. Relative luciferase activities shown here are from a representative experiment, and similar results were obtained from three further independent experiments. All transfections were performed in duplicate for each experiment, and the values between the duplicates were within 10% of the mean.

dominant-negative N17-Ras and N19-Rho completely eliminated the ability of TFII-I to enhance the c-*fos* promoter in response to serum stimulation. The dominant-negative N17-Rac and N17-Cdc42 had only partial effects. Dose-response experiments indicated that the inhibitory effects of N17-Rac and N17-Cdc42 on TFII-I were nearly maximal at the amounts used in these experiments and that even 20-fold-higher doses fail to inhibit completely (data not shown). Consistent with the results from Fig. 1, these results also indicate that the Ras and Rho pathways are more significant for the activity of TFII-I than Rac or Cdc42.

Since Ras efficiently synergized with TFII-I, we sought to determine whether the downstream kinases of the Ras-ERK pathway are capable of cooperating with TFII-I. A cotransfection assay similar to that in Fig. 1 was carried out with activated BXB-Raf or Mek1/EE. Results are shown in Fig. 3. TFII-I overexpression was able to enhance c-*fos* promoter activation with either BXB-Raf or Mek1/EE, suggesting that the Ras/MAP kinase pathway is specifically able to functionally cooperate with TFII-I. The effector of RhoA leading to the activation of the c-*fos* SRE has not been clearly identified yet (46).

Functional MAP kinase pathway is required for the activity of TFII-I. To further explore the functional relationship between different signal transduction pathways and TFII-I, we examined the effect of various kinase inhibitors on the activity of TFII-I. PP1, a specific inhibitor for Src family kinases (15), wortmannin, a specific inhibitor for PI-3-kinase (34), or PD98059, a specific inhibitor for Mek1 (1), were used in the reporter assay. The c-*fos*–luciferase reporter construct was transfected into NIH 3T3 fibroblasts in the absence or presence of TFII-I, and the three different inhibitors were preincubated prior to serum stimulation. Luciferase assay was carried out after serum stimulation. As can be seen in Fig. 4, none of these specific inhibitors dramatically decreased the overall activity of the c-*fos* promoter. Since a number of different pathways can cooperate to induce c-*fos*, specific inhibition of a single pathway may not be sufficient to inhibit the overall promoter response. Nevertheless, the enhancement of the c-*fos* promoter by TFII-I was completely abolished by Mek1 inhibitor PD98059, which blocks ERK activation. This result suggests that TFII-I functions in a manner that is dependent on a

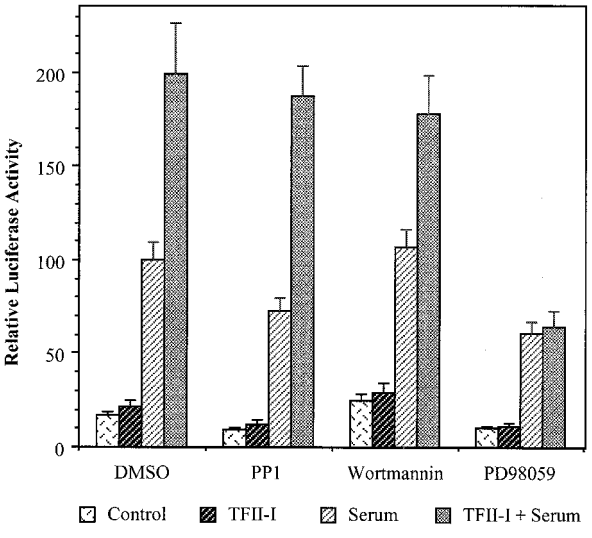

FIG. 4. PD98059 abolishes the activity of TFII-I on the c-*fos* promoter. NIH 3T3 cells were transfected with the c-*fos*–luciferase reporter construct in the absence or presence of TFII-I and serum starved for 36 h in 0.5% CS following the transfection. The transfected cells were then incubated with 100 nM PP1, 100 nM wortmannin, or 25 μM PD98059 for 1 h prior to serum stimulation. Each inhibitor was also included during 4 h FCS stimulation. Cell extracts were then processed for luciferase activity. The relative luciferase activities shown here are from a representative experiment, and similar results were obtained from three further independent experiments. All transfections were performed in duplicate for each experiment, and the values between the duplicates were within 10% of the mean.

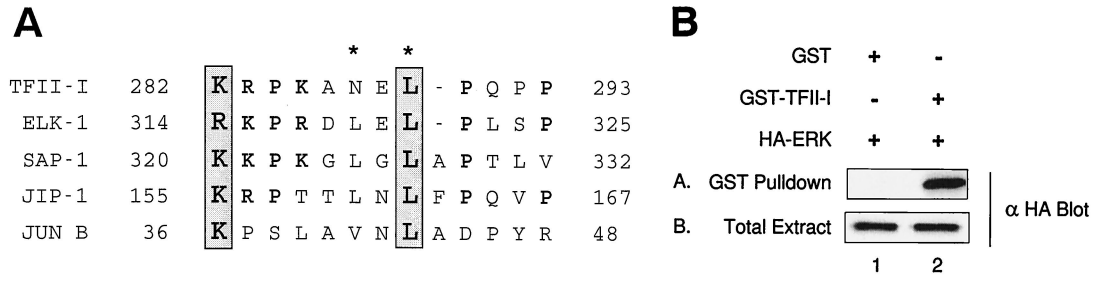

FIG. 5. TFII-I forms a complex with ERK. (A) A consensus MAP kinase interaction domain (D box) of TFII-I (53). (B) TFII-I can form an in vivo complex with the MAP kinase, ERK1. HA-ERK1 expression plasmid was cotransfected into COS-1 cells with pEBG (lane 1) or pEBG–TFII-I (lane 2) expression plasmid. Transfected COS-1 cells were lysed and subjected to GST pulldown assay. Glutathione-Sepharose bead-bound proteins (A) and total extracts (B) were fractionated by SDS–12% PAGE and analyzed by anti-HA antibody Western blotting.

functionally intact MAP kinase pathway. PP1 and wortmannin did not have significant effects on the activity of TFII-I, indicating that Src kinase and phosphatidylinositol 3-kinase pathways are not necessary for TFII-I potentiation of the c-*fos* promoter.

TFII-I can form a complex with ERK. Since the Ras/MAP kinase pathway is important for the activity of TFII-I, we sought to determine whether TFII-I might directly bind to ERK. Interestingly, sequence comparison of TFII-I with other known MAP kinase binding proteins reveals a striking homology between one region of TFII-I and known MAP kinase interaction domains (D box), as shown in Fig. 5A. Thus, GST or GST–TFII-I expression plasmids were cotransfected with an HA-ERK expression plasmid into COS-1 cells to detect in vivo interaction between TFII-I and ERK. Protein complexes were isolated by GST pulldown, and the resulting complexes were separated by SDS-PAGE and analyzed by Western blotting by using anti-HA antibody. As can be seen in Fig. 5B, significant complex formation was observed between TFII-I and ERK (subpanel A, lane 2), whereas no interaction was detected between GST and ERK (subpanel A, lane 1). Figure 5B, subpanel B, shows that there were comparable levels of expression of the transfected ERK between the samples in this experiment.

The D box of TFII-I is required for its interaction with ERK and activation of c-*fos* promoter. To further characterize the interaction between TFII-I and ERK, we asked whether the TFII-I D box is required for its interaction with ERK. To do this, N287 and L289 (residues marked in Fig. 5A) of TFII-I were changed to alanines to disrupt the consensus motif for MAP kinase interaction as previously described with Elk-1 (52, 53). This mutant henceforth is referred to as L289A–TFII-I. The non-PCR-based QuickChange mutagenesis method (Stratagene) was used for mutagenesis to avoid introduction of other mutations into the protein, and the mutation was verified by sequencing. To compare ERK binding capability between the wild-type and the mutant TFII-I, GST-fused wild-type–TFII-I or L289A–TFII-I expression plasmids were cotransfected with HA-ERK expression plasmid into COS-1 cells. GST pulldown assay and Western blotting with anti-HA antibody were carried out to determine the interaction between the mutant L289A–TFII-I and ERK. As can be seen in Fig. 6A, the mutant L289A-TFII-I completely lost its ability to form a complex with ERK (lane 2 [subpanel A]) compared to the wild-type TFII-I (lane 1 [subpanel A]). Subpanel B shows that there was comparable expression of the transfected ERK between samples, and subpanel C confirms the expression of the transfected L289A–TFII-I (lane 2). These results clearly indicate that the putative MAP kinase binding motif (D box) found in TFII-I is necessary for the interaction with ERK.

Next we examined whether the D box of TFII-I is required for its activity. L289A–TFII-I was transfected into NIH 3T3 fibroblasts with the c-*fos*–luciferase reporter construct, and the luciferase activity was measured after serum stimulation. Empty vector and the wild-type TFII-I were also used in the

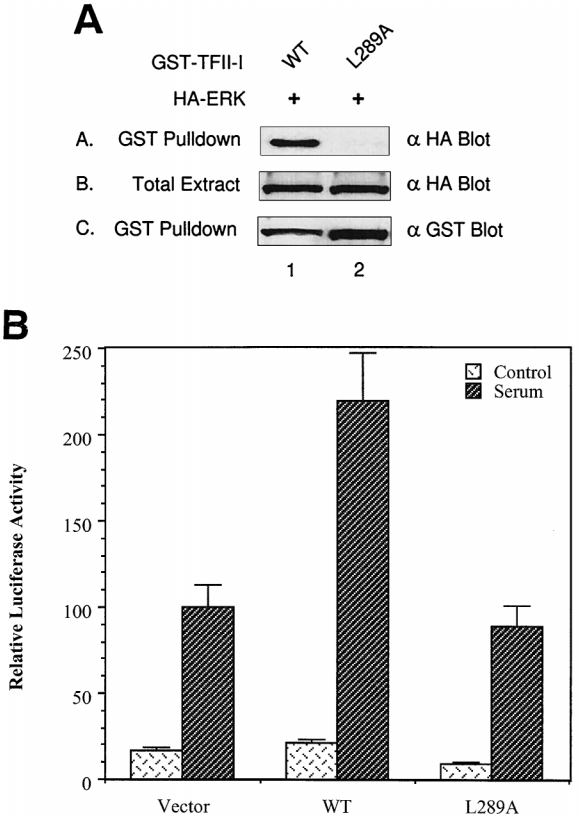

FIG. 6. The D box of TFII-I is required for its interaction with ERK and activation of the c-*fos* promoter. (A) An intact TFII-I D box is required for ERK interaction. pEBG–TFII-I (wild type) (lane 1) or pEBG–L289A–TFII-I (lane 2) expression plasmid was cotransfected with HA-ERK expression plasmid into COS-1 cells. Transfected COS-1 cells were lysed and subjected to GST pulldown assay. Glutathione-Sepharose bead-bound proteins (A and C) and total extracts (B) were fractionated by SDS–12% PAGE and blotted by use of anti-HA (A and B) or anti-GST (C) antibody. (B) TFII-I requires ERK interaction for its enhancement activity on the c-*fos* promoter. pEBG, pEBG–TFII-I (wild type), or pEBG–L289A–TFII-I plasmid were transfected into NIH 3T3 fibroblasts with the c-*fos*–luciferase reporter construct. Cells were serum starved for 36 h in 0.5% CS and then stimulated for 4 h with 10% FCS. Cell extracts were then processed for luciferase activity.

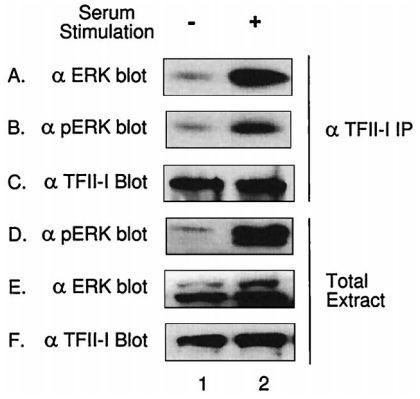

FIG. 7. Serum stimulation enhances complex formation between TFII-I and ERK. NIH 3T3 fibroblasts were serum starved for 36 h (lane 1) and stimulated with 15% FCS for 10 min (lane 2). Total extracts were immunoprecipitated with anti-TFII-I antibody. The immunoprecipitates (A, B, and C) and total extracts (D, E, and F) were fractionated by SDS–12% PAGE and blotted with anti-ERK (A and E), anti-phosphoERK (B and D), or anti-TFII-I (C and F) antibody.

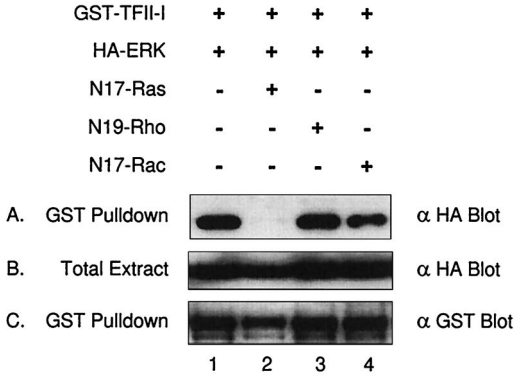

FIG. 8. N17-Ras abrogates the interaction between TFII-I and ERK. pEBG–TFII-I and HA-ERK1 expression plasmids were cotransfected into COS-1 cells in the presence of pCDNA3 (lane 1), N17-Ras (lane 2), N19-RhoA (lane 3), or N17-Rac1 (lane 4). Transfected COS-1 cells were lysed and subjected to GST pulldown assay. Glutathione-Sepharose bead-bound proteins (A and C) and total extracts (B) were fractionated by SDS–12% PAGE and analyzed by anti-HA (A and B) or anti-GST (C) antibody Western blotting.

same experiment as negative and positive controls. The results are shown in Fig. 6B. Interestingly, the mutant L289A–TFII-I, which is defective for ERK binding due to the D-box mutation, was not able to potentiate the c-*fos* promoter upon serum stimulation, whereas the wild-type TFII-I enhanced the promoter response as expected. Thus, these results suggest that the interaction between TFII-I and ERK is required for the activity of TFII-I on the c-*fos* promoter.

Regulation of the interaction between TFII-I and ERK by signal transduction pathways. To further analyze the interaction between TFII-I and ERK, we investigated whether TFII-I–ERK complex formation can be observed between endogenous proteins in a signal-dependent manner. NIH 3T3 fibroblasts were serum starved for 36 h and then stimulated with 15% FCS for 10 min. Total cell extracts were immunoprecipitated with anti-TFII-I antibody and blotted with anti-ERK (or anti-phosphoERK) antibody to detect the interaction between endogenous TFII-I and ERK. As shown in Fig. 7A, TFII-I did form a stable complex with ERK upon serum stimulation. In this assay, ERK activation by serum was verified by anti-phosphoERK (pERK) antibody blotting (panel D), and the activated ERK was indeed found in the complex (panel B). The anti-TFII-I (panels C and F) and anti-ERK (panel E) Western blots show that the expression level of TFII-I and ERK are comparable between samples. These results demonstrate that endogenous TFII-I and ERK interact with each other and that complex formation between TFII-I and ERK can be regulated by serum stimulation.

It was shown in Fig. 1 and 2 that Ras and Rho pathways cooperate with TFII-I for c-*fos* promoter activation. To further investigate the roles of the Ras or Rho pathway in TFII-I function, we examined whether dominant-negative N17-Ras, N19-Rho, or N17-Rac can affect the interaction between TFII-I and ERK. To do this, GST–TFII-I and HA-ERK expression plasmids were cotransfected into COS-1 cells with various expression plasmids encoding dominant-negative N17-Ras, N19-Rho, or N17-Rac. Protein complexes were isolated by GST pulldown and analyzed by Western blot analyses. The interaction between TFII-I and ERK was detected by Western blotting with anti-HA antibody. The results are shown in Fig. 8. Coexpression of the dominant-negative N17-Ras greatly reduced the interaction between TFII-I and ERK, as can be seen in lane 2 of panel A. Panels B and C show that there was comparable expression of the transfected TFII-I and ERK between the samples. This suggests that a functional Ras pathway is required for the interaction between TFII-I and ERK. This also indicates that the Ras may regulate the activity of TFII-I by the modulation of MAP kinase interaction. Interestingly, dominant-negative N19-Rho did not significantly affect the interaction between TFII-I and ERK (Fig. 8A, lane 3), although it did inhibit the activity of TFII-I on the c-*fos* promoter (Fig. 2). This indicates that the Rho pathway regulates TFII-I through a mechanism other than MAP kinase interaction.

Previously it has been suggested that interaction of the Elk-1 D domain with ERK is dependent on the activated form of ERK (53). To determine whether the same may be true for TFII-I, we examined whether the Mek1 inhibitor PD98059 can interfere the interaction between TFII-I and ERK by inhibiting ERK activation. Thus, GST–TFII-I and HA-ERK expression plasmids were cotransfected into COS-1 cells and treated with PD98059 for 1 h before harvest. SB202190, a specific inhibitor for p38 kinase was also used as a negative control. Protein complexes were isolated and analyzed as in Fig. 8, and the results are shown in Fig. 9. Interestingly, as can be seen in lane 2 of panel A, MAP kinase inhibition did not abolish the ability of TFII-I to interact with ERK. The inhibitory effect of PD98059 in this experiment was verified by anti-phosphoERK Western blotting (Fig. 9B, lane 2). The inhibitors did not affect the overall expression of transfected ERK and TFII-I (Fig. 9C and D). This suggests that the interaction between TFII-I and ERK is dependent on a Ras-regulated pathway that is distinct from the MAP kinase pathway.

The D box is required for ERK-dependent phosphorylation of TFII-I. We have found that functionally active ERK is required for the activity of TFII-I (Fig. 4) and that the interaction between TFII-I and ERK is not affected by MAP kinase inactivation (Fig. 9). In order to understand how ERK is required for the activation of TFII-I, we examined whether TFII-I may be a direct substrate of ERK. This is suggested by the presence of two consensus MAP kinase substrate sites as shown in Fig. 10A. Moreover, the sequence of this region of TFII-I is very similar to the known MAP kinase phosphorylation sites of Elk-1 (16, 29). To test this, HA-tagged ERK was overexpressed in EGF-treated COS-1 cells and immunoprecipitated by using anti-HA antibody, and an in vitro kinase

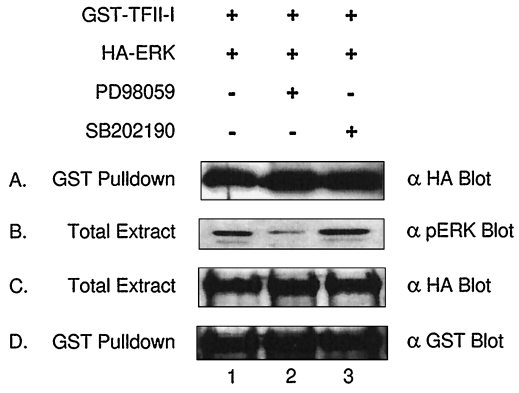

FIG. 9. PD98059 does not disrupt the interaction between TFII-I and ERK. pEBG–TFII-I and HA-ERK1 expression plasmids were cotransfected into COS-1 cells, and the transfected COS-1 cells were then incubated with 25 μM PD98059 (lane 2) or SB202190 (lane 3) for 1 h prior to harvest. The cells were lysed and subjected to GST pulldown assay. GSH-Sepharose bead-bound proteins (A and D) and total extracts (B and C) were fractionated by SDS–12% PAGE and analyzed by anti-HA (A and C), anti-phosphoERK (B), or anti-GST (D) antibody Western blotting.

assay was carried out in the presence of [γ-^{32}P]ATP. GST–TFII-I protein was purified as described in Materials and Methods and added to the reaction as a substrate. As can be seen in Fig. 10B, TFII-I was efficiently phosphorylated in vitro by ERK (lane 2 of subpanel A). To assess whether these putative MAP kinase substrate sites of TFII-I are in fact phosphorylated by ERK, two serine point mutations of TFII-I were generated by changing serine 627 or 633 of TFII-I to alanines as previously described in Fig. 6. These mutants are referred to here as S627A–TFII-I or S633A–TFII-I. To determine whether these two serine mutants affected the ability of ERK to phosphorylate TFII-I, purified S627A–TFII-I or S633A–TFII-I was also analyzed by the same in vitro kinase assay. The mutant S633A–TFII-I exhibited greatly reduced phosphorylation by ERK (lane 5 of subpanel A), and S627A–TFII-I also showed a substantial decrease in its phosphorylation (lane 4 of subpanel A). These results suggest that S627 and S633 are the major MAP kinase phosphorylation sites on TFII-I. It was shown in Fig. 6 that the intact D box of TFII-I is required for its interaction with ERK and also for its activity in vivo. Thus, we examined whether L289A-TFII-I, which is defective of ERK interaction due to its mutations in the D box, can be normally phosphorylated by ERK. As can be seen in lane 3 of subpanel A, L289A–TFII-I was poorly phosphorylated by ERK compared to the wild-type TFII-I. Phosphorylation efficiency of different TFII-I mutants for ERK in comparison with the wild type was presented also in subpanel B by quantitating the ^{32}P incorporation of each band. There were equal amounts of purified TFII-I protein used in each reaction as shown by anti-GST antibody Western blotting in subpanel C. None of these point mutations disrupted the overall structure of TFII-I since none of these mutations affected the ability of TFII-I to bind DNA (data not shown). These results indicate that the TFII-I interaction with ERK is necessary for its optimal phosphorylation by ERK as was also found for Elk-1 (53).

Next, we investigated whether ERK phosphorylation sites of TFII-I are required for its activity. As previously described in Fig. 6B, S627A–TFII-I or S633A–TFII-I were transfected into NIH 3T3 fibroblasts to determine their in vivo functionality for the c-*fos* promoter. The results are shown in Fig. 10C. Both S627A–TFII-I and S633A–TFII-I, which showed reduced phosphorylation by ERK (Fig. 10B), failed to potentiate the

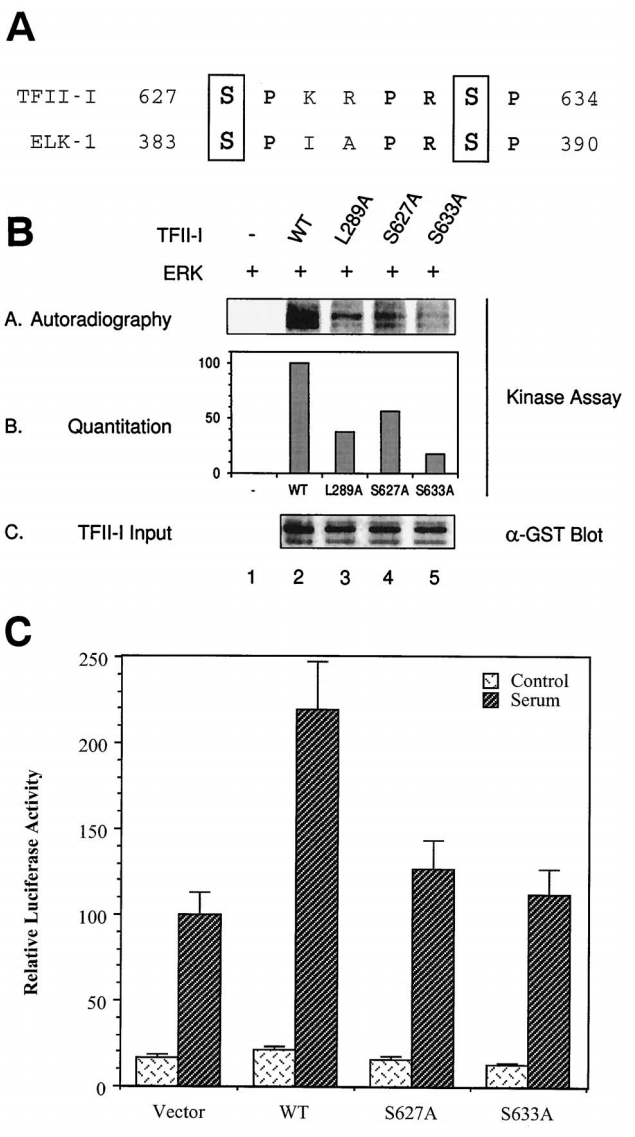

FIG. 10. TFII-I is an ERK substrate, and the consensus phosphorylation sites are required for its activity. (A) Consensus MAP kinase phosphorylation sites found in TFII-I and comparison to the known Elk-1 phosphorylation sites (16, 29, 53). (B) TFII-I phosphorylation by ERK. HA-ERK expression plasmid was transfected into COS-1 cells. After transfection, cultures were maintained for 40 h in 10% FCS containing DMEM before 10 min of EGF stimulation. Cell extracts were then immunoprecipitated by using anti-HA antibody, and an in vitro kinase assay was carried out in the presence of [γ-^{32}P]ATP. The wild-type–TFII-I (lane 2), L289A–TFII-I (lane 3), S627A–TFII-I (lane 4), or S633A–TFII-I (lane 5) protein was purified as described in Materials and Methods and added to the reaction as a substrate. No substrate except [γ-^{32}P]ATP was added to the reaction in lane 1. ^{32}P incorporation into each band was quantitated with a phosphorimager in the middle panel. The comparable amount of the purified TFII-I protein used in each reaction was shown by anti-GST antibody Western blotting in the bottom panel. (C) TFII-I requires ERK phosphorylation sites for its enhancement activity on the c-*fos* promoter. pEBG, pEBG–TFII-I (wild type), pEBG–S627A–TFII-I, or S633A–TFII-I plasmid was transfected into NIH 3T3 fibroblasts with a c-*fos*–luciferase reporter construct. Cells were serum starved for 36 h in 0.5% CS and then stimulated for 4 h with 10% FCS. Cell extracts were then processed for luciferase activity.

c-*fos* promoter in response to serum as the wild-type TFII-I had done. Therefore, serines 627 and 633 of TFII-I are required for its in vivo activity on the c-*fos* promoter.

Both Ras and Rho pathways are required for the activity of TFII-I. Since Ras and Rho pathways regulate TFII-I through

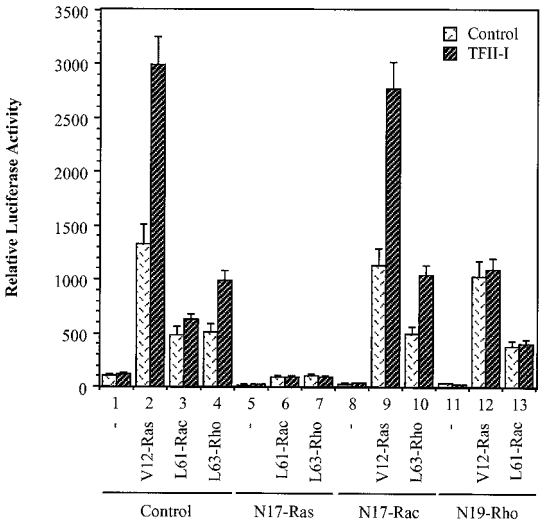

FIG. 11. Both Ras and Rho pathways are required for the activity of TFII-I on the c-*fos* promoter. V12-Ras (lane 2, 9, and 12), L61-Rac (lane 3, 6, and 13), or L63-Rho (lane 4, 7, and 10) expression plasmid were transfected into NIH 3T3 fibroblasts with the c-*fos*–luciferase reporter construct in the absence or presence of TFII-I. pCDNA3 (lanes 1 to 4), N17-Ras (lanes 5 to 7), N17-Rac (lanes 8 to 10), or N19-Rho (lanes 11 to 13) plasmid was also included in the transfection as indicated. After the transfection, the cells were maintained for 40 h in 0.5% CS before harvest. Cell extracts were then processed for luciferase activity.

distinct mechanisms, we attempted to demonstrate whether TFII-I requires both pathways for its activity or whether either of these pathways is sufficient to give rise to the activation of TFII-I for the c-*fos* promoter enhancement. To do this, we carried out c-*fos* reporter assay after the transfection with different combinations of activated or dominant-negative Ras and Rho expression plasmids in the absence or presence of TFII-I, and luciferase activity was measured without serum stimulation. Rac expression plasmids were also used as controls. The results are shown in Fig. 11. In control samples, activated V12-Ras (lane 2) and L63-Rho (lane 4) efficiently synergized with TFII-I for c-*fos* promoter activation in the presence of other normally functioning endogenous signal transduction pathways. However, interestingly, the activated L63-Rho was not able to cooperate with TFII-I in the presence of dominant-negative N17-Ras (lane 7) and, likewise, coexpression of the activated V12-Ras and TFII-I could not further enhance the activity of the c-*fos* promoter in the presence of dominant-negative N19-Rho (lane 12). The dominant-negative N17-Rac did not block the cooperation between TFII-I and the activated V12-Ras or L63-Rho (lane 9 and 10). These results indicate that both the Ras and the Rho pathways need to be functioning for the effects of TFII-I on the c-*fos* promoter and further indicate that the Ras and Rho pathways function in distinct parallel pathways to regulate TFII-I.

DISCUSSION

Ras- and Rho-mediated signal transduction pathways play pivotal roles in transducing signals for a variety of cellular responses. The Ras pathway controls cell proliferation and differentiation in many different cell types, and the Rho family small G proteins regulate intracellular cytoskeletal structures as well as cell proliferation and gene expression (14). It has been demonstrated that Ras efficiently regulates the Raf/Mek1/ERK MAP kinase cascade among other signal transduction pathways, whereas Rho signaling is less well understood (17, 18, 28).

In this report, we have found that both Ras and RhoA modulate the ability of TFII-I to stimulate the c-*fos* promoter. Both activated or dominant-negative Ras and RhoA coexpression have dramatic effects on the ability of TFII-I to enhance c-*fos* promoter activity, whereas Rac and Cdc42 showed only partial effects. Other activators of ERK, Raf and Mek1, also effectively synergize with TFII-I for the activation of the c-*fos* promoter, whereas a specific Mek1 inhibitor, PD98059, which leads to the inactivation of ERK, eliminated the activity of TFII-I on the c-*fos* promoter. We thus conclude that TFII-I is functionally dependent on the Ras/ERK pathway.

Consistent with these findings, we have found that ERK binds directly to TFII-I and that this interaction is modulated by both serum and Ras. Importantly, this interaction can be detected not only with transfected proteins but also with the endogenous proteins. We have found that ERK interacts with TFII-I through the consensus ERK binding motif known as the D box (52, 53). Abrogation of ERK binding to TFII-I by mutation of the D box on TFII-I disrupts the ability of TFII-I to enhance c-*fos* promoter activation. This result strongly suggests that ERK binding is required for its activity. This is likely due to the fact that TFII-I is a substrate for ERK. We have found that TFII-I can be phosphorylated by ERK in vitro. Moreover, mutation of the consensus MAP kinase phosphorylation sites on TFII-I reduces both its ability to be phosphorylated by ERK in vitro and its ability to activate the c-*fos* promoter. These data suggest that the role of the D box in TFII-I is to target ERK to TFII-I as a substrate. Consistent with this, we have found that mutation of the D box of TFII-I also reduces its ability to be phosphorylated by ERK. This is similar to findings from other proteins such as Elk-1 that contain a D box (52, 53).

Our findings that either mutation of the potential ERK phosphorylation sites or inhibition of ERK activity with PD98059 inhibits the ability of TFII-I to enhance c-*fos* promoter activation suggests that phosphorylation of TFII-I modulates its transactivation function. Consistent with this, the S627A and S633A mutants retained the ability to bind DNA despite losing activity (data not shown). S627 and S633 are in a region of striking homology to the Elk-1 phosphorylation sites which are known to regulate the transactivation function of Elk-1 (29). Thus, it seems likely that phosphorylation of these serines in TFII-I regulates its transactivation function as well.

Substrate binding has been implicated as a mechanism for ensuring kinase specificity (20, 51–53). However, there may be other roles for ERK targeting. It is possible that by binding to a transcription factor like Elk or TFII-I, ERK is targeted to specific promoters, where it then can phosphorylate other transcription factors in the promoter enhancer complex. For instance, direct interaction between SRF and MAP kinases has not been demonstrated, and SRF does not contain the conserved D box motif. However, SRF can be phosphorylated on serine 103 by pp90^{rsk}, which is a kinase immediately downstream of ERK (38). Since SRF and TFII-I interact each other at the protein level and bind to the c-*fos* SRE (13, 24), TFII-I could target MAP kinase to SRF. It is of note that the SRF-related proteins MEF2A and MEF2C which, unlike SRF itself, contain the D box, can bind to p38 MAP kinase directly and serve as substrates as well (51). Although, in case of the c-*fos* promoter, the TCFs could target MAP kinases to the promoter, there are other immediate-early gene promoters which contain the SRE but do not bind TCFs (49). TFII-I might play

a more important role for targeting MAP kinases to these promoters than the c-*fos* promoter.

We have found that ERK binding to TFII-I is regulated both by serum and dominant-negative Ras. It remains an open question as to how ERK binding to TFII-I is regulated. One possibility is that this is simply a consequence of cellular relocalization of ERK such that when activated ERK translocates to the nucleus it is now able to bind to nuclear TFII-I. However, this is not a possibility we favor. We find that a substantial fraction of TFII-I is cytoplasmic and in B cells the localization of TFII-I is itself regulated (33). Thus, when dominant-negative Ras inhibits TFII-I–ERK interaction, both proteins are cytoplasmic yet unassociated. Interestingly, PD98059 eliminates the activity of TFII-I on the c-*fos* promoter but did not affect the ability of TFII-I to bind ERK. This indicates that TFII-I requires functional ERK for its activity but not for its interaction with ERK per se. This result also distinguishes TFII-I from Elk-1 in that Elk-1 only binds to the activated form of ERK (53). The failure of PD98059 to inhibit TFII-I–ERK interaction further implies that Ras regulates the ERK–TFII-I interaction via an ERK-independent signaling pathway. Interestingly, in preliminary experiments, we have found that the dominant-negative Ras reduces TFII-I tyrosine phosphorylation (data not shown). This raises the possibility that signal-dependent tyrosine phosphorylation of TFII-I regulates its interaction with ERK.

It is significant that the activity of TFII-I can be modulated by RhoA. There is strong evidence for a signal transduction pathway that leads from RhoA to SRF (18). Though the detailed mechanism of the RhoA/SRF pathway has yet to be fully elucidated, it seems possible that TFII-I functions in this pathway. TFII-I binds to SRF (13, 24), and its activity is modulated by RhoA (this study). Even though the RhoA/SRF pathway is independent of Elk-1, this pathway remains dependent on Ras activation as we have found for TFII-I (18). Interestingly, despite the fact that RhoA affects the activity of TFII-I on the c-*fos* promoter, dominant-negative RhoA fails to disrupt the interaction between TFII-I and ERK, whereas dominant-negative Ras does. This indicates that Ras and RhoA regulate TFII-I by distinct mechanisms. Because dominant-negative RhoA inhibits the signaling of activated Ras to TFII-I and vice versa, the Rho and Ras pathways appear to function in parallel rather than in a linear pathway for TFII-I activation. Thus, TFII-I may also play a role in the RhoA/SRF pathway, as well as in the Ras/ERK pathway. We are currently investigating this possibility further.

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REFERENCES

1. **Alessi, D. R., A. Cuenda, P. Cohen, D. T. Dudley, and A. R. Saltiel.** 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J. Biol. Chem. **270:**27489–27494.
2. **Cheriyath, V., C. D. Novina, and A. L. Roy.** 1998. TFII-I regulates Vbeta promoter activity through an initiator element. Mol. Cell. Biol. **18:**4444–4454.
3. **Chihara, K., M. Amano, N. Nakamura, T. Yano, M. Shibata, T. Tokui, H. Ichikawa, R. Ikebe, M. Ikebe, and K. Kaibuchi.** 1997. Cytoskeletal rearrangements and transcriptional activation of c-fos serum response element by Rho-kinase. J. Biol. Chem. **272:**25121–25127.
4. **Cochran, B. H.** 1993. Regulation of immediate early gene expression, p. 3–24. *In* R. Grzanna and R. Brown (ed.), Activation of immediate early genes by drugs of abuse, vol. 125. National Institutes of Health, Rockville, Md.
5. **Cochran, B. H., J. Zullo, I. M. Verma, and C. D. Stiles.** 1984. Expression of the c-*fos* gene and of a *fos*-related gene is stimulated by platelet-derived growth factor. Science **226:**1080–1082.
6. **Dalton, S., and R. Treisman.** 1992. Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. Cell **68:**597–612.
7. **Derijard, B., M. Hibi, I. H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R. J. Davis.** 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell **76:**1025–1037.
8. **Feig, L. A., and G. M. Cooper.** 1988. Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. Mol. Cell. Biol. **8:**3235–3243.
9. **Feig, L. A., B. T. Pan, T. M. Roberts, and G. M. Cooper.** 1986. Isolation of *ras* GTP-binding mutants using an in situ colony-binding assay. Proc. Natl. Acad. Sci. USA **83:**4607–4611.
10. **Graham, R., and M. Gilman.** 1991. Distinct protein targets for signals acting at the c-*fos* serum response element. Science **251:**189–192.
11. **Grammatikakis, N., J.-H. Lin, A. Grammatikakis, P. N. Tsichlis, and B. H. Cochran.** 1999. $p50^{cdc37}$ acting in concert with Hsp90 is required for Raf-1 function. Mol. Cell. Biol. **19:**1661–1672.
12. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. Nature **311:**433–442.
13. **Grueneberg, D. A., R. W. Henry, A. Brauer, C. D. Novina, V. Cheriyath, A. L. Roy, and M. Gilman.** 1997. A multifunctional DNA-binding protein that promotes the formation of serum response factor/homeodomain complexes: identity to TFII-I. Genes Dev. **11:**2482–2493.
14. **Hall, A.** 1994. Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu. Rev. Cell Biol. **10:**31–54.
15. **Hanke, J. H., J. P. Gardner, R. L. Dow, P. S. Changelian, W. H. Brissette, E. J. Weringer, B. A. Pollok, and P. A. Connelly.** 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J. Biol. Chem. **271:**695–701.
16. **Hill, C. S., R. Marais, S. John, J. Wynne, S. Dalton, and R. Treisman.** 1993. Functional analysis of a growth factor-responsive transcription factor complex. Cell **73:**395–406.
17. **Hill, C. S., and R. Treisman.** 1995. Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors. EMBO J. **14:**5037–5047.
18. **Hill, C. S., J. Wynne, and R. Treisman.** 1995. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. Cell **81:**1159–1170.
19. **Hipskind, R. A., V. N. Rao, C. G. F. Mueller, E. S. P. Reddy, and A. Nordheim.** 1991. Ets-related protein Elk-1 is homologous to the c-*fos* regulatory factor $p62^{TCF}$. Nature **354:**531–534.
20. **Jacobs, D., D. Glossip, H. Xing, A. J. Muslin, and K. Kornfeld.** 1999. Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. Genes Dev. **13:**163–175.
21. **Johansen, F. E., and R. Prywes.** 1994. Two pathways for serum regulation of the c-fos serum response element require specific sequence elements and a minimal domain of serum response factor. Mol. Cell. Biol. **14:**5920–5928.
22. **Kallunki, T., T. Deng, M. Hibi, and M. Karin.** 1996. c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. Cell **87:**929–939.
23. **Kallunki, T., B. Su, I. Tsigelny, H. K. Sluss, B. Derijard, G. Moore, R. Davis, and M. Karin.** 1994. JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. Genes Dev. **8:**2996–3007.
24. **Kim, D.-W., V. Cheriyath, A. L. Roy, and B. H. Cochran.** 1998. TFII-I enhances activation of the c-*fos* promoter through interactions with upstream elements. Mol. Cell. Biol. **18:**3310–3320.
25. **Kruijer, W., J. A. Cooper, T. Hunter, and I. M. Verma.** 1984. Platelet-derived growth factor induces rapid but transient expression of the c-*fos* gene and protein. Nature **312:**711–716.
26. **Lee, J. C., J. T. Laydon, P. C. McDonnell, T. F. Gallagher, S. Kumar, D. Green, D. McNulty, M. J. Blumenthal, J. R. Heys, S. W. Landvatter, et al.** 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature **372:**739–746.
27. **Manzano-Winkler, B., C. D. Novina, and A. L. Roy.** 1996. TFII-I is required for transcription of the naturally TATA-less but initiator-containing Vbeta promoter. J. Biol. Chem. **271:**12076–12081.
28. **Marais, R., and C. J. Marshall.** 1996. Control of the ERK MAP kinase cascade by Ras and Raf. Cancer Surv. **27:**101–125.
29. **Marais, R., J. Wynne, and R. Treisman.** 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell **73:**381–393.
30. **Montaner, S., R. Perona, L. Saniger, and J. C. Lacal.** 1999. Activation of serum response factor by RhoA is mediated by the nuclear factor-kappaB and C/EBP transcription factors. J. Biol. Chem. **274:**8506–8515.
31. **Muller, R., R. Bravo, J. Burckhardt, and T. Curran.** 1984. Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. Nature **312:**716–720.

32. **Nobes, C. D., and A. Hall.** 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell **81:**53–62.
33. **Novina, C. D., S. Kumar, U. Bajpai, V. Cheriyath, K. Zhang, S. Pillai, H. H. Wortis, and A. L. Roy.** 1999. Regulation of nuclear localization and transcriptional activity of TFII-I by Bruton's tyrosine kinase. Mol. Cell. Biol. **19:**5014–5024.
34. **Okada, T., Y. Kawano, T. Sakakibara, O. Hazeki, and M. Ui.** 1994. Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. J. Biol. Chem. **269:**3568–3573.
35. **Perez-Jurado, L. A., Y. K. Wang, R. Peoples, A. Coloma, J. Cruces, and U. Francke.** 1998. A duplicated gene in the breakpoint regions of the 7q11.23 Williams-Beuren syndrome deletion encodes the initiator binding protein TFII-I and BAP-135, a phosphorylation target of BTK. Hum. Mol. Genet. **7:**325–334.
36. **Ridley, A. J., and A. Hall.** 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell **70:**389–399.
37. **Ridley, A. J., H. F. Paterson, C. L. Johnston, D. Diekmann, and A. Hall.** 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell **70:**401–410.
38. **Rivera, V. M., C. K. Miranti, R. P. Misra, D. D. Ginty, R.-H. Chen, J. Blenis, and M. E. Greenberg.** 1993. A growth factor-induced kinase phosphorylates the serum response factor at a site that regulates its DNA-binding activity. Mol. Cell. Biol. **13:**6260–6273.
39. **Rivera, V. M., M. Sheng, and M. E. Greenberg.** 1990. The inner core of the serum response element mediates both the rapid induction and subsequent repression of c-*fos* transcription following serum induction. Genes Dev. **4:**255–268.
40. **Robbins, D. J., M. Cheng, E. Zhen, C. A. Vanderbilt, L. A. Feig, and M. H. Cobb.** 1992. Evidence for a Ras-dependent extracellular signal-regulated protein kinase (ERK) cascade. Proc. Natl. Acad. Sci. USA **89:**6924–6928.
41. **Robertson, L., T. Kerppola, M. Vendrell, D. Luk, R. Smeyne, C. Bocchiaro, J. Morgan, and T. Curran.** 1995. Regulation of c-*fos* expression in transgenic mice requires multiple interdependent transcription control elements. Neuron **14:**241–252.
42. **Roy, A. L., C. Carruthers, T. Gutjahr, and R. G. Roeder.** 1993. Direct role for Myc in transcription initiation mediated by interactions with TFII-I. Nature **365:**359–361.
43. **Roy, A. L., H. Du, P. D. Gregor, C. D. Novina, E. Martinez, and R. G. Roeder.** 1997. Cloning of an Inr- and E-box-binding protein, TFII-I, that interacts physically and functionally with USF1. EMBO J. **16:**7091–7104.
44. **Roy, A. L., S. Malik, M. Meisterernst, and R. G. Roeder.** 1993. An alternative pathway for transcription initiation involving TFII-I. Nature **365:**355–359.
45. **Roy, A. L., M. Meisterernst, P. Pognonec, and R. G. Roeder.** 1991. Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. Nature **354:**245–248.
46. **Sahai, E., A. S. Alberts, and R. Treisman.** 1998. RhoA effector mutants reveal distinct effector pathways for cytoskeletal reorganization, SRF activation and transformation. EMBO J. **17:**1350–1361.
47. **Simon, A., U. Rai, B. Fanburg, and B. Cochran.** 1998. Activation of the JAK-STAT pathway by reactive oxygen species. Am. J. Physiol. **44:**C1640–C1652.
48. **Treisman, R.** 1996. Regulation of transcription by MAP kinase cascades. Curr. Opin. Cell Biol. **8:**205–215.
49. **Treisman, R.** 1990. The SRE: a growth factor responsive transcriptional regulator. Semin. Cancer Biol. **1:**47–58.
50. **Whitmarsh, A. J., P. Shore, A. D. Sharrocks, and R. J. Davis.** 1995. Integration of MAP kinase signal transduction pathways at the serum response element. Science **269:**403–407.
51. **Yang, S. H., A. Galanis, and A. D. Sharrocks.** 1999. Targeting of p38 mitogen-activated protein kinases to MEF2 transcription factors. Mol. Cell. Biol. **19:**4028–4038.
52. **Yang, S. H., A. J. Whitmarsh, R. J. Davis, and A. D. Sharrocks.** 1998. Differential targeting of MAP kinases to the ETS-domain transcription factor Elk-1. EMBO J. **17:**1740–1749.
53. **Yang, S. H., P. R. Yates, A. J. Whitmarsh, R. J. Davis, and A. D. Sharrocks.** 1998. The Elk-1 ETS-domain transcription factor contains a mitogen-activated protein kinase targeting motif. Mol. Cell. Biol. **18:**710–720.
54. **Yang, W., and S. Desiderio.** 1997. BAP-135, a target for Bruton's tyrosine kinase in response to B cell receptor engagement. Proc. Natl. Acad. Sci. USA **94:**604–609.